Genes, Genomes and Genomics ©2007 Global Science Books

Genetics and Genomics of the Heavy Metal Hyperaccumulator Model Species *Thlaspi caerulescens*

Antoine X. Deniau[1] • Henk Schat[1] • Mark G. M. Aarts[2*]

[1] Ecology and Physiology of Plants, Vrije Universiteit Amsterdam, de Boelelaan 1087, 1081 HV Amsterdam, The Netherlands
[2] Laboratory of Genetics, Wageningen University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands

Corresponding author: * **mark.aarts@wur.nl**

ABSTRACT

In the last decade heavy metal hyperaccumulator plants have been increasingly studied, mainly because of their potential use in phytoremediation. *Thlaspi caerulescens* is an attractive model hyperaccumulator plant, because it accommodates a high of intra-specific variation in the degrees and metal-specificity patterns of tolerance and accumulation. In this review we give an overview of recent progress made in the genetics and genomics of heavy metal hyperaccumulation in this species. QTL analysis for zinc and cadmium accumulation in segregating inter-accession crosses demonstrated that these traits are controlled by multiple genes and that there are accession-specific accumulation mechanisms with distinct metal-affinity patterns. Cross-species transcriptome analyses have revealed a large number of genes with differential expression between hyperaccumulators and non-hyperaccumulators. Many of those genes are known to be involved in metal homeostasis, and an even larger number might play a role in this process. However, most of the differentially expressed genes have probably no role in metal homeostasis, owing to the fact that species with different life history and ecology are compared. To confirm the role of candidate genes, mutant research is necessary, but not yet done in hyperaccumulators. In the absence of physical maps and full genome sequences of hyperaccumulators, comparative genomics are indispensable. Co-linearity and micro-synteny analysis should enable the identification of the genes responsible for QTL for accumulation traits in intra- and inter-specific crosses.

Keywords: *Arabidopsis halleri*, *Arabidopsis thaliana*, cadmium, hyperaccumulation, micro-arrays, proteomics, QTL analysis, transcriptomics, zinc

Abbreviations: GA, Ganges; **LC**, La Calamine; **LE**, Lellingen; **PR**, Prayon

CONTENTS

INTRODUCTION

A relatively small number of plant species, generally referred to as metal hyperaccumulators (Brooks *et al.* 1977), can accumulate very high levels of a limited number of metals in their foliage (Baker and Brooks 1989). Metal-hyperaccumulating plants have been defined as plants that accumulate more than 1000 μg g^{-1} nickel, 10 000 μg g^{-1} zinc, or 100 μg g^{-1} cadmium in their leaves, on a dry weight basis, when growing in nature (Reeves 1988; Baker and Brooks 1989; Reeves 1992; Baker *et al.* 2000). The perspective of phytoremediation, i.e. the use of plants to remediate polluted soil by extracting or detoxifying the pollutant, has greatly increased the interest for metal-hyperaccumulator plants, due to their superior ability to accumulate metals in their above-ground parts (Baker *et al.* 1991; Pollard 2002). Much progress has been made over the last decade in understanding the physiology and molecular mechanisms of metal hyperaccumulation (see reviews by Pollard *et al.* 2002; Macnair 2003; McGrath and Zhao 2003). However, a robust evolutionary explanation of the hyperaccumulation traits is still lacking. Boyd and Martens (1992) and Boyd (1998) discussed several hypotheses concerning the possible selective advantage of hyperaccumulation, including metal disposal, drought resistance, elemental allelopathy, elemental biotic stress defence, and inadvertent uptake. Of these hypotheses the "metal defence" hypothesis is best supported by experimental data, showing that several invertebrate herbivores and fungi prefer low metal containing plants over high metal containing ones (Boyd *et al.* 2002; Hanson *et al.* 2003; Huitson and Macnair 2003; Jiang *et al.* 2005; Noret *et al.* 2005). Metals can protect plants from biotic stresses in different ways, acting either as an anti-feedant or as a plant systemic pesticide (Poschenrieder *et al.* 2006). However, the different evolutionary explanations for hyperaccumulation are not mutually exclusive, and it is conceivable that various selective factors have contributed to the evolution of the trait.

Many of the European hyperaccumulators belong to the Brassicaceae plant family, in particular to the genera *Thlaspi* and *Alyssum*. Among the Brassicaceae, *Thlaspi caerulescens* J. and C. Presl is suggested to be an attractive genetic model species because of its small plant size, relatively short generation time, abundant seed production, self-com-

Received: 28 February, 2007. Accepted: 16 April, 2007.

Invited Review

patibility and diploidy (Assunção *et al.* 2003a; Peer *et al.* 2003, 2006). Another advantage is its close relationship with the universal plant model species *Arabidopsis thaliana* L. Heynh. (with on average 88.5% DNA identity in coding regions (Rigola *et al.* 2006)), which provides the opportunity to use molecular genetic tools originally developed for Arabidopsis such as micro-array analysis, gene function analysis and genome analysis for (comparative) analyses in *T. caerulescens*. Another reason to choose *T. caerulescens* as a model hyperaccumulator species is that it is a facultative metallophyte with many metallicolous and non-metallicolous accessions, each with a different degree and metal-specificity pattern with regard to accumulation and tolerance (Assunção *et al.* 2003a; Peer *et al.* 2003, 2006). This facilitates a detailed genetic analysis using intraspecific crosses segregating for tolerance- and/or hyperaccumulation-associated traits. The primary objective of this review is to concisely evaluate the progress made in the recent years in unravelling the genetic basis and the molecular mechanisms of zinc, cadmium and nickel hyperaccumulation in plants, with a special emphasis on *Thlaspi caerulescens*.

GENETIC ANALYSIS OF NATURAL VARIATION

Zinc hyperaccumulation is a constitutive trait in *T. caerulescens*, with so far no accessions reported that do not hyperaccumulate zinc. However, under controlled conditions plants from non-metallicolous populations usually accumulate zinc to higher concentrations in their foliage than plants from metallicolous populations (Escarré *et al.* 2000; Dechamps *et al.* 2007). The phenotype distribution patterns in progenies of reciprocal crosses between plants of different origin suggested that low zinc accumulation is generally dominant over high zinc accumulation and that there inheritance of zinc accumulation is all nuclear encoded (Frérot *et al.* 2003). In all the studies done so far, the phenotype distributions for root or shoot zinc accumulation in genetically segregating progenies obtained by interaccession crosses were continuous rather than bimodal, suggesting that the intra-specific variation in zinc accumulation is controlled by multiple genes (Zha *et al.* 2004; Assunção *et al.* 2006; Deniau *et al.* 2006). In two of these studies, the phenotypes of the F_2 and F_3 progenies obtained after interaccession crosses indicated significant transgression beyond the parental phenotypes (Zha *et al.* 2004; Deniau *et al.* 2006), suggesting that the trait enhancing alleles at the different zinc accumulation loci originated from both of the parental accessions.

