Heavy metal tolerance and accumulation in *Thlaspi caerulescens*, a heavy metal hyperaccumulating plant species

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Heavy metal tolerance and accumulation in *Thlaspi caerulescens*, a heavy metal hyperaccumulating plant species

Zware metalen tolerantie en accumulatie in *Thlaspi caerulescens*, een zware metalen hyperaccumulerende plantensoort

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Chapter 1

General Introduction

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Micronutrients in plants

Micronutrients are elements, which are essential for plant growth and play an important role in physiological and metabolic processes of the plant (Ramesh et al., 2004). However, micronutrients are required in much smaller amounts than macronutrients (nitrogen, phosphorus, potassium, calcium, magnesium and sulfur). The micronutrients are boron (B), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), zinc (Zn), cobalt (Co) and chloride (Cl). The awareness of micronutrient deficiencies in humans, animals and crops has increased and has been identified through increased use of soil and plant analysis. Micronutrients can also be very toxic when available to the plant in high concentrations. As a result of human activities such as mining and smelting of metals, electroplating, gas exhaust, energy and fuel production, the use of fertilizers, sewage and pesticide application, municipal waste generation, etc. (Kabata-Pendias and Pendias, 1989), metal pollution has become one of the most severe environmental problems today.

Although iron is one of the most abundant elements of the earth's crust, iron deficiency is a serious problem both in human nutrition and in agriculture (Guerinot, 2001). Thirty to sixty percent of the world's population is potentially affected by iron deficiency, a leading cause of maternal death in African and Asian countries where people rely mostly on plants for their daily intake of iron (Graham et al., 2001). Iron can also be a limiting factor in the growth of economically important crop plants because of inadequate soil chemistry. Such deficiencies can be corrected by amending the soil. Improving the plant's ability to absorb iron under adverse conditions and increasing its overall content could also offer solutions to these problems.

Zinc is a common element in nature and plays an important part in many biological processes. Zinc is an essential trace element, or micronutrient, which is essential for the normal growth and the reproduction of all higher plants, animals, and humans. In addition, it plays a key role during growth and fulfills an immune function for humans and animals. It is vital for the functionality of more than 300 enzymes, for the stabilization of DNA, and for gene expression as a structural element in transcription factors. Zinc deficiency is the most common crop micronutrient deficiency affecting 50% of cultivated soils in India and Turkey, one third of the cultivated soils in China, and most soils in Western Australia (Broadley et al., 2007).

Boron is believed to be involved in carbohydrate transport in plants; it also assists in metabolic regulation.

Chlorine is necessary for maintaining the osmotic status and ionic balance of cells. Although chlorine deficiency is rare, it can occur in plants growing in highly leached soils. A much larger problem is chlorine toxicity in arid and semi-arid regions. In sensitive plants, low concentrations of chlorine in solution already limit plant growth. The availability of chlorine in soil depends largely on source materials containing chlorine and deposition of marine aerosols. Availability of chlorine increases, and can become toxic, in acidic soils that have a high Anion Exchange Capacity (AEC). Chlorine that causes problems is largely restricted to inorganic sources due to the high solubility of chlorine in water.

Copper is a cofactor of some enzymes and of vitamin A. Copper deficiency can be found in plants grown on calcareous soils or in soils with high levels of organic matter. The availability of copper in soil depends on several factors including soil pH, texture, and on interactions with other nutrients. Increased soil pH reduces the availability of copper to plants through increased adsorption at cation exchange sites. Leached podzolic sands and calcareous sands tend to have lower copper levels. There are many complex interactions of zinc, iron and copper with other nutrients and many of these interactions can cause deficiency. Plants vary widely in their tolerance to zinc, iron and copper and chlorosis is a common response to toxic levels of these micronutrients.

Manganese activates some important enzymes involved in chlorophyll formation. Manganese deficient plants will develop chlorosis between the veins of its leaves. The availability of manganese is partially dependent on soil pH. Toxic levels of manganese vary widely between plants and often lead to brown spots on mature leaves. There are numerous factors that control the availability of manganese to plants; including soil pH, hydrology, organic matter, interactions with other nutrients, and climatic effects. High levels of other cations (Cu, Fe, and Zn) can reduce plant uptake of manganese, probably through competition at the level of uptake transporters.

Molybdenum is essential to plant growth. Molybdenum is used by plants to reduce nitrate because it is a component of the Moco cofactor of nitrate reductase. Some plants use it for

nitrogen fixation, and therefore it may need to be added to some soils before sowing legumes. Toxicity of molybdenum can occur at concentrations of 5-10 mg/kg dry weight of plant tissue and can result in the malformation or discoloration of shoot tissue. Soil pH, Fe and Al oxides, interactions with other nutrients, and climatic factors control the availability of molybdenum. Unlike other micronutrients, molybdenum availability increases with increasing soil pH. Iron and aluminum oxides adsorb molybdenum, thereby reducing its availability for plants. Phosphorus and magnesium tend to enhance molybdenum uptake by plants while nitrogen, copper, and manganese tend to reduce its uptake. Furthermore dry conditions tend to reduce the uptake of molybdenum.

Nickel was established as an essential micronutrient for the growth. The detrimental effects of excessive Ni on plant growth have been well known for many years. More recent evidence indicates that Ni is required in small amounts for normal plant growth and development. Ni is an essential component of urease in plants and microorganisms. A deficiency of Ni in plants is reported to result in necrotic lesions in leaves in response to toxic accumulations of urea (Brown et al., 1987).

Metal hyperaccumulating plant species

There are species that can accumulate large amounts of metals without any sign of toxicity. These plant species are so-called metal hyperaccumulators. Since the 1970's, many new hyperaccumulator species have been identified. The term hyperaccumulator was first defined as plants that contain nickel at concentrations higher than 1000 μg g^{-1} dry weight in their aboveground parts (Brooks et al., 1977). With the discovery of plants accumulating large amounts of other metals, Baker and Brooks (1989) defined additional threshold concentrations for these metals, including 100 μg g^{-1} dry weight for cadmium, 1000 μg g^{-1} dry weight for copper, cobalt, lead and 10,000 μg g^{-1} dry weight for zinc and manganese. Currently over 400 metal hyperaccumulator species, belonging to 45 different families, have been described, with the highest occurrence in the Brassicaceae family (Reeves and Baker, 2000). The majority of these metal hyperaccumulators, at least 317, are nickel hyperaccumulators. These species, containing 0.1 to 1% nickel in their aerial tissues, are mainly found on serpentine soils. Zinc

hyperaccumulators represent the second largest group of hyperaccumulators, although there are only about fifteen of such species known (Baker et al., 1992; Brooks, 1994). They are mainly, though not exclusively, found to grow on calamine soils contaminated with lead, zinc or cadmium (Bert et al., 2002; Meerts and Van Isacker, 1997; Schat et al., 2000). Only four plant species have been identified as being cadmium hyperaccumulators thus far, *Thlaspi caerulescens* J. & C. Presl (further referred to as *T. caerulescens*), *Thlaspi praecox*, *Arabidopsis halleri* (L.) O'Kane and Al-Shehbaz (further referred to as *A. halleri*) and *Sedum alfredii* (Baker et al., 1992; Brooks, 1994; Vogel-Mikuš et al., 2005; Deng et al., 2006).

