Nickel hyperaccumulation in Thlaspi caerulescens: a rare micro-evolutionary event

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Nickel hyperaccumulation in *Thlaspi caerulescens*: a rare micro-evolutionary event

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everything you do in haste
goes hastely waste

für Opa und Papa

Content

Chapter 1

General introduction

Heavy metal hyperaccumulation in plants

The first reference to heavy metal hyperaccumulation of plants was made in 1865 by Risse in Sachs (1865), who reported that *Thlaspi calaminare* (nowadays *Thlaspi caerulescens*) grown on Zn-rich soils close to the German/Belgium border contained 17% Zn in its ash. However, only the discovery of extreme levels of Ni accumulation in *Alyssum bertolonii* from serpentine soils in Italy, with up to 10,000 μg Ni/gDW (1%), made by Minguzzi and Vergano (1948), marked the beginning of an increasing interest of plant scientists in this rare phenomenon. Brooks et al. (1977) defined metal hyperaccumulating plants as plants that accumulate more than 1% zinc (Zn) or manganese (Mn), 0.1% nickel (Ni), copper (Cu) or cobalt (Co), or 0.01% cadmium (Cd) in their above ground parts (on a dry weight basis) when growing on their native soils (Baker and Brooks, 1989, Baker et al., 2000). These concentrations are lethal to normal plants (Marschner, 1995). The relatively small yet diverse group of about 400 heavy metal hyperaccumulators belong to a broad range of unrelated families and occur mainly on metal rich soils, both in tropical as well as temperate regions. Heavy metal hyperaccumulators represent less than 2% of all angiosperms, and even though they occur in a broad range of unrelated plant families, the Brassicaceae family is particularly rich in them, especially the genera *Alyssum* and *Thlaspi*. Most hyperaccumulators, approximately 320 species, are Ni hyperaccumulators (Baker et al. 2000). These species can accumulate concentrations of Ni in excess of 2% on a dry matter basis in their foliage and are found on serpentine soils, derived from ultramafic rocks, typically containing 0.1 to 1% (w/w) Ni. Zn hyperaccumulators, of which 15 species have been identified yet, represent the second largest group of

hyperaccumulator plants. They are mainly found on calamine soils enriched in Zn, Pb and Cd, either naturally, or due to human activities such as mining and metal smelting. Hyperaccumulation of Cd was found in two species thus far, *Thlaspi caerulescens* and *Arabidopsis halleri,* both of which hyperaccumulate Zn too (Brown et al., 1995; Küpper et al., 2000; Lombi et al., 2000). Whereas most hyperaccumulators grow on soils enriched in heavy metals, some species, including *Thlaspi caerulescens* and *Arabidopsis halleri*, are facultative metallophytes, with metallicolous and non-metallicolous populations.

Heavy metals, regardless of whether they are biologically essential or beneficial or not, act harmful within the plant when taken up in excess, through inhibiting enzyme activity, growth, metabolism and mineral nutrition. Therefore, the ability to render excessive foliar metal burdens harmless is a major characteristic of a hyperaccumulating plant, next to enhanced rates of root metal uptake and root to shoot metal translocation (Zhao et al., 2002; Pollard et al., 2002).

The question of how the phenomenon of metal hyperaccumulation has been evolved has not been resolved yet. Boyd and Martens (1992) suggested that metal disposal from the plant, drought resistance, interference with neighbouring plants, inadvertent uptake, or defence against foliar herbivores and pathogens are the potential reasons-to-be of the hyperaccumulation phenotype. Several authors have shown that Ni hyperaccumulation affords protection against a broad range of organisms, such as parasitic bacteria, fungi and herbivorous insects (Boyd and Martens, 1994; Martens and Boyd, 1994; Jhee et al., 1999; Davis and Boyd, 2000; Ghaderian et al., 2000; Davis et al., 2001), whereas Zn or Cd accumulation is effective against some insect

herbivores, at least (Pollard and Baker, 1997; Jiang et al., 2005). However, although these results do demonstrate that hyperaccumulation can be effective against parasites or herbivores, they do not prove that the phenomenon has been evolved in response to parasite or herbivore pressure.

Thlaspi caerulescens

Thlaspi caerulescens J. and C. Presl is an annual, biennial or short-lived perennial self-compatible species of the Brassicaceae family. It has a wide geographical distribution in Europe, from Scandinavia to the Mediterranean area, from sea level to high altitudes in the Alps and the Pyrenees mountains (Tutin et al., 1993; Koch et al., 1998). Remarkably, the species is found on heavy metal (mainly zinc, lead and cadmium) contaminated soils, on serpentine outcrops and on normal calcareous and acidic soils, and it is morphologically highly polymorphic (Ingrouille and Smirnoff, 1986). *T. caerulescens* is one of the most studied hyperaccumulators, and is known to hyperaccumulate Zn, Ni and Cd. Reported foliar metal concentrations amount to 30,000 μg Zn/gDW (Brown et al., 1995), 4,000 μg Ni/gDW (Reeves and Brooks, 1983) and 2,700 μg Cd/gDW (Lombi et al., 2000) in healthy plants growing on metallicolous soils.

Reeves et al. (2001) and Assunção et al. (2003c) found that *Thlaspi caerulescens* has a remarkable ability to accumulate Zn even from soils in which Zn is present at normal or low concentrations, such as serpentine soils. The foliar Zn contents in natural non-metallicolous *T. caerulescens* populations are consistently many-fold higher than in non-hyperaccumulators (Reeves et al., 2001) and when grown in the

same soil, the Zn accumulation rates in non-metallicolous plants are often higher than in metallicolous ones. Thus, the species' apparent ability to accumulate extreme, normally lethal concentrations of Zn in its foliage must represent a constitutive species level trait, at least to a large extent (Meerts and Van Isacker, 1997; Escarré et al.; 2000; Lombi et al., 2000; Assunção et al., 2003c), such as also shown for *A. halleri* (Bert et al., 2002; Macnair, 2002). In contrast to the constitutive nature of Zn hyperaccumulation, Ni hyperaccumulation seems to be population-specific, at least at the level of uptake from the soil. Assunção et al. (2003c) compared populations from different soil types under controlled conditions and showed that the root to shoot translocation rates for Ni were consistently higher in *T. caerulescens* than in non-hyperaccumulator plants, whereas, on a total plant weight basis, some populations accumulated even less rather than more Ni compared to the non-hyperaccumulating congener, *T. arvense*. They suggested that for Ni, enhanced root to shoot translocation, rather than enhanced total accumulation as such, represents a constitutive species level trait in *Thlaspi caerulescens* (Assunção et al., 2003c).

Evidently, hyperaccumulators are inherently able to tolerate the extremely high foliar concentrations of the metals accumulated at their natural population sites. However, this does not necessarily mean that they would also be tolerant to high soil metal concentrations. Although hyperaccumulating plants are apparently more tolerant than non-metallophytes, plants from non-metallicolous populations of *T. caerulescens* and *A. halleri* do suffer from metal toxicity when grown in metalliferous soils, as compared to plants from metallicolous populations (Meerts and Isacker, 1997; Escarré et al., 2000; Schat et al., 2002; Bert et al., 2000; Jiménez-Ambriz et al., 2007).

Moreover, local metallicolous populations appear to be specifically adapted to the metals that are toxically enriched in the soil at their place of origin, just like non-hyperaccumulator metallophytes (Meerts and Van Isacker, 1997; Schat et al., 2002; Assunção et al., 2003c; Pauwels et al., 2007).

In general, from comparisons under controlled conditions it appeared that there is considerable independent variation in the degrees of metal accumulation, translocation and tolerance, both within and among populations, in *T. caerulescens* as well as in *A. halleri*. The patterns of this variation are clearly metal-specific (Pollard and Baker, 1996; Meerts & Van Isacker, 1997; Escarré et al., 2000; Assunção et al., 2001, 2003c; Macnair, 2002; Meerts et al., 2003). This intraspecific variation permits a genetic analysis of these traits using segregating families generated from intraspecific crosses. Assunção et al. (2006) and Deniau et al. (2006) mapped quantitative trait loci for Zn accumulation in roots and shoots in different *T. caerulescens* intraspecific crosses. In both studies, Zn accumulation, in so far as it segregated, appeared to be determined by multiple genes, with the trait-enhancing alleles originating from both parents, indicating that the mechanisms of Zn hyperaccumulation may be population-specific, at least in part. In agreement with this, transgressive segregation of Zn accumulation has been found in different intraspecific *T. caerulescens* crosses (Zha et al., 2004; Deniau et al., 2006). Deniau et al. (2006) found QTLs specific for either Cd accumulation or Zn accumulation, but also common ones, demonstrating that the accumulation of these metals is in part under independent genetic control, such as suggested by the largely independent variation of these traits among natural populations (Assunção et al., 2003c).

The precise relationships between tolerance, translocation and accumulation in hyperaccumulators are not fully understood yet. Macnair et al. (1999) found independent segregation of Zn tolerance and Zn accumulation in an interspecific F_2 cross between the metal hyperaccumulator *A. halleri* and the non-hyperaccumulating, non-metallophyte congener *A. petraea*, which suggests that these traits are under independent genetic control, at least largely. However, the tolerance and accumulation loci segregating in an interspecific cross are likely to be different from those segregating in intraspecific crosses, leaving the possibility that there might be genetically correlated variation in these properties within a hyperaccumulator species. Comparison among metallicolous and non-metallicolous *Thlaspi caerulescens* populations under controlled conditions suggested a negative rather than a positive phenotypic correlation between Zn accumulation capacity and Zn tolerance (Meerts and Van Isacker, 1997; Escarré et al., 2000; Schat et al., 2002). In an intraspecific F_3 cross segregating for both properties, Zn tolerance and Zn accumulation were largely uncorrelated (Assunção et al., 2003b), suggesting that the overall negative phenotypic correlation among local populations may be due to linkage disequilibrium, caused by selection on multiple loci, rather than to pleiotropic genetic control. On the other hand, although information is scarce, it seems that positive phenotypic correlations between tolerance and accumulation among populations may exist for Cd and Ni in *T. caerulescens* (Escarré et al., 2000; Schat et al., 2002). Whilst Zha et al. (2004) could show that Cd tolerance and Cd accumulation do not co-segregate in an intraspecific cross, there is no genetic evidence yet for Ni tolerance and accumulation. Even though controlled experiments suggest little or no phenotypic correlation, the relationships

between translocation and tolerance, or translocation and accumulation are still unclear so far yet. Future analysis of crosses could deliver direct genetic evidence for the relationships between these traits within the hyperaccumulation syndrome.

Like other hyperaccumulating plants, *T. caerulescens* exhibits in comparison with non-hyperaccuulators an enhanced uptake of heavy metal(s), as well as an enhanced ability to translocate these metals efficiently from root to shoot, and an increased capacity to render them harmless at the cellular level in the leaves (Lasat et al., 1996; Shen et al., 1997; Schat et al., 2000). However, the physiological mechanisms underlying these traits are still incompletely known to date.

An enhanced ability to mobilize metals from the rhizosphere has been suggested to play an important role in hyperaccumulation. However, case studies showed that hyperaccumulating and non-hyperaccumulating plants use the same soil Zn pool, and that hyperaccumulator plants do not mobilize Zn more efficiently from the rhizosphere, e.g. through acidification or exudation of organic compounds, than non-hyperaccumulating species do (Whiting et al., 2001; McGrath et al., 2000; Zhao et al., 2001). However, Whiting et al. (2000), comparing different *Thlaspi* populations with different abilities to hyperaccumulate Cd, observed preferential root proliferation in Cd-rich soil patches, i.e. “root foraging”, in a Cd hyperaccumulating population, but not in a population with low Cd accumulation. They suggested that the root metal foraging trait might be associated with high levels of metal tolerance, rather than with high accumulation efficiency itself, because the most tolerant populations demonstrated the foraging response, whereas the more sensitive ones did not (Whiting et al., 2000). The phenomenon of root foraging has also been demonstrated for Zn.

However, the shoot Zn accumulation in the heterogeneous Zn treatment did not significantly differ from that in the homogeneous Zn treatment, and therefore it was concluded that root Zn foraging is not essential for the hyperaccumulation of Zn as such (Schwarz et al., 1999; Haines 2002).

Lasat et al. (1996) used radiotracer flux techniques to monitor concentration-dependent Zn^{2+} uptake in hydroponically grown seedlings of *T. caerulescens* and the non-hyperaccumulating congener *T. arvense* and found a saturable component following Michaelis-Menten kinetics. The Zn^{2+} influx in the roots of both species showed the same K_m value, but the V_{max} was much higher in *T. caerulescens*, suggesting that the Zn transporters in both species are the same, but that they are more expressed in *T. caerulescens* roots. Time-dependent Zn uptake experiments carried out by Lasat and Kochian (2000) revealed that *T. caerulescens* accumulated two times more Zn in the roots than *T. arvense* after 3 hours of metal exposure, in spite of much higher rates of Zn translocation to the shoot in the former species (Lasat et al., 1996).

Further progress was made by identifying a Zn transporter gene (*ZNT1*) from *T. caerulescens* (Lasat et al., 2000; Pence et al., 2000) through functional complementation of the *zhy3* yeast mutant, which is defective in Zn uptake (Zhao et al., 1996). *ZNT1*, a member of the ZIP family of metal transporters (Grotz et al., 1998), with members in the fungal, plant and animal kingdoms (Eng et al., 1998; Guerinot, 2000; Mäser et al., 2001), was highly expressed in *T. caerulescens* roots and shoots, both under Zn deficiency as well as under normal and high Zn supply. In contrast, in *Thlaspi arvense ZNT1* was expressed under Zn deficiency, but strongly down-regulated at normal and high Zn supply. Furthermore, the V_{max} of Zn influx in

the roots of *T. caerulescens*, growing under different Zn concentrations correlated well with the root *ZNT1* transcript levels, and the K_m values were the same at all Zn exposure levels tested (Pence et al., 2000). Even though an explanation of the high expression of *ZNT1* under all Zn supply conditions in *T. caerulescens* is still lacking, Lasat et al. (2000) and Pence et al. (2000) suggested that it would be due to a lower degree of Zn-imposed transcriptional down regulation, possibly due to some alteration in a Zn-responsive element in the *T. caerulescens ZNT1* promoter. However, Assunção et al. (2003a) stated that the much more efficient plant-internal sequestration machinery of *T. caerulescens* might work constitutively and not just under Zn excess, thus leading to a constitutive state of "physiological Zn deficiency", which would in turn lead to a constitutive high expression level of the Zn uptake machinery. Recently, Hanikenne et al. (2008) showed that triplication and altered cis-regulation of the 1b P-type ATPase, *HMA4*, which is thought to function in Zn xylem loading (Hussain et al., 2004), is essential for Zn hyperaccumulation in *A. halleri*. Heterologous expression of this gene under the *A. halleri* promoter resulted in increased Zn root to shoot translocation and a concomitant enhanced expression of Zn-deficiency induced genes in *A. thaliana*, among which *ZIP4*, which is the *Arabidopsis* orthologue of *ZNT1*. These results suggest that the more or less constitutively enhanced expression of *ZNT1* in *T. caerulescens* might be caused by the strongly enhanced rate of Zn xylem loading, leading to a Zn deficiency response in the root.

The mechanisms of Cd uptake in *T. caerulescens* are still elusive. Lombi et al. (2001) and Zhao et al. (2002) established the kinetic parameters of Cd and Zn influx into the

roots of *T. caerulescens* calamine populations (Ganges and Prayon) with different Cd accumulation capacities, and found that Cd uptake was significantly suppressed in the presence of equimolar concentrations of Zn and Mn in the low-Cd-accumulating Prayon accession, but not in the high-Cd-accumulating Ganges population. They suggested that Cd uptake in these two populations may be largely mediated by different transporters, i.e. one with a preference for Cd over Zn or Mn in Ganges, and one with a preference for Zn or Mn over Cd in Prayon (Lombi et al., 2001; Zhao et al., 2002).

Metal hyperaccumulators are characterized by an enhanced translocation of the metal from the root to the leaves resulting in high metal concentrations in the xylem sap and shoot to root metal concentration ratios higher than unity (Lasat et al., 1996; 1998; Krämer at al., 1996; Shen et al., 1997; Schat et al., 2000a). The underlying mechanisms and the transporters involved are incompletely known. Even though Hanikenne et al. (2008) recently showed that a high level of expression of *HMA4* is absolutely required for the high rate of Zn translocation in *A. halleri*, as compared to *A. thaliana* (see above), it is doubtful however, whether enhanced expression of *HMA4* alone is sufficient to produce the full hyperaccumulator translocation phenotype. Variation in Zn and Cd translocation among *T. caerulescens* accessions appeared to be uncorrelated with *HMA4* expression but instead, at least for Cd, correlated with vacuolar retention in root cells, as evidenced by radiotracer efflux analysis (Xing et al., 2008). Lasat et al. (1998) investigated Zn compartmentation by radiotracer efflux analysis and found a faster efflux of Zn out off the vacuoles of root cells of *T. caerulescens* as compared with the non-hyperaccumulator *T. arvense*.

Furthermore, it was shown that Zn accumulation in roots of *T. arvense* was much higher than in *T. caerulescens* when compared after a long exposure (96h) (Lasat et al., 1996). In addition, the hyperaccumulator showed a smaller influx of metal into the root cell vacuoles, compared to its non-hyperaccumulating congener (Lasat and Kochian, 2000). As a result, a larger amount of Zn remained readily available for immediate loading into the xylem and thus translocation to the leaves in the hyperaccumulator, compared to the non-hyperaccumulator (Lasat et al., 1998). Additionally, Lasat et al. (1998) revealed that Zn accumulation in leaf sections of *T. caerulescens* was higher in comparison to *T. arvense* when exposed to relatively high external Zn concentrations. Based on these results, Lasat and Kochian (2000) suggested that next to enhanced xylem loading and a decreased rate of uptake into the root vacuoles, enhanced uptake into leaf cells might play a role in Zn hyperaccumulation in *T. caerulescens*.

Former studies revealed that high concentrations low-molecular chelators, such as histidine and various organic acids, such as citrate, malate and malonate are constitutive in metal hyperaccumulators (Tolrá et al., 1996; Shen et al., 1997; Salt et al., 1999; Sarret et al., 2002; Krämer, 2005; Freeman et al., 2005). The functional significance of these chelators is not entirely clear yet. Whereas the stability of metal-citrate or metal-malate as well as metal-malonate complexes seems to be too low to prevent toxicity within the cytoplasm it is possible that these acids might function to trap metals in leaf vacuoles (Sarret et al., 2002). Alternatively, their presence at high concentrations in hyperaccumulators might merely serve to maintain charge balance, in the face of high rates of cation accumulation. On the other hand, histidine would be

expected to form highly stable complexes, particularly at cytoplasmic pH, suggesting that histidine accumulation might contribute to Ni tolerance (Krämer et al. 1996, 2000; Salt et al. 1999; Kerkeb and Krämer, 2003).

Ingle et al. (2005) showed a constitutively higher expression of the histidine biosynthetic pathway component, ATP-phosphoribosyltransferase (*ATP-PRT*), in the Ni hyperaccumulator *Alyssum lesbiacum*, as compared to the non hyperaccumulating *Alyssum montanum*. Persans et al. (1999), comparing the Ni hyperaccumulator *Thlaspi goesingense* and the non-hyperaccumulator *T. arvense*, found that the histidine concentrations in xylem sap and shoots did not differ significantly between the two species, but that the His concentrations in the roots were much higher in the hyperaccumulator. Although large dose-dependent increases in histidine concentrations in the xylem in response to Ni exposure were not found in *T. goesingense* (Persans et al.,1999), they have been reported for hyperaccumulating *Alyssum* species. Krämer et al. (1996) found a clear accumulation of xylem histidine in response to Ni exposure in *Alyssum lesbiacum*, a Ni hyperaccumulator, but not in *Alyssum montanum*, a non-hyperaccumulator. This response in *A. lesbiacum* was also induced by cobalt, but not by Zn. Moreover, they showed that exogenously supplied histidine improved Ni tolerance and resulted in a 5-fold increase in the Ni loading into the xylem in *A. montanum*, suggesting that elevated root His concentrations are responsible for the enhanced Ni root to shoot translocation and most likely also for the Ni tolerance phenotype of the hyperaccumulating species (Krämer et al., 1996). In line with that, Kerkeb and Krämer (2003) showed that increasing the free His pool in the roots of the non-hyperaccumulator plant, *Brassica juncea*, through exogenous

supply, increased the concentration of Ni in the xylem, without affecting the Ni uptake rate. On the other hand, ectopic over-expression of *ATP-PRT* in *Arabidopsis thaliana* did not result in enhanced Ni allocation to the shoot, although it did produce enhanced root His concentrations and improved the Ni tolerance (Ingle et al., 2005). This suggests that an enhanced root His concentration is not universally sufficient to produce the hyperaccumulator Ni translocation phenotype. The mechanism of His action on Ni translocation is still elusive, but it is conceivable that His and Ni xylem loading, as such, are directly coupled, at least in some species (Kerkeb and Krämer, 2003). Alternatively, Ni-histidine complex formation might as well interfere with tonoplast transport and, therefore, decrease the vacuolar retention in peripheral root tissues.

Another important candidate ligand for heavy metal homeostasis in plants is the non-proteinogenic amino acid nicotianamine (NA), which has been proposed to function as an iron carrier in intra- and inter-cellular transport and in phloem transport. It also acts as a copper carrier in xylem transport and it is a strong chelator of Ni (Scholz et al., 1992; Pich and Scholz, 1996; von Wirén et al., 1999; Douchkov et al., 2002; Mizuno et al., 2003; Pianelli et al., 2005). Roots of *A. halleri* were found to contain around 3-fold higher levels of NA than roots of *A. thaliana* (Weber et al., 2004). A similar result has been obtained for *T. caerulescens* in comparison with *T. arvense*, albeit after exposure to non-toxic Ni concentrations (Mari et al., 2006). These results imply a strong evidence for an important role of NA in hyperaccumulators. Moreover, Weber et al. (2004) found strong evidence for an important role of NA in Zn homeostasis and hyperaccumulation by comparing *A. thaliana* and *A. halleri*

transcript expression patterns under various growth conditions. NA synthase was one of the most highly expressed genes in *A. halleri* when compared to *A. thaliana.* In addition, the hyperaccumulator also showed much higher NAS protein levels (*NAS2* and *NAS4*) in its root tissue (Weber et al., 2003). Former work demonstrated that the overexpression of NA synthases in *A.thaliana* resulted not only in improved accumulation, but also improved the tolerance to Ni (Douchkov et al., 2005; Pianelli et al., 2005).

Next to NA synthases, two major groups of metal transporters are considered to contribute to heavy metal tolerance in hyperaccumulating plants, i.e. the cation diffusion family (CDF) and the P_{1B}-type subfamily of P-type ATPases.

Transporters of the CDF family appear to mediate the cytoplasmic efflux of transition metal cations, such as Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+} or Mn^{2+} and have been named Metal Tolerance Proteins (MTPs). Compared to non-hyperaccumulator species, three genes encoding CDFs are highly expressed in *A. halleri* and *T. caerulescens*, *MTP1*, *MTP8* and *MTP11*. *MTP1* has been identified as a candidate tolerance gene in shoots and roots of *A. halleri* and in roots of *T. caerulescens* by transcriptomic approaches (Becher et al., 2004; van de Mortel et al., 2006; Talke et al., 2006). Expression of *AhMTP1* is constitutively high over a range of external Zn treatments, especially in leaves. In *A. thaliana* the much lower expressed *AtMTP1* was shown to contribute to basic levels of Zn tolerance and Zn accumulation in leaf tissue (Dräger et al., 2005, Krämer, 2005). Ectopic over-expression in *A. thaliana* resulted in enhanced Zn tolerance and increased Zn accumulation in the roots (Van der Zaal et al., 1999). However, until now little is known about the proteins encoded by *MTP8* and *MTP11*

in hyperaccumulating plant species. When (over-)expressed in yeast or *A. thaliana*, these genes seem to contribute to the tolerance and homeostasis of metals other than Zn, such as Fe, Cu and Mn (Delhaize et al., 2003). Both genes have been suggested to play a role in the adjustment of the homeostasis of other metals in Zn hyperaccumulators (Becher et al., 2004; Talke et al., 2006; van de Mortel et al., 2006). The heavy metal transporting P_{1B}- ATPases (*HMA*s) translocate metal cations out of the cytoplasm across membranes using energy from the hydrolysis of ATP. Genes encoding two of them, *HMA3* and *HMA4*, are strongly expressed in *A. halleri* and *T.caerulescens*, as compared to *A. thaliana* (Becher et al., 2004; Talke et al., 2006; van de Mortel et al., 2006; Hammond et al., 2006). *AtHMA4* and *AhHMA4* are both localized to the plasmamembrane and seem to be involved in Zn xylem loading (Hussain et al., 2004; Verret et al., 2004; Hanikenne et al., 2008), whereas *AhHMA3* might be involved in vacuolar sequestration of Zn (Becher et al., 2004), although *AtHMA3* is a transporter of Cd and Pb, rather than Zn (Gravot et al., 2004). In *A. halleri* and *T. caerulescens HMA4* expression is 2-3 times higher in roots than in shoots. Whereas the expression levels of *HMA4* in *A. halleri* are relatively constant over different external Zn concentrations, the levels of expression in *T. caerulescens* are increased in response to high Zn and Cd and also under Zn deficiency (Papoyan and Kochian; 2004; Bernard et al., 2004; Talke et al., 2006). It is known that in *A. thaliana HMA2* and *HMA4* are mainly expressed in xylem parenchyma and play a role in root to shoot translocation of Zn probably through mediating xylem loading (Mills et al., 2003; Hussain et al., 2004). As mentioned above, the high expression of *HMA4* in *A. halleri* has been shown to be essential for its hyperaccumulation phenotype,

confirming its postulated role in root to shoot metal transport (Hanikenne et al., 2008). However, the same gene was found to co-located with a major QTL for Cd and Zn tolerance in an *A. halleri* x *A. petraea* cross (Courbot et al., 2006; Willems et al., 2007). Also, RNAi-mediated silencing of *HMA4* in *A.halleri* decreased its Zn and Cd tolerance (Hanikenne et al., 2008), suggesting that it also acts as a major tolerance gene in hyperaccumulators. Although the underlying mechanism is unclear however, it is conceivable that the translocation of metals from the roots to the shoots, as such, may enhance root tolerance, particularly in hyperaccumulators where metal sequestration primarily takes place in the leaves. Alternatively, expression in tissues other than xylem parenchyma, e.g. in the root tips, albeit at a lower level, might directly confer tolerance through enhanced efflux from the cytosolic compartment of non-vascular tissues (Courbot et al., 2006; Willems et al., 2007).

Outline of the thesis

This work was done in order to obtain a better understanding of heavy metal hyperaccumulation and tolerance traits and their interrelationships. Therefore we investigated the heritabilities and the phenotypic and genetic correlations between Zn accumulation, Ni accumulation and Ni tolerance in *T. caerulescens*, based on variance and co-variance analyses of F_3 families derived from a cross between plants from a serpentine and a calamine population (chapter 2). The role for histidine in plant-internal metal transport, both at the levels of root to shoot translocation and tonoplast transport, were investigated in chapter 3. To this end we compared (1) root and shoot histidine concentrations in serpentine and calamine *T. caerulescens* and *T. arvense*,

(2) Ni tonoplast transport in energized root and shoot derived vesicles of the same populations, with Ni supplied as free Ni, Ni-citrate or Ni-histidine, (3) the effect of exogenous histidine supply on Ni xylem loading in these populations, and (4) the distribution of Ni in roots of serpentine and calamine *T. caerulescens* and in *T. arvense*. In chapter 4 we report on transcriptome comparisons between *T. caerulescens* populations and F_4 lines with contrasting tolerance and accumulation characteristics, using full genome Arabidopsis Agilent microarrays. The analyses were carried out on root as well as shoot material. Our attempt was to find candidate genes responsible for the intraspecific differences in Zn and Ni accumulation and Ni tolerance. The major results of the experiments described in the previous chapters are discussed in detail in chapter 5 and perspectives for future research are outlined.