Some accessions of *T. caerulescens* are also cadmium and nickel hyperaccumulators, but whether hyperaccumulation of these metals is also constitutive at the species level is a matter of definition. In all the accessions studied thus far the concentrations of these metals were higher in the leaves than in the roots, which typify a hyperaccumulator. When expressed on a total plant weight basis, however, the total plant cadmium and nickel burdens of e.g. the accession LE (Luxemburg, non-metallicolous) were not always higher than in the non-hyperaccumulator congener *T. arvense* (Assunção *et al.* 2003b). Cadmium hyperaccumulation is particularly pronounced in the accessions from South-Eastern France, with accessions accumulating more than 1000 μg cadmium g^{-1} d.w. in the leaves (Escarré *et al.* 2000; Reeves *et al.* 2001). Analysis of progeny of a reciprocal F_2 cross between a plant from PR (metallicolous, Belgium) and one from the Ganges region (metallicolous, SE France) showed that high cadmium accumulation was partially dominant over low cadmium accumulation. Like for zinc accumulation, the phenotype distribution was continuous rather than bimodal, suggesting multiple genes were involved. In contrast to those for zinc accumulation, the segregation patterns for cadmium accumulation did not exhibit significant transgression, suggesting that the trait-enhancing alleles of the responsible loci originated exclusively from the GA parent (Zha *et al.* 2004). Similar results were obtained by Deniau *et al.* (2006), using a LC (metallicolous, Belgium) x GA interaccession cross. Detailed segregation analyses of nickel accumulation in inter-accession crosses have not been published yet.

One of the reasons that metal hyperaccumulators are able to accumulate some, but not all metals, is that the accumulated elements are either essential elements, and as such indispensable for plant growth (zinc, nickel and manganese), or they share similar chemical properties with essential elements, such as cadmium with zinc. It therefore seems obvious to assume some association between the molecular mechanisms controlling zinc and cadmium accumulation and tolerance and consequently, significant overlap in their genetic control. So far, this has been partially confirmed in genetic studies. Both in the LC x GA and the PR x GA crosses there was a significant, but far from strict, co-segregation of cadmium and zinc accumulation. This suggests that the metals are taken up by common transporter(s), at least in part, or that the metal transporters are controlled by common regulators. The possibility of common transporters for different metals has been reinforced by the occurrence of clear-cut antagonisms between different metals with regard to their accumulation (Zha *et al.* 2004; Deniau *et al.* 2006). Remarkably, these antagonisms are to a large extent accession-specific, suggesting that multiple accumulation systems with different metal-affinity patterns and differential expression among accessions are involved in the hyperaccumulation trait (Deniau *et al.* 2006). Based on the transgressive segregation pattern for zinc accumulation and the significant but weak genetic correlation of zinc and cadmium accumulation in the F_2 progenies, as well as the differential zinc/cadmium antagonisms in the parent accessions, Zha *et al.* (2004) proposed that zinc accumulation is largely governed by accession-specific systems. PR seems to use a system with high affinity for zinc but low affinity for cadmium, whereas GA predominantly uses a system with high affinity for cadmium, but low affinity for zinc. The latter system is likely to be responsible for the superior cadmium accumulation capacity in the GA accession, but it also accounts for its zinc accumulation capacity.

The quantitative genetic variation in accumulation capacity among accessions has been has been exploited to map the loci contributing to zinc (Assunção *et al.* 2006; Deniau *et al.* 2006) and cadmium (Deniau *et al.* 2006) hyperaccumulation using quantitative trait loci (QTL) analysis (Alonso-Blanco and Koornneef 2000). Assunção *et al.* (2006) provided the first genetic map of *T. caerulescens*, based on a cross between plants from the accessions LC and LE. No significant QTL were found to explain the observed variation for zinc accumulation in shoots in the segregating population, probably due to the small size of the mapping population. However, for zinc accumulation in roots two QTL were found with trait-enhancing alleles originating from both parents. Deniau *et al.* (2006) mapped QTL for cadmium and zinc accumulation in shoots and roots in a LC x GA cross, which confirmed that zinc and cadmium accumulation in roots and zinc accumulation in shoots, are controlled by more than one gene. As expected based on previous analysis, one of the zinc accumulation loci co-localized with a cadmium accumulation locus, with the trait-enhancing alleles originating from the GA parent. These QTL explained most of the genetic variance, both for zinc and for cadmium accumulation. In biological terms, this QTL may represent a gene which enhances the expression of a zinc/ cadmium uptake transporter with relatively high affinity for cadmium, such as proposed to be predominant in the high cadmium-accumulating accession GA (Zha *et al.* 2004). The nature of such gene is speculative, but most likely it encodes a transcription factor or the transporter itself, with differences in the promoter causing differences in expression. The remaining QTL, explaining less of the genetic variance, were metal specific, with the trait enhancing alleles for cadmium accumulation originating exclusively from the GA pa-rent and those for zinc accumulation coming from both GA and LC. The two QTL for zinc accumulation in roots were different from those found by As-

sunção *et al*. (2006), even though both populations shared one parent (LC). Taken together, the results of Assunção *et al.* (2006) and Deniau *et al.* (2006) revealed that there are at least four loci determining the inter-accession variation in zinc accumulation in roots. Depending on the origin of the parents, either of these loci may or may not segregate in inter-accession crosses. To pinpoint the genes responsible for the QTL it is crucial to identify the corresponding chromosomal regions. At the moment this is not straightforward in *T. caerulescens*, since there is no physical map of the *T. caerulescens* genome. However, considering the often well-preserved genome co-linearity of members of the Brassicaceae family (Schranz *et al.* 2006), it is possible to identify an Arabidopsis genomic region co-linear to the *T. caerulescens* QTL interval by using flanking common genes as genetic markers. Additional markers are needed to fine-map the region further or the Arabidopsis genomic region can be search directly for candidate genes based on a presumed shared function in metal homeostasis in both species. The present genetic maps and the accompanying mapping populations open up new avenues for the further identification of genes involved in zinc and cadmium hyperaccumulation in *T. caerulescens*.