T. caerulescens (Figure 1) is one of these natural zinc, cadmium and nickel hyperaccumulator species. It is a self-compatible species, showing variable rates of outcrossing in nature. *T. caerulescens* is closely related to *A. thaliana* (L.) Heynh., with on average 88.5 % DNA identity in coding regions (Rigola et al., 2006) and 87 % DNA identity in the intergenic transcribed spacer regions (Peer et al., 2003). As in most metal hyperaccumulators, the zinc, cadmium and nickel concentration in shoot tissue of *T. caerulescens* is often higher than in root tissue (Lasat et al., 1996; Schat et al., 2000; Shen et al., 1997).

Figure 1. *Thlaspi caerulescens*

A. halleri is another metal hyperaccumulating species and is known to hyperaccumulate zinc and cadmium (Bert et al., 2000; Weber et al., 2004). Also *A. halleri* is, like *T. caerulescens*, able to accumulate very high concentrations of zinc in the leaves, between 3,000 and 22,000 μg g^{-1} dry biomass within a wide dynamic range of external zinc concentrations in the field (Bert et al., 2000). The leaves of some individuals within the populations have been reported to contain hyperaccumulator levels of cadmium of more than 100 μg g^{-1} dry biomass (Dahmani-Muller et al., 2000; Talke et al., 2006). *A. halleri* and *A. thaliana* share on average approximately 94% nucleotide sequence identity within the coding regions (Becher et al., 2004). *A. halleri* is exclusively found on metal contaminated sites in Western Europe, though in Eastern Europe it is found on both contaminated and uncontaminated sites (Bert et al., 2000).

Plant metal homeostasis

The complex network of homeostatic mechanisms evolved in plants to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001) (Figure 2) also applies for metal hyperaccumulators. In general this network involves three major components: a) transport, b) chelation, and c) sequestration (Figure 2). Plants use two methods to take up metals from the soil: (1) acidification of the rhizosphere through plasma membrane pumps and (2) secretion of ligands, which chelate the metal such as phytosiderophores. The soluble metals can enter the root symplast by crossing the plasma membrane (PM) of the root endodermal cells or they enter the root apoplast through the space between cells. Entering the root symplast and crossing these PMs is mediated by transporters such as channel proteins and/or H^+-coupled carrier proteins (Clemens et al., 2002).

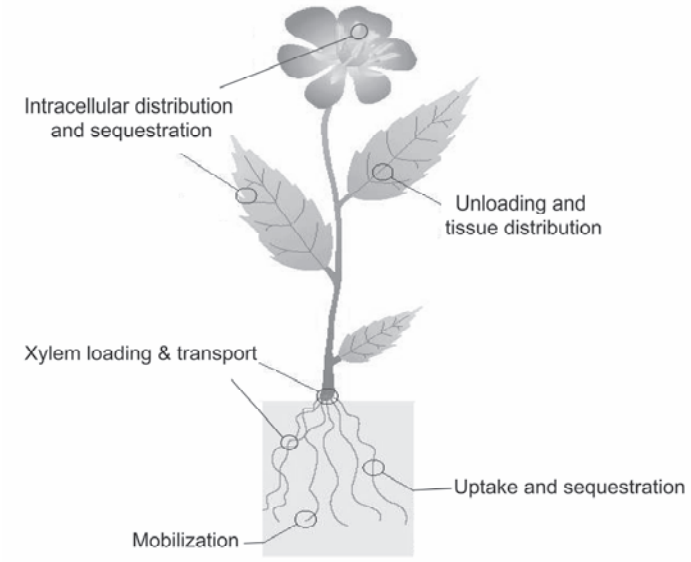

Figure 2. Plant metal homeostasis (Clemens et al., 2002).
Homeostatic mechanisms evolved in plants to control the uptake, accumulation, trafficking and detoxification of metals. This network involves three major components: a) transport, b) chelation, and c) sequestration.

The solutes can subsequently enter the xylem from where it is translocated to aerial tissues. Before entering the xylem, these metals must pass through the cells of the endodermis through the action of membrane pumps or channels. The transport of ions into the xylem generally is a tightly controlled process mediated by membrane located transport proteins. Chelation with certain ligands such as histidine, nicotianamine, mugineic acid (Pich et al., 1994), citrate, malate or oxalate (Senden et al., 1995) appears to be required for transport within the xylem. Xylem-unloading processes are the first step in controlled distribution and detoxification of metals in the shoot, as well as in a possible re-distribution of metals via the phloem (Schmidke and Stephan, 1995). The metals reach the apoplast of the leaves via the xylem sap, from where they are unloaded and imported into the leaf tissue (Marschner, 1995), from where the metals are distributed via the apoplast or the symplast (Clemens et al., 2002). Once in the leaf tissue, the metal can be sequestered into different subcellular compartments (cell wall, cytosol and vacuole). Storage and detoxification of metals appears to occur preferentially in the trichomes (Clemens et al., 2002).

Metal transporters

To govern metal translocation throughout the plant, plant genomes encode large families of metal transporters that vary in their substrate specificities, expression patterns, and cellular localization. Metal transporters are of major importance in metal homeostasis and can either be involved in metal uptake or metal efflux within the plant. Proteins belonging to the ZRT-, IRT-like (ZIP) family play a role in uptake of metals within the plant (Fox and Guerinot, 1998). ZIPs have eight transmembrane domains and a histidine-rich variable loop between transmembrane domains III and IV (Colangelo and Guerinot, 2006). The ZIP family contains 15 members; *ZIP1-12*, *IRT1*, *IRT2*, and *IRT3*. The family derives its name from the founding members, *ZRT1* and *ZRT2* of *Saccharomyces cerevisiae* (Zhao and Eide, 1996a, 1996b). The cloning of *IRT1* from Arabidopsis (Eide et al., 1996) has lead to the discovery of the ZIP family in plants. *IRT1* is known to mediate the uptake of iron (Eide et al., 1996), zinc and manganese (Korshunova et al., 1999) and has a high affinity for iron and a low affinity for zinc, manganese and cadmium. The assumed role of ZIP transporters in metal accumulation is supported by additional research on

ZIP homologs, like *ZNT1* (Pence et al. 2000), a putative *AtZIP4* orthologue, and its homologue *ZNT2* (Assunção et al. 2001), a putative *AtIRT3* orthologue, in the hyperaccumulating species *T. caerulescens*. Other members of the ZIP family have specific substrate specificities and affinities. In support of a role in metal homeostasis, expression studies demonstrated that steady-state mRNA levels for some of the ZIP transporters are metal responsive under certain conditions.