Chapter 2

Intraspecific variation of nickel and zinc accumulation and tolerance in the hyperaccumulator *Thlaspi caerulescens*

Kerstin H. Richau & Henk Schat (2009). *Plant Soil* 314, 253-262.

Summary

Plants from two contrasting populations of the hyperaccumulator *Thlaspi caerulescens*, one from the serpentine area of Monte Prinzera (MP) in northern Italy and a Belgian calamine population, La Calamine (LC), were crossed to study the genetic correlation of Ni and Zn accumulation as well as Ni accumulation and Ni tolerance. Parental populations and F_3 and F_4 progeny of the interpopulation cross were phenotyped. The phenotype distributions for Zn and Ni accumulation of the parental populations were non-overlapping, with MP having higher foliar metal concentrations than LC. Ni tolerance was also higher in MP, but the parental distributions were overlapping. The F_3 and F_4 progeny exhibited a clear segregation for the Ni and Zn accumulation trait as well as for Ni tolerance. Variance and covariance analysis of the F_3 progeny demonstrated significant heritability values (h^2) for Ni and Zn foliar accumulation (0.70 and 0.59, respectively) and Ni tolerance (0.47), as well as a significant positive genetic correlation between the foliar accumulation of Ni and Zn (r_A^2 = 0.77). Ni tolerance and Ni accumulation were uncorrelated. Regressing the F_4 family means on the F_3 parent values yielded similar estimates for the heritabilities of Ni and Zn accumulation in the leaves (0.66 and 0.55, respectively).

Introduction

Heavy metal hyperaccumulation in higher plants is a rare phenomenon, represented by less than 0.2% of all angiosperm species (Baker et al., 2000). Hyperaccumulators (Brookes et al. 1977) are plants able to accumulate metal(s) in their foliage to concentrations of two to three orders of magnitude higher than in normal plants. Enhanced root metal uptake and enhanced root to shoot metal translocation, with a shoot to root metal concentration ratio exceeding unity, are the major characteristics of a hyperaccumulator (McGrath et al. 1993; Pollard et al., 2002). Most of the approximately 400 known hyperaccumulator species are Ni hyperaccumulators, all of which occur on serpentine soils, derived from ultramafic rocks, typically containing 0.1-1% of Ni. Around 15 species are known to hyperaccumulate Zn under natural conditions. Both groups include several members of the *Brassicaceae* family, like *Thlaspi* and *Alyssum* species. Some hyperaccumulators are facultative metallophytes, occurring both on metalliferous and non-metalliferous soil, and two of them, *Thlaspi caerulescens* and *Arabidopsis halleri* are more or less widely distributed throughout Europe (Escarré et al., 2000; Bert et al., 2002, Macnair, 2002). These species maintain high Zn concentrations in their leaves over a wide range of metal concentrations in the soil (Meerts and Van Isacker, 1997; Escarré et al., 2000). When growing in non-metalliferous soil, their foliar Zn concentrations are usually below the arbitrary threshold for hyperaccumulation, but still one order of magnitude higher than in normal plants, at least (Reeves et al., 2001). When grown under controlled conditions at the same Zn supply, non-metallicolous populations often appear to accumulate more Zn than metallicolous ones (Meerts and Van Isacker, 1997; Escarré et al., 2000; Schat et al., 2002), showing that these species' apparent ability to accumulate

extreme, normally lethal concentrations of Zn in their foliage is constitutive at the species level, although there is a significant variation in degree between populations (Meerts and Van Isacker, 1997; Escarré et al., 2000). Both *T. caerulescens* and *A. halleri* can also hyperaccumulate Cd, and serpentine populations of *T. caerulescens* hyperaccumulate Ni (Reeves and Brookes, 1983; McGrath et al., 1993; Brown et al., 1995). It is still a matter of debate whether these Cd and Ni hyperaccumulation abilities are also constitutive at the species level. Assunção et al (2003c) compared *T. caerulescens* populations from different soil types under controlled conditions and showed that some populations, on a total dry weight basis, did not accumulate more Ni or Cd than the non-hyperaccumulating congener *T. arvense*, whereas others hyperaccumulated either Cd or Ni, or both. However, all of the populations showed the strongly enhanced leaf to root metal concentration ratio, both for Ni and Cd, suggesting that enhanced root to shoot translocation of these metals, rather than the enhanced total accumulation as such, represents a constitutive species level trait in *T. caerulescens* (Assunção et al., 2003a,c). Several authors claim that hyperaccumulation is a metal tolerance strategy, and, consequently, that high levels of tolerance to the hyperaccumulated metals are constitutive in hyperaccumulators (Krämer et al., 1997). However, although it is obvious that hyperaccumulation presupposes tolerance to high leaf-internal concentrations of the naturally hyperaccumulated metals, this does not necessarily mean that hyperaccumulators would inherently tolerate high concentrations of these metals in the soil. For example, Pauwels et al. (2007), using root growth inhibition as a toxicity end point, found significantly higher Zn tolerance in *A. halleri* populations from Zn-toxic, calamine soils than in the non-metallicolous ones. Similarly, using a variety of toxicity end points, metallicolous *T. caerulescens*

populations have been shown to be more Zn tolerant than non-metallicolous ones (Assunção et al., 2001; Jiménez-Ambriz et al. 2007). Thus, although there might be a degree of constitutive tolerance, particularly in *A. halleri*, it is evident that calamine populations exhibit enhanced levels of Zn tolerance, compared to non-metallicolous populations. In general, as shown by Assunção et al. (2003c), such enhanced tolerances in metallicolous populations are confined to those metals that are toxically enriched at the population's sites, i.e. Cd and Zn at calamine sites and Ni at serpentine sites, just as in non-hyperaccumulator metallophytes (Schat and Vooijs, 1997). Among *T. caerulescens* populations there is a negative, rather than a positive phenotypic correlation between Zn accumulation and tolerance, as found by Assunção et al. (2003b) in a segregating intraspecific cross between metallicolous and non-metallicolous *T. caerulescens* accessions. This suggests that Zn hyperaccumulation, in so far as it segregates in intraspecific *T. caerulescens* crosses is not merely a Zn tolerance strategy. However, one might maintain that, in so far as Zn tolerance and Zn accumulation are constitutive at the species level, they may not segregate in an intraspecific cross, which would obscure any positive genetic correlation. Nevertheless, Macnair et al. (1999) found independent segregation of Zn tolerance and hyperaccumulation of the metal in an interspecific F_2 cross between the metal hyperaccumulator *A. halleri* and the non-hyperaccumulating, non-metallophyte congener *A. lyrata* ssp. *petraea*, which suggests hat the traits are under independent genetic control indeed.

With regard to Cd and Ni, however, the phenotypic correlation between accumulation and tolerance among *T. caerulescens* populations seem to differ from that of Zn. Calamine populations from southern France combine exceptionally high levels of

tolerance and Cd accumulation, both in the field and under controlled conditions (Lombi et al., 2000; Roosens et al., 2003; Zha et al., 2004). Likewise, serpentine populations combine enhanced levels of Ni accumulation and Ni tolerance (Schat et al., 2000a). To elucidate the genetic relationship of Ni hyperaccumulation and Ni tolerance in *T. caerulescens*, we investigated the co-segregation of these traits in F_3 families derived from a cross between a plant from a Belgian calamine population named La Calamine (LC), which is lacking Ni hyperaccumulation capacity and one from an Italian serpentine population, Monte Prinzera (MP), which exhibits high degrees of Ni hyperaccumulation and Ni tolerance (Assunção et al. 2003c). We also established the heritability values for Ni and Zn hyperaccumulation, as well as the genetic correlation between Zn and Ni foliar accumulation rates.

Materials and Methods

Plant origin and crossing scheme

A *Thlaspi caerulescens* J. and C. Presl plant grown from seeds collected at a strongly Pb/Cd/ Zn-enriched site near La Calamine, Belgium (LC) was crossed to a plant grown from seeds collected at Monte Prinzera, Italy (MP), an ultramafic site with a high soil Ni concentration. The cross was made by emasculating flower buds of the MP mother plant, followed by repeated hand pollination for three days. F_1 seeds were obtained and the seeds of randomly selected F_1 plants were collected after allowing them to self-pollinate. Two F_2 families, numbered 5 and 9, were sown out and 80 plants (61 F_2 (9) and 19 F_2 (5)) were allowed to self-pollinate, resulting in two sets of F_3 families called F_3 (9) and F_3 (5), respectively. Furthermore, phenotyped individuals (see below) of 42 F_3 families (27 F_3(9) and 15 F_3(5)) were randomly selected and allowed to self-pollinate, to investigate the heritability of Ni and Zn accumulation

through mid-offspring/parent regression.

Plant culture and vernalisation

Plants were grown from seeds sown on moist peat. Three week old seedlings were transferred to 1 liter polyethylene pots, filled with modified half-strength Hoagland's nutrient solution, containing 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1mM $NH_4H_2PO_4$, 0.5 μM $MgSO_4$, 1μM KCL, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA. After adding the pH buffer MES in a 2 mM concentration the pH was adjusted to 5.5, using KOH. Pots were randomised within the growth chamber and the nutrient solution was changed twice a week. The crossings and experiments were carried out in a climate chamber (20/15^{o}C day/night; 250 μmoles/m^2/s at plant level; 14 h/d; 75 % RH). Plants were vernalized at 4/4^{o}C day/night; 200 μmoles/m^2/s at plant level; 12h/d; +/- 60% RH for 5-6 weeks in a growth cabinet, while changing the nutrient solution once a week, and then returned to the climate chamber.

Ni and Zn accumulation

Three week old seedlings from the parent populations LC and MP (20 LC plants and 20 MP plants), from the F_3 families (1 to 5 plants per family) and from the F_4 families (5 plants per family) were grown in nutrient solution (1 seedling per pot) supplemented with 10 μM $NiSO_4$ (this concentration was found to yield the highest relative difference in Ni accumulation between LC and MP in previous experiments). The pots were randomised within the growth chamber and the nutrient solution was the same as during preculture and was replaced twice a week. No zinc was added in addition to the standard concentration in the Hoagland solution (2μM). After three

weeks of exposure even aged leaves were harvested (3 per plant). The material was dried overnight at 70^{o}C in a stove, followed by digestion in Teflon bombs in a 1:4 mixture of HNO_3 (65%) and HCL (37%) at 140^{o}C for 7 h. The metal compounds were analyzed by flame atomic absorption spectrometry (Perkin Elmer 1100B), and the Ni and Zn concentrations were calculated on a dry weight basis.

Ni tolerance

After harvesting the leaves for the determination of Ni and Zn accumulation, the F_3 plants, and those of the parental populations were additionally tested for Ni tolerance, by exposing them to weekly increasing $NiSO_4$ concentrations [100, 200, 500, 750, 1000 μM], over a time course of 5 weeks. At the end of each exposure step, the plants with visible chlorosis were registered and then returned to normal nutrient solution to check the reversibility of the chlorosis. In all cases the chlorosis appeared to be reversed, showing that the chlorosis was induced by excessive Ni exposure.

Statistics

Heritability values for Ni and Zn foliar accumulation and Ni tolerance were calculated from the F_3 data by means of variance partitioning, according to Falconer (1981). The genetic and environmental correlations between the Ni and Zn foliar accumulation rates were calculated from covariance partitioning (Falconer, 1981). The heritability values for Ni and Zn foliar accumulation were also calculated through regressing the mid-offspring (F_4) values on the parent (F_3) ones. The correlation between Ni accumulation and Ni tolerance was analysed by regressing the Ni accumulation rates on tolerance, as well as by a non-parametric 2x2 contingency test, using the median values as class borders.

Results

Ni and Zn accumulation

Foliar Ni accumulation was established in plants exposed to 10 μM $NiSO_4$ in hydroponics. The LC and MP populations exhibited non-overlapping phenotype distributions for Ni accumulation in leaves, with MP accumulating about 25 times more Ni than LC, on average (3.98 to 12.58, and 0.15 to 0.50 μmol/g DW, respectively) (Table 1 and Fig. 1).

Table 1: Ni and Zn concentration ranges in the foliage of the parental populations and their F_3 progeny.

	n	μg Ni g^{-1}DW	Median	μg Zn g^{-1}DW	Median
F_3(9)	133	27- 3733	707	313- 5892	1380
F_3(5)	139	112- 4393	912	822- 4857	1778
MP	20	1280- 3215	1819	3426- 9612	4786
LC	20	37- 108	79	503- 1654	1148

The Zn accumulation distributions of the parent populations were neither overlapping, with MP accumulating about 5 times more Zn than LC, on average (7.94 - 39.81 μmol Zn /g DW and 1.58 -7.94 μmol Zn /g DW, respectively) (Fig. 2). Zn and Ni accumulation clearly segregated in the F_3 population. Both for Ni and Zn accumulation, there was no significant transgression beyond the parental phenotype distributions. There were no significant differences in leaf Ni and Zn concentrations between F_3(5) and F_3(9) families (Fig. 3 and Fig. 4) and therefore both groups were analysed together. Analyses of variance showed significant variation between families

(P<0.001 and P<0.01 for Ni and Zn accumulation, respectively). Also the covariance of Ni and Zn accumulation differed significantly between families (P<0.001).

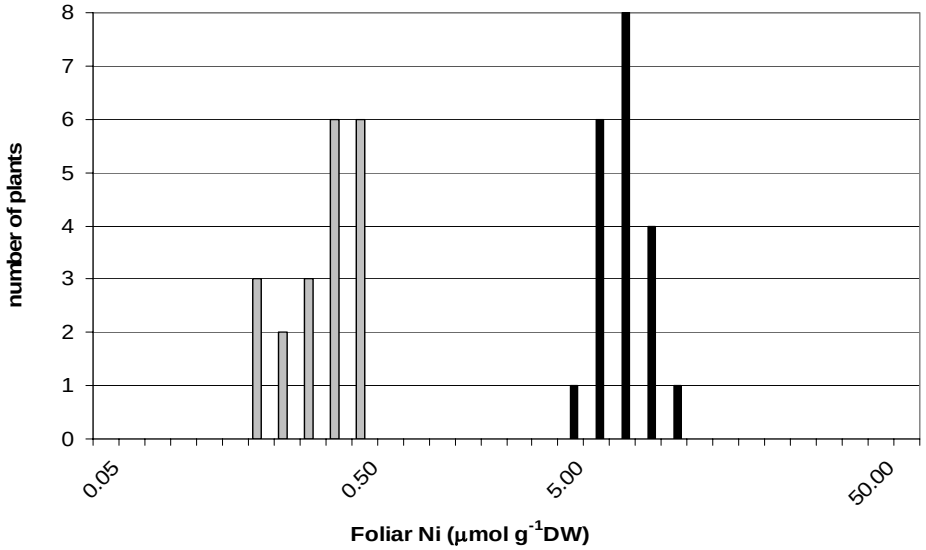

Figure 1: Frequency distribution over Ni accumulation classes (log scale) among individuals of the parental populations LC (grey bars) and MP (black bars) (n= 40).

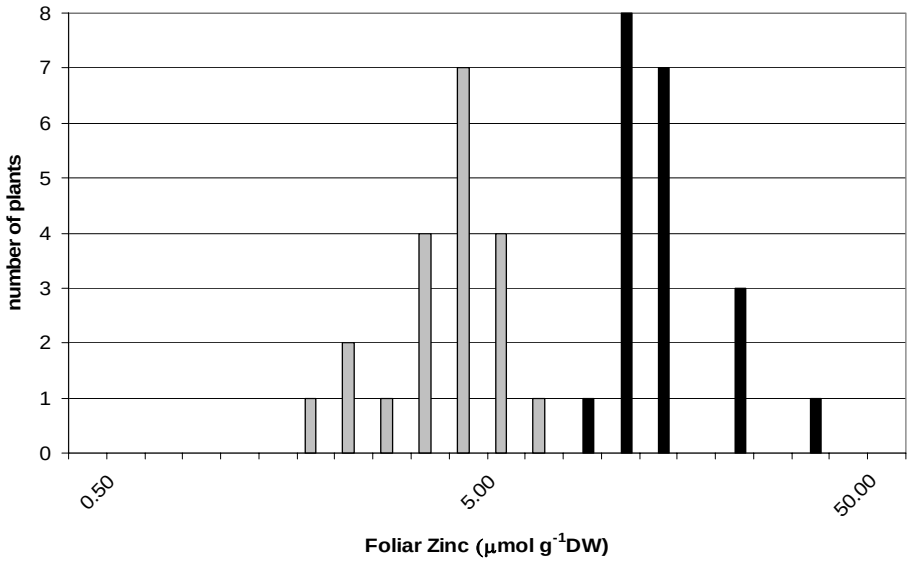

Figure 2: Frequency distribution over Zn accumulation classes (log scale) among individuals of the parental populations LC (grey bars) and MP (black bars) (n= 40).

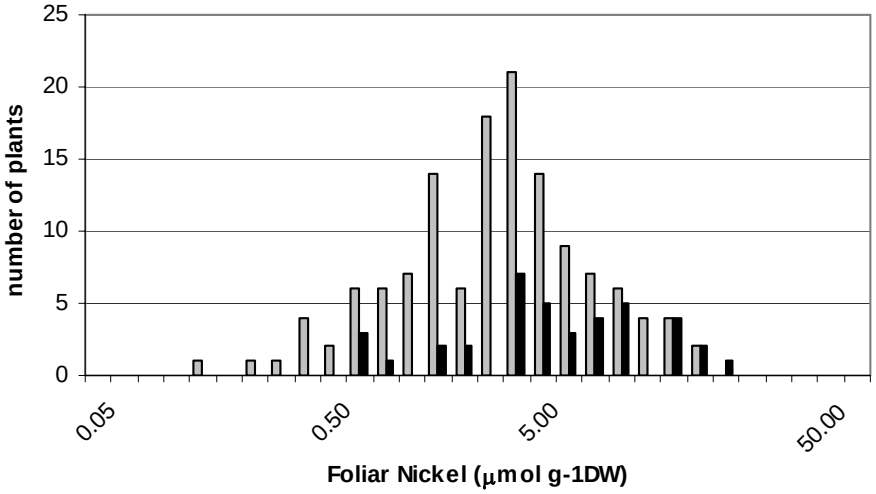

Figure 3: Frequency distribution over Ni accumulation classes (log scale) of individuals in F_3(9) (grey bars)(n= 133) and F_3(5) families (black bars) (n=39).

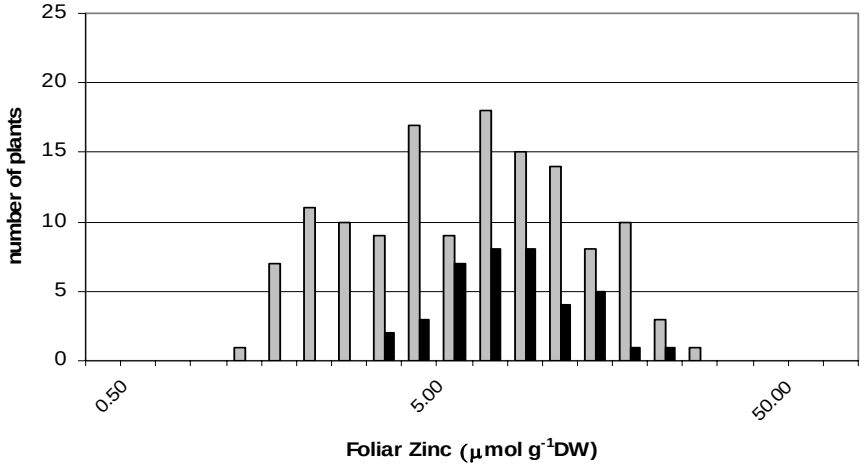

Figure 4: Frequency distribution over Zn accumulation classes (log scale) of individuals in F_3(9) (grey bars)(n= 133) and F_3(5) families (black bars) (n=39).

The Ni and Zn concentrations in the leaves of the parental populations were significantly correlated (r = 0.691 and r = 0.574 for MP and LC, respectively). The F_3 progeny showed a significant correlation between Ni and Zn accumulation (r = 0.652) (Fig. 5). Also the F_4 progeny exhibited a lower but still significant correlation between

Ni and Zn accumulation (r = 0. 49) (data not shown). Partitioning of the phenotypic correlation, based on the covariance and variance partitioning, yielded a highly significant positive genetic correlation (r_A^2 = 0.77) and an insignificant negative environmental correlation (r_E^2 = - 0.11).

From the variance partitioning of the F_3 data, the heritabilities of the Ni and Zn accumulation phenotypes were calculated as 0.70 and 0.59, respectively. Regressing the mid-offspring values of F_4 families on the corresponding F_3 parent values yielded very similar heritability estimates, 0.66 and 0.58 for Ni and Zn accumulation, respectively (P<0.01) (Fig. 6 and Fig. 7).

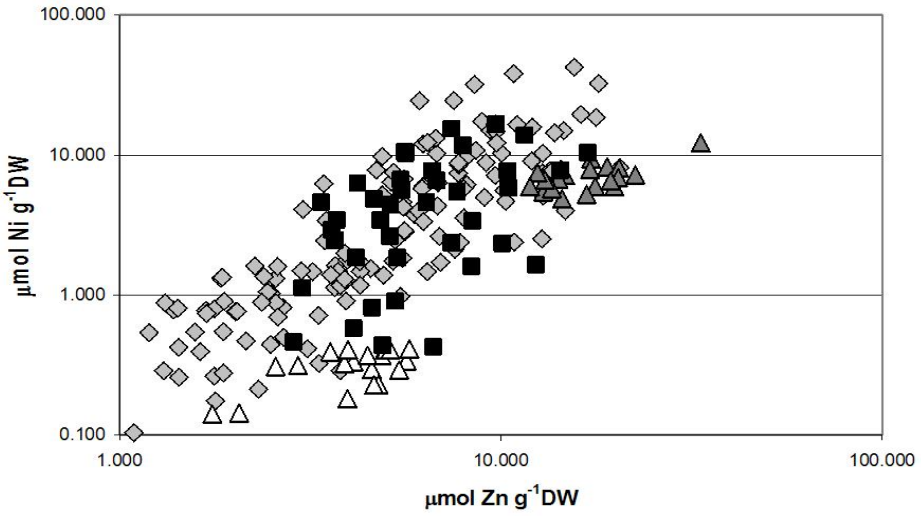

Figure 5: Correlation of Ni and Zn accumulation in the F_3(5), the F_3(9) set of families (black squares, r = 0.66 and grey squares, r = 0.51, respectively) (n=172) and the parental populations LC (white triangles, r = 0.57) and MP (grey triangles, r = 0.69).

Ni tolerance

After having been phenotyped for Ni and Zn accumulation, plants were exposed to weekly increasing $NiSO_4$ concentrations, and after each exposure step, the plants with

visible chlorosis were registered. Of the parental populations MP showed higher Ni tolerance than LC ($P<0.01$), on average, although the distributions were broadly overlapping (Table 2). There was a clear segregation of the tolerance trait in the F_3 progeny, and both family sets, $F_3(5)$ and $F_3(5)$, contained individuals that were in the lowest tolerance classes, which were not represented among MP parent populations, as well as in the highest tolerance class, which was not represented among the LC parent population.

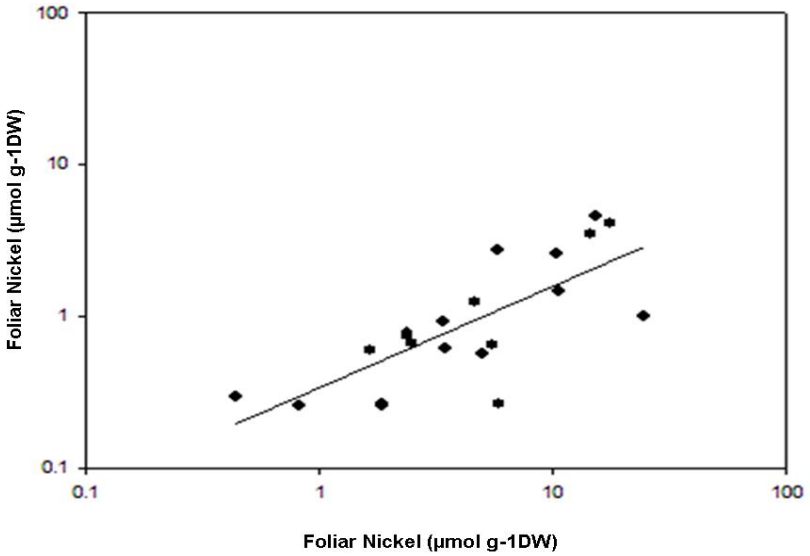

Figure 6: Mid-offspring/parent regression for Ni concentration in the leaves of F_3 and F_4 progeny ($h^2= 0.6668$) (n=20).

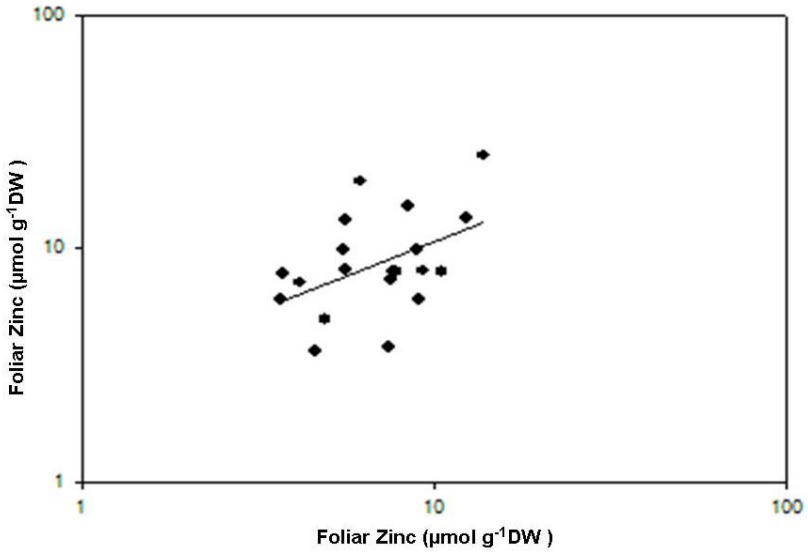

Figure 7: Mid-offspring/parent regression for Zn concentration in the leaves of F_3 and F_4 progeny ($h^2 = 0.5870$) (n=20).

Table 2: Numbers of Individuals in EC_{100} classes for Ni induced chlorosis for the parental populations LC and MP (n=70 per parent) and their F_3 progeny (n=172).

	EC_{100} for chlorosis µM Ni in nutrient solution					
	100	200	500	750	1000	>1000
MP				31	16	23
LC	3	2	7	52	6	
F_3(9)	8	3	8	26	54	36
F_3(5)	5		16	4	8	4

Analysis of variance demonstrated significant variation between families ($P<0.05$) and a heritability value of 0.49. However, there was no correlation between the

median Ni concentration in the leaves, measured after 3 weeks of growth at 10 μM Ni (see Material and Methods), and the tolerance to this metal (Fig. 8). Also, a 2x2 non-parametric-contingency test with the median tolerance and accumulation values as class borders did not yield any significant association of high accumulation and high tolerance.

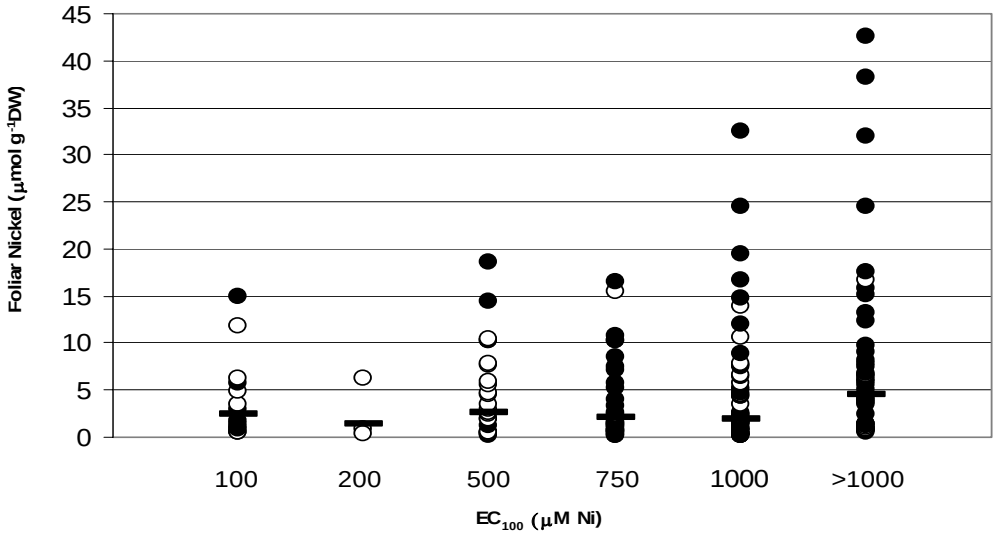

Figure 8: Ni accumulation and tolerance of the F_3 progeny (F_3(5) open circles and F_3(9) closed circles). Plants were grown five weeks in nutrient solution with weakly increasing Ni concentrations. EC_{100} values correspondent to week number of treatment. Black bars represent the median Ni accumulation.