TRANSCRIPTOMICS

Genome-wide expression analysis is a powerful tool to obtain clues about the genes that are involved in adaptive traits or in responses to environmental conditions. Designing dedicated "whole-genome" micro-arrays for metal hyperaccumulating species is not yet an option, because of the associated high costs (van de Mortel and Aarts 2006a). Fortunately there are alternatives. For *T. caerulescens*, spotted cDNA arrays have been developed, with a limited number of approximately 1900 genes (Plessl *et al.* 2005; Hassinen *et al.* 2007). More information has been obtained by using cross-species, heterologous hybridization with labelled cDNAs from the metal hyperaccumulators *Arabidopsis halleri* and *T. caerulescens* to genome-wide Arabidopsis micro-arrays (Becher *et al*. 2004; Weber *et al.* 2004; Hammond *et al.* 2006; van de Mortel *et al.* 2006b). By comparative transcript profiling of shoots and roots of *A. halleri* and *A. thaliana*, Weber *et al.* (2004) used an early version of the Affymetrix Arabidopsis genome array (www.Affymetrix.com), containing multiple 25-bp oligonucleotide probes representing little over 8000 genes, to establish a strongly enhanced expression and organ-specific regulation of different members of the zinc transporting ZIP protein family and the nicotianamine synthase (NAS) metal homeostasis gene family in the zinc hyperaccumulator *A. halleri*. The higher expression of these genes in the hyperaccumulator might account for a higher rate of cellular zinc uptake, with a predominant role for *ZIP9* in roots and *ZIP6* in shoots, and for a higher rate of nicotianamine synthesis to achieve enhanced cytoplasmic zinc buffering (Weber *et al.* 2004) and intercellular metal mobility (Ling *et al.* 1999) with major roles for *NAS2* in roots and possibly *NAS3* in shoots. There were striking differences between root and shoot metal homeostasis transcript profiles, which may reflect the different functions of the root and the shoot in metal hyperaccumulation. *NAS2* and *NRAMP3*, a member of the natural resistance-associated macrophage protein gene family encoding a protein involved in iron, manganese and cadmium transport from vacuoles, are highly expressed in roots of *A. halleri* and may have a role in sustaining root-to-shoot mobility of zinc through vascular and intercellular transport (*NAS2*) (Thomine *et al.* 2000, 2003; Filatov *et al.* 2006). *NRAMP3* has been found not to be able to transport Zn, or at a very low rate in yeast complementation experiments. The function of *NRAMP3* seems to be related to protection against ROS and Fe homeostasis. On the other hand, *TcNRAMP4* which is also higher expressed in *Thlaspi* than Arabidopsis thus does transport Zn (Oomen *et al.* pers. comm.). The cation diffusion facilitator (CDF) family members, such as *ZAT/MTP1*, and the heavy metal transporting ATPase family member, *HMA3*, which were predominant in the shoot transcript profiles, are important for the vacuolar sequestration and efflux of metals from the leaf cells, respectively (Becher *et al*. 2004), thereby generating a metal sink in the shoot that would be an important driving force for metal hyperaccumulation. Recently, by using a combination of genome-wide cross species micro-array analysis (ATH1 GeneChip® array) and real-time reverse transcription-PCR, Talke *et al.* (2006) identified a set of candidate genes for zinc hyperaccumulation, zinc and cadmium hypertolerance, and the adjustment of micronutrient homeostasis in *A. halleri*. Eighteen putative novel metal homeostasis genes were found to be more expressed in *A. halleri* than in *A. thaliana*, and 11 previously identified candidate genes were confirmed. The encoded proteins included *HMA4*, initially identified in Arabidopsis by Mills *et al.* (2003) and later shown to act in root to shoot Zn transport together with *HMA2* by Hussain *et al.* (2004). The transporter is also able to transport Cd (Bernard *et al.* 2004; Verret *et al.* 2004; Mills *et al.* 2005) as was also the case for the orthologous transporter from *T. caerulescens* (Bernard *et al.* 2004; Papoyan and Kochian 2004). Expression of either *AtHMA4* or *AhHMA4* conferred cellular zinc and cadmium tolerance in yeast (*Saccharomyces cerevisiae*) (Talke *et al*. 2006). Among the newly identified proteins were also *IRT3* and *ZIP10*, which have been proposed to contribute to cytoplasmic zinc influx, and *FRD3* which has been shown to be required for iron transport in *A. thaliana* (Green and Rogers 2004) and recent experimental evidence suggests it to be a citrate efflux transporter (Durett *et al.* 2006; Puig *et al.* 2007). The presence of multiple gene copies in *A. halleri*, when compared to *A. thaliana*, is a hallmark of several highly expressed candidate genes with possible roles in metal hyperaccumulation, such as *HMA4* and *MTP1* (Talke *et al.* 2006). The transcriptional regulation of marker genes suggested that in the steady state, *A. halleri* roots, but not the shoots, act as physiologically zinc deficient under conditions of moderate zinc supply (Talke *et al*. 2006).

The transcriptome of *T. caerulescens* has recently been profiled using a custom cDNA spotted microarray representing 1900 expressed sequence tags (ESTs) and comparing accessions LC and LE of *T. caerulescens* grown under different zinc exposure conditions (Plessl *et al.* 2005). Although differences were observed between the two accessions and between metal exposure conditions, these differences were not very large, despite the differences in zinc uptake and tolerance of the tested accessions. Most remarkable was the difference in expression of genes involved in synthesis of nicotianamine precursors, which were much higher expressed in LC compared to LE, at elevated zinc concentrations (100 and 1000 μM). Hammond *et al.* (2006) provided a more comprehensive transcriptional profile of shoots of *T. caerulescens* compared to that of the non-hyperaccumulator congener, *T. arvense*. These species have often been used in comparative physiological and molecular studies on metal hyperaccumulators (Lasat *et al.* 1996; Pence *et al.* 2000; Assunção *et al.* 2001; Pineros and Kochian 2003), although phylogenetic studies have shown that the *Thlaspi* genus is polyphyletic, with *T. arvense* not closely related to *T. caerulescens*. *T. caerulescens* is probably best compared to the non-accumulator *T. perfoliatum* which is a more closely related species than *T. arvense* (Koch and Mummenhoff 2001). In this study Affymetrix ATH1 GeneChip® arrays were used for heterologous hybridisation. To avoid a strong increase in (false) negative probes due to insufficient DNA identity, a new gDNA-based probe-selection and probe-masking strategy (Hammond *et al.* 2005) was used to profile and compare the transcriptomes of *T. caerulescens* and *T. arvense*. This strategy was shown to be robust and valid, based on *in silico* alignments of the array probe sequences with *T. caerulescens* and *T. arvense* sequences and on quantitative real-time PCR. In total, 4947 transcripts (representing homologues, possibly orthologues, of genes in *A. thaliana*) were identified as differentially (>2-fold or <0.5-fold) expressed in the shoots of *T. caerulescens* com-

pared with *T. arvense*. The abundance of 3349 transcripts was higher in *T. caerulescens* than in *T. arvense* and the abundance of 1598 transcripts was lower. Among them many transcripts encoding proteins with putative roles in cellular zinc homeostasis, including several genes previously shown to be involved in zinc transport and zinc compartmentalization. There was differential expression of five genes homologous to the *A. thaliana* ZIP transporter family, including higher expression of *AtIRT3*, *AtZIP6* and *AtZIP7*, and lower expression of *AtZIP3* and *AtZIP10* in shoots of *T. caerulescens* compared with *T. arvense*. Transcripts with homology to three CDF/MTP transporters (*AtMTP11*, *AtMTP12* and *AtMTP5* (Delhaize *et al.* 2003)) were expressed at higher levels in *T. caerulescens* than in *T. arvense*. Also, four genes of the P-type ATPase family had higher expression in shoots of *T. caerulescens* compared with *T. arvense*: homologues of the P1B-type ATPases *AtHMA3* and *AtHMA4*, and the Ca^{2+}-transporting ATPases *AtACA13* and *AtACA12*. Curiously, in the study of Hammond *et al.* (2006) the transcript abundance of *TcZTP1* (corresponding to *AtMTP1/ZAT)* was found to be lower in *T. caerulescens* than in *T. arvense.* Assunção *et al*. (2001), using Northern analysis, found this gene to be much higher expressed in three *T. caerulescens* accessions than in *T. arvense*, both in roots and in shoots. Such discrepancies might result from a lack of correlation between probe/target homology in the study of Hammond *et al.* (2006). The probe signal strength may be a result of the formation of secondary structures, which have the potential to increase or decrease the signal from a particular probe (Grigoryev *et al.* 2005). So, despite the elegant probe masking technique, unreliable data are likely to occur. Of the genes identified as differentially expressed between *A. halleri* and *A. thaliana* in the studies of Becher *et al.* (2004) and Weber *et al.* (2004) using the Affymetrix *A. thaliana* AG GeneChip® array, only 16 genes were common to the genes identified as significantly differentially expressed between *T. caerulescens* and *T. arvense* in the study of Hammond *et al.* (2006), including transcripts homologous to *AtCAX2*, *AtHMA3*, *AtZIP6*, *AtZAT/MTP1*, and a cytochrome P450. Nevertheless, a recent re-analysis of all available *A. thaliana* GeneChip® array experiments involving metal hyperaccumulators (largely shoot expression) indicated some 60 genes to be significantly differentially expressed between hyperaccumulator and non-accumulator species (Broadley *et al.* 2007). Only six of these encode proteins with a specific role in zinc homeostasis, including three CDF/MTP transporters: *AtZAT/MTP1*, *AtMTP8* and *AtMTP11*. Others are *AtIRT3* (*TcZNT2*), *AtHMA3* and the nicotianamine synthase gene *AtNAS3*. This obvious lack of correspondence might in part result from technical issues related to probe/target homology (see above). On the other hand, it is well possible that the mechanisms of hyperaccumulation are largely different in *T. caerulescens* and *A. halleri*.