Another family of proteins involved in metal uptake and transport is the natural resistance associated macrophage protein family (NRAMP). This family has been identified in bacteria, fungi, plants, and animals. Arabidopsis contains six genes encoding proteins with high homology to NRAMPs (Mäser et al., 2001). Many additional ESTs indicate that genes from the *NRAMP* family are present in other dicots and monocots (tomato, soybean, *Medicago truncatula*, rice, maize). The function of AtNRAMP proteins in metal transport has been demonstrated in heterologous yeast expression systems and *in planta* (Alonso et al., 1999; Curie et al., 2000; Thomine et al., 2000). In yeast, expression of *AtNRAMP1, 3* and *4* can complement the phenotype of yeast strains deficient for manganese or iron uptake (Curie et al., 2000; Thomine et al., 2000). It was also found that *NRAMP1* and *NRAMP3* are expressed under iron-limiting conditions (Thomine et al., 2000). Furthermore, the expression of *AtNRAMP1, 3* and *4* in yeast increased their cadmium sensitivity and cadmium accumulation (Thomine et al., 2000). Further research showed that *AtNRAMP3* controls iron-acquisition, manganese and zinc accumulation upon iron starvation, and cadmium sensitivity by mobilizing iron, cadmium and other metals from the vacuoles in Arabidopsis (Thomine et al., 2003; Lanquar et al., 2005). This indicates that these *AtNRAMP* genes encode transporters with limited metal specificity.

The alkali metal potassium is a major macronutrient and is the most abundant cation in plants. Potassium transporters are required for the accumulation of potassium ions from soil and for their distribution throughout the diverse plant tissues (Mäser et al., 2001). A large number of genes in Arabidopsis encode potassium transporters, which fall into either of four to five families; two distinct potassium channel families, Trk/HKT transporters; KUP/HAK/KT transporters and K^+/H^+ antiporter homologs (Mäser et al., 2001). The potassium channel family contains 15 genes and is perhaps the best understood transporter family in plants in terms of gating, second messenger regulation, transport properties and predicted functions in different plant cells and

membranes (Mäser et al., 2001). The selectivity for potassium is structurally defined by four highly conserved pore-forming loops on the outer surface of the channel, each P-loop being embedded between two transmembrane domains (Mäser et al., 2001). The functional channel is either a tetramer of α-subunits with one P-loop per subunit or possibly a dimer of α-subunits carrying two P-loops (Mäser et al., 2001). Furthermore, α-subunits differ in the number of transmembrane domains, with either two, four, six or eight transmembrane domains (Mäser et al., 2001). The first potassium channels cloned from Arabidopsis were *KAT1* (Anderson et al., 1992) and *AKT1* (Sentenac et al., 1992).

Trk/HKT transporters are reminiscent of potassium channels in that they possess in a single polypeptide chain four domains resembling P-loops. These P-loop-like domains are only weakly conserved to potassium channel P loops. The *Trk/HKT* gene family in Arabidopsis is represented by a single member *AtHKT1*. The AtHKT1 protein does not transport potassium but sodium (Uozumi et al., 2000). Therefore, AtHKT1 might function in sodium transport in Arabidopsis, and plant Trk/HKT transporters have been proposed to contribute to sodium transport and sensitivity in plants (Uozumi et al., 2000). HKT transporters have two functions: (i) to take up Na^+ from the soil solution to reduce K^+ requirements when K^+ is a limiting factor, and (ii) to reduce Na^+ accumulation in leaves by both removing Na^+ from the xylem sap and loading Na^+ into the phloem sap (Rodríguez-Navarro and Rubio, 2006)

Bacterial potassium uptake permeases named KUPs and fungal high-affinity potassium transporters named HAKs form an additional family of potassium transporters that has also been identified in plants (Mäser et al., 2001). The plant genes were named *AtKT*, *AtHAK* or *AtKUP*. Knowledge on the function of KUP/HAK/KTs remains limited (Mäser et al., 2001). For *AtHAK5* it was demonstrated that there is an in vivo function for *AtHAK5* in the inducible high-affinity K^+ uptake system in Arabidopsis roots (Gierth et al., 2005).

The K^+/H^+ antiporters have first been described from gram-negative bacteria, where they are gated by glutathione-S conjugates and inactivated by glutathione ((Mäser et al., 2001). Arabidopsis contains six putative potassium efflux antiporters, named *KEA1-6* (Mäser et al., 2001). None of these *KEA*s has been experimentally characterized.

Another family of transporters is the cation/proton antiporter group. These antiporters extrude cations from the cytosol to the outside across the PM or into intracellular compartments, including the vacuole. The vacuole is an important storage organelle for many ions. The concentration gradient of cations is established across the plant tonoplast, in part, by high-capacity cation/H^+ (CAX) exchange activity. Arabidopsis has four genes encoding cation/H^+ exchangers, *CAX1-4*. *CAX1* and *CAX2* were identified (Hirschi et al., 1996) by their ability to sequester calcium into yeast vacuoles in *S. cerevisiae* mutants deleted for the vacuolar high-affinity Ca^{2+}-ATPase (*PMC1*) and low-affinity H^+/Ca^{2+} antiporter (*VCX1*). *CAX2* appears to be a plant vacuolar metal transporter (Hirschi et al., 2000). *CAX3* cannot suppress the Ca^{2+} sensitive phenotype of yeast mutants defective in vacuolar Ca^{2+} transport and the precise function of this transporter remains unknown (Shigaki and Hirschi, 2000). The *CAX4* gene product does not suppress yeast defects in vacuolar calcium transport. However, *CAX4* expression weakly suppressed the Cd^{2+} sensitivity of an *IRT1* harboring strain. Although this suppression was not strong, it suggests that CAX4 functions in providing Cd^{2+} tolerance in this strain, indicating that CAX4 may transport Cd^{2+}.