Discussion

In agreement with a previous study (Assunção et al., 2003c), our results revealed a striking difference between the populations LC and MP with regard to their foliar Ni accumulation capacity. MP plants accumulated, on average, 25 times more Ni in their leaves than LC plants did. Zn accumulation was also higher in MP, but no more than 5-fold, on average. Both populations accumulated much more Zn than Ni, although Ni

was supplied at a 5-fold higher concentration (10 µM Ni versus 2 µM Zn).

The F_3 progeny of the intrapopulation cross showed a broad segregation for foliar Ni and Zn accumulation but significant transgression beyond the parent phenotypes was not found. Our heritability estimates, 0.70 and 0.59 for Ni and Zn foliar accumulation respectively, demonstrate that the phenotypic variation among the F_3 progeny was mainly attributable to genetic variation.

The segregation patterns in the F_3 progeny, both for Ni and Zn accumulation, were continuous rather than bimodal, with a large fraction of the individuals having Zn and Ni accumulation rates intermediate between those of the parental phenotypes. This may be taken to suggest that the difference in metal accumulation between LC and MP is controlled by more than one gene, and that high accumulation is only partially dominant over low accumulation. However, the heritabilities obtained in this study are too low to exclude the possibility of a single codominant gene.

In previous studies on different intraspecific *T. caerulescens* crosses, Assunção et al. (2003b,c, 2006), Zha et al. (2004), and Deniau et al. (2006) obtained evidence of polygenic control of the intraspecific variation in Zn and Cd accumulation, Zha et al. (2004) and Deniau et al. (2006) observed significant transgression for Zn accumulation and Assunção et al. (2006) and Deniau et al. (2006) found that both parents contributed trait enhancing alleles at different loci. The absence of significant transgression in the present study suggests that the trait-enhancing allele or alleles of the genes governing the segregation of Ni and Zn accumulation in the present study were all contributed by the MP parent.

The significant genetic correlation between Zn and Ni accumulation in the F_3 progeny suggests that the superior Ni and Zn accumulation capacities in MP, as compared to

LC, are mediated by a combined Zn/Ni accumulation system that is not expressed in LC, at least in part. In agreement with this, Assunção et al. (2001) showed that at equimolar Zn and Ni supply in the nutrient solution, Ni accumulation was strongly inhibited in MP, but not in LC. On the other hand, Zn accumulation was barely affected by equimolar Ni supply, both in MP and LC, suggesting that the Ni hyperaccumulation system in MP has a strong preference for Zn over Ni. Similar results have been obtained for other serpentine Ni-hyperaccumulating *Thlaspi* species (Taylor and Macnair, 2006). The 10-fold higher foliar concentration of Ni, as compared to that of Zn, found in MP plants growing in their natural habitat, can only be explained by the 50-fold excess of Ni over Zn in the soil at the Monte Prinzera site (Assunção et al., 2003c). Since LC shows far higher shoot to root concentration ratios for both Ni and Zn (Assunção et al., 2003c), it is likely that the higher foliar accumulation of both metals in MP is due to an enhanced capacity for uptake, rather than for root to shoot transport.

In agreement with Assunção et al. (2003a,c), MP was found to be more Ni tolerant than LC, on average. However, the phenotype distributions showed overlap, and were suggestive of intrapopulation variation, although the testing method could have played a role in addition. In any case, there was clearly heritable variation for Ni tolerance in the F_3 progeny. Furthermore, we did not find any significant correlation, nor association, between Ni tolerance and foliar Ni accumulation. It is remarkable however, that virtually all of the highly Ni accumulating plants are found among the more Ni tolerant ones, although the mean Ni accumulation level does not increase with tolerance (Fig. 6). This might be taken to suggest that high Ni tolerance may be based either on low accumulation or on a mechanism associated with enhanced rather

than decreased accumulation. However, the low plant numbers in the low tolerance classes make it difficult to draw any firm conclusion at this point. In different intraspecific *T. caerulescens* crosses there was neither consistent co-segregation of Zn tolerance and Zn accumulation, nor of Cd tolerance and Cd accumulation (Assunção et al., 2003b,c; Zha et al., 2004). Thus, in general, foliar metal hyperaccumulation in *T. caerulescens* doesn't seem to be a strategy for metal tolerance as such.

The evolutionary origin of Ni hyperaccumulation in *T. caerulescens* remains elusive. It is definitely not a constitutive trait, such as in many serpentine *Alyssum* species (Krämer et al., 1996, 2000). It is apparently a low-affinity phenomenon, since it is only possible where the soil Ni availability greatly exceeds that of Zn, i.e. in serpentine soil. In *T. caerulescens*, and possibly in other serpentine *Thlaspi* and *Alyssum* species, the trait could have been evolved through direct selection for Ni accumulation via some pre-existent Zn transporter. Alternatively, selection for Zn accumulation from serpentine soils, which are often poor in Zn, might have produced high degrees of inadvertent Ni accumulation, due to insufficient transporter specificity.

Present results show that Ni tolerance and Ni accumulation segregated independently and therefore we conlude that these traits are under independent genetic control in a *T. caerulescens* intraspecific cross. Furthermore, Ni and Zn accumulation were genetically correlated, confirming the hypothesis that Ni hyperaccumulation is accomplished through a Zn accumulation system.

Chapter 3

Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*

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Summary

The mechanisms of enhanced root to shoot metal transport in heavy metal hyperaccumulators are incompletely understood. We compared the distribution of Ni over root segments and tissues in the hyperaccumulator *Thlaspi caerulescens* and the non-hyperaccumulator *T. arvense*, and investigated the role of free histidine in Ni xylem loading and Ni transport across the tonoplast. We found Ni accumulation in mature cortical root cells in *T. arvense* and in a high-Ni-accumulating *T. caerulescens* accession, but not in a low-accumulating *T. caerulescens* accession. Compared to *T. arvense*, the concentration of free histidine in *T. caerulescens* was 10-fold enhanced in roots, but was only slightly higher in leaves, regardless of Ni exposure. Ni uptake in MgATP-energized root- and shoot-derived tonoplast vesicles was almost completely blocked in *T. caerulescens* when Ni was supplied as a 1:1 Ni-His complex, but was uninhibited in *T. arvense*. Exogenous histidine supply enhanced Ni xylem loading in *T. caerulescens* but not in *T. arvense*. The high rate of root to shoot translocation of Ni in *T. caerulescens* as compared to *T. arvense* seems to depend on the combination of two distinctive characters, i.e. a greatly enhanced root histidine concentration and a strongly decreased ability to accumulate histidine-bound Ni in root cell vacuoles.

Introduction

A minority of plant species accumulate one or more heavy metals in their foliage at concentrations that are one or two orders of magnitude higher than in normal plants growing in the same environment. These plants, called hyperaccumulators, are further characterized by shoot to root metal concentration ratios above unity, which is, for most metals, about an order of magnitude higher than in normal plants (Brooks et al., 1977; Baker and Walker, 1990; Baker et al., 2000). World-wide more than 400 species of hyperaccumulators have been identified thus far. The great majority of these species hyperaccumulate Ni and are endemic to ultramafic, serpentine soils (Reeves and Brookes, 1983). In the examples tested so far, these species appear to possess high levels of tolerance to Ni (Krämer et al., 1996; Assunção et al., 2003c).

The mechanisms responsible for metal hyperaccumulation and high-level metal tolerance have not been completely identified thus far. In general, these traits are thought to be attributable to altered patterns of transmembrane metal transport and of metal chelator synthesis (Clemens, 2001). Among the potential non-protein metal chelators, the amino acid histidine (His) has been suggested to be implicated in Ni hyperaccumulation in *Alyssum* species. In three hyperaccumulating species of *Alyssum*, Ni exposure induced a dose-dependent increase of His in the xylem sap, which was not found in the non-hyperaccumulating congeneric species *A. montanum* (Krämer et al., 1996). In addition, exogenously supplied His enhanced Ni tolerance and xylem loading of Ni in *A. montanum*, but not in *A. lesbiacum* (Krämer et al., 1996). Enhanced Ni and His concentrations in the xylem sap upon exogenous His supply were also observed in another non-hyperaccumulator, *Brassica juncea* (Kerkeb and Krämer, 2003). In *A. lesbiacum*, on the other hand, the Ni xylem loading was

constitutively high and unaffected by exogenously supplied His, which was attributed to a five-fold higher constitutive root His concentration in this species, as compared to *B. juncea* (Kerkeb and Krämer, 2003). In agreement with these results, Ingle et al. (2005) found an enhanced expression of the His biosynthetic pathway, particularly for the transcripts encoding the first enzyme of the pathway, ATP-phosphoribosyltransferase, in *A. lesbiacum* as compared to *A. montanum*. Persans et al. (1999), comparing the Ni hyperaccumulator *Thlaspi goesingense* and the non-hyperaccumulator *Thlaspi arvense*, also found much higher free His concentrations in the roots of the hyperaccumulator species.

The above suggests that the strongly enhanced rates of xylem loading of Ni found in Ni hyperaccumulators are achieved through a constitutively enhanced concentration of free His in the roots. On the other hand, Callahan et al. (2007) observed a Ni-induced His accumulation in the roots of a serpentine population of the Zn/Cd/Ni hyperaccumulator *Thlaspi caerulescens*, suggesting that Ni exposure might further increase the root His pool in some hyperaccumulator species or ecotypes. However, increasing the root His pool, as such, is not always sufficient to enhance Ni xylem loading in non-hyperaccumulators. ATP-phosphoribosyltransferase over-expressing *Arabidopsis thaliana* lines displayed increased free His concentrations in roots and shoots, as well as enhanced tolerance to Ni, but did not exhibit enhanced Ni concentrations in the xylem and the leaves, as compared to wild-type (Wycisk et al., 2004; Ingle et al., 2005). A possible explanation is that, on the one hand, *A. thaliana* and, on the other hand, *A. montanum* and *B. juncea*, have different abilities to retain the Ni-His complex in the root, e.g. through vacuolar sequestration.

Recently, Hanikenne et al. (2008) demonstrated that the Zn hyperaccumulation

phenotype in *Arabidopsis halleri* is dependent on enhanced expression of *HMA4*, a stelar 1b P-type ATPase involved in Zn xylem loading (Hussain et al., 2004). However, it cannot be excluded that reduced vacuolar retention in root cells plays an important subsidiary role. There are indications that hyperaccumulation is associated with decreased vacuolar metal sequestration in roots, in addition to enhanced uptake. Using 65Zn compartmental flux analysis, Lasat et al. (1998) found that the fraction of absorbed Zn that was stored in root vacuoles was approximately 2.4-fold higher in the non-hyperaccumulator, *Thlaspi arvense*, than in *T. caerulescens*. Efflux analysis revealed that Zn efflux from root cell vacuoles was almost 2-fold faster in the hyperaccumulator than in the non-hyperaccumulator. Based on these results, the authors concluded that the lower vacuolar retention of Zn in roots of the hyperaccumulator facilitates radial Zn movement across the root to the xylem, thus contributing to a higher Zn accumulation rate in the leaves (Lasat et al., 1998). More recently, Xing et al. (2008), using 109Cd compartmental flux analysis, compared Cd fluxes in roots of two *T. caerulescens* accessions with contrasting degrees of Cd root to shoot translocation and found that low translocation was associated with a 1.5-fold higher vacuolar Cd fraction in the roots, although the half-life times for vacuolar efflux were identical. Moreoever, they showed that Cd and Zn translocation rates were uncorrelated with *HMA4* expression levels, which seem to reinforce the potential importance of root vacuolar retention in root to shoot metal translocation.

In the present work we explore the relationships between root free His concentrations, tonoplast Ni transport, and root to shoot Ni translocation in various Ni hyperaccumulating and non-Ni-hyperaccumulating accessions of *T. caerulescens*. *T. arvense* was used as a non-hyperaccumulator reference species. First, to characterise

the variation in Ni accumulation capacity, root and shoot His and Ni concentrations were measured in Ni-exposed and non-Ni-exposed plants, and the distribution patterns of Ni over different root segments and tissues were established. Second, the effect of exogenous His supply on Ni and His concentrations in the xylem sap was studied. Third, to establish the effect of Ni speciation on vacuolar sequestration of Ni, Ni uptake was compared in MgATP-energized purified root- and shoot-derived tonoplast vesicles, with Ni supplied as Ni-citrate complex (1:1), Ni-His complex (1:1), or as a sulphate salt.

Materials and Methods

Plant material and growth conditions

Seeds were collected from four accessions of *Thlaspi caerulescens* J. and C. Presl originating from La Calamine (LC, Belgium), Lellingen (LE, Luxemburg), Saint Félix de Pallières (SF, France) and Monte Prinzera (MP, Italy), and one *Thlaspi arvense* L. accession at an uncontaminated site near Amsterdam (the Netherlands). LC and SF are both from calamine soil, strongly enriched in Pb, Cd and Zn (Assunção et al., 2003c; Roosens et al., 2003). MP and LE originate from strongly Ni-enriched serpentine soil and from non-metalliferous soil, respectively (Assunção et al., 2003c). Seeds were sown on moist peat and 2-week-old seedlings were transferred to 1-liter polyethylene pots (three seedlings per pot) filled with modified half-strength Hoagland's nutrient solution (Schat et al., 1996). Nutrient solutions were replaced twice per week by fresh ones. All the experiments were performed in a climate room (20/15oC day/night; 250 µmoles m-2 s-1 at plant level, 14 h d-1; 75% RH).

Experimental design

Experiment 1: To characterize the variation in Ni accumulation capacity among the

accessions, plants were grown in hydroponics for three weeks, of which the last two were with 10 µM $NiSO_4$ in the nutrient solution. This concentration proved to yield large relative differences among accessions in previous experiments (Assunção et al., 2003c). Prior to harvest, roots were desorbed in ice-cold $Pb(NO_3)_2$ (5 mM), for 30 min. Then roots and shoots were separated, frozen in liquid nitrogen, lyophilized and stored under vacuum until analysis.

Experiment 2: To assess the Ni distribution over root tips and mature root segments, plants were grown for six weeks in non-metal amended nutrient solution, and then exposed for 5 or 10 d to 5, 25, or 250 µM $NiSO_4$ (*T. arvense* to 5 or 25 µM). Then root systems were harvested after desorption (see above) and 1.5-mm lateral root tips were cut off with a razor blade, blotted dry, weighed and digested for Ni measurement. Intact root systems were processed in the same way.

Experiment 3: To compare the Ni distribution over root tissues, plants were grown for one week in non-metal-amended nutrient solution, and then exposed for six weeks to 25 or 250 µM $NiSO_4$ (*T. arvense* only to 25 µM). Transverse and longitudinal root sections were made with a razor blade. Root sections and segments were stained with dimethylglyoxime and studied under a light microscope.

Experiment 4: To assess the root and shoot His concentrations under different Ni exposure levels, plants were grown for four weeks in non-metal amended nutrient solution and then exposed for two weeks to 0, 25, or 250 µM $NiSO_4$ (*T. arvense* to 0 and 25 µM). Afterwards roots and shoots were separated, frozen in liquid nitrogen, lyophilized and stored under vacuum until analysis.

Experiment 5: To establish the effect of His on Ni tonoplast transport, 6-week-old plants grown in non-metal-amended nutrient solution were harvested. Tonoplast

vesicles were isolated from roots and shoots. Ni uptake was measured in MgATP-energized vesicles, with Ni supplied at 1mM, either as $NiSO_4$, Ni-citrate (1:1), or Ni-histidine (1:1).

Experiment 6: To assess the effect of exogenous His supply on Ni xylem loading, 6-week-old plants were placed for 4 h with their roots in 1 mM L-histidine in a 2 mM Mes/KOH buffer (pH 5.5), always 8 h before the onset of the dark period. A Mes/KOH-buffered 1 mM L-alanine solution and fresh nutrient solution were used as controls. After the pretreatment, the root systems were rinsed in demineralised water, and the leaf rosettes were cut off just below the lowest leaf. Then the root systems were transferred to a fresh nutrient solution amended with 25 or 250 µM $NiSO_4$. Root-pressure exudates were collected overnight in 2 ml vials connected with the excised stems via silicon tubing. The exudates were collected at 2 h after the onset of the light period, and frozen at –20^{o}C until analysis for His and Ni.

Analytical procedures

Ni measurements: Ni concentrations in plant digests or root-pressure exudates were measured using flame (Perkin Elmer 1100B, the Netherlands) or Graphite Furnace Atomic Absorption Spectrophotometry (Perkin Elmer 2100, the Netherlands). Fresh and lyophilized plant material samples (5 to 100 mg) were digested in 2 ml of a 4:1 mixture of HNO_3 (65%) and HCl (37%), in Teflon ® bombs at 140^{o}C for 7 h.

Histochemical staining of Ni: Ni in root sections for light microscopy (Olympus CX41, Japan) was visualized by immersing the sections for 5 min in a solution droplet composed of 1% (w/w) dimethylglyoxime, 1.5% (w/w) NaOH and 50µM borax ($Na_2B_4O_7.10H_2O$) in distilled water (pH 10.1) on a glass slide (Seregin et al., 2003). Microphotographs were made using a colour video camera (Altra20, Olympus,

Japan).

Amino acid measurements: Twenty mg of lyophilized plant material was ground in 2 ml double distilled water, using mortar, pestle and quartz sand. Prior to grinding, 10 µl of 100 µM γ-butyric acid was added as an internal standard. The extracts were centrifuged for 10 min at 10 000 g and the supernatants were filtered. Amino acids were derivatised by adding 10 µl of extract or xylem exudate to 70 µl of 0.2 M Na-borate buffer (pH 8.8) and 10 µl 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC) (Waters), according to the manufacturer's instructions. After vortexing and heating for 10 min at 55^{o}C in a water bath, 5 µl was injected onto a Novapak C18 (4 µm, 3.9 x 150 mm) HPLC column (Waters). Before injection the column was equilibrated with Na-acetate buffer (140 mM Na-Ac, 7 mM triethylamine, pH 5.8, set with 50% H_3PO_4) at a flow rate of 1 ml min^{-1}. Elution was done using a 50-min gradient of 0 to 15% (v/v) acetonitrile in Na-acetate buffer. Amino acid peaks were detected using a scanning fluorescence detector (Waters 474) at excitation and emission wavelengths of 250 and 395 nm, respectively. Peaks were identified by overlay with standard amino acid mixtures and confirmed by standard addition of pure histidine and alanine. Peak areas were integrated using Waters Millennium Software, after correction for recovery, based on the internal standard. Amino acid concentrations were calculated using calibration curves made with dilutions of histidine and alanine solutions.

Tonoplast vesicle isolation: Tonoplast vesicles were isolated from root and shoot materials according to Schumaker and Sze (1986), with minor modifications, as described in Verkleij et al. (1999).

Vesicle integrity, purity and ATPase-latency tests: Prior to the experiments, the

integrity of the vesicles was checked by monitoring acridine orange quenching upon ATP supply (SLM-AMICO-Bouman, series 2, Netherlands), as described by Verkleij et al. (1999). The ATPase activities of the vesicle preparations were measured according to McRae et al. (2002), using the spectrophotometric phosphate determination described by Chifflet et al. (1998), both with and without 0.03% (w/v) Triton-X-100, to establish the sidedness of the vesicles. The degrees of inhibition by KNO_3 (50 mM), Na_3VO_4 (100 µM) and NaN_3 (100 µM) were measured to estimate the contributions of vacuolar V-type, plasma membrane P-type, and mitochondrial ATPases, respectively.

Metal uptake assay: All uptake experiments were performed according to Verkleij et al. (1999) with slight modifications: vesicle suspension aliquots were incubated in 10 ml bathing medium containing 150 mM sorbitol, 200 mM BTP (pH 7.2, set with MES), 50 mM KCl and 2 mM ATP. A proton gradient was induced by addition of $MgSO_4$ at a final concentration of 4 mM. Four min after proton gradient induction, Ni was added to the bathing medium, either as $NiSO_4$, Ni-His (1:1), or Ni-citrate (1:1) complex, at a 1-mM concentration. After 100 sec of incubation with Ni, the vesicles were filtered over a nitrate-cellulose filter (Schleicher & Schuell, NC45). After eight washings with 5 ml sorbitol/BTP buffer, the residue was washed off with 2 ml of 0.1% TFA, and Ni concentrations in plant digests were measured using Graphite Furnace Atomic Absorption Spectrophotometry (Perkin Elmer 2100, the Netherlands). The values were corrected for aspecific binding of Ni to the vesicle membrane, established in a parallel incubation with vesicles that had been inactivated by heating for 5 min at 90^{o}C. Another parallel incubation was done without Mg and ATP.

Statistics: Results were statistically analysed using two-way ANOVA, if necessary after log-transformation of the data. The minimum significant range statistic (MSR) was used for a posteriori comparison of individual means.

Results

Ni accumulation

The Ni concentrations in roots and shoots, as well as in the total plant (amount of total plant Ni per unit of total plant dry weight) varied strongly among the *T. caerulescens* accessions under study, increasing in the order LC < SF < LE << MP (Table 1). Compared to the non-hyperaccumulator *T. arvense*, the whole plant Ni concentration was significantly lower in LC, but about 10-fold higher in MP. In the *T. caerulescens* accessions, the shoot to root Ni concentration ratios were always enhanced compared to *T. arvense*, and close to unity in all cases, demonstrating that variation in Ni uptake, rather than the Ni distribution over root and shoot, is the major determinant of the variation in the shoot and root Ni concentrations among the accessions. It is noteworthy that MP accumulated Ni to 3-fold higher concentrations in its roots than did *T. arvense* (Table 1).

Ni accumulation in root tips versus whole root systems

The Ni accumulation patterns in root tips, as compared with those in whole root systems, varied strongly between the *T. caerulescens* accessions (Fig. 1). In the accessions with the lowest Ni accumulation rates, LC and SF, Ni apparently accumulated more in the root tips than in mature root segments, except after 10 d of exposure to 250 µM Ni, whereas in MP, with the highest Ni accumulation rate, Ni accumulated equally in root tips and mature root segments (after 5 d), or much more in mature root segments (after 10 d). The Ni distribution in LE was intermediate

between LC/SF and MP. In *T. arvense*, there was no significant difference between the Ni concentrations in the root tips and the mature root segments, irrespective of the duration and level of Ni exposure (Fig. 1).

Table 1: Ni concentrations (µmol g^{-1} DW) in roots and shoots and whole plants (total plant Ni per unit of total plant biomass, as µmol g^{-1} DW) in different accessions of *T. caerulescens* and in *T. arvense* after two weeks of exposure to 10 µM Ni in the nutrient solution. Values are the means of 5 pots (3 plants per pot) ± SE; */**/*** = significantly different from *T. arvense* at α = 0.05, 0.01 and 0.001, respectively.

Accession/species	**roots**	**shoots**	**whole plants**
T. arvense	3.8 (0.36)	0.7 (0.10)	1.6 (0.18)
***T. caerulescens* LC**	0.7 (0.18)***	1.1 (0.12)	1.0 (0.11)*
***T. caerulescens* SF**	2.1 (0.21)*	2.1 (0.17)**	2.1 (0.22)
***T. caerulescens* LE**	3.8 (0.53)	3.4 (0.31)***	3.5 (0.42)*
***T. caerulescens* MP**	12.6 (2.41)***	15.0 (2.70)***	14.4 (1.88)***

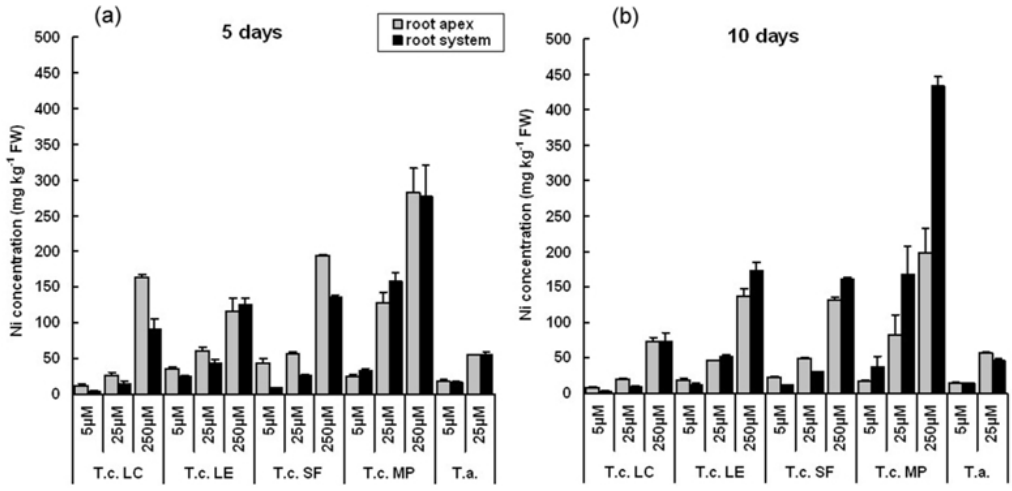

Fig. 1: Ni concentration (mg kg^{-1} FW) in root tips and whole root systems of *T. caerulescens* accessions and *T. arvense* after 5 (a) or 10 d (b) of exposure to 5, 25, or 250 µM Ni in the nutrient solution (means ± SE of 5 pots with 3 plants each).

Ni distribution over root tissues

The Ni distribution over root tissues was studied in the *T. caerulescens* accessions LC and MP and in *T. arvense*. In control plants of both species Ni was not histochemically detectable (Fig. 2a). In both *T. caerulescens* accessions Ni was also not detectable after 6 weeks of growth at 25 µM Ni (data not shown). At 250 µM Ni, the root tips were brightly stained with the red Ni-dimethylglyoxime precipitate, both in MP and LC, particularly the root cap and the rhizodermis of the meristematic zone (Fig. 2b,e,f). In MP roots the stain was also found within the elongation zone, particularly the rhizodermis and the cortex, which were not or barely stained in LC roots. In LC the mature root sections remained completely unstained, occasionally except for root hair-bearing rhizodermal cells (Fig. 2c,d). On the contrary, in MP the mature root sections were brightly stained. In the root hair zone the most intensive staining was observed in the rhizodermal and cortical cells (Fig. 2g), while in the basal root parts Ni was apparent mostly in the stelar tissues, but barely in the cortex and rhizodermis (Fig. 2h). The roots of *T. arvense* displayed a totally different staining pattern. At the 25-µM Ni exposure level the root cap was stained intensely, while the meristematic zone remained almost unstained (Fig. 2i). In the root hair zone the stain was observed mainly in the rhizodermis, cortex (the most intensive) and endodermis (Fig. 2j,k). In the basal root parts Ni was most abundant in the endodermis (Fig. 2l).