More recently, using a 60-mer oligo micro-array whose 40,000 probes were designed to represent full-genome coverage of *A. thaliana* (Arabidopsis 3 oligo micro-array; Agilent Technologies Inc.), van de Mortel *et al.* (2006b) examined the transcription profiles of roots of Arabidopsis and *T. caerulescens* plants grown under zinc deficiency, sufficiency and excess, using a 60-mer oligo Agilent Arabidopsis 3 oligo micro-array with 40 000 probes, which were designed to represent full-genome coverage of *A. thaliana*. A total of 608 zinc-responsive genes with at least 3-fold difference in expression between two of the three zinc treatments, were detected in *A. thaliana* and 352 in *T. caerulescens*. Only 14% of the genes that were zinc-responsive in *T. caerulescens* were also zinc-responsive in *A. thaliana*. When comparing *A. thaliana* with *T. caerulescens* at each of the zinc exposure levels, more than 2,200 genes were significantly higher expressed in *T. caerulescens* (≥5-fold at a false discovery rate <0.05). While a large fraction of these genes are of yet unknown function, and most genes with a higher expression in *T. caerulescens* than in *A. thaliana* appear to function in processes other than metal homeostasis, there is still a considerable group of metal homeostasis related genes that are much higher expressed in *T. caerulescens* compared to *A. thaliana.* Among these were *HMA3* and *HMA4*, *MTP1 (ZTP1)* and *MTP8, ZIP4 (TcZNT1)* and *ZIP10*, *IRT3 (TcZNT2)*, *NRAMP3*, *FRD3* (see above) and two metallothionein genes, *MT2a* and *MT2b.* The function of these MTs might lie in maintaining proper copper homeostasis, rather than in Zn or Cd accumulation or tolerance (Roosens *et al.* 2004). The strong expression of *NAS2* in *A. halleri* compared to *A. thaliana* (Weber *et al.* 2004; Talke *et al.* 2006) was not found in *T. caerulescens*. However, the very different expression profiles of the other three *NAS* genes between *A. thaliana* and *T. caerulescens* suggest a major function of nicotianamine synthesis in metal adaptation in *T. caerulescens*. In addition to the differential expression of the *NAS* genes, van de Mortel *et al.* (2006b) found an unexpectedly high expression of *FRO5*, *FRO4* and *FRD3* under zinc deficiency in *T. caerulescens*. *FRO4* and *FRO5* resemble the ferric chelate reductase gene *FRO2* (Robison *et al.* 1999) but, in contrast to *FRO2*, their expression is not induced in Arabidopsis roots upon iron deficiency (Mukherjee *et al.* 2005; Wu *et al.* 2005), and they are likely to perform an additional function other than iron reduction. Although *FRD3* has been mainly implicated in iron homeostasis (Green and Rogers 2004), the gene has also been identified as highly expressed in *A. halleri* compared to *A. thaliana* (Talke *et al.* 2006). These results suggest a much broader role of these genes in general metal homeostasis than previously thought. Recent work by Durrett *et al.* (2006) suggested that *FRD3* encodes a citrate efflux transporter which may be needed for proper metal cation transport through the plant, not limited to iron transport.

The comparative transcriptomic analyses of the hyperaccumulator *T. caerulescens* and the non-accumulators *A. thaliana* or *T. arvense* emphasize the role of previously implicated zinc homeostasis genes but also suggest a similar role for many more, as yet uncharacterised genes, such as the 24 highly expressed genes with a putative function in lignin biosynthesis (van de Mortel *et al.* 2006). The high expression of lignin biosynthesis genes corresponds to the deposition of lignin in the endodermis, of which there are one or two layers in *T. caerulescens* roots but none in *A. thaliana* (van de Mortel *et al.* 2006) or *T. arvense* (Broadley *et al*. 2007). A similar phenomenon has been observed in the salt tolerant Brassicaceae species *Thelungiella halophila* (Inan *et al.* 2004), and it may indicate the adaptive flexibility that is found in this family especially to abiotic stresses.

Non-targeted approaches, such as differential display (DD) (Liang and Pardee 1992), are viable alternatives for determining differential gene expression, especially for heterologous comparisons, where genes can be identified that are not represented on the array or for which the probes do not match the target properly. For instance, Mandaokar *et al.* (2003) identified additional differentially expressed genes in Arabidopsis with DD compared to micro-array. DD has been applied for the isolation of genes involved in many processes in Arabidopsis, such as the response to cadmium (Suzuki *et al.* 2001). Hassinen *et al.* (2006) compared shoot transcript patterns of two *T. caerulescens* accessions using DD and found 16 differentially expressed genes, three of which had no homology to Arabidopsis genes. Two zinc-responsive metallothionein (*MT*) genes were identified in *T. caerulescens*, *TcMT2a* and *TcMT3*, apparently involved in intracellular metal binding (Roosens *et al.* 2004, 2005). The *TcMT3* expression levels appeared to reflect the shoot zinc levels and may thus have a function in metal homeostasis under zinc exposure. Two more genes with possible roles in metal sequestration were isolated in this study, namely those encoding the *TcMRP10* transporter and a pectine methylesterase (PME). *TcMRP10* was highly homologous to *AtMRP10* and marginally less homologous to *AtMRP4*. The *AtMRPs* belong to a family of membrane-associated glutathione-conjugate transporters, which forms part of the super-family of ABC transporters (Bovet *et al.* 2003). ABC

transporters are involved in the vacuolar sequestration of cadmium in yeast and plants (Ortiz *et al.* 1992; Wemmie *et al.* 1994; Song *et al.* 2003). *TcMRP10* was induced in *T. caerulescens* shoots upon exposure to high zinc concentrations, although transport of zinc has not been shown for these plant ABC transporters. Recently Bovet *et al*. (2006) suggested the sequestration of cadmium in vacuoles of *Arabis alpina* could be attributed to *AtMRP3*, in line with their previous findings in *A. thaliana* (Bovet *et al.* 2003). In *T. caerulescens*, zinc is mainly stored in the vacuole (Küpper *et al.* 1999), but the apoplast is also a major storage compartment (Frey *et al.* 2000). PMEs modify the properties of cell walls by demethylation of pectin residues, thus creating free carboxylic groups for interaction with divalent cations. A higher expression of PME in the zinc-accumulator accession may thus enhance zinc binding in the apoplast. In conclusion, even though a small set of genes was identified using DD, (novel) genes with possible implications in metal adaptation were found.