P_{1B}-ATPases are a subfamily of the P-type ATPases, which are known to transport heavy metals such as copper, zinc, cadmium and lead (Lutsenko and Kaplan, 1995; Axelsen and Palmgren, 2001; Argüello, 2003). P_{1B}-ATPases have eight predicted transmembrane domains, a CPx motif that is thought to play a role in translocation, and putative metal-binding domains. In Arabidopsis there are eight genes encoding P_{1B}-ATPases (*AtHMA1 – AtHMA8*), which differ in their structure, function and regulation. *AtHMA1, AtHMA2, AtHMA3* and *AtHMA4* are involved in the transport of zinc, cobalt, copper and cadmium (Axelsen and Palmgren, 2001; Seigneurin-Berny et al., 2006). *AtHMA1* is involved in copper homeostasis and offers an additional way to the previously characterized chloroplast envelope Cu-ATPase *PAA1* (*AtHMA6*) to import copper in the chloroplast (Seigneurin-Berny et al., 2006). The *AtHMA2* gene encodes a Zn^{2+}-ATPase in the transmembrane and is activated by cadmium and, to a lesser extent, by other divalent metals (lead, nickel, copper and cobalt) (Eren and Argüello, 2004). *AtHMA3* is suggested to play a role in the transport and detoxification of cadmium and lead in Arabidopsis (Gravot et al., 2004). The *AtHMA4* gene is known to be involved in the transport of zinc and cadmium in Arabidopsis. This

gene is up-regulated in roots exposed to a high concentration of zinc and manganese but down-regulated by cadmium (Mills et al., 2003). Bernard et al. (2004) showed that *TcHMA4*, the *AtHMA4* homologue of *T. caerulescens*, is about 20 times higher expressed in the roots of *T. caerulescens* than in the roots of Arabidopsis. In three different accessions of *T. caerulescens, TcHMA4* was constitutively expressed independent of the cadmium concentration. Mutant analysis showed a possible function for *TcHMA4* both in cadmium and zinc transport (Hussain et al., 2004). Other recently published work suggest that *TcHMA4* is involved in pumping cadmium and other heavy metals and micronutrients across the plasma membrane out of the cell, therefore acting as a plasma membrane-localized metal ATPase pump (Papoyan et al., 2004). The authors suggested that the protein functions in metal xylem loading by mediating transport of heavy metals and micronutrients from xylem parenchyma into xylem vessels in the *T. caerulescens* roots (Papoyan et al., 2004). *AtHMA5* is proposed to have a role in copper compartmentalization and detoxification (Andres-Colas et al., 2006) and *AtHMA6* (*PAA1*) is a critical component of a Cu transport system in chloroplasts responsible for cofactor delivery to plastocyanin and Cu/ZnSOD (Shikanai et al., 2003). *AtHMA7* (*RAN1*) is a putative Cu^{+}/Ag^{+}-pump. Experiments by Hirayama et al. (1999) who were looking for mutants that show ethylene phenotypes in response to trans-cyclooctene, an ethylene antagonist, have implicated *AtHMA7* to be involved in ethylene-signaling pathways. They showed that the "ethylene-related" phenotype could be rescued by excess copper and that the plant pump could complement a mutation in yeast of ccc2, a yeast copper ATPase. *AtHMA8* is most similar to *AtHMA6* (Baxter et al., 2003) but no function for this gene has been identified thus far.

Another gene family involved in metal efflux is the cation diffusion facilitator (CDF) protein family, which is found in mammals, bacteria, fungi and plants. This gene family contains six transmembrane domains and encodes proton antiporters that efflux heavy metals out of the cytoplasm (Gaither and Eide, 2001). The first characterized *CDF* gene in Arabidopsis was *ZAT1* (van der Zaal et al., 1999), later renamed *Metal Tolerance Protein 1* (*MTP1*). *MTP1* is homologous to some mammalian zinc transporter genes that are involved in zinc vesicular sequestration (Wenzel et al., 1997). Transgenic Arabidopsis plants overexpressing *MTP1* have an increased resistance to zinc and exhibit increased zinc content in the roots, which suggest that

MTP1 is involved in the sequestration of zinc (van der Zaal et al., 1999; Kobea et al., 2004; Desbrosses-Fonrouge et al., 2005; Krämer, 2005). *TcZTP1*, the orthologue of *MTP1* in *T. caerulescens*, might contribute to zinc tolerance (Assunção et al., 2001) in the hyperaccumulator. Northern blot analysis showed that *ZTP1* is clearly overexpressed in *T. caerulescens* acc. La Calamine when compared to the non-accumulator *T. arvense*. The same was shown for *AhMTP1*, the orthologue in *A. halleri* (Dräger et al., 2004), where transcript abundance of *MTP1* is up-regulated in response to high zinc concentrations in the roots of *A. halleri*.

Yellow Stripe-like (YSL) proteins are thought to play a role in long-distance transport of metals that are complexed with plant-derived phytosiderophores (PS) or nicotianamine (NA) (Didonato et al., 2004). In Arabidopsis there are eight predicted YSL proteins and these are thought to transport metal-NA complexes (Curie et al., 2001). The *YSL1* and *YSL3* genes are similar to the maize (*Zea mays*) *YS1* phytosiderophore transporter (*ZmYS1*) and the *AtYSL2* Fe-nicotianamine transporter gene, and are predicted to encode proteins that transport metal-nicotianamine complexes into cells (Waters et al., 2006). *YSL1* and *YSL3* mRNAs are expressed in both root and shoot tissues, and both are regulated in response to the iron status of the plant (Waters et al., 2006). Beta-glucuronidase reporter expression, driven by *YSL1* and *YSL3* promoters, reveals expression patterns of the genes in roots, leaves, and flowers. Expression was highest in senescing rosette leaves and cauline leaves (Waters et al., 2006). Whereas the single mutants *ysl1* and *ysl3* had no visible phenotypes, the *ysl1 ysl3* double mutant exhibited iron deficiency symptoms, such as interveinal chlorosis (Waters et al., 2006). Leaf iron concentrations are decreased in the double mutant, whereas manganese, zinc, and especially copper concentrations are elevated (Waters et al., 2006). In seeds of double-mutant plants, the concentrations of iron, zinc, and copper were low (Waters et al., 2006) and Le Jean et al. (2005) showed that iron and nicotianamine levels in seeds rely in part on *AtYSL1* function. Mobilization of metals from leaves during senescence is impaired in the double mutant. In addition, the double mutant has reduced fertility due to defective anther and embryo development (Waters et al., 2006). The proposed physiological roles of YSL1 and YSL3 are in delivery of metal micronutrients to and from vascular tissues (Waters et al., 2006). Didonato et al. (2004) suggested that *YSL2* transports metals that are chelated by NA; *AtYSL2* has 62% identity to *ZmYSL1,* which