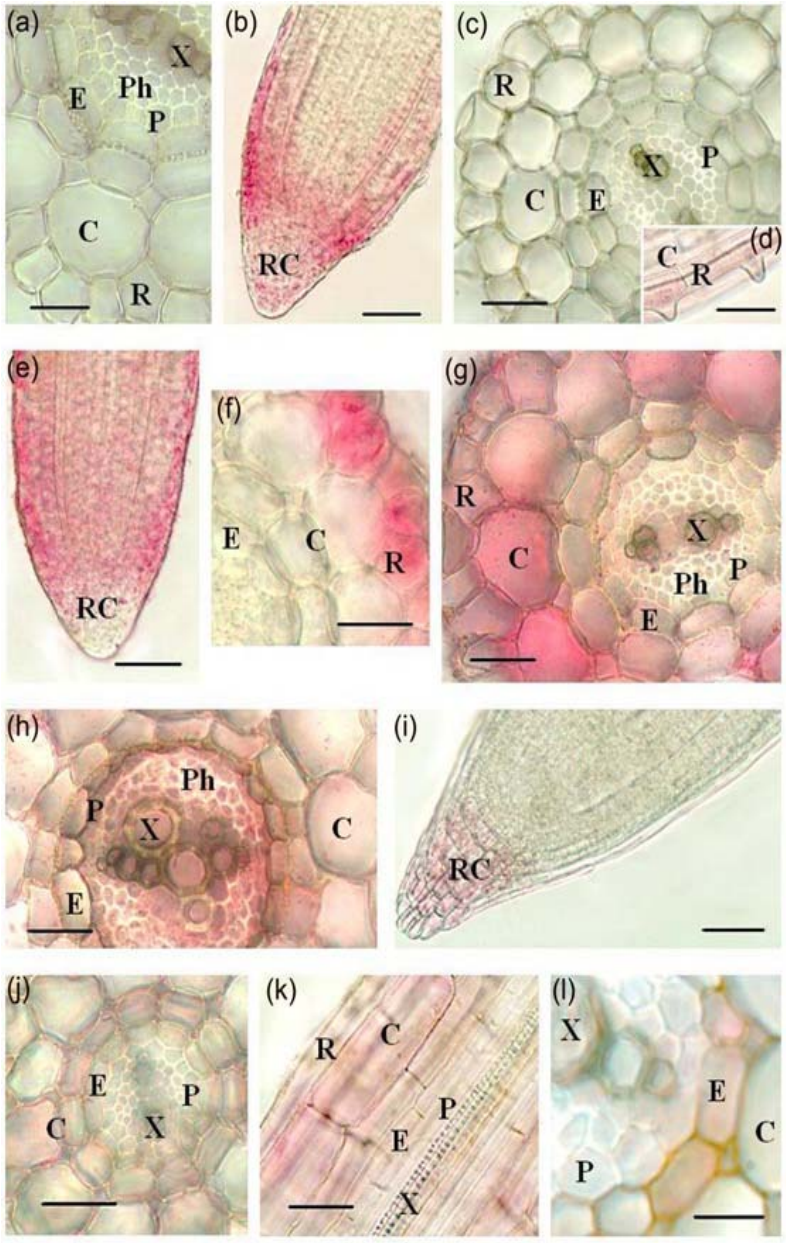

Fig. 2: Ni distribution over the root tissues in the *T. caerulescens* accessions LC (a-d) and MP (e-h), exposed to 250 µM Ni for 6 weeks (b-h) and *T. arvense*, exposed to 25µM Ni for 6 weeks (i-l). No visible staining with dimethylglyoxime was found in the roots of control plants (a). Apical part of the root (b, e, f, i),– root hair zone (a,c,d,g,j,k), basal part of the root (h,l). Each picture is a representative selection out of six plants per plant type per treatment.
C – cortex; E – endodermis; P – pericycle; Ph – phloem; R – rhizodermis; RC – root cap; X – xylem. Scale bars: (a,c,d,f,g,h,j,k) – 25 µm; (b,e,i) – 50 µm; (l) – 10 µm.

Histidine concentrations in roots and shoots

There was hardly any variation in the root and shoot His concentrations within *T. caerulescens*. In all the accessions the root His concentrations were 8- to 10-fold higher, and the shoot concentrations equal to, or only up to two-fold higher than those in *T. arvense*. In all the *T. caerulescens* accessions, as well as in *T. arvense*, Ni exposure did not significantly affect the root and shoot His concentrations (Fig. 3).

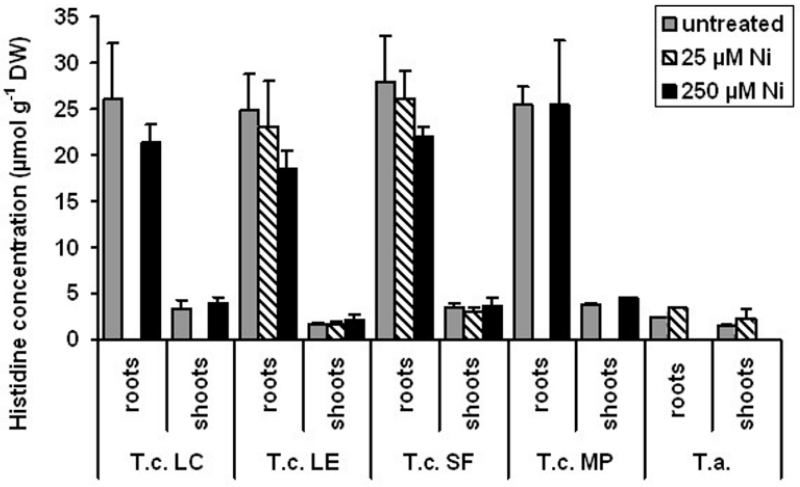

Fig. 3: Free His concentrations (µmol $^{g-1}$ DW) in roots and shoots of *T. caerulescens* accessions and in *T. arvense*, after 2 weeks of exposure to 0, 25 or 250 µM Ni in the nutrient solution (means ± SE of 3 pots with 3 plants each). Accessions LC and MP were only exposed to 0 and 250 µM Ni; *T. arvense* only to 0 and 25 µM Ni.

Tonoplast transport assays

Tonoplast transport assays were performed with root- and shoot-derived vesicles isolated from LC, MP and *T. arvense*. All the tonoplast vesicle preparations were capable of forming a proton gradient that remained stable for at least 40 min, as evidenced by acridine orange fluorescence quenching upon MgATP supply, indicating that the vesicles were sealed and transport-competent (data not shown).

Additions of 1 mM $NiSO_4$, Ni-His, or Ni-citrate were without any effect on the proton gradient of MgATP-energized vesicles, demonstrating that, even if a proton-linked Ni antiport system were active at the tonoplast (Ingle *et al.*, 2008), the capacity of the tonoplast ATPase was sufficient to maintain the proton gradient across this membrane. In the presence of 50 mM KNO_3 there was no detectable fluorescence quenching upon MgATP addition, indicating a complete inhibition of the proton gradient formation. The total ATPase activity varied between 8.4 and 12.5 µmol Pi mg^{-1} protein h^{-1} (average 11.4), without significant differences between species, accessions, or leaf- and root-derived vesicles. The latency varied between 58 and 36% (average 47%), and significant differences between *T. arvense* and one or both of the *T. caerulescens* populations were found for leaf-derived (58% in *T. arvense* versus 44% in MP and LC), as well as root-derived vesicles (36% in *T. arvense* versus 43 and 58% in LC and MP, respectively). The total ATPase activity was not significantly inhibited by Na_3VO_4 (-0.1 to 1.2%), or by NaN_3 (0 to 1.5%), but strongly by KNO_3, i.e. between 69 and 74% (average 72%), without significant differences between vesicle origins. These results suggest that all the vesicle preparations were only slightly contaminated with non-vacuolar membrane fractions.

Significant Ni uptake was found in all the vesicle preparations (Fig. 4), though only in presence of Mg and ATP (data not shown). Overall, there was no significant difference between the vesicles derived from LC and MP. In both accessions the leaf-derived vesicles took up significantly more Ni than did the root-derived ones, irrespective of whether Ni was supplied as Ni-citrate, Ni-His, or $NiSO_4$. In both accessions and in both root- and leaf-derived vesicles, Ni uptake was highest when Ni was supplied as Ni-citrate and lowest when Ni was supplied as Ni-His. In *T. arvense*,

on the other hand, root-derived vesicles took up significantly more Ni than did leaf-derived ones, irrespective of the Ni speciation in the bathing solution, and Ni uptake was highest when Ni was supplied as Ni-His, and lowest when Ni was supplied as Ni-citrate (Fig.4). Remarkably, when supplied as Ni-His, the Ni uptake in root-derived vesicles was about 7-fold higher in *T. arvense* than in *T. caerulescens* (Fig. 4a).

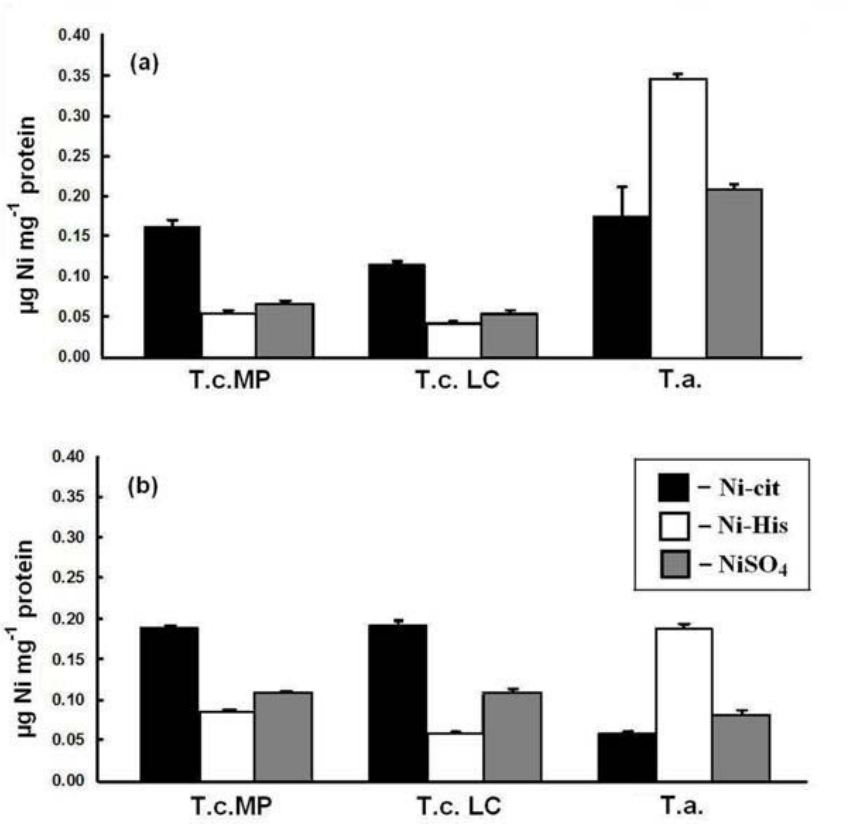

Fig. 4: Ni concentrations (µg g^{-1} protein) in MgATP-energized root-derived (a) and leaf-derived (b) tonoplast vesicles of *T. caerulescens*, accessions MP and LC, and *T. arvense* after 100 s incubation with 1 mM Ni-citrate (1:1), Ni-His (1:1), and $NiSO_4$ (means ± SE of 3 independent batches of plants). Values were corrected for aspecific binding through subtraction of the heat-denaturated controls.

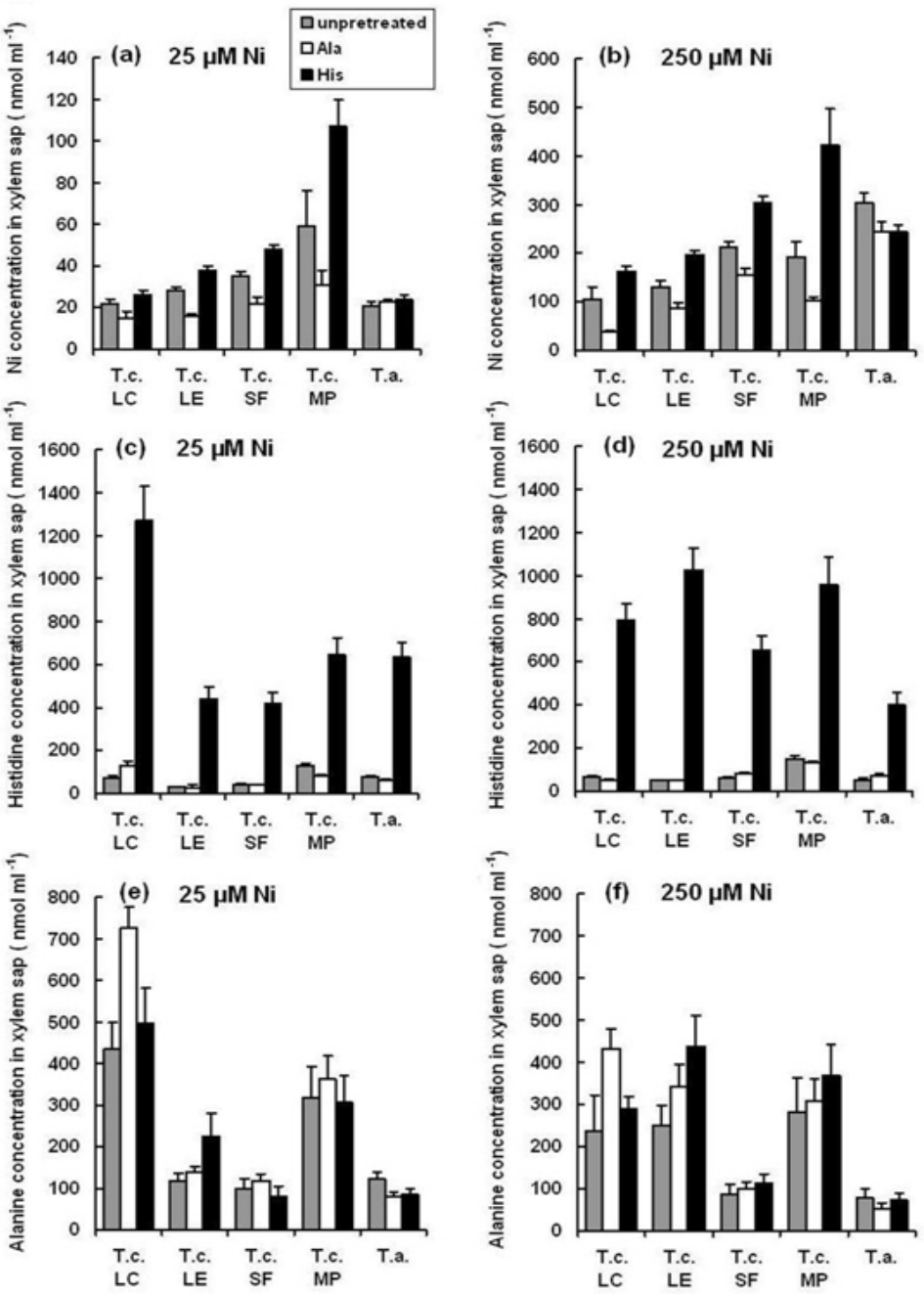

Fig. 5: Ni (a, b), histidine (c, d) and alanine (e,f) concentrations (nmol ml-1) in root-pressure exudates after pretreatment with 1 mM L-His, L-Ala, or control nutrient solution in different *T. caerulescens* accessions and *T. arvense*, at 25 (a, c, e) or 250 µM Ni (b, d, f) in the nutrient solution (means ± SE of 6 – 12 plants; one plant per pot).

Effects of exogenous His supply on Ni xylem loading

Exogenous His supply, prior to Ni exposure, significantly enhanced the Ni concentration in the root-pressure exudates, except for LC at the 25-µM Ni exposure level and *T. arvense* at both exposure levels (Fig. 5a,b). Remarkably, at both Ni exposure levels Ala supply significantly decreased Ni xylem loading in all the *T. caerulescens* accessions, compared to the nutrient solution control, but not in *T. arvense*. In all cases, there was no significant effect of the level of Ni exposure or exogenous amino acid supply on the exudation volumes, i.e. 0.5-1.5 ml in *T. caerulescens* and 1.5-2.5 ml in *T. arvense* (data not shown). In all the *T. caerulescens* accessions as well as in *T. arvense*, exogenous His supply strongly and comparably increased the His concentration in the xylem exudates (Fig. 5c,d), indicating a common capability to take up exogenously supplied His and load it into the xylem. On the other hand, the xylem Ala concentrations were barely or not at all increased upon exogenous Ala supply, except in LC, indicating either a generally lower uptake or lower xylem loading of this amino acid (Fig. 5e,f).

Discussion

Our results are in agreement with the previously reported broad variation in Ni accumulation capacity among *T. caerulescens* accessions (Assunção et al., 2001, 2003c, 2008; Richau and Schat, 2009a). They also confirm the hypothesis that enhanced Ni root to shoot transport, rather than enhanced Ni uptake, is constitutive at the species level in *T. caerulescens* (Assunção et al., 2003c, 2008). The hypothesis that the high rates of metal uptake in hyperaccumulators would be driven by enhanced xylem loading, inducing a deficiency response in the roots (Hanikenne et al., 2008), does not seem to apply to Ni hyperaccumulation in *T. caerulescens*, since at least MP

accumulates Ni to much higher concentrations in its roots than *T. arvense* (Table 1). MP also accumulates much higher concentrations of Zn in its roots than *T. arvense*, even at external Zn concentrations as low as 1 µM (Assunção et al., 2003c). This is not surprising in view of the strong genetic correlation between Zn and Ni accumulation in LC × MP crosses, which demonstrates that the combination of high Zn accumulation and high Ni accumulation in this accession is due to common genetic determinants (Richau and Schat, 2009a).

The variation in root Ni accumulation among the *T. caerulescens* accessions is associated with markedly different patterns of Ni distribution over root segments, varying from preferential accumulation in the root tips in the low-Ni-accumulating accession LC to preferential accumulation in mature root segments in the high-Ni-accumulating accession MP (Fig. 1). It is tempting to assume that this variation in Ni distribution is largely a consequence of the variation in the rate of Ni accumulation, because the relative differences in the Ni distribution patterns between the accessions tend to decrease with increasing Ni exposure level (Fig. 1), more or less in parallel with the relative differences in root Ni accumulation (Assunção et al., 2003c). In *T. arvense* the Ni distribution over root tips and mature root segments is approximately even, like in the moderately Ni-accumulating accession LE, with comparable root Ni concentrations (Fig. 1, Table 1).

At the tissue level there are also marked differences in the Ni distribution patterns, as revealed by the histochemical analyses. A remarkable feature in *T. caerulescens* is the accumulation of Ni to stainable concentrations in the meristematic zone of the root tip, which is not apparent in *T. arvense* (Fig. 2b,e,i). The functional significance of this phenomenon, if any, is as yet elusive. Another remarkable phenomenon is the

absence of Ni at stainable concentrations (LC), or the predominantly stelar localisation of Ni (MP) in the basal root parts of *T. caerulescens*, as compared to the prevalent occurrence of stainable Ni in the peripheral tissues, including the endodermis, of the mature root sections of *T. arvense* (Fig. 2). The differences among *T. caerulescens* accessions (hardly any staining in LC, and intensive staining in MP) are mainly concerned with the presence of stainable Ni the mature root parts. These differences seem to be related with the inter-accession differences in the rates of Ni accumulation.

Compared to *T. arvense*, all the *T. caerulescens* accessions under study exhibit strongly enhanced free His concentrations, though exclusively in the roots (Fig. 3). In our experiments the root His concentrations in *T. caerulescens* are neither affected by Ni exposure (Fig. 3) nor by Zn exposure (K. H. Richau, unpublished results), in contrast with results obtained by Callahan et al. (2007), who found Ni-inducible His accumulation in another (serpentine) *T. caerulescens* population. The reason for this discrepancy is not yet clear, but Ingle et al. (2005) observed that a Ni-induced increase in shoot histidine concentration in *Alyssum lesbiacum* was dependent on the duration of exposure of the plants to Ni. In any case, due to its high concentration and high affinity for metals, His is a very good candidate chelator for Ni and Zn in *T. caerulescens* roots. In fact, Salt et al. (1999), using X-ray absorption spectroscopy, demonstrated that Zn is largely co-ordinated with nitrogen, most probably with His, in *T. caerulescens* roots. In view of its high affinity for His, the same would be expected for Ni (Krämer et al., 1996).

The patterns of vacuolar Ni sequestration in *T. caerulescens* seem to be fundamentally different from those in *T. arvense* (Fig. 4). First, regardless of the Ni source in the

bathing solution, shoot-derived tonoplast vesicles accumulate more Ni than root-derived ones in *T. caerulescens*, whereas the reverse is found in *T. arvense*, in line with the organ-specific Ni allocation patterns in intact plants. It is evident that this difference is not quantitatively explained by the difference in the vesicles' sidedness between the species (see Results section). Our results are in full agreement with those obtained for Zn (Lasat et al., 1998). Second, the patterns of preference for the the different Ni sources are reversed, i.e. Ni-citrate >> $NiSO_4$ > Ni-His in *T. caerulescens* and Ni-His >> $NiSO_4$ > Ni-citrate in *T. arvense*, both in root- and shoot-derived vesicles, with the largest difference in the case of Ni-His. Although it is difficult to interpret the Ni source effects on the vesicular Ni uptake rates, due to the different complex stabilities and total Ni solubilities in the bathing solution, and due to the absence of direct evidence regarding the transport of Ni in complexed form, it is likely that the very large inter-specific differences in Ni uptake from the Ni-His source result from differential abilities to transport the Ni-His complex in undissociated form, particularly because in *T. caerulescens* vesicles Ni uptake is lower in the case of Ni-His supply, as compared to $NiSO_4$ supply. In any case, regardless of the details of the mechanisms, the transport assays strongly suggest that chelation of Ni by His in the cytoplasm will strongly inhibit vacuolar Ni accumulation in *T. caerulescens*, but not in *T. arvense*. This applies both to roots and leaves, but, in view of the large difference between the root and shoot His concentrations in *T. caerulescens*, it seems likely that His-imposed inhibition of vacuolar Ni sequestration *in planta* will be much stronger in roots than in leaves.

The positive effect of exogenous His supply on the Ni xylem concentrations in *T. caerulescens* suggests that in this species Ni xylem loading can be limited by the His

concentrations in the roots, depending on the Ni exposure level and the metal accumulation capacities of the accession in question. In this respect it is interesting that, at the 25-µM Ni exposure level, exogenous His does not significantly increase Ni xylem loading in LC, but most strongly does so in MP, i.e. the accessions with the lowest and highest rates of Ni accumulation, respectively (Fig. 5a). At the 250 µM exposure level, the effect of exogenous His supply tends to be overall stronger and becomes also significant in LC (Fig. 5b). These results suggest that Ni xylem loading in *T. caerulescens* is limited by the root His concentrations, both at 25 µM and 250 µM Ni in the nutrient solution, possibly except for LC at 25 µM Ni, where the endogenous His concentration may be sufficient for maximum Ni xylem loading. In *T. arvense*, on the other hand, there is no positive effect of exogenous His supply on Ni xylem loading, irrespective of the Ni exposure level. This is certainly not caused by an inability to take up exogenously supplied His and load it into the xylem (Fig. 5d) but, probably, by the absence of His-mediated inhibition of the vacuolar accumulation of Ni in root cells (see above). The absence of any positive effect of exogenous Ala on Ni xylem loading suggests that the His effect in *T. caerulescens* is not merely a general amino acid effect, but specific to His. Moreover, exogenously supplied Ala was barely recovered in the xylem exudates (Fig. 5e,f), suggesting that its uptake into the root might have been lower than in case of His, on average. On the other hand, compared to the normal nutrient solution control, exogenous Ala did significantly affect Ni xylem loading in *T. caerulescens*, but negatively, rather than positively (Fig. 5a,b). The reason for this is unknown yet.

It is remarkable that the *T. caerulescens* accessions are not different with regard to their root His concentrations. One might expect elevated concentrations in the

naturally Ni-hyperaccumulating accession MP at least, the more so because in this accession Ni translocation was most strongly enhanced by exogenous His. However, it is arguable that the Ni concentrations in natural serpentine soil solutions will generally be lower, rather than higher than the 25-µM level chosen in our experiment, suggesting that the constitutive root His level in MP may be sufficient to sustain maximum Ni translocation in the natural environment. This would also explain why natural selection seems to have acted on Ni uptake capacity, rather than on Ni translocation capacity.

In several respects, our results conflict with those obtained with *Alyssum montanum*, *A. lesbiacum* and *Brassica juncea* (Krämer et al., 1996; Kerkeb and Krämer, 2003). First, in the non-hyperaccumulators *A. montanum* and *B. juncea*, exogenous His supply enhanced Ni xylem loading, which is not apparent in *Thlaspi arvense* (Fig. 5a,b). It remains to be established whether this is due to interspecific variation in the capacity to sequester Ni-His in root cell vacuoles among non-hyperaccumulator species. However, it is notable that *Arabidopsis thaliana* plants with enhanced root histidine concentrations (produced by overexpression of ATP-phosphoribosyltransferase) do not show either elevated root Ni concentrations or increased Ni transport into the xylem (Ingle et al., 2005), suggesting the existence of important interspecific differences in transmembrane Ni transport. Second, in connection with this, the loading of His and Ni into the xylem seem to be completely independent in *T. arvense* (Fig. 5a-d), in contrast to the observations made in *B. juncea* (Kerkeb and Krämer, 2003). Third, significantly enhanced Ni xylem loading upon exogenous His supply was not apparent in *A. lesbiacum* (Krämer et al., 1996), but it is in *T. caerulescens* (Fig. 5a,b). It is conceivable that this discrepancy may

result from differences in Ni uptake rates and constitutive root His concentrations (see above).

In conclusion, based on the combination of the results of all the experiments, it is strongly suggested that the high constitutive root His concentration in *T. caerulescens* counteracts the vacuolar storage of Ni in mature peripheral root tissues, which seems to be essential for the high rates of metal xylem loading, compared to non-hyperaccumulator species. Compared with *T. arvense*, it seems that two major evolutionary events have contributed to the evolution of His-mediated metal xylem loading in *T. caerulescens*: first, a loss of the ability to sequester His-chelated metals in the vacuole and, second, a strong enhancement of the root His concentration. The molecular mechanisms underlying these phenomena are as yet far from understood. Transcriptomics studies in *T. caerulescens* (Hammond et al., 2006; van de Mortel et al., 2006) did not reveal enhanced expression of the histidine synthetic pathway, such as found in *A. lesbiacum* (Ingle et al., 2005), and vacuolar transporters responsible for the transport of His-bound Ni have not yet been identified.

Chapter 4

Microarray analysis as a tool to find candidate genes for metal tolerance and accumulation in hyperaccumulator plants: transcriptomic comparison of two contrasting *Thlaspi caerulescens* accessions and of selected F_4 offspring of an interaccession cross

Abstract

The aim of this study was to establish candidate genes responsible for contrasting accumulation and tolerance phenotypes among accessions of the hyperaccumulator, *Thlaspi caerulescens*. Therefore, using the Agilent3 full genome Arabidopsis array, we compared the transcriptional profiles of a calamine (LC) and a serpentine (MP) accession, with low and high nickel and zinc accumulation and low and high nickel tolerance, respectively. We also compared pooled F_4 offspring of a LC x MP interaccession cross, selected for contrasting nickel and zinc accumulation and nickel tolerance capacities. Genes thought to be somehow involved in metal homeostasis, e.g. those encoding transmembrane metal transporters or genes involved in metal chelator synthesis, were considered to be candidates. However, we did not find any metal homeostasis-related genes that were more than three-fold differentially expressed between the accessions, nor between the F_4 lines with contrasting accumulation or tolerance phenotypes. In particular, comparisons between contrasting F_4 lines barely revealed any differentially expressed genes. In this respect, our results are strikingly different from those reported for cross-species comparisons between hyperaccumulators and congeneric non-hyperaccumulators, where metal homeostasis-related genes were strongly over-represented among the more then 3-fold differentially expressed genes. We conclude that the high expression level of these genes is constitutive at the species level in *Thlaspi caerulescens*, at least largely. The contrasting accumulation and tolerance phenotypes among accessions are not brought about by comparably strong expression differences of metal homeostatic genes, but rather by more subtle gene expression differences, or by structural variation or differential post-translational regulation of proteins.

Introduction

Plants capable of accumulating exceptionally high foliar concentrations of metals such as Ni, Zn, Cd, and Pb have been termed hyperaccumulators (Brooks et al., 1977). Hyperaccumulators are characterized by enhanced rates of metal uptake and translocation to the shoot, typically resulting in leaf to root metal concentration ratios above unity, as well as an increased ability to render these metals harmless in their leaves. Hyperaccumulation of metals under natural conditions has been reported in more than 400 plant species (Baker et al., 1989), the majority of which are Ni hyperaccumulators. About 15 species have been reported to hyperaccumulate Zn and 4 of these can hyperaccumulate Cd in addition, among which *Arabidopsis halleri* and *Thlaspi caerulescens*. In contrast to most of the Ni hyperaccumulators, which are endemic to serpentine soil, both *A. halleri* and *T. caerulescens* are facultative metallophytes, occurring on metalliferous as well as non-metalliferous soils (Baker et al. 1989; Escarré et al., 2000; Bert et al., 2002; Macnair, 2002). Serpentine accessions of *T. caerulescens* also hyperaccumulate Ni in their natural environment (Brooks et al., 1977; Assunção et al., 2003c).