PROTEOMICS

The major advantage of proteomics over transcriptomics is that it focuses on the actual proteins, rather than the potential to make proteins. The importance of post-transcriptional regulation has been underscored by several studies in yeast showing only a weak or moderate correlation between mRNA and protein levels, except for very abundant proteins (Gygi *et al.* 1999; Ideker *et al.* 2001). A disadvantage of proteomics is currently still the reduced resolution compared to transcriptomics. Nevertheless, protein profiling has been used to study the effects of several biotic and abiotic stress factors on plants. Only a few reports on the effects of metal ions on non-hyperaccumulator species proteomes are available to date (Thiellement *et al.* 1999, 2002). A study on the rice proteome indicated that some metals disrupt the photosynthetic machinery (Hajduch *et al.* 2001). Additionally, Repetto *et al.* (2003) have shown that cadmium-induced changes in the pea root proteome were shown to be modulated by mycorrhizal symbiosis. In both studies only a limited number of detected proteins were clearly affected by exposure to heavy metals. The fraction affected by heavy metal exposure is relatively low, compared to the fraction of transcripts affected, due to post-regulation and the lower sensitivity of protein profiling. Besides differences in protein spot intensity, also genetic differences between plant accessions leading to allelic diversity can result in different protein isoforms, which can alter mobility in protein profiles.

The study of Ingle *et al.* (2005) is probably the first attempt to use proteomic profiling to explore the molecular mechanisms related to heavy metal accumulation in a hyperaccumulator species. These authors tried to identify proteins that play a role in nickel accumulation (or tolerance) in the nickel hyperaccumulator *Alyssum lesbiacum* by analysing changes in protein abundance occurring in response to short- and long-term exposure to nickel. Short-term exposure led to a change in the abundance of several proteins associated with sulphur-containing amino acid metabolism. Together these changes indicated a re-allocation of sulphur towards the increased production of cystein. The increase in proteins such as oxidoreductase, mannitol-6-phosphate reductase and a glutathione *S*-transferase may reflect an antioxidant system to prevent membrane damage, especially when the nickel concentration is close to the maximum that the plants can tolerate without growth inhibition. In the light of the small number of proteins seen to alter in abundance after a long-term exposure to nickel, Ingle *et al.* have made an attempt to compare the proteome of *A. lesbiacum* with that of *A. montanum* (a related non-accumulator) in the absence of added nickel. Unfortunately, the protein patterns of the two species were insufficiently similar to allow any analysis. This clearly suggests that the proteomic approach is most successful for intra-specific comparisons.

Recently, Tuomainen *et al.* (2006) compared proteins patterns of three metal hyperaccumulator accessions of *T. caerulescens* with distinct characteristics of metal uptake and transport. The strongest differences were seen when comparing accessions, while the effects of metal exposures were less pronounced. The 48 tentatively differential spots represented proteins with core metabolic functions (e.g. photosynthesis, nitrogen assimilation, carbohydrate metabolism) as well as putative signalling and regulatory proteins. As no distinction could be made between differences due to expression differences or isoform differences best would be to study this in a genetically segregating population of the investigated accessions to find a true connection to metal accumulation or tolerance (Tuomainen *et al.* 2006). Especially with the increasing resolution power of proteomics, proteome data will provide an increasingly important contribution to understand the systems biology behind metal hyperaccumulation in plants.

CONCLUSIONS AND FUTURE RESEARCH PERSPECTIVES

Cross-species transcriptomic analyses have identified many genes with differential expression in hyperaccumulating and non-hyperaccumulating species. However, since the species compared have a very different life history and ecology, it is likely that most of the observed differences in transcript abundances are unrelated to metal adaptation or hyperaccumulation. Moreover, cross-species comparisons using heterologous probes suffer from the draw-back of uncorrelated probe/target homology. In particular, when using *A. thaliana* probes, comparisons of hyperaccumulators with *A. thaliana* are likely to only identify genes with higher expression in the hyperaccumulator species, and it is well conceivable that hyperaccumulation may be in part due to a decreased, rather than enhanced expression of particular genes. In spite of this, cross-species transcriptomic comparisons have identified candidate hyperaccumulation genes, a large number of which with a proven involvement in metal homeostasis and a much larger group with a putative role in metal homeostasis. It is unlikely, however, that the evolution of hyperaccumulation would require genetic changes at hundreds of loci. It can be expected that many differences in transcript abundance may be a consequence, rather than a cause of hyperaccumulation. The regulation of metal homeostasis genes in plants is almost completely unexplored. Only for iron homeostasis the first transcription factors are found (Colangelo and Guerinot 2004; Jakoby *et al.* 2004; Yuan *et al.* 2005). The observed large-scale differences in gene expression profiles between hyperaccumulator and non-hyperaccumulator species warrant a more dedicated look towards potential transcriptional regulators and gene promoter sequences of candidate genes. Also, since many differentially expressed genes appear to encode unknown proteins, functional characterization of transcriptional regulators and structural genes through mutant research is indispensable, but has never been done in a hyperaccumulator yet. Another future effort should be dedicated to the development of physical maps of hyperaccumulators, accompanied with large-scale genome sequencing, which will easily reveal the expected differences in *cis* regulatory sequences as well as the presence of multiple copies of key structural genes. In the absence of such physical maps, comparative genomics remains the viable alternative. It is most important to establish the chromosomal colinearity and micro-synteny between existing *T. caerulescens* and *A. halleri* genetic maps and the Arabidopsis physical map, in order to identify the genes responsible for the QTL for metal accumulation and tolerance established in *T. caerulescens* inter-accession crosses and *A. halleri* x *A. lyrata* interspecific crosses (Drager *et al.* 2004). In conclusion, the knowledge of the genes controlling specific steps of the metal homeostasis network in hyperaccumulators is still rudimentary, but rapidly increasing, facilitated by advances in high-throughput profiling of the transcriptome, proteome, metabolome and ionome (Salt 2004). This knowledge is expected to efficiently

improve crop yield, crop nutritional value and food safety, three items which are of major global concern (Ghandilyan *et al.* 2006). A multi-disciplinary research effort that integrates the work of molecular and plant biologists, soil chemists and microbiologists is essential for a better understanding of metal hyper-accumulation in plants.

ACKNOWLEDGEMENTS

The authors acknowledge Ronald Oomen for sharing his latest results prior to publication.