is known to transport iron and copper when complexed to NA. *YSL2* could not restore growth of a zinc-uptake-defective yeast strain, suggesting that *YSL2* is not transporting zinc when available at low concentrations. *YSL3* shows 69% identity to *YSL2* and has been shown to be the closest relative to *YSL2* (Didonato et al., 2004). In *T. caerulescens* also three *YSL* genes have been characterized, *TcYSL3*, *TcYSL5* and *TcYSL7* (Gendre et al., 2007). The three *YSL* genes are expressed at high levels compared with their Arabidopsis homologs but with distinct patterns. While *TcYSL7* was highly expressed in the flowers, *TcYSL5* was higher expressed in the shoots, and the expression of *TcYSL3* was similar in all the organs tested (Gendre et al., 2007). In situ hybridizations showed that *TcYSL7* and *TcYSL5* are expressed around the vasculature of the shoots and in the central cylinder in the roots (Gendre et al., 2007). The exposure to heavy metals (zinc, cadmium, nickel) did not affect the high and constitutive expression of the *TcYSL* genes (Gendre et al., 2007). It was also demonstrated by mutant yeast complementation and uptake measurements that TcYSL3 is an iron/nickel-NA influx transporter (Gendre et al., 2007).

Metal chelators

Another group of compounds, important for metal homeostasis, are metal chelators, which include phytochelatins, metallothioneins, organic acids and amino acids. Metal chelators play an important role in metal detoxification (Ernst et al., 1992). Phytochelatins (PCs) are metal-binding peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n=2-11) (Kondo et al., 1984; Grill et al., 1985; Jackson et al., 1987). PCs are non-translationally synthesized from reduced glutathione (Grill et al., 1989). PCs were first discovered in the fission yeast *Schizosaccharomyces pombe* (Kondo et al., 1984) and thereafter in plants (Grill et al., 1989). Metal ions like Cd^{2+}, Cu^{2+}, Ag^{+} and As^{3+} have been shown to form complexes with PCs in vivo (Maitani et al., 1996; Schmöger et al., 2000).

Metallothioneins (MTs) chelate metal ions like cadmium, zinc and copper. MTs are small, Cys-rich proteins, which are present in animals, fungi, cyanobacteria, and plants (Zhou and Goldsbrough, 1994). To date more than 50 MT-like sequences have been described in various plants (Rauser, 1999), which differ from the mammalian and fungal MTs in their Cys motifs. It has been hypothesized that these differences in Cys motifs are responsible for the differences in

metal specificities (e.g. type-1: C-X-C, type-2: combination of C-X-C, C-C and C-X-X-C) (Robinson et al., 1993). In *A. thaliana*, at least three type-1 sequences, two type-2 sequences and at least two sequences of a different type, designated as *MT3*, have been identified (Zhou and Goldsbrough, 1995; Murphy et al., 1997). Evidence for developmental regulation and responsiveness to a variety of stimuli including metal exposure were found (Rauser, 1999). *TcMT3* was constitutively higher expressed in different accessions of *T. caerulescens* compared to Arabidopsis (Roosens et al., 2004). These authors also suggested that *TcMT3* is not responsible for cadmium tolerance itself but that the high expression of these genes is likely to be an adaptation of the plant to ensure an adequate copper homeostasis in the presence of excess zinc or cadmium in the leaves. In many plants including Arabidopsis, MT RNAs are strongly induced by copper and slightly by zinc and cadmium (Zhou and Goldsbrough, 1995).

Nicotianamine (NA) is another metal chelating compound. NA is a non-proteinaceous amino acid that binds mainly iron but also other divalent metal ions (von Wirén et al., 1999). One molecule of NA is synthesized by trimerization of S-adenosylmethionine (SAM), which is catalyzed by the enzyme nicotianamine synthase (NAS). NA is a precursor of the phytosiderophore mugeneic acid (MA) but it is not excreted by the roots like MA. It has been found in all plants investigated (Stephan and Scholz, 1993). NA chelates metals like iron, zinc, copper and nickel and is thought to play a role in long distance metal transport, possibly involved in the entry of metals into the phloem or xylem. The role of NA in metal homeostasis has mainly been studied in the tomato mutant *chloronerva*, which is deficient in NA (Ling et al., 1999). This mutant has the characteristics of iron deficiency as indicated by its interveinal chlorosis. The enzyme NAS was first described in tobacco (Noma et al., 1971) and has been identified also in plants like *Hordeum vulgare* (Herbik et al., 1999; Higuchi et al., 1999; Kim et al., 2005), *Lycopersicon esculentum* (Ling et al., 1999), *A. thaliana* (Suzuki et al., 1999), *Oryza sativum* (Higuchi et al., 2001; Inoue et al., 2003), *Zea mays* (Mizuno et al., 2003), *A. halleri* (Becher et al., 2004; Weber et al., 2004), and *T. caerulescens* (Pianelli et al., 2005). The nicotianamine synthase family in Arabidopsis consists of four members, *AtNAS1-AtNAS4*. Expression of *A. halleri NAS2* in zinc hypersensitive *Schizosaccharomyces pombe* cells showed that formation of

NA confers zinc tolerance (Weber et al., 2004). It was also shown that heterologous expression of a *T. caerulescens* NAS gene in yeast caused nickel tolerance (Vacchina et al., 2003).

The organic acids citrate and malate are thought to be potential ligands and have been implicated in metal homeostasis (Rauser, 1999). Organic acids have been proposed as important cellular ligands for iron, zinc, cadmium and nickel (Rauser et al 1999; von Wirén et al., 1999). By X-ray absorption spectrometry (XAS) and extended X-ray absorption fine structure (EXAFS) analysis, citrate was identified as the predominant ligand for zinc in the leaves of *T. caerulescens* (Salt et al., 1999; Kupper et al., 2004) while malate was the major zinc ligand in aerial tissues of *A. halleri* (Sarret et al., 2002). Similarly, nickel-citrate accounted for one quarter of the nickel species in leaves of the nickel hyperaccumulator *T. goesingense* and in the related nonaccumulator *T. arvense* (Krämer et al., 2000). Citrate and malate are probably only relevant as ligands for these metals within the vacuoles (Krämer et al., 2000).