Former studies on different *A. halleri* and *T. caerulescens* populations consistently showed high foliar Zn concentrations, regardless of the soil type at the population site, demonstrating that Zn hyperaccumulation is a constitutive trait in these species (Meerts and Isacker 1997; Bert et al., 2002; Macnair, 2002). However, Ni and Cd hyperaccumulation are confined to specific local accessions, at least when considering the rate of root metal uptake, which may even be lower in some *T. caerulescens* accessions than in the non-hyperaccumulator congener species, *T. arvense*. However, enhanced Ni and Cd translocation to the shoot seems to be constitutive at the species

level in *T. caerulescens* (Assunção et al., 2003a,c). *A. halleri* is not known to hyperaccumulate Ni, and shows a strong variation in Cd accumulation between accessions. The shoot to root Cd concentration ratios in this species is usually far below unity, which in fact casts doubt on the Cd hyperaccumulator status of this species (Bert et al., 2002). In *A. halleri* the Zn tolerance levels of non-metallicolous accessions were lower than those of metallicolous populations, on average, but much higher than normally reported for non-metallicolous plants (Pauwels et al., 2006). This also seems to apply to *T. caerulescens* (Schat et al., 2000; Assunção et al., 2000c), suggesting that both species are relatively Zn-tolerant at the species level, but that further adaptation to calamine soil has repeatedly occurred at a local scale (Pauwels et al., 2006). As a species, *T. caerulescens* seems to be relatively tolerant to Ni too, but there is significant variation between accessions (Schat et al., 2000; Assunção et al., 2003c). Cd tolerance, on the other hand, is apparently not constitutive at the species level, since some *T. caerulescens* accessions can be more sensitive than *T. arvense* (Assunção et al., 2003c). In general, metallicolous accessions seem to exhibit enhanced tolerance, compared to non-metallicolous accessions, but exclusively to the metals that are present at toxic concentrations in the soil at the population sites (Assunção et al., 2003c).

Hyperaccumulation is believed to result mainly from altered patterns of expression of component traits of the plant metal homeostatic network (Clemens, 2001). Therefore many investigations were carried out to identify metal chelators and metal transporter proteins with differential expression between hyperaccumulators and related non-hyperaccumulators, using cross-species microarraying. Transcriptome comparisons have been made between *Arabidopsis thaliana* and *T. caerulescens* (van de Mortel et

al., 2006, 2008), *T. arvense* and *T. caerulescens* (Hammond et al., 2006), *A. thaliana* and *A. halleri* (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006), and between selected lines from an *A. halleri* x *A. lyrata* cross (Filatov et al., 2006). These studies have identified a core set of genes with a more or less consistently enhanced expression in the hyperaccumulators and, therefore, with a putative function in hyperaccumulation. Among these are genes encoding plasmamembrane transporters responsible for metal uptake into cells (*ZIP*s/*IRT*s), genes involved in vacuolar metal sequestration (*MTP*s, *CAX*2, *HMA*3), metal remobilization from the vacuole (*NRAMP*s), xylem loading and metal ligand transport (*HMA4*, *YSL*s, *FRD*3), and metal ligand synthesis (*NAS*s) (Verbruggen et al., 2009). Of these genes *HMA4* has been shown to be essential for Zn hyperaccumulation and Zn and Cd hypertolerance in *A. halleri*, by means of RNAi-mediated silencing (Hanikenne et al., 2008). Moreover *HMA4* co-localized with a major QTL for both Cd and Zn tolerance, and two copies of *MTP1* co-localized with two QTLs for Zn tolerance in a backcross derived from an inter-specific cross between *A. halleri* and its non-accumulating, non-tolerant congener, *A. lyrata* (Willems et al., 2007; Courbot et al., 2007). However, all these studies dealt with interspecific comparisons, or interspecific crosses, and the extent to which these genes are also responsible for the intraspecific variation among hyperaccumulator accessions remains to be established.

The aim of the present study was to address the question whether the pronounced intraspecific variation in metal accumulation and tolerance between *T. caerulescen*s accessions is due to expression variation of the candidate genes identified in inter-specific comparisons, or to others. To this end we compared the transcriptomes of two contrasting accessions of *Thlaspi caerulescens*, as well as contrasting F_4 progenies,

selected for low and high degrees of Ni and Zn accumulation and Ni tolerance. The Monte Prinzera (MP) population originates from a Ni-enriched serpentine soil in northern Italy and is known to hyperaccumulate Ni and to be more tolerant towards that metal (Richau and Schat, 2009a). La Calamine (LC) is an accession from calamine soil, which does not hyperaccumulate Ni, although it does show the enhanced root to shoot Ni translocation rate, typical of a hyperaccumulator (Assunção et al., 2003).

Materials and Methods

Plant origin and crossing scheme

Plants were grown from seeds collected from two *Thlaspi caerulescens* J. and C. Presl accessions, one originating from a strongly Pb/Cd/ Zn-enriched site in Belgium called La Calamine (LC), and the other from the area of Monte Prinzera, Italy (MP), an ultramafic site with a high soil Ni concentration. A cross was made by emasculating flower buds of the MP mother plant, followed by repeated hand pollination for three days. F_1 seeds were obtained and the seeds of randomly selected F_1 plants were collected after allowing them to self-pollinate. Two F_2 families, numbered 5 and 9, were sown out and about 80 plants (61 F_2 (9) and 19 F_2 (5)) were allowed to self-pollinate, resulting in two sets of F_3 families called F_3 (9) and F_3 (5), respectively. Furthermore, 20 randomly selected LC and MP plants (10 of each accession) as well as 42 F_3 individuals were selected according to their phenotypes for Ni tolerance or Ni and Zn accumulation, and allowed to self-pollinate to establish F_4 progeny for gene expression analysis.

Plant culture and vernalisation

Plants were grown from seeds sown on moist peat before transferring three week old seedlings to 1 liter polyethylene pots (3 seedlings per pot), filled with modified half-strength Hoagland's nutrient solution, containing 3 mM KNO_3, 2 mM Ca $(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 μM $MgSO_4$, 1 μM KCL, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA. After adding the pH buffer MES at a 2 mM concentration the pH was adjusted to 5.5, using KOH. The nutrient solution of the randomly arranged pots was changed twice a week and all crossings and experiments were carried out in a climate chamber (20/15^{o}C day/night; 250 μmoles/m^2/s at plant level; 14 h/d; 75 % RH). Plants were vernalised at 4/4^{o}C day/night; 200 μmoles/m^2/s at plant level; 12h/d; +/- 60% RH for 5-6 weeks in a growth cabinet, while changing the nutrient solution once a week, and then returned to the climate chamber.

Ni and Zn accumulation

Three week old seedlings of the parental populations LC and MP and the F_3 families (1 to 5 plants per family) were grown in nutrient solution (1 seedling per pot) supplemented with 10 μM $NiSO_4$ (this concentration was found to yield the highest relative difference in Ni accumulation between LC and MP in previous experiments). The nutrient solution was the same as during preculture and was replaced twice a week. No zinc was added in addition to the standard concentration in the Hoagland solution (2 μM). After three weeks of exposure three full-grown leaves of comparable age were harvested per plant and dried overnight at 70^{o}C in a stove. About 100 mg of root or leaf the material was digested in Teflon bombs in a 1:4 mixture of HNO_3

(65%) and HCL (37%) at 140^{o}C for 7 h. The metal compounds were analyzed by flame atomic absorption spectrometry (Perkin Elmer 1100B), and the Ni and Zn concentrations were calculated on a dry weight basis. The extremes in accumulation were used to establish a F_4 progeny by selfing. F_4 plants were phenotyped in the same way. Immediately after three weeks of exposure to 10 µM Ni about half of the root system and three leaves per plant were frozen in liquid nitrogen and stored at -80 ^{o}C until selection for microarraying.

Ni tolerance assessment

After harvesting the leaves for the determination of Ni and Zn accumulation the F_3 plants and those of the parental populations were additionally tested for Ni tolerance, by exposing them to weekly increasing $NiSO_4$ concentrations [100, 200, 500, 750, 1000 µM], over a time course of 5 weeks. At the end of each exposure step, the plants with visible chlorosis were registered and then returned to normal nutrient solution to check the reversibility of the chlorosis. In all cases the chlorosis appeared to be reversed, showing that the chlorosis was induced by excessive Ni exposure. Phenotype extremes in Ni tolerance were allowed to self pollinate to establish a F_4 progeny. F_4 plants were phenotyped for tolerance in the same way. After the first step in the tolerance test, i.e. the 100-µM step, three leaves per plant were harvested, frozen in liquid nitrogen and stored at -80 ^{o}C until selection for microarraying.

Microarray analysis of Ni tolerance and Zn/ Ni accumulation

Within both experiments, we used a so-called common reference model (Yang and Speed, 2002), with LC in the tolerance arrays experiments and the low Ni/low Zn accumulating phenotype group as common reference in the accumulation arrays. The common references were hybridized onto every slide and labelled with the

fluorescence dye Cyanine 5 (Cye 5), whereas the other samples were labelled with Cyanine 3 (Cye 3).

Leaf material of parental plants as well as F_4 plants were homogenized in liquid nitrogen and total RNA of approximately 100 mg leaf tissue was extracted with Trizol (Invitrogen, Carlsbad, Ca, USA). After extraction, total RNA was purified, using the RNeasy kit (Qiagen Benelux B.V., Venlo, The Netherlands). For the hybridization 3000 ng of labelled RNA was used on an Arabidopsis3 60-mer oligonucleotide microarray (Agilent Technologies Inc. Palo Alto, Ca, USA). The Agilent3 oligo microarray contains approximately 40.000 probes representing more than 27.000 annotated genes and more than 10.000 non-annotated genes, almost representing the complete *Arabidopsis* transcriptome.

F_4 plants were selected and pooled according to their Ni and Zn accumulation phenotypes. These groups are further referred to as high Ni/high Zn, high Ni/low Zn, low Ni/high Zn, low Ni/low Zn (Fig. 1). Each group was represented by three replicates obtained by randomly pooling the individual plants. One sample of the low Ni/low Zn group was used as a common reference in these arrays. For the comparison between high and low Ni tolerance, F_4 plants that became chlorotic at 100 µM Ni and plants that remained green at 1000 µM Ni were selected and pooled to three replicate samples per group.

After hybridization the slides were scanned, analyzed and normalized for spot intensity with the Rosetta Luminator software (Rosetta Biosoftware, Seattle, USA) by Service XS (Leiden, the Netherlands). Moderate t-statistics were applied to the normalized data, using the limma package (Smyth, 2005), available from R/BioConductor (Gentleman, 2004). To assure comparability of our results with other

published (cross-species) analyses between hyperaccumulator and non-hyperaccumulator congeners we applied two criteria for differential expression, (1) significance at $P < 0.05$, and (2) a 3-fold difference threshold. We also classified the differentially expressed genes according to function and the pathways in which they function, using the BABELOMICS FatiGO-program (Al-Shahrour et al. 2006) and performed hierarchical clustering, using Cluster/Treeview (Eisen et al., 1998).

Results

Ni tolerance

After normalization and applying the customized criteria of (1) fold difference in expression ≥ 3 (between groups or accessions) and (2) $P< 0.05$, we found 47 and 24 genes that were higher or lower expressed in the leaves of MP than in those of LC, respectively. Of these genes, only two and one were also more than 3-fold higher or lower expressed in highly tolerant F_4 than in LC (Table. 1). The comparison between the low and the high tolerant F_4 showed just 21 genes to be differentially expressed (Fig 1). None of these were also more than 3 fold differentially expressed between MP and LC within the accumulation array data.

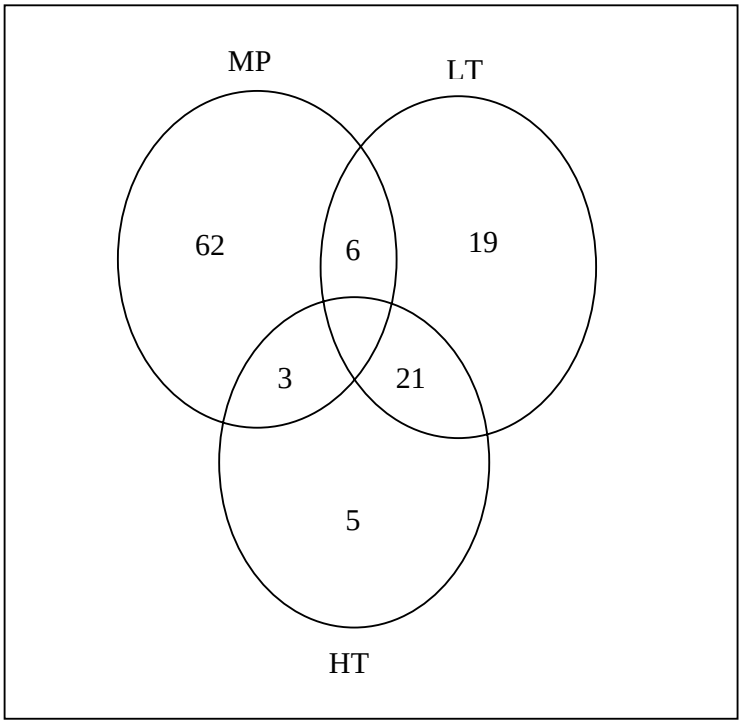

Figure 1: Genes differentially expressed in *T. caerulescens* leaves of selected tolerance groups (Monte Prinzera (MP), low tolerant (LT), high tolerant (HT) after exposure to 100 µM Ni SO_4. La calamine (LC) acted as common reference.

Table 1: Differentially expressed genes in MP, the high tolerant (HT) and low tolerant (LT) group of the F_4 offspring in comparison to LC as common reference. Plants were treated for one week with 100µM $NiSO_4$. Genes were selected after applying customized criteria: (1) minimum of > 3 fold change in expression (between groups or accessions) and (2) a P-value < 0.05.

up / down	LC	MP	LT	MT
LC		47	21	18
MP	24		5	2
LT	25	1		14
MT	11	1	7	

We checked the biological function of all the genes that were more than 3-fold

differentially expressed in MP leaves versus LC, in total 62, on the basis of their GO annotations (The Gene Ontology Consortium, 2000) (Fig. 2) and hence of their involvement in certain biological pathways (Al-Shahrour et al., 2006). None of the more than 3-fold differentially expressed genes seem to be directly or indirectly involved in heavy metal homeostasis, as to our knowledge.

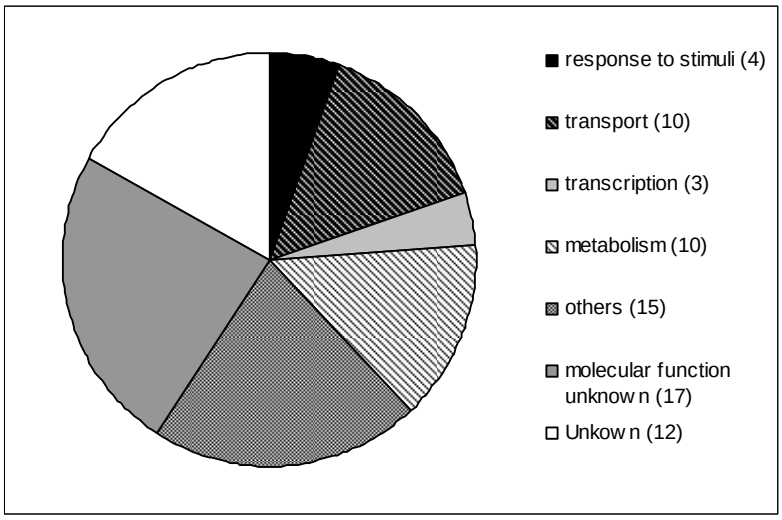

Figure 2: Biological functions of genes differentially up- and down-regulated in leaves of MP exposed to 100µM Ni in comparison to LC. Functions according to GO annotations.

Finally we performed hierarchical clustering according to Eisen et al. (1998) to evaluate the similarity in gene expression between the F_4 samples and the LC and MP accessions, since genes showing similarity in expression patterns are supposed to be functionally related or controlled by the same regulators. Significant clustering of genes with putative involvement in metal homeostasis was not found.

Ni and Zn Accumulation

F_4 root and shoot material was compared by transcript profiling as described above. F_4

material with contrasting Ni and Zn accumulation and Ni to Zn concentration ratios was selected as indicated in Fig. 3.

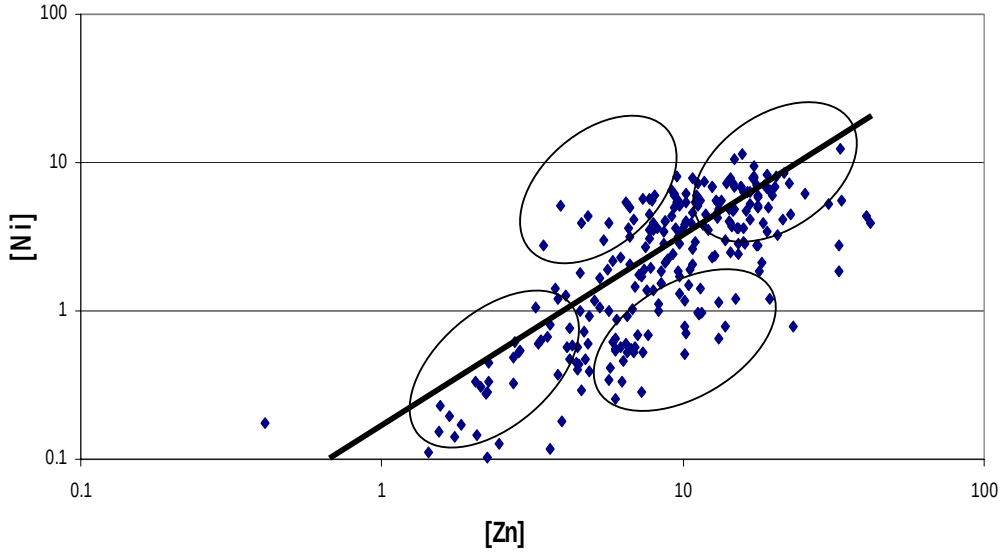

Figure 3: Extreme phenotype groups in Zn and Ni accumulation within the F_4 progeny.

The selected accumulation phenotypes (high Ni/high Zn, high Ni/low Zn, low Ni/high Zn, low Ni/low Zn) were compared using low Ni/low Zn as a common reference. After applying the customized criteria we found 163 genes differentially expressed in roots of the selected F_4 accumulation phenotypes in comparison to the low Ni and low Zn accumulating common reference, of which 50 genes were up- and 113 genes were down-regulated. The leaf transcriptome comparisons between extreme groups of Ni and Zn accumulation revealed almost two times as many differentially expressed genes as the tolerance arrays (215 versus 116). In general the comparison between the high Ni/high Zn accumulating group and the low Ni/low Zn group showed the highest number of genes differentially expressed, that is in the leaves 163 genes of which 94

up- and 69 down-regulated (Fig. 4). In the roots of the high Ni/high Zn group only 64 genes were differentially expressed, of which 2 were up- and 62 down-regulated, respectively (Fig. 4).

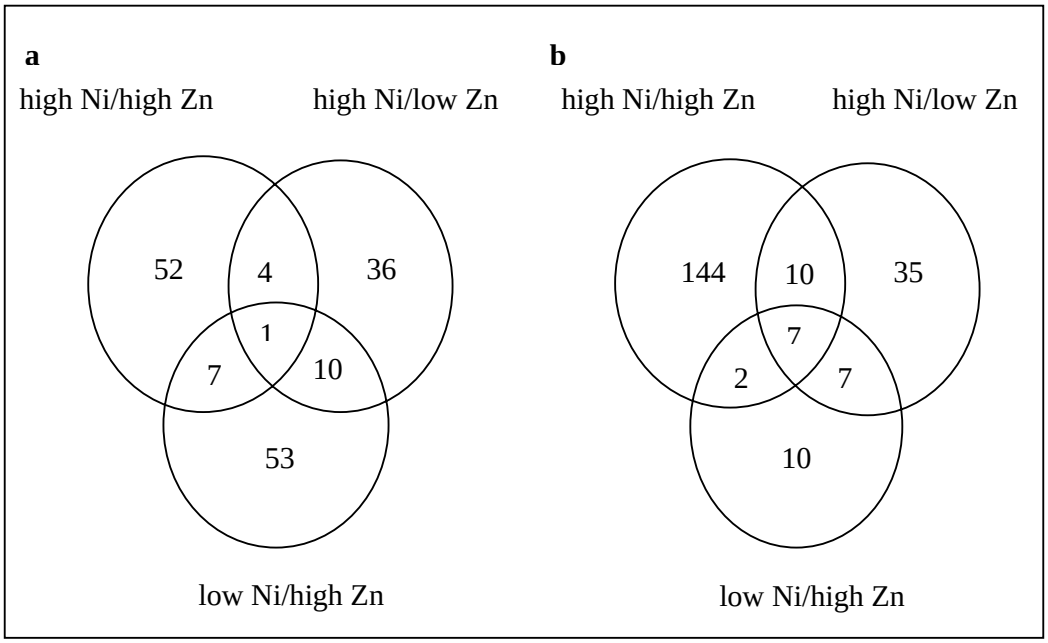

Figure 4: Genes differentially expressed in *T. caerulescens* roots (a) and leaves (b) of selected accumulation groups.

Out of all the genes that were more than 3-fold differentially expressed in leaves in either of the comparisons between accumulation phenotypes, only five were also differentially expressed between LC and MP within the tolerance array results. Two of them were higher expressed in the high Ni/high Zn group than in the low Ni/low Zn one, but lower expressed in MP than in LC, and one of them the other way around. One was down- and one was up- regulated in both MP and the high Ni/high Zn group, respectively as compared with LC and the low Ni/low Zn group (Table 2).

Clustering of the differentially expressed genes, either in root or leaf material did not

reveal any particular pathway of interest, as found for the comparison of tolerance phenotypes.

Table 2: List of all the genes that were commonly > 3-fold differentially expressed in leaves of either of the accumulation phenotypes and MP, with the low Ni/low Zn group and LC as common reference, respectively.

AGI gene code	Putative function	Go annotation	M	fold change	differentially expressed in
At1g51830	protein kinase	calcium ion binding	2.085	4.242	high Ni/high Zn
			-2.649	0.159	MP
At1g53990	lipase/hydrolase family protein	molecular function unknown	1.658	3.156	high Ni/high Zn
			-2.835	0.140	MP
At1g72290	trypsin and protease inhibitor family protein		-2.203	0.217	high Ni/high Zn
			2.083	4.238	MP
At3g48930	40S ribosomal protein	hormone activity	-1.928	0.263	high Ni/high Zn
			-2.149	0.225	MP
At5g67100	DNA polymerase alpha catalytic subunit	porin activity	1.902	3.738	high Ni/high Zn
			1.624	3.082	high Ni/low Zn
			1.773	3.418	MP

Discussion

To identify candidate genes for Ni and Zn accumulation and tolerance to Ni in *T. caerulescens*, we performed transcript profiling, using the full genome Arabidopsis3 oligo-array, which is feasible due to the high DNA identity of *T. caerulescens* and *A. thaliana* (van de Mortel et al., 2006; Rigola et al., 2006). Using the same platform, cross-species comparisons between the root transcriptomes of *Arabidopsis thaliana* and *T. caerulescens* have yielded more than 2000 genes that were more than 5 times higher expressed in *T. caerulescens* than in *A. thaliana*, many of them being putatively involved in metal homeostasis, stress response and lignin synthesis (van de Mortal et al., 2006). Genes involved in metal homeostasis and stress response were

also over-represented among the ones that were differentially expressed in *A. halleri* versus *A. thaliana* cross-species comparisons (Weber et al., 2004; Talke et al., 2006). We expected that comparisons between the *T. caerulescens* accessions LC and MP, and between F_4 lines selected for opposed tolerance and accumulation phenotypes for Ni and Zn should identify genes that are involved in the control of these traits, assuming that differential regulation of otherwise similar genes would be responsible for the phenotypic differences. However, only very few genes appeared to be differentially expressed by a factor of three or more. Among these, there were no genes with putative functions in metal homeostasis, and stress response genes were not over-represented. In either of the comparisons, over-representation of specific functional classes was not found. In general, only few genes that were more than 3-fold higher expressed in the highly Ni-tolerant, and highly Ni/Zn accumulating F_4 lines, in comparison with the phenotypically contrasting lines, were also more than 3-fold higher expressed in MP accession, compared with the LC one. This emphasizes once more that the genes that were differentially expressed between the contrasting F_4 groups were probably not directly related to the high accumulation and high tolerance phenotype of the MP parent accession. The reasons for the apparent lack of strong differential expression of metal homeostatic genes between the accessions or selected F_4 groups with contrasting tolerance and accumulation phenotypes might be various. With regard to Ni tolerance, it should be admitted that the average difference in degree between the LC and MP accessions is rather low and, in general, the heritability of the Ni tolerance character is barely significant (Richau and Schat, 2009a), suggesting that we may have selected largely non-genetically based phenotypes. In addition, it may be argued that we only compared the leaf

transcriptomes, whereas the tolerance character might conceivably reside mainly in the roots. However, it has been shown that root growth is not the most suitable tolerance end point for a hyperaccumulator. Overall, in *T. caerulescens* the interaccession variation in the root growth response to metal exposure is rather inconsiderable, compared with the foliar chlorosis response that we used as a toxicity end point in this study (Assunção et al., 2003c). Furthermore, it is arguable that the phenotypic difference between the high Ni/low Zn and low Ni/high Zn F_4 groups will be largely non-genetic, in view of the high genetic correlation between Zn and Ni accumulation (Richau and Schat, 2009a). However, this argument does most probably not apply to the difference between the high Ni/high Zn and the low Ni/low Zn F_4 groups, which is much bigger (Fig. 3), and doubtlessly largely genetically based (Richau and Schat, 2009a). Therefore, it seems that the majority of the genes that appeared to be differentially expressed between hyperaccumulators and non-hyperaccumulators, at least in so far as they are involved in accumulation, are generally not more then 3-fold differentially expressed among different *T. caerulescens* accessions. This was previously found for the zinc transporters *ZNT1* and *ZNT2*, for example, which are expressed to comparable levels in LC, MP and the non-metallicolous accession LE (Assunção et al., 2001). It is possible that less than 3-fold expression differences in metal homeostatic genes could account for the phenotypic differences. If so, then much more replication would be required to make intraspecific comparisons with sufficient resolution. Alternatively, the proteins responsible for the phenotypic variation considered here might be regulated post-transcriptionally, rather than by their transcript level. This possibility is exemplified by the vacuolar Mg and Zn/proton antiporter protein, *MHX*, which was found to be

present at much higher levels in leaves of *A. halleri* than in those of *A. thaliana* and, therefore, proposed to be involved in hyperaccumulation. The transcript levels, however, were not different (Elbaz et al., 2006). It is also conceivable that part of the variation results from quantitative or qualitative alterations of the metal affinity patterns of metal processing proteins, due to non-synonymous mutations in their coding sequences.

Chapter 5

General discussion

Enhanced metal uptake and root to shoot translocation in combination with the ability to render high amounts of heavy metals harmless at the cellular level are the main characteristics of heavy metal hyperaccumulating plants. Although significant progress has been made, the molecular and biochemical mechanisms underlying this intriguing phenomenon is only partly understood to date. This is in part because hyperaccumulation is usually constitutive at the species level (Bert et al., 2002, Macnair, 2002), and therefore the identification of underlying mechanisms through intraspecific comparisons and analysis of intraspecific crosses between plants possessing or lacking the trait is precluded.