REFERENCES

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in Arabidopsis: an underexploited resource for plant genetics. *Trends in Plant Science* **5**, 22-29

Assunção AGL, da Costa Martins P, de Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. *Plant, Cell and Environment* **24**, 217-226

Assunção AGL, Schat H, Aarts MGM (2003a) *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. *New Phytologist* **159**, 351-360

Assunção AGL, Ten Bookum WM, Nelissen HJM, Vooijs R, Schat H, Ernst WHO (2003b) A co-segregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. *New Phytologist* **159**, 383-390

Assunção AGL, Pieper B, Vromans J, Lindhout P, Aarts MGM, Schat H (2006) Construction of a genetic linkage map of *Thlaspi caerulescens* and quantitative trait loci analysis of zinc accumulation. *New Phytologist* **170**, 21-32

Baker AJM, Brooks R (1989) Terrestrial and higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. *Biorecovery* **1**, 81-126

Baker AJM, Reeves RD, McGrath SP (1991) *In situ* de-contamination of heavy metal polluted soils using crops of metal accumulating plants – a feasibility study. In: Hinchee RE, Olfenbuttel RF (Eds) *In Situ Bioreclamation*, Stoneham, MA, USA: Butterworth-Heinemann, pp 539-544

Baker AJM, McGrath SP, Reeves DR, Smith JAC (2000) Metal hyperaccumulators plants: a review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry N, Banuelos G (Eds) *Phytoremediation of Contaminated Soil and Water*, CRC Press LLC, Boca Raton, FL, pp 85-107

Becher M, Talke IN, Krall L, Kramer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. *Plant Journal* **37**, 251-268

Bernard C, Roosens N, Czernic P, Lebrun M, Verbruggen N (2004) A novel CPx-ATPase from the cadmium hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **569**, 140-148

Bert V, Meerts P, Saumitou-Laprade P, Salis P, Gruber W, Verbruggen N (2003) Genetic basis of Cd tolerance and hyperaccumulation in *Arabidopsis halleri*. *Plant and Soil* **249**, 9-18

Bovet L, Eggman T, Meylan-Bettex M, Polier J, Kammer P, Marin E, Feller U, Martinoia E (2003) Transcript levels of AtMRPs after cadmium treatment: induction of AtMRP3. *Plant Cell and Environment* **26**, 371-381

Bovet L, Kammer PM, Meylan-Bettex M, Guadagnuolo R, Matera V (2006) Cadmium accumulation capacities of *Arabis alpina* under environmental conditions. *Environmental and Experimental Botany* **57**, 80-88

Boyd RS (1998) Hyperaccumulation as a plant defensive strategy. In: Brooks RR (Ed) *Plants that Hyperaccumulate Heavy Metals*, CAB International, Wallingford, UK, pp 181-201

Boyd RS, Martens SN (1992) The raison d'être for metal hyperaccumulation by plants. In: Baker AJM, Proctor J, Reeves RD (Eds) *The Vegetation of Ultramafic (Serpentine) Soils,* Intercept, Andover, UK, 279-289

Boyd RS, Davis MA, Wall MA, Balkwill K (2002) Nickel defends the South African hyperaccumulator *Senecio coronatus* (Asteraceae) against *Helix aspersa* (Mollusca: Pulmonidae). *Chemoecology* **12**, 91-97

Broadley MR, White PJ, Hammond JP, Zelko I, Lux A (2007) Zinc in plants. *New Phytologist* **173**, 677-702

Brooks RR, lee J, Reeves RD, Jaffre T (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *Journal of Geochemical Exploration* **7**, 49-57

Colangelo EP, Guerinot ML (2004) The essential basic helix-loop-helix protein FIT1 is required for the iron deficiency response. *Plant Cell* **16**, 3400-3412

Dechamps C, Lefèbvre C, Noret N, Meerts P (2007) Reaction norms of life history traits in response to zinc in *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites. *New Phytologist* **173**, 191-198

Delhaize E, Kataoka E, Hebb DM, White RG, Ryan PR (2003) Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. *Plant Cell* **15**, 1131-1142

Deniau AX, Pieper B, Ten Bookum WM, Lindhout P, Aarts MGM, Schat H (2006) QTL analysis of cadmium and zinc accumulation in the heavy metal hyperaccumulator *Thlaspi caerulescens*. *Theoretical and Applied Genetics* **113**, 907-920

Drager DB, Desbrosses-Fonrouge AG, Krach C, Chardonnens AN, Meyer RC, Saumitou-Laprade P, Kramer U (2004) Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. *Plant Journal* **39**, 425-439

Dubois S, Cheptou PO, Petit C, Meerts P, Poncelet M, Vekemans X, Lefebvre C, Escarré J (2003) Genetic structure and mating systems of metallicolous and nonmetallicolous populations of *Thlaspi caerulescens*. *New Phytologist* **157**, 633-641

Durrett TP, Gassmann W, Rogers EE (2006) Functional characterization of FRD3, a novel organic acid effluxer involved in iron homeostasis. Available online: http://abstracts.aspb.org/pb2006/public/M17/M1701.html

Escarré J, Lefèbvre C, Gruber W, Leblanc M, Lepart J, Rivière Y, Delay B (2000) Zinc and cadmium hyperaccumulation by *Thlaspi caerulescens* from metalliferous and nonmetalliferous sites in the Mediterranean area: implications for phytoremediation. *New Phytologist* **145**, 429-437

Filatov V, Dowdle J, Smirnoff N, Ford-Lloyd B, Newbury HJ, Macnair MR (2006) Comparison of gene expression in segregating families identifies genes and genomic regions involved in a novel adaptation, zinc hyperaccumulation. *Molecular Ecology* **15**, 3045-3059

Frérot H, Petit C, Lefèbvre C, Gruber W, Collin C, Escarré J (2003) Zinc and cadmium accumulation in controlled crosses between metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **157**, 643-648

Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionnally different leaf epidermal cells in the hyperaccumulator *Thlaspi caerulescens*. *Plant Cell and Environment* **23**, 675-687

Ghandilyan A, Vreugdenhil D, Aarts MGM (2006) Progress in the genetic understanding of plant iron and zinc nutrition. *Physiologia Plantarum* **126**, 407-417

Green LS, Rogers EE (2004) FRD3 Controls iron localization in Arabidopsis. *Plant Physiology* **136**, 2523-2531

Gygi Sp, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. *Molecular Cell Biology* **19**, 1720-1730

Hajduch M, Rakwal R, Agrawal GK, Yonekura M, Pretová A (2001) High-resolution two-dimensional electrophoresis separation of proteins from metal-stressed rice (*Oryza sativa* L.) leaves: drastic reductions/fragmentation of ribulose-1,5-bisphosphate carboxylase/oxygenase and induction of stress-related proteins. *Electrophoresis* **22**, 2824-2831

Hammond JP, Broadley MR, Craigon DJ, Higgins J, Emmerson ZF, Townsend HJ, White PJ, May ST (2005) Using genomic DNA-based probe-selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. *Plant Methods* **1**, 10

Hammond JP, Bowen HC, White PJ, Mills V, Pyke KA, Baker AJM, Whiting SN, May ST, Broadley MR (2006) A comparison of the *Thlaspi caerulescens* and *T. arvense* shoot transcriptome. *New Phytologist* **170**, 239-260

Hanson B, Garifullina GF, Lindblom SD, Wangeline A, Ackley A, Kramer K, Norton AP, Lawrence CB, Pilon-Smits EAH (2003) Selenium accumulation protects *Brassica juncea* from invertebrate herbivory and fungal infection. *New Phytologist* **159**, 461-469

Hassinen VH, Tervahauta AI, Halimaa P, Plessl M, Peräniemi S, Schat H, Aarts MGM, Servomaa K, Kärenlampi SO (2007) Isolation of Zn-responsive genes from two accessions of the hyperaccumulator plant *Thlaspi caerulescens*. *Planta* **225**, 977-989