The amino acid histidine is also a metal ligand and is thought to be involved in the chelation of nickel (Krämer et al., 1996). Studies of a range of aerial tissues of *T. caerulescens* at various developmental stages indicated that the speciation of zinc is dynamic and suggested that histidine may be more important in chelating zinc in older tissues which may be related to a shift in the cellular or subcellular distribution of zinc at different developmental stages.

Comparative transcriptomics – model species lead the way

Whereas the physiology of metal hyperaccumulation is already understood fairly well (Clemens et al., 2002), the underlying molecular control is still not investigated in detail. To understand more about the genetic mechanisms conferring the hyperaccumulation trait, comparative genomics seems an attractive approach.

Plant biology has reached the stage that more and more molecular tools are developed for species other than the general reference species like Arabidopsis or rice. Unfortunately this often does not include the construction of “whole genome” microarrays. Although genome-wide expression analysis is very instructive to obtain clues about (novel) genes that are active under the studied conditions, the costs for designing and producing microarrays for the wide variety of species with interesting traits are too high. That is also why in recent years several successful

attempts have been made to perform heterologous microarray hybridizations to study the transcriptome of different non-model species. Recently Hammond et al. (2006) presented an improved method of using Arabidopsis Affymetrix array to determine the transcript profile of two related Thlaspi species with contrasting metal accumulation and tolerance phenotypes.

Comparative transcriptomics in plant biology

Two main reasons to perform heterologous transcript profiling are: 1) To compare two closely related species with contrasting traits and 2) to compare different tissues, stages or conditions in a species for which no specific arrays are available. Often these two objectives are combined, as has also been done by Hammond et al. (2006). The need for comparative transcriptomics, which inherently involves heterologous microarray hybridization, is often fueled by the presence of interesting traits and properties in one species that are not found in any of the current model species. If the right conditions and tissues are chosen for sampling and assuming that many of the basic processes in plants are similar, the differences found by comparative transcriptomics will include detection of genes involved in the studied trait or property. Good examples are adaptive traits, such as physiological adaptation to continuous adverse environmental conditions (low or high light intensity, high altitude, low or high temperatures, extreme soil composition (pH, salt, heavy metals, nutrient deficiencies, regular flooding, etc.) or developmental adaptations (leaf or root hair density, leaf shape, flowering time, vernalization response, wax deposition, etc.). Other examples are differences in general plant architecture like fruit or flower size, tuberization, etc.; differences in plant defense or differences in primary or secondary metabolite production. In order to obtain informative hybridization results, the two species that are compared should be closely related to allow sufficient probe-cDNA cross hybridization.

Especially Arabidopsis arrays have been used for comparative transcriptomics in the past to compare Arabidopsis (*A. thaliana*) to related Brassicaceae species like *A. halleri* (Becher et al., 2004; Weber et al., 2004), *T. caerulescens* (Hammond et al., 2006; van de Mortel et al., 2006), *Thellungiella halophila* (Taji et al., 2004; Gong et al., 2005), *Brassica oleracea* (Hammond et al., 2005) and *B. napus* (Li et al., 2005), but recently also a tomato array has been successfully

used to examine fruit ripening and development in tomato, eggplant and pepper (Moore et al., 2005) and no doubt more of such experiments will soon follow. Three of the Brassicaceae species were studied because of their special adaptive traits, such as Zn and/or Cd hyperaccumulation and hypertolerance of *A. halleri* and *T. caerulescens* and the salt and cold tolerance of *T. halophila*. Hammond et al. (2006) compared two Thlaspi species to each other but not to Arabidopsis. An interesting general trend that emerged from the analysis of the adapted species was that many orthologues of genes that were induced by stress in Arabidopsis were higher expressed in the adapted species, especially in the absence of the stressor.

As the Arabidopsis genome was the first plant genome to be fully sequenced, the largest variety of array platforms is available for this model species. Generally four different types can be distinguished: (1) spotted (full-length) cDNA microarrays; (2) spotted PCR-amplified gene-specific sequence tag (GST) or genomic amplicon arrays; (3) on-slide synthesized short oligonucleotide arrays; and (4) spotted or on-slide synthesized long oligonucleotide arrays (Rensink and Buell, 2005). Spotted cDNA microarrays are the least sophisticated, containing a collection of clones from different cDNA libraries, which are PCR-amplified using vector specific primers. Short oligonucleotide arrays contain probes up to 25 bases in length. Long oligonucleotide arrays contain probes of between 50-70 bases. The spotted gene-specific fragment arrays contain unique segments of the gene, which are amplified from genomic DNA (gDNA) or gDNA libraries using specific primers for each gene fragment. Oligo arrays are generally more technologically advanced and are therefore often only commercially available, while the spotted PCR fragment arrays are often developed within academia. Next to Arabidopsis arrays there is a growing list of arrays for other species, mainly crops like barley, *Brassica*, *Citrus*, grape, lily, maize, potato, rice, soybean, sugar cane, tomato and wheat, but also for trees such as poplar, pine and spruce and for the legume model *M. truncatula* (Rensink and Buell, 2005; Huang et al., 2006; Ralph et al., 2006).

Considering that the successful use of heterologous microarray hybridization depends largely on the level of sequence similarity and considering that this is highest among members of the same plant families, the current range of arrays already covers many agronomically important plant families (Brassicaceae, Leguminosae, Graminae, Solanaceae). Of course it will still be

possible that there is too much sequence diversity for members of the same family to permit heterologous micro-array hybridization. Even if the average level of sequence identity is sufficient, it is important to note that subset of probes may not hybridize efficiently due to lower sequence identity. In the Thlaspi interspecies comparison method described by Hammond et al. (2006), the Affymetrix arrays were used that contain several 25-bp oligonucleotide probes per gene. *T. caerulescens* has an average coding region DNA identity with Arabidopsis of 88.5 % (Rigola et al., 2006). Upon heterologous hybridization, many probes did not perfectly match the orthologous Thlaspi sequence resulting in a relatively low number of "present" calls. After hybridization with Thlaspi gDNA, non-fitting probe pairs could be discarded without discarding the entire probe-set and thus still get acceptable expression data. This method is only useful for microarray platforms that contain multiple probes for one gene. Also, when comparing two species care should be taken to include the same probes in the final set for comparison. If not, using one probe for the gene in one species and another for the orthologous gene in the other species can lead to false conclusions on expression levels. When using arrays with only one probe per gene, discarding probes that do not hybridize properly to the target cDNAs will result in fewer genes for which expression can be determined. This may be a disadvantage compared to short oligonucleotide arrays, but on the other hand, longer oligos permit less sequence conservation between species, so less probes will be discarded compared to the short oligo arrays.