However, *T. caerulescens* accommodates a high level of intraspecific variation in the degree of metal tolerance, uptake and translocation of heavy metals, and it is a small self-compatible plant with a relatively small genome with approximately 89-% DNA identity with *Arabidopsis thaliana* in the coding sequences, which make the species highly useful as a model for further analysis of hyperaccumulation-related traits. However, since at least Zn hyperaccumulation and high root to shoot translocation rates of Zn, Cd and Ni are constitutive at the species level, at least largely, it could well be that important genetic determinants of these traits may not segregate in intraspecific crosses, even though there is considerable genetic variation in the levels of either of these traits (Assunção et al. 2003a,b,c; Richau and Schat 2009a).

A first aim of the present study was to unravel the genetic relationship between heavy metal hyperaccumulation and heavy metal tolerance through comparisons between *Thlaspi caerulescens* accessions and analysis of progenies of a cross between plants from accessions with contrasting levels of these traits. Therefore we investigated the segregation patterns of Ni accumulation, the co-segregation of Ni and Zn accumulation, and the co-segregation of Ni tolerance and Ni accumulation in the F_3

and F_4 progenies of a single intraspecific cross between plants from serpentine and from calamine soil.

The results clearly confirmed the calamine population (LC) as a low-Ni-tolerant/low-Ni-accumulating population compared to the serpentine one (MP) with a high-tolerance/high-accumulation phenotype for Ni (Assunção et al. 2003a,c). We showed that MP accumulated almost 30 times more Ni in the leaves than did LC. Zn accumulation measurements, on the other hand, confirmed the suggested constitutive nature of the Zn accumulation trait (Meerts and Van Isacker, 1997; Escarré et al., 2000). Both populations under study accumulated much more Zn than Ni, although Ni was supplied at a 5-fold higher concentration (10 µM Ni versus 2 µM Zn). Furthermore, the foliar Zn concentration in MP was more than two-fold higher than that of Ni, and more than 5-fold higher than in LC (chapter 2, table 1). Assunção et al. (2001) showed that at equimolar Zn and Ni supply in the nutrient solution, Ni accumulation was strongly inhibited in MP, but not in LC. Based on this, these authors proposed that there is a common uptake system for Ni and Zn in MP, which is barely or not expressed in LC (Assunção et al., 2001, 2008). If so, according to the so-called "inadvertent uptake hypothesis" (Boyd & Martens, 1992), the high-Zn-accumulation phenotype in MP might then reflect a direct selection on Ni accumulation via a pre-existing Zn accumulation system, or alternatively, the high-Ni-accumulation phenotype might represent a by-product of selection for enhanced Zn accumulation in a high-Ni environment. However, additional study is necessary to test these hypotheses.

Accumulation data for the F_3 and F_4 progenies under study showed a vast segregation of both traits, with a positive phenotypic correlation of Ni and Zn accumulation, which appeared to be solely due to genetic correlation. This strongly confirms the

hypothesis of a common uptake system for Zn and Ni in MP (chapter 2). The more or less continuous rather than bimodal segregation patterns, with a large fraction of the individuals having Zn and Ni accumulation rates intermediate between those of the parental phenotypes, may be taken to suggest that the difference in metal accumulation between LC and MP is governed by more than one gene and that high accumulation is only partially dominant over low accumulation. However, the heritabilities obtained in this study were too low to exclude the possibility of a single codominant gene. In previous studies on different intraspecific *T. caerulescens* crosses, Assunção et al. (2003b, 2006), Zha et al. (2004), and Deniau et al. (2006) obtained evidence of polygenic control of the intraspecific variation in Zn and Cd accumulation, Zha et al. (2004) and Deniau et al. (2006) observed significant transgression for Zn accumulation and Assunção et al. (2006) and Deniau et al. (2006) found that both parents contributed trait enhancing alleles at different loci. The absence of significant transgression in the present study suggests that the trait-enhancing allele or alleles of the genes governing the segregation of Ni and Zn accumulation in the present study were all contributed by the MP parent.

Studies on metal tolerances in *T. caerulescens* accessions revealed that enhanced levels of tolerance have been evolved in metallicolous populations, in response to the soil metal enrichments in their environment, exactly as in non-hyperaccumulator facultative metallophytes (Schat & Vooijs, 1997). Moreover, Assunção et al. (2003b) established that variations in tolerance are correlated with soil metal compositions at the population site, and thus proved that tolerance to heavy metals is not a fully constitutive trait in *Thlaspi caerulescens*, but subject to evolutionary change at a local scale. In line with that, our results showed that MP, originating from serpentine soil, was considerably more tolerant to Ni than the calamine population (LC), on average,

although the distributions of the parental accessions were broadly overlapping, either due to genetic variation within the accessions, or owing to non-genetic sources, associated with the testing methodology. However, the heritability of the variation among the F_3 progeny of the MP x LC cross was significant, which proves that the variation for Ni tolerance within this progeny was due to genetic segregation, at least in part. Studies on inheritance of metal tolerances in a first-generation backcross progeny of a cross between *Arabidopsis halleri* and *A. lyrata* ssp. *petraea* revealed high broad-sense heritabilities of 89% and 69% for Cd and Zn tolerance, respectively (Courbot et al., 2007, Willems et al., 2007). We found a relatively low heritability value for Ni tolerance in the LC x MP cross ($h^2 = 0.47$). This is not surprising in view of the relatively small difference in Ni tolerance between LC and MP as compared to the difference in Zn and Cd tolerance between *A. lyrata* and *A. halleri*. From the combination of a higher Ni tolerance and a much higher Ni accumulation in MP, as compared to LC, it can be concluded that the plant-internal sequestration capacity represents a major determinant of the Ni hypertolerance phenotype in this accession.

Former studies on different intraspecific *T. caerulescens* crosses reported neither consistent co-segregation of Zn tolerance and Zn accumulation, nor of Cd tolerance and Cd accumulation (Assunção et al., 2003b; Zha et al., 2004). In line with that there was no correlation between the Ni concentration in the leaves, measured after 3 weeks of growth at 10 µM Ni, and the tolerance to this metal in the present study. Moreover, a 2x2 non-parametric contingency test with the median tolerance and accumulation values as class borders did not yield any significant association of high accumulation and high tolerance (Chapter 2, Fig. 8). It is remarkable however, that virtually all of the highly Ni accumulating plants were found among the more Ni tolerant ones, although the mean Ni accumulation level did not increase with tolerance (Chapter 2,

Fig. 6). This might suggest that high Ni tolerance is based either on low accumulation or on a mechanism associated with enhanced rather than decreased accumulation. However, the low plant numbers in the low tolerance classes made it difficult to draw any conclusion with reasonable certainty at this point. Further analysis of bigger progenies will be required to resolve the details of the relationship between Ni accumulation and Ni tolerance in LC x MP crosses. In any case, there is no straight-forward correlation between Ni accumulation and Ni tolerance within the segregating progeny, in agreement with the cases for Zn and Cd (Assunção et al., 2003b; Zha et al., 2004).

In the present study we also compared Ni accumulation in root and shoot, and Ni localization patterns in roots among other *T. caerulescens* accessions, next to MP and LC, and between *T. caerulescens* and *T. arvense* (Chapter 3). Our results confirmed the broad variation in Ni accumulation capacity among *T. caerulescens* accessions (Assunção et al., 2003c; Richau and Schat, 2009a). However, shoot to root Ni concentration ratios were always enhanced in *T. caerulescens*, compared to *T. arvense*, and close to unity in most cases, demonstrating that variation in uptake, rather than translocation, was the major determinant of the variation in the shoot and root Ni concentrations among the accessions, at least when grown for 2 weeks at 10 µM Ni. The least Ni-accumulating accession, LC, even took up less Ni, per unit of total plant weight, than did *T. arvense*. Therefore we can confirm the hypothesis that enhanced Ni translocation from the roots to the shoots, rather than enhanced Ni uptake, is constitutive at the species level in *T. caerulescens* (Assunção et al., 2003c, 2008). However, since MP accumulated much more Ni in roots than did *T. arvense*, we can not confirm the hypothesis that the high metal uptake in hyperaccumulators

would be driven by enhanced xylem loading, inducing a deficiency response in the roots (Hanikenne et al., 2008).

We found a strong association between the variation in Ni accumulation among the *T. caerulescen*s and the patterns of Ni distribution over the root tip and the mature root segments. In the low-Ni-accumulating accessions Ni accumulated primarily in the root tip and much less in the mature root segments, whereas the reverse was found in the high-Ni-accumulating accession MP, as compared to an even distribution of Ni over the tip and the mature sections in *T. arvense* and a moderately Ni-accumulating *T. caerulescens* accession (LE). These differences in Ni distribution among the *T. caerulescens* accessions seemed to be largely a consequence of the variation in their Ni accumulations rates, the more so because they tended to decrease with increasing Ni exposure. Also at the tissue level, very marked differences in Ni distribution were found. Ni storage in mature peripheral root tissues, like the cortex, rhizodermis and endodermis was evident in MP and *T. arvense*, but not in the low-Ni-accumulating accession LC, where Ni appeared to be mainly present in the rhizodermis of the meristemic zone of the root tips and in root hair-bearing mature rhizodermal cells. These results, obtained through histochemical staining and microscopic investigation, are quantitatively in agreement with the Ni measurements in intact root segments, although the detection limit of the staining method is considerably higher than that for the graphite furnace AAS method.

In this study we also tried to characterize the role for histidine in Ni hyperaccumulation in *T. caerulescens* (Chapter 3). Among the nonprotein metal chelators, the amino acid histidine (His) has been suggested to play an important role in Ni hyperaccumulation in *Alyssum* species, based on the observations that exogenous His supply can strongly enhance Ni tolerance and Ni xylem loading in

non-hyperaccumulators such as *Alyssum montanum* and *Brassica juncea*, but not in Ni-hyperaccumulating Alyssum species, which already have a strongly enhanced constitutive root His pool (Krämer et al., 1996; Kerkeb and Krämer, 2003). In our study, all the *T. caerulescens* accessions exhibited constitutively strongly enhanced free His concentrations in their roots (10-fold higher than in *T. arvense*, irrespective of Ni exposure). The effect of exogenous His supply on the Ni and His concentrations in the xylem sap were also studied, and the results revealed that in all accessions, apart from LC under low Ni exposure, but never in *T. arvense*, exogenous supply of His increased the Ni concentration in the xylem exudates, although exogenously supplied His was comparably recovered in the xylem sap of both species. Our results suggest that Ni xylem loading in *T. caerulescens* can be limited by the His concentration in the roots, depending on the Ni exposure level and the metal accumulation capacities of the accession in question. The questions of why Ni-hyperaccumulating accessions and non-Ni-hyperaccumulating accessions have almost identical root-internal His concentrations, or why Ni exposure does not further increase the root His pool in the Ni-hyperaccumulating accessions, remain to be answered. It seems possible that the constitutive His level is sufficient to allow maximum Ni translocation in the natural, serpentine environment, where the Ni concentrations in the soil solution might not exceed the low micromolar range. Furthermore, recent pilot experiments showed that exogenous Ni supply did not only enhance the xylem loading of Ni, but, even more so, that of Zn (H. Schat and A.D. Kozhevnikova, unpublished), which is not surprising in view of the fact that Zn is predominantly bound to His in *T. caerulescens* roots (Salt et al., 1999). Since Zn hyperaccumulation is constitutive at the species level in *T. caerulescens*, and at least much less variable in degree than Ni

accumulation, this might explain the low degree of variation among *T. caerulescens* accessions with regard to their root His concentrations.

To unravel the mechanism of His-mediated Ni xylem loading in *T. caerulescens*, or more specifically, to check whether His could inhibit the uptake and retention of Ni in root cell vacuoles, we measured Ni uptake in MgATP-energized tonoplast vesicles derived from leaves and roots of the accessions MP and LC, with the highest and the lowest degrees of Ni hyperaccumulation among all accessions tested, respectively, and from *T. arvense* (Chapter 3). For both *T. caerulescens* accessions leaf-derived vesicles took up more Ni than did root-derived ones, whereas the reverse was found for *T. arvense* vesicles, irrespective of whether Ni was supplied as Ni-citrate, Ni-His or $NiSO_4$.. Complexation with His strongly decreased Ni uptake, in comparison with complexation by citrate and no complexation, in *T. caerulescens* vesicles, but not in *T. arvense* vesicles, regardless of whether the vesicles were leaf- or root-derived. However, because high His concentrations are confined to the root in *T. caerulescens*, it seems likely that His-imposed inhibition of vacuolar Ni sequestration in planta will be much stronger in roots than in leaves. It is suggested, by the combination of experimental results, that the combination of a high root His concentration and a loss of the ability to transport His-complexed Ni into the vacuole is essential to sustain the high root to shoot Ni translocation rates in *T. caerulescens*. Also, it seems that the high His concentration in *T. caerulescens* roots is sufficient to prevent any significant accumulation of Ni in mature peripheral root tissues under low Ni exposure in low-Ni-accumulating accessions, such as LC. The same His-based mechanism also seems to account for the translocation of Zn (see above), and, in view of the high degree of correlated variation of Zn and Cd translocation among accessions (Xing et al., 2008), possibly for that of Cd in *T. caerulescens*. To explain the latter correlation,

experimental data on the effects of exogenous His supply on Cd xylem loading are urgently required. An interesting aspect of the effects of exogenous His supply on hyperaccumulators is that they vary among species. In the Ni hyperaccumulator, *Alysssum lesbiacum*, it had no effect on Ni xylem loading (Krämer et al., 1996), which was attributed to a constitutively enhanced root-internal concentration (Kerkeb and Krämer, 2003). However, in Ni-hyperaccumulating *T. caerulescens* accessions, in which the constitutive root-internal His concentration is as high as in *A. lesbiacum*, exogenous His supply did significantly enhance xylem loading, as shown in this study. The reason for this is elusive, but it might have something to do with the rates of uptake of Ni or exogenous His, or alternatively, with the rates of *de novo* His synthesis under Ni exposure, because these factors are expected to determine whether Ni xylem loading will be limited by the root His pool or not. To test this hypothesis, comparative experiments with different rates of Ni and His supply, including measurements of the Ni and His fluxes, would be required. On the other hand, there is also variation among non-hyperaccumulator Brassicaceae. Exogenous His supply increased both Ni tolerance and Ni xylem loading, the latter up to hyperaccumulator level, in *Alyssum montanum* (Krämer et al., 1996), whereas it had no effect on Ni xylem loading in *T. arvense* in this study. Enhanced Ni xylem loading upon exogenous His supply was also observed in *Brassica juncea* (Kerkeb and Krämer, 2003). On the other hand, upregulation of the His synthetic pathway in *Arabidopsis thaliana* through over-expression of ATP-phosphoribosyl transferase did not result in increased concentrations of Ni in the leaves, nor in the xylem sap, although it did produce increased His concentrations in roots and leaves, as well as enhanced Ni tolerance (Ingle et al., 2005). It is tempting to assume that these interspecific differences in the response to exogenous His are due to correlated differences in the

capacity to sequester His-complexed Ni in the root cell vacuoles. Comparative transport assays with purified tonoplast vesicles are urgently required to test this assumption.

Decreased vacuolar metal sequestration in hyperaccumulator roots, as compared with non-hyperaccumulator roots, has been reported for Zn by Lasat et al. (1998), who found a lower accumulation in, and a faster release from root cell vacuoles in *T. caerulescens*, as compared to *T. arvense*. Yang et al. (2006) reported that 2.7 times more Zn was retained in root cell vacuoles of non-hyperaccumulating *Sedum alfredii*, as compared to the hyperaccumulating ecotype of the same species. Xing et al. (2008) found a higher vacuolar Cd fraction in a slowly Cd-translocating *T. caerulescens* accession, as compared to a rapidly Cd-translocating one, although the release rates were not different in this case. These studies clearly suggest that decreased vacuolar sequestration in root cell vacuoles is essential for the high rates of metal translocation in hyperaccumulators. There is strong evidence, however, that enhanced expression of the heavy metal transporting 1b P-type ATPase, *HMA4*, is also essential for the high rates of metal xylem loading in hyperaccumulators, at least for Zn and, probably, Cd (Hanikenne et al., 2008). There is no evidence, however, that *HMA4* would play a role in the xylem loading of Ni in Ni hyperaccumulators. Such evidence should come from gene silencing experiments in Ni hyperaccumulators. However, Ni hyperaccumulators have not been successfully transformed thus far. Although the prominent role for *HMA4* in Cd and Zn hyperaccumulation in *Arabidopsis halleri* is beyond doubt (Hanikenne et al., 2008), it is possible that the intra-specific variation in hyperaccumulation capacity in this species or in *T. caerulescens* is completely unrelated to variation in *HMA4* expression, which might be constitutively enhanced at the species level, but rather to variation in vacuolar retention of the metals in root

cells (Xing et al., 2008). On the other hand, in the present study we found almost identical Ni uptake rates, irrespective of the Ni speciation in the bathing solution, in tonoplast vesicles derived from the two *T. caerulescens* accessions with maximally contrasting shoot to root Ni concentration ratios after longer exposure, LC and MP (Assunção et al., 2003a). Moreover, our study suggests that significant Ni storage in mature root segments, presumably in vacuoles, may not occur until the root His pool becomes limiting for xylem loading. Since the His pool is of a comparable size in all the accessions, this could mean that the variation in root vacuolar metal fractions among hyperaccumulator accessions might merely reflect variation in uptake rates (His-mediated xylem loading will sooner saturate at higher uptake rates). More extensive comparisons of vacuolar compartmentalization at different metal exposure levels and of *HMA4* expression patterns among hyperaccumulator accessions will be needed to test these hypotheses.

To identify candidate genes for Ni and Zn accumulation and tolerance to Ni in *T. caerulescens*, we performed in chapter 4 transcript profiling, using the full genome Arabidopsis3 oligo-array, which is feasible due to the high DNA identity of *T. caerulescens* and *A. thaliana* (van de Mortel et al., 2006; Rigola et al., 2006). Van de Mortel et al. (2006) carried out cross-species comparisons between the root transcriptomes of *Arabidopsis thaliana* and *T. caerulescens* using the same platform, and have yielded more than 2000 genes that were more than 5 times higher expressed in *T. caerulescens* than in *A. thaliana*, of which many are known to be putatively involved in metal homeostasis, stress response and lignin synthesis. Genes involved in metal homeostasis and stress response were also over-represented among the ones that were differentially expressed in *A. halleri* versus *A. thaliana* cross-species comparisons (Weber et al., 2004; Talke et al., 2006). We expected that comparisons

between the *T. caerulescens* accessions LC and MP, and between F_4 lines selected for opposed tolerance and accumulation phenotypes for Ni and Zn should identify genes that are involved in the control of these traits, assuming that differential regulation of otherwise similar genes would be responsible for the phenotypic differences. However, only very few genes appeared to be differentially expressed by a factor of three or more, and moreover, among these, there were no genes with putative functions in metal homeostasis, and stress response genes were not over-represented. Overall, the present results lead to the conclusion that the majority of the genes that appeared to be differentially expressed between hyperaccumulators and non-hyperaccumulators are generally not differentially expressed among different hyperaccumulator accessions. This is in line with the previous findings for the zinc transporters *ZNT1* and *ZNT2*, for example (Assunção et al., 2001). This may be not surprising for genes involved in Ni tolerance, in view of the small difference in Ni tolerance between LC and MP, and the lack of correlation between Ni tolerance and accumulation among the F_4 progeny. However, the Ni accumulation capacities are different by more than one order of magnitude, both among the accessions and the progeny selected for high and low Ni accumulation. Therefore, we expected to find at least one or a few metal transporters to be differentially expressed. If it is possible that less than 3-fold expression differences could account for the phenotypic differences, much more replication would be required to make intraspecific comparisons with sufficient resolution. Alternatively, the proteins responsible for the phenotypic variation considered here might be regulated post-transcriptionally, rather than by their transcript concentrations itself. This possibility is represented by the vacuolar Mg and Zn/proton antiporter protein, *MHX*, which was found to be present at much higher levels in leaves of *A. halleri* than in those of *A. thaliana* and, therefore,

proposed to be involved in hyperaccumulation. Whereas, nevertheless, the transcript levels of this protein were not different between this species (Elbaz et al., 2006). It is also conceivable that part of the variation results from quantitative or qualitative alterations of the metal affinity patterns of metal processing proteins, due to non-synonymous mutations in their coding sequences.

In conclusion, the analysis of segregating progenies of an intraspecific *T. caerulescens* cross reveals a strong genetic correlation between Ni and Zn hyperaccumulation, demonstrating that their segregation is governed by the same gene or genes. On the other hand, Ni tolerance and Ni accumulation are uncorrelated, but bigger progenies should be analyzed to assess the precise relationship between these traits. Root histidine concentrations are strongly and constitutively enhanced in *T. caerulescens*, compared to *T. arvense*, which seems to prevent Ni retention in root cell vacuoles, thus promoting Ni xylem loading. More detailed physiological experimentation is required to explain the intraspecific variation in the vacuolar retention of different metals in roots and the consequences of this for their translocation to the shoot. The molecular determinants of the variation in Ni tolerance and Ni hyperaccumulation among *T. caerulescens* accessions are elusive. Microarraying does not seem to reveal the responsible genes, although more replication could be helpful. However, the great majority of metal homeostasis genes that are differentially expressed in cross-species comparisons seem to be expressed at the same level in different *T. caerulescens* accessions. Therefore, candidate gene approaches might be more rewarding.

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Summary

Nickel hyperaccumulation in *Thlaspi caerulescens*: a rare micro-evolutionary event

Heavy metal hyperaccumulation is a comparatively rare trait in the plant kingdom. Although progress has been made, the physiological and molecular basis, as well as the ecological function of the phenomenon is far from being well understood yet. Enhanced capacities for root uptake, root to shoot translocation and sequestration of metals in leaves are the major characteristics of hyperaccumulators. *Thlaspi caerulescens*, a Zn/ Cd/ Ni hyperaccumulator, offers excellent opportunities to study the genetics and physiological mechanisms underlying these traits, since it exhibits a distinct intraspecific variability in metal accumulation, translocation and tolerance. Furthermore, the high degree of DNA sequence identity (89% in coding sequences) between *T. caerulescens* and the plant genetics model, *Arabidopsis thaliana*, presents good opportunities to utilize the molecular tools and genomic information available for *A. thaliana*.

The present work was undertaken to obtain a better understanding of the heavy metal hyperaccumulation and tolerance traits and their interrelationships. We compared plants from two contrasting *T. caerulescens* accessions, one from the serpentine area of Monte Prinzera (MP) in northern Italy and a Belgian calamine accession, La Calamine (LC). A single MP x LC cross was used to study the genetic correlation between Ni and Zn accumulation and between Ni accumulation and Ni tolerance. Therefore, parental accessions as well as F_3 and F_4 progeny of the interaccession cross were phenotyped. The phenotypic distributions for Zn and Ni accumulation of the parental populations were non-overlapping, with MP having higher foliar metal

concentrations than LC. Ni tolerance was also higher in MP, but the parental distributions here were overlapping. The F_3 and F_4 progeny exhibited a clear segregation for the Ni and Zn accumulation trait as well as for Ni tolerance. Variance and covariance analysis of the F_3 progeny demonstrated significant heritability values (h^2) for Ni and Zn foliar accumulation (0.70 and 0.59, respectively) and Ni tolerance (0.47), as well as a significant positive genetic correlation between the foliar accumulation of Ni and Zn ($r_A^2 = 0.77$). However, Ni tolerance and Ni accumulation were uncorrelated. Regressing the F_4 family means on the F_3 parent values yielded similar estimates for the heritabilities of Ni and Zn accumulation in the leaves (0.66 and 0.55, repectively)

Even though in previous studies histidine has been implicated to play an important role in Ni hyperaccumulation, its precise function remained elusive. Therefore, we investigated the role for histidine in plant-internal metal transport, both at the levels of root to shoot translocation and tonoplast transport, between calamine, serpentine and non-metallicolous accessions of *T. caerulescens* and the non-hyperaccumulating non-metallophyte congeneric species, *T. arvense*. We compared (1) root and shoot histidine concentrations in plants grown with and without Ni in the nutrient solution (2) Ni tonoplast transport in energized root- and shoot-derived tonoplast vesicles, with Ni supplied as free Ni, Ni-citrate or Ni-histidine, (3) the effect of exogenous histidine supply on Ni xylem loading, along with, (4) the distribution of Ni over root segments and root tissues. Within the present study we show accumulation of Ni in mature root cortical cells of *T. arvense* and a high-Ni-accumulating *T. caerulescens* accession, but not in low-accumulating *T. caerulescens* accessions. Compared to *T. arvense*, the concentration of free histidine in *T. caerulescens* was 10-fold enhanced in roots, but only slightly higher in leaves, regardless of Ni exposure. Ni uptake in MgATP-

energized root- and shoot-derived tonoplast vesicles was almost completely blocked in *T. caerulescens*, but uninhibited in *T. arvense*, when Ni was supplied as a 1:1 Ni-His complex. Exogenous histidine supply enhanced Ni xylem loading in *T. caerulescens* but not in *T. arvense*. Therefore we conclude that the high rate of root to shoot translocation of Ni in *T. caerulescens* as compared to *T. arvense* depend on the combination of two distinctive characters, i.e. a greatly enhanced root histidine concentration and a strongly decreased ability to accumulate histidine-bound Ni in root cell vacuoles.

We also carried out some transcriptome comparisons between *T. caerulescens* accessions and F_4 lines with contrasting tolerance and accumulation characteristics derived from the LC x MP cross, with the aim to find candidate genes responsible for the intraspecific differences in Zn and Ni accumulation and Ni tolerance. To this end we used the full genome Arabidopsis Agilent3 array. In general, only a few genes appeared to be more than 3-fold differentially expressed, among which not a single metal homeostasis-related gene. It seems that the high number of metal homeostatic genes that are differentially expressed in cross-species comparisons between hyperaccumulators and non-hyperaccumulators, are expressed at similar levels in the LC and MP *T. caerulescens* accessions, in spite of the strongly different phenotypes for Ni and Zn accumulation. These differences are possibly due to post-translational regulation or structural alterations of metal processing proteins. Alternatively, it is conceivable that fairly subtle differences in transcript levels may have rather drastic phenotypic effects.

Samenvatting

Hyper accumulatie van nikkel in *Thlaspi caerulescens*: een zeldzame micro-evolutionaire gebeurtenis

Hyperaccumulatie van zware metalen is een betrekkelijk zeldzame eigenschap in het plantenrijk. Hoewel in de laatste jaren wel degelijk vooruitgang is geboekt, zijn de onderliggende fysiologische en moleculaire mechanismen, maar ook de ecologische aanpassingswaarde van dit fenomeen nog verre van volledig opgehelderd. In vergelijking met 'normale' planten worden hyperaccumulatoren gekarakteriseerd door een verhoogde metaalopnamecapaciteit, een versnelde translocatie van de opgenomen metalen van de wortel naar het blad, en een zeer efficiënte ontgifting van deze metalen door opslag in de vacuoles van bladcellen. De Zinkboerenkers (*Thlaspi caerulescens*), een hyperaccumulator van zink (Zn) maar soms ook van cadmium (Cd) of nikkel (Ni), biedt goede kansen om de overervings- en werkingsmechanismen van het hyperaccumulatiesyndroom verder te analyseren. Deze soort bezit namelijk een hoge mate van erfelijke variatie in metaalopname, metaaltranslocatie, en metaaltolerantie. Bovendien is die variatie grotendeels metaalspecifiek. Verder is de DNA sequentie van de Zinkboerenkers ongeveer 89% identiek met die van de modelsoort van de plantengenetica, *Arabidopsis thaliana* (de Zandraket), en dat maakt het mogelijk om de veelheid aan moleculaire technieken en de genomische informatie die voor de Zandraket beschikbaar zijn, ook op de Zinkboerenkers toe te passen.