Huitson S, Macnair MR (2003) Does zinc protect the zinc hyperaccumulator *Arabidopsis halleri* from herbivory by snails? *New Phytologist* **159**, 453-459

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. *Plant Cell* **16**, 1327-1339

Ideker T, Thorsson V, Ranish JA, Christmas R, Buhler J, Eng JK, Bumgarner R, Goodlet DR, Aebersold R, Hed L (2001) Integrated genomic and proteomic analyses of a systematically perturbed network. *Science* **292**, 929-934

Inan G, Zhang Q, Li PH, Wang Z, Cao Z, Zhang H, Zhang C, Quist TM, Goodwin SM, Zhu J, Shi H, Damsz B, Charbaji T, Gong Q, Ma S, Fredricksen M, Galbraith DW, Jenks MA, Rhodes D, Hasegawa PM, Bohnert HJ, Joly RJ, Bressan RA, Zhu JK (2004) Salt cress. A halophyte and cryophyte Arabidopsis relative model system and its applicability to molecular genetic analyses of growth and development of extremophiles. *Plant Physiology* **135**, 1718-1737

Ingle RA, Smith JAC, Sweetlove LJ (2005) Responses to nickel in the proteome of the hyperaccumulator plant *Alyssum lesbiacum*. *Biometals* **18**, 627-641

Jakoby M, Wang HY, Reidt W, Weisshaar B, Bauer P (2004) *FRU* (*BHLH029*) is required for induction of iron mobilization genes in *Arabidopsis thaliana*. *FEBS Letters* **577**, 528-534

Jiang RF, Ma DY, Zhao FJ, McGrath SP (2005) Cadmium hyperaccumu-

lation protects *Thlaspi caerulescens* from leaf feeding damage by trips (*Frankliniella occidentalis*). *New Phytologist* **167**, 805-813

Jiménez-Ambriz G, Petit C, Bourrié I, Dubois S, Olivieri I, Ronce O (2007) Life history variation in the heavy metal tolerant plant *Thlaspi caerulescens* growing in a network of contaminated and noncontaminated sites in southern France: role of gene flow, selection and phenotypic plasticity. *New Phytologist* **173**, 199-215

Kawecki TJ, Ebert D (2004) Conceptual issues in local adaptation. *Ecology Letters* **7**, 1225-1241

Koch M, Mummenhoff K, Hurka H (1998) Systematics and evolutionary history of heavy metal tolerant *Thlaspi caerulescens*. Western Europe: evidence from genetic studies based on isozyme analysis. *Biochemical Systematics and Ecology* **26**, 823-838

Koch M, Mummenhoff K (2001) *Thlaspi* s. str. (Brassicaceae) versus *Thlaspi* s. l.: morphological and anatomical characters in the light of ITS nrDNA sequence data. *Plant Systematics and Evolution* **227**, 209-225

Kupper, H, Zhao FJ, McGrath SP (1999) Cellular compartimentation of zinc in the leaves of the hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **199**, 305-311

Lasat MM, Baker AJM, Kochian LV (1996) Physiological characterization of root Zn^{2+} absorption and translocation to shoots in Zn hyperaccumulator and nonaccumulator species of *Thlaspi*. *Plant Physiology* **112**, 1715-1722

Ledesma A, Moral V, Villalba M, Salinas J, Rodriguez R (2006) Ca^{2+}-binding allergens from olive pollen exhibit biochemical and immunological activity when expressed in stable transgenic Arabidopsis. *FEBS Journal* **273**, 4425-4434

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of polymerase chain reaction. *Science* **257**, 967-971

Ling HK, Koch G, Baumlein H, Ganal MW (1999) Map-based cloning of *chloronerva*, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. *Proceedings of the National Academy of Sciences USA* **96**, 7098-7103

Long XX, Yang XE, Ni WZ (2002) Current status and perspective on phytoremediation of heavy metal polluted soils. *Journal of Applied Ecology* **13**, 757-762

Macnair MR (1983) The genetic control of copper tolerance in the yellow monkey flower, *Mimulus guttatus*. *Heredity* **50**, 283-293

Macnair MR (1993) The genetics of metal tolerance in vascular plants. *New Phytologist* **124**, 541-559

Macnair MR (2003) The hyperaccumulation of metals by plants. *Advances in Botanical Research* **40**, 63-105

Macnair MR, Gardner M (1999) The evolution of edaphic endemics. In: Howard DJ, Berlocher SH (Eds) *Endless Forms: Species and Speciation*, Oxford University Press, Oxford, UK, pp 157-171

Macnair MR, Tilstone GH, Smith SE (2000) The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G (Eds) *Phytoremediation of Contaminated Soil and Water*, CRC Press LLC, Boca Raton, FL, pp 235-250

Mandaokar A, Kumar VD, Amway M, Browse J (2003) Micro-array and differential display identify genes involved in jasmonate-dependant anther development. *Plant Molecular Biology* **52**, 775-786

McGrath SP, Zhao FJ (2003) Phytoextraction of metals and metalloids from contaminated soils. *Current Opinion in Biotechnology* **14**, 277-282

McIntyre T (2003) Phytoremediation of heavy metals from soils. *Advances in Biochemical Engineering and Biotechnology* **78**, 97-123

Meerts P, van Isacker N (1997) Heavy metal tolerance and accumulation in metallicolous and non-metallicolous populations of *Thlaspi caerulescens* from continental Europe. *Plant Ecology* **133**, 221-231

Mills RF, Francini A, Ferreira da Rocha PS, Baccarini PJ, Aylett M, Krijger GC, Williams LE (2005) The plant P1B-type ATPase *AtHMA4* transports Zn and Cd and plays a role in detoxification of transition metals supplied at elevated levels. *FEBS Letters* **579**, 783-791

Mukherjee I, Campbell NH, Ash JS, Connolly EL (2005) Expression profiling of the Arabidopsis ferric chelate reductase (*FRO*) gene family reveals differential regulation by iron and copper. *Planta* **14**, 1-13

Noret N, Meerts P, Tolrà R, Poschenrieder C, Barceló J, Escarre J (2005) Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosenolates. *New Phytologist* **165**, 763-772

Ortiz DF, Kreppel L, Speiser DM, Scheel G, McDonald G, Ow DW (1992) Heavy metal tolerance in the fission yeast requires an ATP-binding cassette-type vacuolar membrane transporter. *The EMBO Journal* **11**, 3491-3499

Papoyan A, Kochian LV (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. *Plant Physiology* **159**, 421-430

Peer WA, Mamaoudian M, Lahner B, Reeves RD, Murphy AS, Salt DE (2003) Identifying model metal hyperaccumulating plants: germplasm analysis of 20 Brassicaceae accessions from a wide geographical area. *New Phytologist* **159**, 421-430

Peer WA, Mahmoudian M, Freeman JL, Lahner B, Richards EL, Reeves RD, Murphy AS, Salt DE (2006) Assessment of plants from the Brassicaceae family as genetic models for the study of nickel and zinc hyperaccumulation. *New Phytologist* **172**, 248-260

Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. *Proceedings of the National Academy of Sciences USA* **97**, 4956-4960

Piñeros MA, Kochian LV (2003) Differences in whole-cell and single-channel ion currents across the plasma membrane of mesophyll cells from two closely related *Thlaspi* species. *Plant Physiology* **131**, 583-594