Challenges for comparative transcriptomics

Comparative transcriptomics can be a very rewarding tool to discover new gene expression profiles in plant species in the absence of species specific cDNA information or microarrays, on the condition that there are arrays from a sufficiently closely related species. It is clear that such is still not the case for many important families. Currently for instance the Caryophyllaceae, Chenopodiaceae, Compositae, Rosaceae and Umbelliferae are not yet represented among the species for which there are microarrays available. Even though for a growing number of species, including *T. caerulescens* (Plessl et al., 2005), spotted cDNA micro-arrays are being developed, the number of genes represented on such arrays are often limited and probably more information will be obtained by hybridizing to a heterologous, but genome-wide,

microarray. Rather than trying to complete as many as possible of the species specific arrays, it will probably be more efficient to focus on the development of a family specific array. This may well be based on the transcriptome of one reference species, like Arabidopsis for the Brassicaceae, supplemented with probes representing genes not found in Arabidopsis but present in other Brassicaceae. The growing collection of Expressed Sequence Tags that are generated for very many different species would be an excellent source of information for careful design of plant-family-oriented gene-specific long oligonucleotide probes. This will not be easy and it will be a true challenge to develop the bioinformatic tools to accomplish the design of gene-specific probes that are able to detect orthologues while distinguishing paralogues, a problem that is also not solved in designing species-specific microarrays.

Another important item that should be dealt with to improve comparative transcriptomics is to be able to account for all possible transcripts that can be found in a plant cell. Even for Arabidopsis, of which the full genome sequence is known and for which gene expression has been studied by many groups all over the world, the recent use of whole-genome tiling arrays (WGAs) showed that there were still many regions with transcriptional activity although there was no gene annotated (Mockler and Ecker, 2005). WGAs contain non- or partially overlapping probes that are tiled to cover the entire genome. They are instructive to identify rarely or low expressed genes or miRNAs that are hard to identify or predict otherwise and to design probes to add to the current arrays. Although very informative for plant genomics, including transcriptomics, the high costs associated with making such arrays makes it unlikely that this kind of arrays will soon be available for many gene families. Also for these, model species will lead the way.

Outline of the thesis

This thesis describes the comparative transcriptome analysis of *T. caerulescens* and *A. thaliana* in order to obtain a better understanding of heavy metal hyperaccumulation and tolerance. The transcriptome comparison of roots of *T. caerulescens* to those of the related metal-non-accumulating species *A. thaliana* under various zinc exposure conditions is described in chapter 2. In chapter 3 a similar approach was taken to compare the transcriptome of roots of *T. caerulescens* to those of the related metal-non-accumulating species *A. thaliana* under various cadmium exposure conditions and another transcriptome comparison is described in chapter 4 in which roots and shoots of *T. caerulescens* genotypes La Calamine and Ganges, with contrasting cadmium accumulation phenotypes, are compared when exposed to cadmium. The functional analysis of the nicotianamine synthase protein family, which was found to be very differently expressed in *A. thaliana* and *T. caerulescens*, is described in chapter 5. The main results of the experiments described in the previous chapters and the perspectives for future research are discussed in chapter 6.

Chapter 2

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*

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ABSTRACT

The micronutrient zinc has an essential role in physiological and metabolic processes in plants as a cofactor or structural element in 300 catalytic and non-catalytic proteins, but it is very toxic when available in elevated amounts. Plants tightly regulate their internal zinc concentrations in a process called zinc homeostasis. The exceptional zinc hyperaccumulator species *Thlaspi caerulescens* can accumulate up to 3% of zinc on a dry weight basis, but also high amounts of nickel and cadmium, without any sign of toxicity. This should have drastic effects on the zinc homeostasis mechanism. We examined in detail the transcription profiles of roots of Arabidopsis and *T. caerulescens* plants grown under deficient, sufficient and excess supply of zinc. A total of 608 zinc-responsive genes with at least a threefold difference in expression level were detected in Arabidopsis and 352 in *T. caerulescens* in response to changes in zinc supply. Only 14% of these genes were also zinc responsive in Arabidopsis. When comparing Arabidopsis with *T. caerulescens* at each zinc exposure, over 2200 genes were significantly differentially expressed ($\geq$ 5-fold and false discovery rate $<$ 0.05). While a large fraction of these genes are of yet unknown function, many genes with a different expression between Arabidopsis and *T. caerulescens* appear to function in metal homeostasis, in abiotic stress response and in lignin biosynthesis. The high expression of lignin biosynthesis genes corresponds to the deposition of lignin in the endodermis, of which there are two layers in *T. caerulescens* roots and only one in Arabidopsis.

INTRODUCTION

Micronutrients are essential for humans, plants and animals. The micronutrient zinc plays an important role in physiological and metabolic processes of plants (Ramesh et al., 2004). Zinc serves as a cofactor for more than 300 enzymes including RNA polymerase, alcohol dehydrogenase, Cu/Zn superoxide dismutase, and carbonic anhydrase (Guerinot and Eide, 1999). Zinc is essential, but toxic when available to the plant in elevated amounts, therefore plants need to keep very tight control over the internal concentrations of zinc in a process called zinc homeostasis.

Although the zinc homeostasis mechanism is supposed to be universal within plants, there are species that can accumulate large amounts of zinc without any sign of toxicity. Species accumulating more than 10,000 μg zinc g^{-1} of dry weight (DW) (1%, w/w) are called zinc hyperaccumulators (Baker and Brooks, 1989). As a comparison, most plants contain between 30 and 100 μg zinc g^{-1} DW and concentrations above 300 μg zinc g^{-1} DW are generally toxic (Marschner, 1995). Over 400 metal hyperaccumulator species from a wide range of unrelated families have been described. About fifteen of these are zinc hyperaccumulators (Baker et al., 1992; Brooks, 1994). They are mainly, though not exclusively, found to grow on calamine soils contaminated with lead, zinc or cadmium (Meerts and Van Isacker, 1997; Schat et al., 2000; Bert et al., 2002). *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) is one of these natural zinc hyperaccumulator species. In addition to zinc it can also hyperaccumulate cadmium and nickel. It is a self-compatible species, showing variable rates of outcrossing in nature. *T. caerulescens* is closely related to *Arabidopsis thaliana* (L.) Heynh., with on average 88.5 % DNA identity in coding regions (Rigola et al., 2006) and 87 % DNA identity in the intergenic transcribed spacer regions (Peer et al., 2003). As in most metal hyperaccumulators, the zinc concentration in shoot tissue of *T. caerulescens* is often higher than in root tissue (Lasat et al., 1996; Shen et al., 1997; Schat et al., 2000).