Het in dit proefschrift beschreven werk was er in eerste instantie op gericht om een beter inzicht te verkrijgen in de genetische basis van de variatie in de hyperaccumulatie van, en de tolerantie voor Zn en Ni binnen de Zinkboerenkers. Daartoe werden twee lokale populaties met contrasterende eigenschappen vergeleken,

namelijk één van een sterk met zink en cadmium vervuilde bodem bij La Calamine (LC, België), en één van een nikkelrijke 'serpentijnbodem' op de berg Monte Prinzera (MP, Italië). Ook werd een LC plant gekruist met een MP plant. Het nageslacht van deze kruising werd gebruikt om vast te stellen in hoeverre de accumulatie van Zn en Ni, en de accumulatie van en de tolerantie voor Ni, door dezelfde of door verschillende genen bepaald wordt. Uit de resultaten bleek dat MP planten veel meer Ni, maar ook meer Zn in hun bladeren accumuleren dan LC planten. MP planten waren gemiddeld ook meer nikkeltolerant dan LC planten. Uit een analyse van de variantie en de covariantie van deze eigenschappen in de F3 generatie van de kruising bleek dat de variatie in nikkel- en zinkaccumulatie binnen deze generatie voor respectievelijk 70 en 59% door erfelijke factoren (genen) bepaald wordt. De onderlinge genetische correlatie van deze eigenschappen was 77%, d.w.z. de variatie in beide eigenschappen wordt grotendeels door identieke genen gecontroleerd. Ook de variatie in nikkeltolerantie bleek deels erfelijk te zijn (± 50%), maar was in het geheel niet gecorreleerd met de variatie in nikkelaccumulatie.

Een tweede doel van het onderzoek was het ophelderen van de rol van het aminozuur histidine (His) in het hyperaccumulatiesyndroom. Daartoe werden de His concentraties in wortel en blad en het effect van extern aangeboden His op het nikkeltransport van wortel naar blad, via het xyleem, vergeleken bij een aantal lokale populaties van de Zinkboerenkers en een populatie van de Witte Krodde (*Thlaspi arvense*), een verwante niet-hyperaccumularende soort. Ook werden vacuolaire membranen geisoleerd uit twee contrasterende populaties van de Zinkboerenkers en uit de Witte Krodde. De nikkeltransportcapaciteit van deze 'tonoplastfracties' werd vergeleken, waarbij Ni aangeboden werd als Ni-citraat complex, Ni-His complex, of als Ni-sulfaat.

Ook werd, bij dezelfde populaties, de verdeling van Ni over verschillende wortelsegmenten en wortelweefsels vastgesteld, het laatste met behulp van een histochemische kleuring van Ni met dimethylglyoxime. Het bleek dat alle populaties van de Zinkboerenkers een ongeveer tien maal zo hoge concentratie His in hun wortels hebben dan de Witte Krodde. De concentratie His in het blad was veel lager dan in de wortel en nauwelijks verschillend van de Witte Krodde. Extern aangeboden His verhoogde het Ni transport via het xyleem bij de Zinkboerenkers, maar niet bij de Witte Krodde, ondanks het feit dat beide soorten de aangeboden His in gelijke mate opnamen. De nikkeltransportcapaciteit van de gezuiverde 'tonoplastfracties' was vooral verschillend wanneer Ni aangeboden werd als Ni-His complex. In dit geval was er nauwelijks meetbaar transport bij de Zinkboerenkers, maar ongehinderd transport bij de Witte Krodde. De verdeling van Ni over wortelsegmenten en –weefsels was zeer verschillend, namelijk geconcentreerd in de rhizodermis van de meristematische zone van de worteltoppen bij LC, en min of meer verspreid over de gehele wortel, inclusief de cortex, bij MP en de Witte Krodde. Uit de combinatie van experimenten kan geconcludeerd worden dat de hoge nikkeltranslocatie van de wortel naar het blad bij de Zinkboerenkers mogelijk gemaakt wordt door een hoge concentratie His in de wortels, in combinatie met een lage capaciteit om met His gecomplexeerd Ni over de tonoplast te transporteren, waardoor de vacuolaire retentie van Ni in volwassen perifere wortelweefsels tegengewerkt wordt.

Tenslotte werden, om kandidaatgenen voor nikkelaccumulatie en –tolerantie te identificeren, de transcriptprofielen vergeleken van LC en MP, en van hoog- en laagaccumulerende, en hoog- en laagtolerante F4 families van de LC x MP kruising. Hiertoe werd gebuik gemaakt van de Agilent3 micro-array, die het gehele genoom van Arabidopsis bestrijkt. Meer dan drievoudige verschillen in expressie werden

slechts bij een klein aantal genen aangetroffen. Daaronder bevonden zich geen genen die op enigerlei wijze met de metaalhuishouding in verband gebracht kunnen worden. Het lijkt er op dat de sterke verschillen in de expressie van grote aantallen metaalgerelateerde genen, zoals die in vergelijkingen tussen hyperaccumulatoren en niet-hyperaccumulatoren gevonden is, niet gevonden worden bij intraspecifieke vergelijkingen van verschillende populaties of selectielijnen van een hyperaccumulerende soort, ondanks de soms grote fenotypische verschillen in accumulatie en tolerantie. Het is denkbaar dat expressieregulatie op eiwitniveau of subtiele structurele veranderingen in de eiwitstructuur hierbij een rol spelen.

Zusammenfassung

Nickel-Hyperakkumulation in *Thlaspi caerulescens*: ein seltenes mikro-evolutionäres Ereignis

Schwermetall-hyperakkumulation ist eine vergleichsweise seltene Eigenschaft innerhalb des Pflanzenreiches. Obwohl in den letzten Dekaden grosse Fortschritte auf diesem Gebiet gemacht worden sind, ist die physiologische und genetische Basis sowie die ökologische Funktion dieses Phänomens noch nicht komplett aufgeklärt.

Im Vergleich zu „normalen Pflanzen“ stellen eine gesteigerte Schwermetall-aufnahmekapazität, ein erhöhter Transport der Metalle von der Wurzel in die Blätter und eine sehr effiziente Entgiftung der Metallen in den Blattvakuolen, die wichtigsten Charakterzüge einer hyperakkumulierenden Pflanze dar. Das Gebirgs-Täschelkraut (*Thlaspi caerulescens*), eine Pflnze, die Zink (Zn), Cadmium (Cd) oder auch Nickel (Ni) hyperakkumuliert, ist ein exzellentes Modell zur weiteren Erforschung des physiologischen und genetischen Hintergrundes der Hyperakkumulation in Pflanzen, und das nicht nur, weil diese natürliche Hyperakkumulatorspezies ausgeprägte intraspezifischen Unterschiede in Metallakkumulation and Toleranz aufweist. Durch die hohe Sequenzidentität zwischen dem Gebirgs- Täschelkraut und dem allgemein anerkannten Model der Pflanzengenetik, *Arabidopsis thaliana* (Ackerschmalwand), von 89% in den kodierenden Regionen, ist es ausserdem möglich molekulare Techniken und genetische Informationen, die für die Ackerschmalwand bereits vorhandene sind, zu nutzen.

Diese Arbeit wurde in erster Linie durchgeführt, um eine bessere Einsicht in die molekularen Hintergründe der Hyperakkumulation von Zn und Ni und der Toleranz gegenüber Ni zu erhalten, insbesondere in die Variationen dieser Eigenschaften, wie

sie innerhalb verschiedener Populationen des Gebirgs-Täschelkrautes auftreten. Aus diesem Grund wurden zwei lokale Populationen mit verschiedenartige Eigenschaften verglichen, die eine von einem stark mit Zn und Cd kontaminierten Boden bei La Calamine (LC, Belgium) und die andere von einem nickelreichen Serpentinenboden vom Monte Prinzera (MP, Italien). Ausserdem wurde eine MP Pflanze mit einer LC Pflanze gekreuzt, um Nachkommen für Experimente bezüglich des Vererbungsgrades von Akkumulation und Toleranz zu erhalten, und darzustellen, ob die Akkumulation von Zn und Ni, sowie die Akkumulation von Ni und die Toleranz gegenüber diesem Metal durch das selbe oder von verschieden Genen gesteuert wird.

Unsere Ergebnisse zeigen, dass die MP Pflanzen nicht nur viel mehr Ni, sondern auch mehr Zn in ihren Blättern akkumuliert, als die LC Pflanzen. Im Durchschnitt wiesen die MP Pflanzen auch eine höhere Ni Toleranz auf, als die LC Pflanzen. Aus Varianz/Covarianz-Analysen dieser Eigenschaften innerhalb der F_3 Generation unserer intraspezifischen Kreuzung konnte ermittelt werden, dass die Variationen der Nickel- und Zink-Akkumulation innerhalb dieser Generation zu 70, beziehungsweise 59% aus vererbbare Faktoren hervorgeht. Auch die Variationen der Ni -Toleranz scheint teils erblich zu sein (±50%), wobei die Toleranz in keiner Weise in Zusammenhang gebracht werden konnte mit der Kapazität, dieses Metal zu akkumulieren.

Ferner wurden innerhalb dieser Arbeit Untersuchungen bezüglich der Rolle der Aminosäure Histidin (His) innerhalb des Hyperakkumulations-Phänomens angestellt. Zu diesem Zweck wurden die His Konzentrationen in Wurzeln und Blättern und der Effekt von extern dargereichtem His auf den Nickeltransport von den Wurzeln über das Xylem in die Blätter, zwischen verschiedenen lokalen Populationen des Gebrigs-Täschelkrauts und einer Population des Acker-Täschelkraut (*Thlaspi arvense*), eines

nicht hyperakkumulierenden Verwandten, verglichen. Zusätzlich wurden Tonoplastvesikel von MP-, LC- sowie von Acker-Täschelkraut-Pflanzen isoliert, die dann entweder in Ni-Citrat-Komplex, Ni-Histidine Komplex oder in Ni-Sulfat inkubiert wurden, um deren Nickeltransportkapazität zu vergleichen. Außerdem wurde für die untersuchten Populationen die Verteilung von Ni innerhalb verschiedener Wurzelsegmenten und Geweben mit Hilfe von histochemischer Färbung, untersucht.

Wir konnten zeigen, dass alle Populationen des Gebirgs-Täschelkrauts ungefähr zehn Mal so viel His in ihren Wurzeln besitzen, wie das Acker-Täschelkraut. Die His-Konzentrationen in den Blättern war viel niedriger als in den Wurzeln, und kaum abweichend von denen der Acker-Täschelkraut-Pflanzen. Extern über die Nährstofflösung angebotenes His erhöhte den Ni-Transport über das Xylem erheblich bei den hyperakkumulierenden Populationen, jedoch nicht im Acker-Täschelkraut, obwohl beide Arten das angebotene His in gleicher Menge aufnahmen. Die Transportkapazität der gereinigten Tonoplastvesikel war stark abhängig von der Darreichungsform des Nickels und unterschied sich vor allem, wenn Nickel als Ni-His Komplex vorlag. In diesem Fall, wies das Gebrigs-Täschelkraut kaum einen Transport über die Tonoplastmembran auf, wobei die Acker-Täschelkraut Vesikel ungehindert Ni-His aufnahmen. Die Verteilung von Nickel innerhalb des verschiedenen Wurzelsegmente und Gewebe brachte ein sehr unterschiedliches Bild. Während in MP und dem Acker-Täschelkraut das Metall mehr oder weniger über die gesamte Wurzel verteilt ist, war es in den LC Wurzeln in der Rhizodermis, sowie der meristematischen Zone der Wurzelspitze konzentriert. Die Kombination dieser Ergebnisse führt zu der Erkenntnis, dass der hohe Nickeltransport in die Blätter der Hyperakkumulatoren durch eine Kombination von hohen His-Konzentration in den

Wurzeln sowie durch eine niedrige Kapazität den Ni-His Komplex über die Tonoplastmembran zu transportieren, ermöglicht wird. Diese gehemmte Ni-His Transportkapazität, unterbindet eine Speicherung der Metalle in den Vakuolen der Hyperakkumulator-Wurzeln.

Abschliessend wurden Transkriptprofile von LC, MP, sowie F_4 Pflanzen der MPxLC Kreuzung mit gegensätzlichen Akkumulations- und Toleranzeigenschaften, verglichen. Zu diesem Zweck wurde von dem Arabidopsis3 Microarray Gebrauch gemacht, der das gesamte Genom von *Arabidopsis thaliana* abdeckt. Eine mehr als dreifach höhere oder niedrigere Expression von Genen wurden nur in einer kleinen Anzahl beobachtet, wobei sich unter diesen keine Gene befanden, die mit Schwermetallakkumulation oder Toleranz in Verbindung gebracht werden konnten. Es kann geschlussfolgert werden, dass die grosse Anzahl von unterschiedlich expremierten Genen, die mit Akkumulation und Toleranz in Verbindung gebracht werden können, also die, die bei Vergleichen zwischen hyperakkumulierenden und nicht-hyperakkumulierenden Arten ermittelt werden können, nicht durch einen Vergleich zwischen Populationen oder selektierten Phenotypen von Hyperakkumulatoren gefunden werden können, obwohl diese grosse Variationen in Akkumulation und Toleranz aufweisen. Es wird vermutet, dass die Regulation dieser Eigenschaften eher auf Proteinebene vorgenommen wird, eventuell spielen bestimmte strukturell Veränderungen innerhalb der Proteine hier eine Rolle.

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Tolerance arrays

down in MP

GeneName	Description	M	fold change
At1g07600.1	metallothionein-like protein	-3.606	0.082
At1g09510.1	cinnamyl-alcohol dehydrogenase family	-3.004	0.125
At1g53990.1	GDSL-motif lipase/hydrolase family protein	-2.835	0.140
At1g09400.1	12-oxophytodienoate reductase	-2.684	0.156
At1g51830.1	leucine-rich repeat protein kinase	-2.649	0.159
CK121013	Unknown	-2.364	0.194
At1g51850.1	leucine-rich repeat protein kinase	-2.250	0.210
At3g48930.1	40S ribosomal protein	-2.149	0.225
IR2:013278994-013278	Unknown	-2.112	0.231
At2g31360.1	delta 9 desaturase (ADS2)	-2.069	0.238
At1g32130.1	IWS1 C-terminus family protein	-2.024	0.246
At1g04270.1	\40S ribosomal protein S15 (RPS15A)	-1.999	0.250
At3g26150.1	cytochrome P450 71B16, putative (CYP71B16)	-1.907	0.267
At4g28780.1	GDSL-motif lipase/hydrolase family protein	-1.851	0.277
At3g11560.1	expressed protein [At3g11560.1]	-1.805	0.286
At4g08300.1	nodulin MtN21 family protein	-1.739	0.300
At4g23680.1	major latex protein-related	-1.739	0.300
At2g33470.1	glycolipid transfer protein-related	-1.683	0.311
At3g29030.1	expansin, putative (EXP5)	-1.668	0.315
IR1:011194427-011194	Unknown	-1.664	0.316
At1g19530.1	expressed protein [At1g19530.1]	-1.593	0.332
At5g13310.1	expressed protein [At5g13310.1]	-1.588	0.333

down in HT

GeneName	Description	M	fold change
At2g14580.1	pathogenesis-related protein	-2.526	0.174
At3g02040.1	glycerophosphoryl diester phosphodiesterase family protein	-1.725	0.302
BP622370	Arabidopsis thaliana cDNA clone RAFL17-03-A05 3', mRNA sequence [BP622370]	-1.591	0.332

down in LT

GeneName	Description	M	fold change
NP306585	transposase, putative [NP306585]	-3.035	0.122
IR1:022816643-022816	Unknown	-2.695	0.154
At5g52140.1	zinc finger protein-related [At5g52140.1]	-2.567	0.169
At3g30290.1	\cytochrome P450 family protein	-2.398	0.190
At1g18960.1	myb family transcription factor	-2.352	0.196
At3g42155.1	hypothetical protein [At3g42155.1]	-2.291	0.204
At3g09180.1	expressed protein [At3g09180.1]	-2.098	0.234
BE844688	A. thaliana (Col-0 gl1) library enriched for salt-induced transcripts	-2.094	0.234
At2g30700.1	expressed protein [At2g30700.1]	-1.990	0.252
At2g18880.1	fibronectin type III domain-containing protein	-1.989	0.252
At4g09150.1	T-complex protein 11	-1.949	0.259
At5g22470.1	poly (ADP-ribose) polymerase family protein	-1.861	0.275
At5g09225.1	expressed protein [At5g09225.1]	-1.846	0.278
At3g11170.1	omega-3 fatty acid desaturase, chloroplast (FAD7) (FADD)	-1.759	0.295
At3g61190.1	BON1-associated protein 1 (BAP1)	-1.709	0.306
AV564904	Arabidopsis thaliana green siliques Columbia Arabidopsis thaliana cDNA clone	-1.685	0.311
At5g44005.1	expressed protein [At5g44005.1]	-1.589	0.332

down in MP and HT

GeneName	Description	M MP5	fold change	M MT	fold change
At4g19120.1	early-responsive to dehydration stress protein (ERD3)	-2.273	0.207	-2.125489454	0.229

down in MP and LT

GeneName	Description	M MP5	fold change	M LT	fold change
At1g17910.1	wall-associated kinase	-2.600	0.165	-1.586801656	0.333

down in HT and LT

GeneName	Description	M MT	fold change	M LT	fold change
At3g09450.1	hypothetical protein [At3g09450.1]	-2.950	0.129	-2.221611564	0.214
At1g17710.1	expressed protein [At1g17710.1]	-2.157	0.224	-1.915072001	0.265
At5g27350.1	sugar-porter family protein 1 (SFP1)	-1.918	0.265	-1.659073154	0.317
At3g22160.1	VQ motif-containing protein	-1.769	0.293	-1.615497275	0.326
At4g15530.1	pyruvate phosphate dikinase family protein	-1.760	0.295	-1.6466357	0.319
At5g15500.1	ankyrin repeat family protein	-1.640	0.321	-1.78378007	0.290
At5g64000.1	3'(2'),5'-bisphosphate nucleotidase, putative / inositol polyphosphate 1-phosphatase	-1.610	0.328	-1.584829594	0.333

up in MP

GeneName	Description	M	fold change
At5g61410.1	ribulose-phosphate 3-epimerase, chloroplast	5.033	32.733
At1g52400.1	glycosyl hydrolase family 1 protein / beta-glucosidase	4.151	17.763
At2g26980.1	CBL-interacting protein kinase 3 (CIPK3)	3.309	9.908
At2g27420.1	cysteine proteinase	3.166	8.973
IR5:019956393-019956	Unknown	2.868	7.302
At2g37770.1	aldo/keto reductase family protein	2.772	6.830
At2g46300.1	expressed protein [At2g46300.1]	2.713	6.558
At3g29630.1	glycosyltransferase family protein	2.528	5.766
At1g50580.1	glycosyltransferase family protein	2.505	5.678
At2g33080.1	leucine-rich repeat family protein	2.503	5.670
At1g12060.1	IQ domain-containing protein / BAG domain-containing protein	2.436	5.413
At1g32740.1	expressed protein [At1g32740.1]	2.401	5.283
At2g27315.1	hypothetical protein [At2g27315.1]	2.397	5.268
At5g43910.3	pfkB-type carbohydrate kinase family protein	2.248	4.751
At1g47310.1	expressed protein [At1g47310.1]	2.245	4.739
At3g49340.1	cysteine proteinase	2.164	4.481
At1g72290.1	trypsin and protease inhibitor family protein / Kunitz family protein	2.083	4.238
At1g10070.1	branched-chain amino acid aminotransferase 2	2.061	4.172
At2g27230.1	transcription factor-related	2.030	4.084
At2g47130.1	short-chain dehydrogenase/reductase (SDR) family protein	2.000	4.000
At3g29130.1	expressed protein ; expression supported by MPSS [At3g29130.1]	1.889	3.703
At2g22360.1	DNAJ heat shock family protein	1.867	3.649
At2g31560.1	expressed protein [At2g31560.1]	1.865	3.643
At3g25020.1	disease resistance family protein contains leucine rich-repeat (LRR)	1.852	3.609
At2g27395.1	cysteine protease-related	1.807	3.500
At3g52870.1	calmodulin-binding family protein	1.801	3.485
At5g67100.1	DNA-directed DNA polymerase alpha catalytic subunit	1.773	3.418
At1g11440.1	expressed protein [At1g11440.1]	1.745	3.351
At5g48540.1	33 kDa secretory protein-related	1.733	3.324
At3g14080.1	small nuclear ribonucleoprotein	1.716	3.286
BP661428	Arabidopsis thaliana cDNA clone RAFL06-87-B05 3	1.711	3.275
At1g24150.1	formin homology 2 domain-containing protein	1.707	3.265
At3g56170.1	Ca(2+)-dependent nuclease	1.675	3.194
At5g26200.1	mitochondrial substrate carrier family protein	1.672	3.186
At3g12320.1	expressed protein [At3g12320.1]	1.670	3.181
At2g21650.1	myb family transcription factor	1.645	3.127
At4g20110.1	vacuolar sorting receptor	1.636	3.108
At5g04590.1	sulfite reductase / ferredoxin (SIR)	1.619	3.073
At1g26800.1	zinc finger (C3HC4-type RING finger) family protein	1.618	3.070
At5g25770.1	expressed protein [At5g25770.1]	1.604	3.039

up in HT

GeneName	Description	M	fold change
At1g06080.1	delta 9 desaturase (ADS1)	3.264	9.607
At1g56070.1	elongation factor 2	1.802	3.487

up in LT

GeneName	Description	M	fold change
At3g14230.2	AP2 domain-containing protein RAP2.2 (RAP2.2)	2.145	4.421
At3g10020.1	expressed protein [At3g10020.1]	1.674	3.192

up in HT and LT

GeneName	Description	M MT	fold change	M LT	fold change
At5g60100.1	pseudo-response regulator 3 (APRR3)	3.600	12.123	3.675079589	12.773
At5g54190.1	protochlorophyllide reductase A, chloroplast	3.355	10.230	2.303745124	4.937
At4g30650.1	hydrophobic protein	2.914	7.534	1.899864873	3.732
At5g24070.1	peroxidase family protein	2.686	6.435	2.785894875	6.897
At1g23205.1	invertase/pectin methylesterase inhibitor family protein	2.277	4.845	1.643970433	3.125
At4g25050.1	acyl carrier family protein	2.275	4.841	2.194527073	4.577
At4g03210.1	xyloglucan:xyloglucosyl transferase	2.228	4.686	2.343367194	5.075
At2g38900.1	serine protease inhibitor	2.136	4.396	2.484305314	5.596
At2g38720.1	microtubule associated protein (MAP65/ASE1) family protein	2.081	4.231	2.183570811	4.543
IR3:010474182-010474	Unknown	2.057	4.162	1.640312036	3.117
At4g39260.1	glycine-rich RNA-binding protein 8 (GRP8)	2.015	4.041	1.869758347	3.655
At5g18600.1	glutaredoxin family protein	1.946	3.853	1.782730313	3.441
At2g45180.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	1.915	3.770	1.6742412	3.192
At4g23820.1	glycoside hydrolase family 28 protein	1.865	3.642	1.765001677	3.399

up in MP and HT

GeneName	Description	M MP5	fold change	M MT	fold change
At1g58080.1	ATP phosphoribosyl transferase 1 (ATP-PRT1)	2.794	6.934	1.893760674	3.716
At1g69610.1	expressed protein [At1g69610.1]	2.063	4.180	1.202462087	2.301

up in MP and LT

GeneName	Description	M MP5	fold change	M LT	fold change
At4g28920.1	hypothetical protein	3.538	11.614	2.466622979	5.527
At4g38360.1	expressed protein	3.460	11.003	1.931267549	3.814
At3g11990.1	expressed protein [At3g11990.1]	2.287	4.880	1.613165022	3.059
At1g17890.1	GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase	2.045	4.126	1.676737799	3.197
At1g21550.1	calcium-binding protein	2.018	4.049	1.59158039	3.014

Accumulation arrays (leaf material)

down in high Ni/high Zn

GeneName	Description	M	fold change
At4g22870.1	eucoanthocyanidin dioxygenase	-4.409	0.047
At5g17220.1	glutathione S-transferase, putative [At5g17220.1]\""	-3.801	0.072
At5g54060.1	glycosyltransferase family protein	-3.697	0.077
At1g12030.1	expressed protein	-3.583	0.083
At4g10770.1	oligopeptide transporter OPT family protein	-3.384	0.096
At5g13930.1	chalcone synthase / naringenin-chalcone synthase identical to SP|P13114 [At5g13930.1]	-2.828	0.141
At5g48880.1	acetyl-CoA C-acyltransferase 1 / 3-ketoacyl-CoA thiolase 1 (PKT1)	-2.765	0.147
At1g23110.1	hypothetical protein [At1g23110.1]	-2.747	0.149
At1g73010.1	expressed protein	-2.640	0.160
At4g22880.1	leucoanthocyanidin dioxygenase	-2.629	0.162
At4g11820.1	hydroxymethylglutaryl-CoA synthase	-2.616	0.163
At3g48140.1	senescence-associated protein	-2.453	0.183
At3g54140.1	proton-dependent oligopeptide transport (POT) family protein	-2.448	0.183
At5g07990.1	flavonoid 3'-monooxygenase	-2.444	0.184
At3g57740.1	protein kinase family protein	-2.436	0.185
At1g73325.1	trypsin and protease inhibitor family protein / Kunitz family protein	-2.435	0.185
At1g01250.1	AP2 domain-containing transcription factor	-2.412	0.188
At4g04750.1	sugar transporter family protein	-2.370	0.193
At3g61880.1	cytochrome P450	-2.267	0.208
At4g14090.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	-2.211	0.216
At1g72290.1	trypsin and protease inhibitor family protein / Kunitz family protein	-2.203	0.217
At3g59140.1	ABC transporter family protein	-2.199	0.218
IR2:007087636-007087	Unknown	-2.153	0.225
At3g48410.1	hydrolase, alpha/beta fold family protein	-2.134	0.228
At5g44040.1	expressed protein	-2.118	0.230
At3g46670.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	-2.112	0.231
At1g14240.1	nucleoside phosphatase family protein	-2.090	0.235
At1g30040.1	gibberellin 2-oxidase / GA2-oxidase (GA2OX2)	-2.088	0.235
At5g26220.1	ChaC-like family protein	-2.077	0.237
At3g52820.1	purple acid phosphatase (PAP22)	-2.065	0.239
At4g33040.1	glutaredoxin family protein	-2.055	0.241
At5g14140.1	zinc finger (C2H2 type) family protein	-2.027	0.245
At5g20150.1	SPX (SYG1/Pho81/XPR1) domain	-2.010	0.248
At2g23000.1	serine carboxypeptidase S10 family protein	-1.995	0.251
At5g09220.1	amino acid permease 2 (AAP2)	-1.969	0.255
BX835149	Arabidopsis thaliana Flowers and buds Col-0 Arabidopsis thaliana cDNA clone	-1.950	0.259
At1g24140.1	matrixin family protein	-1.945	0.260
At3g48930.1	40S ribosomal protein S11 (RPS11A) [At3g48930.1]	-1.928	0.263
At3g48540.1	cytidine/deoxycytidylate deaminase family protein	-1.926	0.263
At5g42180.1	peroxidase 64 (PER64) (P64) (PRXR4)	-1.922	0.264
At3g12750.1	zinc transporter (ZIP1)	-1.919	0.264
At5g59220.1	protein phosphatase 2C	-1.900	0.268
At3g62400.1	expressed protein	-1.879	0.272
At1g32100.1	pinoresinol-lariciresinol reductase	-1.875	0.273
At1g05300.1	metal transporter	-1.860	0.276
At3g45140.1	lipoxygenase (LOX2)	-1.847	0.278
At5g64410.1	oligopeptide transporter OPT family protein	-1.781	0.291
At2g25625.1	expressed protein [At2g25625.1]	-1.745	0.298
At3g52060.1	expressed protein	-1.743	0.299
At1g80760.1	major intrinsic family protein / MIP family protein	-1.734	0.301
At2g24190.1	short-chain dehydrogenase/reductase (SDR) family protein	-1.728	0.302
At1g10970.1	metal transporter	-1.723	0.303
At2g43580.1	chitinase	-1.721	0.303
At3g53880.1	aldo/keto reductase family protein	-1.718	0.304
IR3:020384747-020384	Unknown	-1.709	0.306
TC255286	Q9LR73 (Q9LR73) F21B7.12, complete [TC255286]\""	-1.706	0.306
At3g52780.1	purple acid phosphatase (PAP20)	-1.682	0.312
At3g47420.1	glycerol-3-phosphate transporter	-1.679	0.312
At3g47000.1	glycosyl hydrolase family 3 protein beta-D-glucan exohydrolase	-1.629	0.323
At3g55060.1	expressed protein	-1.629	0.323
At5g55730.1	fasciclin-like arabinogalactan-protein (FLA1)	-1.614	0.327
At1g07220.1	expressed protein [At1g07220.1]	-1.611	0.327
At4g21910.1	MATE efflux family protein	-1.611	0.327
At4g34850.1	chalcone and stilbene synthase family protein	-1.600	0.330

down in high Ni/low Zn

GeneName	Description	M	fold change
At1g12030.1	expressed protein	-3.374	0.096
At4g10770.1	oligopeptide transporter OPT family protein	-2.786	0.145
At5g57440.1	haloacid dehalogenase-like hydrolase family protein]	-2.694	0.155
At3g16857.2	two-component responsive regulator family protein	-2.617	0.163
At5g55730.1	fasciclin-like arabinogalactan-protein (FLA1)	-1.879	0.272
At1g09930.1	oligopeptide transporter OPT family protein	-1.870	0.274
At3g11990.1	expressed protein [At3g11990.1]	-1.850	0.277
At5g22830.1	magnesium transporter CorA-like family protein	-1.765	0.294
At3g19260.1	longevity-assurance (LAG1) family protein	-1.757	0.296
At5g57900.1	SKP1/ASK1 interacting partner 1 (SKIP1)	-1.755	0.296
At5g58560.1	phosphatidate cytidylyltransferase family protein	-1.731	0.301
At1g17710.1	expressed protein [At1g17710.1]	-1.730	0.301
IR3:019997787-019997	Unknown	-1.717	0.304
At3g57740.1	protein kinase family protein	-1.601	0.330
At3g61880.1	cytochrome P450	-1.593	0.331
At3g18830.1	mannitol transporter	-1.590	0.332

down in low Ni/high Zn

GeneName	Description	M	fold change
At1g78000.1	sulfate transporter (Sultr1;2)	-1.773	0.293

down in high Ni/high Zn and high Ni/low Zn

GeneName	Description	M high Ni/high Zn	fold change	M high Ni/low Zn	fold change
At1g12030.1	expressed protein	-3.583	0.083	-3.374	0.096
At4g10770.1	oligopeptide transporter OPT family protein	-3.384	0.096	-2.786	0.145
At3g57740.1	protein kinase family protein	-2.436	0.185	-1.601	0.330
At3g61880.1	cytochrome P450	-2.267	0.208	-1.593	0.331

down in high Ni/high Zn and low Ni/high Zn

GeneName	Description	M high Ni/high Zn	fold change	M low Ni/high Zn	fold change
At1g78000.1	sulfate transporter (Sultr1;2)	-1.173	0.443	-1.773	0.293

