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 analyses 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

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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). Metal-tolerant 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-
patability 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 why T. caerulescens was 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 F2 and F3 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 lead hyperaccumulators, but whether hyperaccumulation of these metals is also constitutive at the species level and nickel hyperaccumulators, but whether hyperaccumulation of these metals is also constitutive at the species level is also constitutive at the species level and whether 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 F2 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 explored 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 the significant zinc/cadmium antagonisms in the parent accessions, one QTL was 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 LOI (Assunção 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-
sunčan 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 chromosome regions. At the moment, this is 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 searched 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; Hansinen et al. 2007). More information has been obtained by using cross-species, heterologous hybridization with labeled 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 nicotineamine 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 nicotamine synthesis to achieve enhanced cytoplasmic zinc buffering (Weber et al. 2004) and intercellular metal mobility (Ling et al. 1997) with major roles for NAS2 in roots and possibly NAS3 in shoots, which have been expressed in 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, TrNRAMP4, which is also higher expressed in *Thlaspi* than Arabidopsis thus does transport Zn (Oomen et al. pers. comm.). The cathodic diffusion facilitator (CDF) family members, such as ZAT1/MITP1, and the heavy metal transport 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) we 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 Huisman 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). An overall more closely related species was *T. arvense*, 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 the hyperaccumulator than in *A. halleri*. The Thlaspi genus is polyphyletic, with *A. arvense* closely related to *T. caerulescens*. *T. caerulescens* is probably best compared to the non-accumulator *T. perforatum* which is a more closely related species than *A. thaliana* (Koch and Mümvenhoff 2001). In this study Affymetrix ATH1 GeneChip® arrays were used for heterologous hybridisation. To avoid a strong increase in (false) negative probes due to inhomogeneity of the precursors, a stringent DNA identity and probe-masking strategy (Hammond et al. 2005) was used to profile and compare the transcriptomes of *T. caerulescens* and *A. arvense*. This strategy was shown to be robust and valid, based on in silico alignments of the array probe sequences with *T. caerulescens* and *A. 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* MT and ZIP transporter family, including *AtMTP1*, *AtZIP1*, *AtZIP4*, and *AtZIP10*. Transcripts with lower abundance of *AtZIP10* and lower expression of *AtZIP3* and *AtZIP10* in shoots of *T. caerulescens* compared with *T. arvense*. Transcripts with homology to three CDF/MTP transporters (*AtMTP1*, *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⁺⁺-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 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*, *AtZIP10*, *AtMTP1*, 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 nonaccumulator species (Broadley et al. 2007). Only six of these encode proteins with a specific role in zinc homeostasis, including three CDF/MTP transporters: *AtZIP10*, *AtMTP8* and *AtMTP11*. Others are *AtHMA3* (*AtMT2a*), *AtHMA4* and the nicotianamine synthase gene *AtNAS3*. The obvious lack of correspondents 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. (2005) 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 using 79 genes in *A. thaliana* and 125 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 2200 genes were significantly higher expressed in *T. caerulescens* (25-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*, *IRT5* (*TcZNT2*), *NARP3*, *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 (Green et al. 2004). In addition, strong expression of *NAS2* (*TcMT2a*), *AtZIP7* (*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 *FRD5*, *FRO4* and *FRO5* 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 et al. 2004), they might also be 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 the salt tolerant species *Thelungiella halophila* (Suzuki et al. 2001). Hassenen 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 remained to be determined to cadmium levels and may thus have a function in metal homeostasis under zinc excess. Two more genes with possible roles in metal sequestration were isolated in this study, namely those encoding the *TcMRP10* transporter and a pectate methyl-esterase (PME). *TcMRP10* was highly homologous to *AtMRP10* and marginally less homologous to *AtMTP4*. The *ATMPs* 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 (Ortíz 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 AMrP3, in line with the observed protection of A. thaliana seedlings in A. thaliana hybrid plants in T. caerulescens, zinc is mainly stored in the vacuole (Küp- per et al. 1999), but the apoplastic 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-accumul- ator accession may thus enhance zinc binding in the apoplastic. In conclusion, even though a small set of genes was identified using DD, (novel) genes with possible implic- ations in metal adaptation were found.

PROTEOMICS

The major advantage of proteomics over transcriptomics is that it focuses on the actual proteins, rather than the poten- tial to make proteins. The importance of post-transcrip- tional 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 pro- teins (Gygí et al. 1999; Idéker et al. 2001). A disadvantage of proteomics is currently still the reduced resolution com- pared 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). Addi- tionally, 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 profiling. Likely, 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 pro- teins that play a role in nickel accumulation (or tolerance) in the nickel hyperaccumulator Alysson lesbiacum by analy- ising changes in protein abundance occurring in response to short- and long-term exposure to nickel. Short-term exposure experiments can lead to changes in the expression levels of genes associated with sulphur-containing amino acid metabolism. Together these changes indicated a re-allocation of sulphur towards the increased production of cysteine. The increase in proteins such as oxidoreductase, mannitol-6-phosphate re- ductase and a glutathione S-transferase may reflect an anti- oxidant 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 abun- dance 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 metabo- lism) as well as putative signalling and regulatory proteins. The observed large-scale differences in transcript abundances are unrelated to metal adaptation or hyperac- cumulation. Cross-species transcriptomic comparisons have identified distinct 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 homeo- stasis. It is unlikely, however, that the evolution of hyperac- cumulation 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 hy- peraccumulation. The regulation of metal homeostasis genes in plants is almost completely unexplored. Only for iron homeostasis the first transcription factors are found (Colan- gelo and Gueriniot 2004; Jakoby et al. 2004; Yuan et al. 2005). The observed large-scale differences in gene expres- sion profiles between hyperaccumulator and non-hyperac- cumulator species warrant a more dedicated look towards potential transcriptional regulators and gene promoter se- quences of candidate genes. Also, since many differentially expressed genes appear to encode unknown proteins, func- tional 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 on a more detailed level, with the aid of 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 thaliana 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. lyrae interspecific crosses (Dra- ger 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 in- creasing, facilitated by advances in high-throughput pro- filing of the transcriptome, proteome, metabolome and io- nome (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.

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