Plessl M, Rigola D, Hassinen V, Aarts MGM, Schat H, Ernst D (2005) Transcription profiling of the metal-hyperaccumulator *Thlaspi caerulescens* (J. and C. PRESL). *Zeitschrift für Naturforschung* **60c**, 216-223

Pollard AJ (2000) Metal hyperaccumulation: a model system for co-evolutionary studies. *New Phytologist* **146**, 179-181

Pollard A, Baker AJM (1996) Quantitative genetics of zinc hyperaccumulation in *Thlaspi caerulescens*. *New Phytologist* **132**, 113-118

Pollard A, Baker AJM (1997) Deterrence of herbivory by zinc hyperaccumulation in *Thlaspi caerulescens* (Brassicaceae). *New Phytologist* **135**, 655-658

Pollard AJ, Powell KD, Harper FA, Smith JAC (2002) The genetic basis of hyperaccumulation in plants. *Critical Reviews in Plant Science* **21**, 539-566

Poschenreider C, Tolra R, Barcelo J (2006) Can metals defend plants against abiotic stress? *Trends in Plant Science* **11**, 288-295

Puig S, Andres-Colas N, Garcia-Molina A, Penarrubia L (2007) Copper and iron homeostasis in Arabidopsis: responses to metal deficiencies, interactions and biotechnological applications. *Plant, Cell and Environment* **30**, 271-290

Reeves RD (1988) Nickel and zinc accumulation by species of *Thlaspi* L., *Cochlaria* L., and other genera of the Brassicaceae. *Taxon* **37**, 309-318

Reeves RD (1992) New Zealand serpentines and their flora. In: Baker AJM, Proctor J, Reeves RD (Eds) *The Vegetation of Ultramafic (Serpentine) Soils*, Intercept, Andover, UK, pp 129-137

Reeves RD, Schwartz C, Morel JL, Edmondson J (2001) Distribution and metal-accumulating behavior of *Thlaspi caerulescens* and associated metallophytes in France. *International Journal of Phytoremediation* **3**, 145-172

Repetto O, Bestel-Corre G, Dumas-Gaudot E, Berta G, Gianinazzi-Pearson V, Gianinazzi S (2003) Targeted proteomics to identify cadmium-induced protein modifications in *Glomus mosseae*-inoculated pea roots. *New Phytologist* **157**, 555-267

Reznick DN, Ghalambor CK (2001) The population ecology of contemporary adaptations: what empirical studies reveal about the conditions that promote adaptive evolution. *Genetica* **112**, 183-198

Rigola, D, Fiers M, Vurro E, Aarts MGM (2006) The heavy metal hyperaccumulator *Thlaspi caerulescens* expresses many species-specific genes as identified by comparative EST analysis. *New Phytologist* **170**, 753-766

Roosens NH, Bernard C, Leplae R, Verbruggen N (2004) Evidence for copper homeostasis function of metallothionein (MT3) in the hyperaccumulator *Thlaspi caerulescens*. *FEBS Letters* **577**, 9-16

Roosens NH, Bernard C, Leplae R, Verbruggen N (2005) Variations in plant metallothioneins: the heavy metal hyperaccumulation *Thlaspi caerulescens* as a study case. *Planta* **222**, 716-729

Robinson NJ, Procter CM, Connolly EL, Guerinot ML (1999) A ferric-chelate reductase for iron uptake from soils. *Nature* **397**, 694-697

Salt DE (2004) Update on plant ionomics. *Plant Physiology* **136**, 2451-2456

Schat H, ten Bookum WM (1992) Genetic control of copper tolerance in *Silene vulgaris*. *Heredity* **68**, 219-229

Schranz E, Lysak MA, Mitchell-Olds T (2006) The ABC's of comparative genomics in the Brassicaceae: building blocks of crucifer. *Trends in Plant Science* **11**, 535-542

Song WY, Sohn E, Martinoia E, Lee Y, Yang YY, Jasinski M, Fotestier C, Hwang I, Lee YM (2003) Development of transgenic plants for phytoremediation of lead and cadmium. *Nature Biotechnology* **21**, 914-919

Suzuki N, Koizumi N, Sano H (2001) Screening of cadmium-responsive genes in *Arabidopsis thaliana*. *Plant Cell and Environment* **24**, 1177-1188

Talke IN, Hanikenne M, Krämer U (2006) Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. *Plant Physiology* **142**, 148-167

Thiellement H, Bahrman N, Damerval C, Plomion C, Rossignol M, Santoni V, de Vienne D, Zivy M (1999) Proteomics for genetic and physiological studies in plants. *Electrophoresis* **20**, 2013-2026

Thiellement H, Zivy M, Plomion C (2002) Combining proteomic and genetic studies in plants. *Journal of Chromatography B* **752**, 137-149

Thomine S, Wang RC, Ward JM, Crawford NM, Schroeder JI (2000) Cadmium and iron transport by members of a plant metal transporters family in *Arabidopsis* with homology to Nramp genes. *Proceedings of the National Academy of Sciences USA* **97**, 4991-4996

Thomine S, Lelievre F, Debarbieux E, Schroeder JI, Barbier-Brygoo H (2003) *AtNRAMP3*, a multispecific vacuolar metal transporter involved in plant responses to iron deficiency. *Plant Journal* **34**, 685-695

Tuomainen MH, Nunan N, Lehesranta SJ, TervahautaAI, Hassinen VH, Schat H, Koistinen KM, Auriola S, McNicol J, Karenlampi SO (2006) Multivariate analysis of protein profiles of metal hyperaccumulator *Thlaspi caerulescens* accessions. *Proteomics* **6**, 3696-3706

Urquhart C (1971) Genetics of lead tolerance in *Festuca ovina*. *Heredity* **26**, 19-33

van de Mortel JE, Aarts MGM (2006a) Comparative transcriptomics – model

species lead the way. *New Phytologist* **170**, 199-201

van de Mortel JE, Almar Villanueva L, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM (2006b) Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. *Plant Physiology* **142**, 1127-1147

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of *AtHMA4* enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. *FEBS Letters* **576**, 306-312

Weber M, Harada E, Vess C, Roepenack-Lahaye E, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as a potential metal hyperaccumulation factors. *Plant Journal* **37**, 269-281

Wemmie JA, Szczypka MS, Thiele DJ, Moyle-Rowley WS (1994) Cadmium tolerance mediated by yeast AP-1 protein requires the presence of an ATP-binding cassette transporter-encoding gene, *YCF1*. *The Journal of Biological Chemistry* **269**, 32592-32597

Wu H, Li L, Yuan Y, Cheng X, Ling HQ (2005) Molecular and biochemical characterisation of the Fe(III) chelate reductase gene family in *Arabidopsis thaliana*. *Plant Cell Physiology* **46**, 1505-1514

Yuan YX, Zhang J, Wang DW, Ling HQ (2005) *AtbHLH29* of *Arabidopsis thaliana* is a functional ortholog of tomato *FER* involved in controlling iron acquisition in strategy I plants. *Cell Research* **15**, 613-621

Zha HG, Jiang RF, Zhao FJ, Vooijs R, Schat H, Barker JHA, McGrath SP (2004) Co-segregation analysis of cadmium and zinc accumulation in *Thlaspi caerulescens* interecotypic crosses. *New Phytologist* **163**, 299-312