Accumulation arrays (root material)

down in high Ni/high Zn

GeneName	Description	M	fold change
At5g15490.1	UDP-glucose 6-dehydrogenase	-3.883	0.068
At2g29910.1	F-box family protein	-3.543	0.086
At5g05700.1	arginine-tRNA-protein transferase 1 / arginyltransferase 1	-3.099	0.117
At5g53450.1	protein kinase family protein	-3.082	0.118
At1g21400.1	2-oxoisovalerate dehydrogenase	-2.939	0.130
At5g13370.1	auxin-responsive GH3 family protein	-2.910	0.133
At1g08630.1	L-allo-threonine aldolase-related	-2.767	0.147
At5g02780.1	In2-1 protein	-2.749	0.149
At4g39510.1	cytochrome P450 family protein	-2.701	0.154
At1g52130.1	jacalin lectin family protein	-2.631	0.161
At5g16790.1	expressed protein [At5g16790.1]	-2.617	0.163
At4g13530.1	expressed protein	-2.600	0.165
At1g24320.1	alpha-glucosidase	-2.406	0.189
At5g57440.1	haloacid dehalogenase-like hydrolase family protein	-2.388	0.191
At5g54370.1	late embryogenesis abundant protein-related / LEA protein-related	-2.357	0.195
At3g12900.1	oxidoreductase, 2OG-Fe(II) oxygenase family protein	-2.252	0.210
At5g20890.1	chaperonin	-2.219	0.215
At5g14180.1	lipase family protein	-2.209	0.216
At4g15530.1	pyruvate phosphate dikinase family protein	-2.196	0.218
At5g01330.1	pyruvate decarboxylase	-2.183	0.220
At1g22780.1	40S ribosomal protein S18 (RPS18A)	-2.134	0.228
At2g43520.1	trypsin inhibitor	-2.115	0.231
At1g47620.1	cytochrome P450	-2.090	0.235
At1g35260.1	Bet v I allergen family protein	-2.075	0.237
At4g17340.1	major intrinsic family protein / MIP family protein	-2.006	0.249
At1g52410.1	caldesmon-related	-2.000	0.250
At3g16120.1	dynein light chain	-1.991	0.252
At3g06850.2	branched chain alpha-keto acid dehydrogenase E2 subunit (din3)	-1.969	0.255
At5g45105.1	metal transporter	-1.966	0.256
At3g48580.1	xyloglucan:xyloglucosyl transferase	-1.953	0.258
At5g35830.1	ankyrin repeat family protein	-1.952	0.259
IR5:025813866-025813	Unknown	-1.941	0.260
At5g66560.1	phototropic-responsive NPH3 family protein	-1.887	0.270
At5g64530.1	no apical meristem (NAM) family protein	-1.850	0.277
At1g73040.1	jacalin lectin family protein	-1.819	0.283
At5g16710.1	dehydroascorbate reductase	-1.815	0.284
At3g51200.1	auxin-responsive family protein	-1.799	0.287
At5g67370.1	expressed protein	-1.753	0.297
At2g38390.1	peroxidase	-1.731	0.301
At1g69490.1	no apical meristem (NAM) family protein	-1.709	0.306
At5g14260.1	SET domain-containing protein	-1.674	0.313
At4g16130.1	GHMP kinase family protein	-1.629	0.323
At2g32610.1	cellulose synthase family protein	-1.628	0.324
At5g02150.1	expressed protein [At5g02150.1]	-1.626	0.324
At3g10720.1	pectinesterase	-1.622	0.325
At5g03830.1	expressed protei	-1.622	0.325
At5g67385.1	phototropic-responsive protein	-1.614	0.327
At1g50060.1	pathogenesis-related protein	-1.612	0.327
At4g01995.1	expressed protein [At4g01995.1]	-1.594	0.331
At4g34030.1	methylcrotonyl-CoA carboxylase beta chain	-1.588	0.333

down in high Ni/low Zn

GeneName	Description	M	fold change
At2g29910.1	F-box family protein	-3.543	0.086
At1g52130.1	jacalin lectin family protein	-2.631	0.161
At5g54370.1	late embryogenesis abundant protein-related / LEA protein-related	-2.357	0.195
At2g43520.1	trypsin inhibitor, putative	-2.115	0.231
At5g66560.1	phototropic-responsive NPH3 family protein	-1.887	0.270
At2g38390.1	peroxidase	-1.731	0.301
At3g10720.1	pectinesterase[At3g10720.1]\""	-1.622	0.325
At1g50060.1	pathogenesis-related protein	-1.612	0.327

down in low Ni/high Zn

GeneName	Description	M	fold change
At2g23960.1	defense-related protein	-4.483	0.045
BP570267	Arabidopsis thaliana green siliques Columbia Arabidopsis thaliana cDNA clone	-3.956	0.064
At3g42540.1	hypothetical protein [At3g42540.1]	-3.237	0.106
At5g65440.1	expressed protein [At5g65440.1]	-3.002	0.125
At5g49360.1	glycosyl hydrolase family 3 protein [At5g49360.1]	-2.882	0.136
At2g25690.1	senescence-associated protein-related	-2.573	0.168
At4g17650.1	aromatic-rich family protein	-2.563	0.169
At1g15040.1	glutamine amidotransferase-related [At1g15040.1]	-2.540	0.172
IR1:002086293-002086	Unknown	-2.451	0.183
At2g46060.2	transmembrane protein-related	-2.308	0.202
At1g68300.1	universal stress protein (USP) family protein	-2.214	0.216
At2g45530.1	zinc finger (C3HC4-type RING finger) family protein	-2.193	0.219
At5g03990.1	expressed protein	-2.190	0.219
At3g19370.1	expressed protein [At3g19370.1]	-2.133	0.228
At1g75190.1	expressed protein [At1g75190.1]	-2.131	0.228
At5g44040.1	expressed protein similar to unknown protein (gb|AAD10667.1) [At5g44040.1]	-2.115	0.231
IR5:004297500-004297	Unknown	-2.061	0.240
At3g54290.1	expressed protein	-2.041	0.243
At5g22920.1	zinc finger (C3HC4-type RING finger) family protein	-2.033	0.244
At1g75260.1	isoflavone reductase family protein	-2.021	0.246
At1g55810.1	uracil phosphoribosyltransferase	-1.995	0.251
At5g46860.1	syntaxin 22 (SYP22) (VAM3)	-1.995	0.251
At5g40740.1	expressed protein [At5g40740.1]	-1.982	0.253
At5g55960.1	expressed protein [At5g55960.1]	-1.964	0.256
At3g11690.1	expressed protein [At3g11690.1]	-1.958	0.257
At4g15910.1	drought-responsive protein	-1.948	0.259
At3g48930.1	40S ribosomal protein S11 (RPS11A) [At3g48930.1]	-1.945	0.260
At5g04750.1	F1F0-ATPase inhibitor protein	-1.845	0.278
IR1:003491913-003491	Unknown	-1.828	0.282
At3g56090.1	ferritin	-1.823	0.283
At1g21980.1	1-phosphatidylinositol-4-phosphate 5-kinase	-1.805	0.286
At5g45020.1	expressed protein [At5g45020.1]	-1.768	0.294
At3g47000.1	glycosyl hydrolase family 3 protein	-1.744	0.299
At3g62400.1	expressed protein	-1.738	0.300
At2g19730.1	60S ribosomal protein L28 (RPL28A) [At2g19730.1]	-1.725	0.303
At5g45500.1	expressed protein	-1.719	0.304
At5g66650.1	expressed protein	-1.711	0.305
IR1:026231405-026231	Unknown	-1.708	0.306
At1g67330.1	expressed protein	-1.706	0.306
At4g21910.1	MATE efflux family protein	-1.657	0.317
At1g01560.1	mitogen-activated protein kinase	-1.623	0.325
At1g09500.1	cinnamyl-alcohol dehydrogenase family / CAD family	-1.608	0.328
At1g69325.1	remorin family protein	-1.592	0.332

down in high Ni/high Zn and high Ni/low Zn

GeneName	Description	M high Ni/high Zn	fold change	M high Ni/low Zn	fold change
At5g02780.1	In2-1 protein	-2.749	0.149	-1.942244495	0.260
At5g20890.1	chaperonin	-2.219	0.215	-1.807710688	0.286
At1g35260.1	Bet v I allergen family protein	-2.075	0.237	-2.708094445	0.153
At1g52410.1	caldesmon-related	-2.000	0.250	-2.116224683	0.231

down in high Ni/high Zn and low Ni/high Zn

GeneName	Description	M high Ni/high Zn	fold change	M low Ni/high Zn	fold change
At4g39510.1	cytochrome P450 family protein	-2.701	0.154	-2.795736358	0.144
At4g13530.1	expressed protein predicted protein	-2.600	0.165	-1.930024895	0.262
At4g15530.1	pyruvate phosphate dikinase family protein	-2.196	0.218	-2.062084813	0.239
At1g22780.1	40S ribosomal protein S18 (RPS18A)	-2.134	0.228	-1.876155646	0.272
At4g17340.1	major intrinsic family protein / MIP family protein	-2.006	0.249	-2.098804568	0.233
At3g16120.1	dynein light chain	-1.991	0.252	-1.683813362	0.311
At5g64530.1	no apical meristem (NAM) family protein	-1.850	0.277	-1.855390644	0.276

down in high Ni/high Zn, high Ni/low Zn and low Ni/high Zn

GeneName	Description	M high Ni/high Zn	fold change	M high Ni/low Zn	fold change	M low Ni/high Zn	fold change
At1g08630.1	L-allo-threonine aldolase-related	-2.767	0.147	-1.860910563	0.275	-2.624269629	0.162

Accumulation arrays (leaf material)

up in high Ni/high Zn

GeneName	Description	M	fold change
At3g14230.2	AP2 domain-containing protein RAP2.2 (RAP2.2)	3.892	14.849
At2g33830.1	dormancy/auxin associated family protein	3.642	12.482
At5g20630.1	germin-like protein (GER3)	3.489	11.227
At3g26740.1	light responsive protein-related	3.220	9.315
At1g26945.1	expressed protein	3.130	8.756
At3g61080.1	fructosamine kinase family protein	3.094	8.540
At2g45180.1	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	3.002	8.014
At1g76430.1	phosphate transporter family protein	2.994	7.969
At3g53670.1	expressed protein [At3g53670.1]	2.974	7.858
At4g15660.1	glutaredoxin family protein	2.731	6.637
At5g54190.1	protochlorophyllide reductase A, chloroplast	2.699	6.493
At3g47630.1	expressed protein [At3g47630.1]	2.667	6.353
At3g53440.1	expressed protein [At3g53440.1]	2.632	6.198
At3g54810.1	zinc finger (GATA type) family protein	2.605	6.082
At3g16240.1	delta tonoplast integral protein (delta-TIP)	2.593	6.032
At3g48560.1	acetolactate synthase, chloroplast / acetohydroxy-acid synthase (ALS)	2.558	5.889
At2g18300.1	basic helix-loop-helix (bHLH) family protein	2.548	5.849
At1g71010.1	phosphatidylinositol-4-phosphate 5-kinase family protein	2.536	5.798
At2g18550.1	homeobox-leucine zipper family protein	2.471	5.544
At1g51200.1	zinc finger (AN1-like) family protein	2.426	5.374
At3g58120.1	bZIP transcription factor family protein	2.411	5.320
At1g73120.1	expressed protein [At1g73120.1]	2.399	5.274
At1g80440.1	kelch repeat-containing F-box family protein	2.337	5.053
At1g77210.1	sugar transporter	2.334	5.041
At1g78380.1	glutathione S-transferase	2.323	5.004
At3g53800.1	armadillo/beta-catenin repeat family protein	2.308	4.953
At2g34080.1	cysteine proteinase	2.307	4.947
At3g62200.1	expressed protein	2.296	4.910
At1g71030.1	myb family transcription factor	2.266	4.809
At1g69610.1	expressed protein [At1g69610.1]	2.254	4.769
At5g42680.1	expressed protein	2.250	4.757
At5g42680.1	expressed protein	2.250	4.757
At5g14740.1	carbonic anhydrase 2 / carbonate dehydratase 2 (CA2) (CA18)	2.243	4.735
At3g46130.1	myb family transcription factor (MYB48)	2.227	4.681
At3g19030.1	expressed protein contains	2.226	4.680
At3g54400.1	aspartyl protease family protein	2.213	4.636
At3g15270.1	squamosa promoter-binding protein-like 5 (SPL5)	2.184	4.546
At3g57520.1	alkaline alpha galactosidase	2.182	4.538
At3g48100.1	two-component responsive regulator / response regulator 5 (ARR5) / response reactor 2 (RR2)	2.167	4.490
IR1:018314380-018314	Unknown	2.162	4.475
At3g62550.1	universal stress protein (USP) family protein	2.114	4.329
At3g54830.1	amino acid transporter family protein	2.101	4.289
At2g07520.1	hypothetical protein [At2g07520.1]	2.096	4.276
At1g68520.1	zinc finger (B-box type) family protein	2.092	4.265
At1g51830.1	\leucine-rich repeat protein kinase	2.085	4.242
At3g51250.1	senescence/dehydration-associated protein	2.082	4.235
At5g17990.1	anthranilate phosphoribosyltransferase	2.041	4.114
At4g39510.1	cytochrome P450 family protein	2.000	4.001
At2g39480.1	ABC transporter family protein	1.976	3.933
At4g39260.1	glycine-rich RNA-binding protein 8 (GRP8) (CCR1)	1.968	3.911
At3g62220.1	serine/threonine protein kinase	1.965	3.905
At2g24240.1	potassium channel tetramerisation domain-containing protein	1.956	3.880
At1g03870.1	fasciclin-like arabinogalactan-protein (FLA9)	1.933	3.818
At3g15450.1	expressed protein	1.914	3.769
At5g67100.1	DNA-directed DNA polymerase alpha catalytic subuni	1.902	3.738
At5g28770.1	bZIP transcription factor family protein	1.897	3.724
At3g50560.1	short-chain dehydrogenase/reductase (SDR) family protein	1.891	3.708
At3g23880.1	F-box family protein contains F-box domain Pfam:PF00646 [At3g23880.1]	1.853	3.613
At1g55630.1	pentatricopeptide (PPR) repeat-containing protein	1.830	3.556
At2g47970.1	NPL4 family protein	1.823	3.538
At3g50830.1	stress-responsive protein	1.817	3.523
At2g36910.1	multidrug resistance P-glycoprotein (PGP1)	1.802	3.488
At2g37130.1	peroxidase 21 (PER21) (P21) (PRXR5)	1.784	3.444
At4g34790.1	auxin-responsive family protein	1.766	3.401
At1g22570.1	proton-dependent oligopeptide transport (POT) family protein	1.765	3.399
At2g01755.1	hypothetical protein [At2g01755.1]	1.754	3.373
At5g66400.1	dehydrin (RAB18)	1.754	3.372
At3g49590.1	expressed protein [At3g49590.1]	1.738	3.335
At5g35740.1	glycosyl hydrolase family protein 17	1.734	3.325
At5g04310.1	pectate lyase family protein	1.713	3.279
At1g68840.1	DNA-binding protein RAV2 (RAV2)	1.706	3.262
At1g47960.1	invertase/pectin methylesterase inhibitor family protein	1.682	3.209
At1g53990.1	GDSL-motif lipase/hydrolase family protein	1.658	3.156
At1g55350.1	calpain-type cysteine protease family	1.642	3.121
At4g28080.1	expressed protein [At4g28080.1]	1.641	3.120
IR1:011194427-011194	Unknown	1.615	3.064
At4g21870.1	26.5 kDa class P-related heat shock protein (HSP26.5-P)	1.610	3.053
At4g03210.1	xyloglucan:xyloglucosyl transferase	1.598	3.028
At4g01870.1	tolB protein-related	1.591	3.013

up in high Ni/low Zn

GeneName	Description	M	fold change
At3g14230.2	AP2 domain-containing protein RAP2.2 (RAP2.2)	3.599	12.113
At1g26945.1	expressed protein	2.575	5.959
At3g53670.1	expressed protein [At3g53670.1]	2.334	5.041
At5g17700.1	MATE efflux family protein	2.300	4.923
At3g47630.1	expressed protein [At3g47630.1]	2.234	4.705
At5g54190.1	protochlorophyllide reductase A, chloroplast	2.201	4.597
At3g16240.1	delta tonoplast integral protein (delta-TIP)	2.107	4.309
At3g53440.1	expressed protein [At3g53440.1]	2.011	4.030
At5g66400.1	dehydrin (RAB18)	1.984	3.955
At5g17990.1	anthranilate phosphoribosyltransferase	1.949	3.861
At3g54810.1	zinc finger (GATA type) family protein	1.921	3.786
At1g19730.1	thioredoxin H-type 4 (TRX-H-4) (GREN)	1.883	3.689
At5g42680.1	expressed protein	1.798	3.478
At3g10020.1	expressed protein [At3g10020.1]	1.788	3.454
At3g48560.1	acetolactate synthase, chloroplast / acetohydroxy-acid synthase (ALS)	1.710	3.272
At3g50830.1	stress-responsive protein	1.665	3.171
At1g71010.1	phosphatidylinositol-4-phosphate 5-kinase family protein	1.631	3.097
At5g67100.1	DNA-directed DNA polymerase alpha catalytic subunit	1.624	3.082
At3g61080.1	fructosamine kinase family protein	1.609	3.050

up in low Ni/high Zn

GeneName	Description	M	fold change
At5g66400.1	dehydrin (RAB18)	3.170	9.003
At1g62760.1	invertase/pectin methylesterase inhibitor family protein	3.035	8.197
At5g54190.1	protochlorophyllide reductase A, chloroplast	2.842	7.168
At1g26945.1	expressed protein	2.368	5.162
At3g54810.1	zinc finger (GATA type) family protein	2.003	4.010
At1g78380.1	glutathione S-transferase	1.938	3.831
At3g48560.1	acetolactate synthase, chloroplast / acetohydroxy-acid synthase (ALS)	1.738	3.336
At3g50830.1	stress-responsive protein	1.684	3.214
At3g53440.1	expressed protein [At3g53440.1]	1.609	3.051

up in high Ni/high Zn and high Ni/low Zn

GeneName	Description	M high Ni/high Zn	fold change	M high Ni/low Zn	fold change
At3g14230.2	AP2 domain-containing protein RAP2.2 (RAP2.2)	3.892	14.849	3.598515265	12.113
At3g61080.1	fructosamine kinase family protein	3.094	8.540	1.608921937	3.050
At3g53670.1	expressed protein [At3g53670.1]	2.974	7.858	2.33368766	5.041
At3g47630.1	expressed protein [At3g47630.1]	2.667	6.353	2.234333078	4.705
At3g16240.1	delta tonoplast integral protein (delta-TIP)	2.593	6.032	2.107421169	4.309
At1g71010.1	phosphatidylinositol-4-phosphate 5-kinase family protein	2.536	5.798	1.630693792	3.097

up in high Ni/low Zn and low Ni/high Zn

GeneName		M high Ni/low Zn	fold change	M low Ni/ high Zn	fold change
At1g26945.1	expressed protein	2.575	5.959	2.367856353	5.162
At3g48560.1	acetolactate synthase, chloroplast	1.710	3.272	1.737915446	3.336
At3g50830.1	stress-responsive protein	1.665	3.171	1.684402658	3.214
At3g53440.1	expressed protein [At3g53440.1]	2.011	4.030	1.609131937	3.051
At3g54810.1	zinc finger (GATA type) family protein	1.921	3.786	2.003440157	4.010
At5g54190.1	protochlorophyllide reductase A, chloroplast	2.201	4.597	2.841585379	7.168
At5g66400.1	dehydrin (RAB18)	1.984	3.955	3.170455183	9.003

up in high Ni/high Zn and low Ni/ high Zn

GeneName	Description	M high Ni/high Zn	fold change	M low Ni/ high Zn	fold change
At1g78380.1	glutathione S-transferase	2.323	5.004	1.937844675	3.831

up in high Ni/high Zn, high Ni/low Zn and low Ni/high Ni

GeneName	Description	M high Ni/ high Zn	fold change	M high Ni/ low Zn	fold change	M low Ni/ high Zn	fold change
At1g26945.1	expressed protein	3.130	8.756	2.574951442	5.959	2.367856353	5.162
At5g54190.1	protochlorophyllide reductase A, chloroplast	2.699	6.493	2.200753461	4.597	2.841585379	7.168
At3g53440.1	expressed protein [At3g53440.1]	2.632	6.198	2.010810334	4.030	1.609131937	3.051
At3g54810.1	zinc finger (GATA type) family protein	2.605	6.082	1.920728966	3.786	2.003440157	4.010
At3g48560.1	acetolactate synthase, chloroplast / acetohydroxy-acid synthase (ALS)	2.558	5.889	1.71030964	3.272	1.737915446	3.336
At3g50830.1	stress-responsive protein	1.817	3.523	1.665050417	3.171	1.684402658	3.214
At5g66400.1	dehydrin (RAB18)	1.754	3.372	1.983692258	3.955	3.170455183	9.003

Accumulation arrays (root material)

up in high Ni/high Zn

GeneName	Description	M	fold change
At4g02580.1	NADH-ubiquinone oxidoreductase 24 kDa subunit	1.779	3.433
At4g13370.1	expressed protein [At4g13370.1]	1.710	3.272

up in high Ni/low Zn

GeneName	Description	M	fold change
At3g26740.1	light responsive protein-related	3.456	10.971
At1g71050.1	heavy-metal-associated domain-containing protein / copper chaperone (CCH)-related	3.405	10.589
At1g05680.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	3.246	9.487
At2g03760.1	steroid sulfotransferase	3.198	9.174
At4g37390.1	auxin-responsive GH3 family protein	3.155	8.910
At1g69410.1	eukaryotic translation initiation factor 5A,	3.082	8.465
At1g78380.1	glutathione S-transferase	2.560	5.898
At3g14630.1	cytochrome P450	2.408	5.307
At1g32350.1	alternative oxidase	2.291	4.893
At4g37720.1	phytosulfokines-related	2.284	4.872
At1g58180.2	carbonic anhydrase family protein / carbonate dehydratase family protein	2.284	4.871
At4g28460.1	hypothetical protein [At4g28460.1]	2.199	4.593
At5g59820.1	zinc finger (C2H2 type) family protein (ZAT12)	2.188	4.556
At5g57220.1	cytochrome P450	2.138	4.402
At1g64660.1	Cys/Met metabolism pyridoxal-phosphate-dependent enzyme family protein	2.099	4.285
At1g10370.1	glutathione S-transferase, putative (ERD9)	2.083	4.238
At1g11925.1	stigma-specific Stig1 family protein	2.025	4.071
At4g25200.1	23.6 kDa mitochondrial small heat shock protein (HSP23.6-M)	1.996	3.989
At2g23170.1	auxin-responsive GH3 family protein	1.855	3.617
At4g37370.1	cytochrome P450	1.845	3.593
At5g64250.1	2-nitropropane dioxygenase family / NPD family	1.786	3.449
At1g11320.1	expressed protein	1.785	3.446
At3g23240.1	ethylene-responsive factor 1 / ethylene response factor 1 (ERF1)	1.772	3.415
At3g53160.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein	1.733	3.325
At3g63380.1	calcium-transporting ATPase, plasma membrane-type	1.657	3.153
At3g46090.1	\zinc finger (C2H2 type) family protein (ZAT7)	1.654	3.146
At4g37320.1	cytochrome P450 family protein [At4g37320.1]	1.616	3.064
At2g38465.1	expressed protein [At2g38465.1]	1.587	3.004

up in low Ni/high Zn

GeneName	Description	M	fold change
At5g65870.1	phytosulfokines 5 (PSK5)	3.027	8.150
At1g04270.1	40S ribosomal protein S15 (RPS15A)	2.216	4.645
NP453084	En/Spm-like transposon protein [Arabidopsis thaliana] [NP453084]	1.974	3.929
At3g25190.1	nodulin	1.914	3.769
At3g29680.1	transferase family protein	1.815	3.519
At5g64410.1	oligopeptide transporter OPT family protein	1.787	3.450
At5g07200.1	gibberellin 20-oxidase identical to GI:1109699 [At5g07200.1]	1.731	3.320
At3g29060.1	EXS family protein / ERD1/XPR1/SYG1 family protein	1.661	3.163
At4g14430.1	enoyl-CoA hydratase/isomerase family protein	1.618	3.069
At5g38100.1	methyltransferase-related	1.614	3.060

up in high Ni/low Zn and low Ni/high Zn

GeneName	Description	M high Ni/low Zn	fold change	M low Ni/high Zn	fold change
At1g71010.1	phosphatidylinositol-4-phosphate 5-kinase family protein	5.091	34.075	5.243548232	37.885
At1g65690.1	harpin-induced protein-related / HIN1-related / harpin-responsive protein-related	3.492	11.253	3.698201037	12.980
At1g19960.1	expressed protein [At1g19960.1]	2.536	5.801	2.162181812	4.476
At1g21310.1	proline-rich extensin-like family protein	2.493	5.630	2.808991234	7.008
At5g59380.1	methyl-CpG-binding domain-containing protein	2.322	5.000	3.060841307	8.345
At1g66260.1	RNA and export factor-binding protein	2.136	4.395	2.463741866	5.516
At1g69640.1	acid phosphatase	2.102	4.292	1.78156605	3.438
At2g17840.1	senescence/dehydration-associated protein-related (ERD7)	1.881	3.683	2.086482395	4.247
At1g68920.1	basic helix-loop-helix (bHLH) family protein	1.698	3.245	1.729149682	3.315
At1g71780.1	expressed protein [At1g71780.1]	1.662	3.165	2.646103903	6.260