The complex network of homeostatic mechanisms that evolved in plants to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001) also applies for

metal hyperaccumulators. In general this network involves three major components: a) transport, b) chelation and c) sequestration. While the physiology of metal hyperaccumulation is already understood fairly well (Clemens et al., 2002), the underlying molecular genetics is still not explored in full detail. Previously published transcript profiling studies on copper, zinc and iron deficiency in *A. thaliana* (Wintz et al., 2003) and comparative analysis of *A. thaliana* with the zinc and cadmium hyperaccumulating *Arabidopsis halleri* (Becher et al., 2004; Weber et al., 2004) using first generation Affymetrix chips representing a subset of only 8300 of the ~30,000 Arabidopsis genes, already identified several genes to respond to zinc deficiency in *A. thaliana*. These analyses also revealed that the transcriptional regulation of many genes is strikingly different in *A. halleri* compared to *A. thaliana*.

In this report we describe the analysis of three transcript profiling experiments, with the main aim to establish which genes are most likely to be relevant for adaptation to high zinc exposure in *Thlaspi caerulescens*. Therefore we examined not only the response of roots of both plant species to zinc deficiency, but also to excess of zinc. We used an Agilent "whole transcriptome" 60-mer oligo DNA microarray representing all annotated genes for *A. thaliana* (further referred to as Arabidopsis) and some 10,000 non-annotated genomic regions with known transcriptional activity, thus covering nearly the complete Arabidopsis transcriptome. In the intraspecific comparison we identified the Arabidopsis and *T. caerulescens* genes that are differentially expressed a week after transferring the plants to low or high zinc supply. These are relevant to determine differences in the zinc homeostasis network between the two species. We also compared the differences in transcription between the two species at zinc deficiency, sufficiency and excess supply conditions to identify the genes that are significantly higher expressed in the hyperaccumulating species compared to Arabidopsis. We finally examined all analyses to identify any particular processes, biochemical pathways or gene classes that could play a particular role in the adaptation to high zinc accumulation.

RESULTS

Experimental design

To analyze the response of Arabidopsis and *T. caerulescens* to different zinc exposures, we aimed at a comparison of the transcript profile of plants grown under sufficient zinc supply with plants grown under zinc deficient conditions and excess zinc conditions. To minimize variation in the bioavailability of zinc or other micronutrients we used a hydroponic rather than soil-based culturing system. For both excess and deficient zinc conditions the induction of severe stress to the plants was avoided by exposing them only for one week to these conditions. Arabidopsis plants (accession Columbia) were established to grow on a nutrient solution containing 2 μM $ZnSO_4$, which is sufficient to yield healthy and robust plants with normal seed set even after prolonged cultivation. After three weeks the plants were transferred to fresh solutions for exposure to zinc deficiency (0 μM $ZnSO_4$) and excess zinc (25 μM $ZnSO_4$). One third of the plants remained at sufficient zinc (2 μM $ZnSO_4$) as a control. From previous experiments (data not shown) we learned that plants continue to grow in zinc deficient media, while deficiency symptoms (chlorosis and necrosis) or toxicity symptoms become obvious only after about three weeks. Upon harvesting root tissue, the plants growing on zinc deficient and sufficient medium did not show any visible phenotypic differences, while plants growing on excess zinc showed a slight growth inhibition in the roots (data not shown). At this stage, plants were not flowering yet.

For *T. caerulescens* a similar approach was taken. However, in order to properly compare the results between the two species we aimed at maintaining comparable physiological conditions. *T. caerulescens* accession La Calamine is zinc tolerant as well as zinc hyperaccumulating and requires more zinc than Arabidopsis for normal growth. Therefore a hydroponic solution containing 100 rather than 2 μM $ZnSO_4$ was used to grow seedlings under zinc sufficient conditions. To avoid any problems with possible precipitation of zinc or other minerals, 1 mM $ZnSO_4$ was used as the excess zinc exposure concentration, although we learned from previous experiments that *T. caerulescens* La Calamine is well able to withstand this exposure for several weeks. When root tissues were harvested after one week exposure, the *T.*

caerulescens plants showed no altered phenotype that could be attributed to exposure to excess zinc or zinc deficiency.

Mineral content in Arabidopsis and *T. caerulescens*

Zinc, iron and manganese concentrations were determined in root and shoots of hydroponically grown Arabidopsis and *T. caerulescens* plants grown at deficient, sufficient and excess zinc. Comparison of the metal concentration levels between these two species already displayed the typical difference between a metal hyperaccumulator and a metal non-accumulator (Figure 1). At low zinc supply (zinc deficiency) the difference is not very pronounced and although *T. caerulescens* clearly contains more zinc in the leaves, the concentration in the roots is for both species between 1.2-1.7 μmol g^{-1} D.W. (Figure 1A). At sufficient zinc supply, *T. caerulescens* accumulates about three times more zinc in the roots than Arabidopsis, and the concentration in shoots is much higher (~70-fold) than in Arabidopsis. At excess zinc, the zinc exclusion strategy of Arabidopsis roots has collapsed and their zinc concentration is now about 4.5-fold higher than in *T. caerulescens*, this is ~ 15-fold higher compared to sufficient conditions in Arabidopsis. At this high zinc supply Arabidopsis is still able to exclude zinc accumulation in the leaves, with a concentration about 9-fold lower than in *T. caerulescens*. For *T. caerulescens* there is not much difference in both root and shoot concentrations of plants growing at sufficient or excess zinc.

Since we expected that differences in zinc supply would also affect the concentration of other metals we measured the iron (Figure 1B) and manganese (Figure 1C) concentrations in the same material. Similar to zinc, the iron concentration in roots of both species increases upon increase in zinc supply (Figure 1B-I). At deficient and sufficient zinc supply the iron concentration in *T. caerulescens* is about 2- to 3-fold higher than in Arabidopsis. At excess zinc supply the root iron concentrations are similar for both species. Generally the iron concentrations are much lower in leaves than in roots. The iron concentration in leaves is similar for the three *T. caerulescens* treatments and this is only marginally higher in Arabidopsis under sufficient zinc supply (Figure 1B-II). In Arabidopsis leaves the iron concentration decreases marginally with increasing zinc supply.

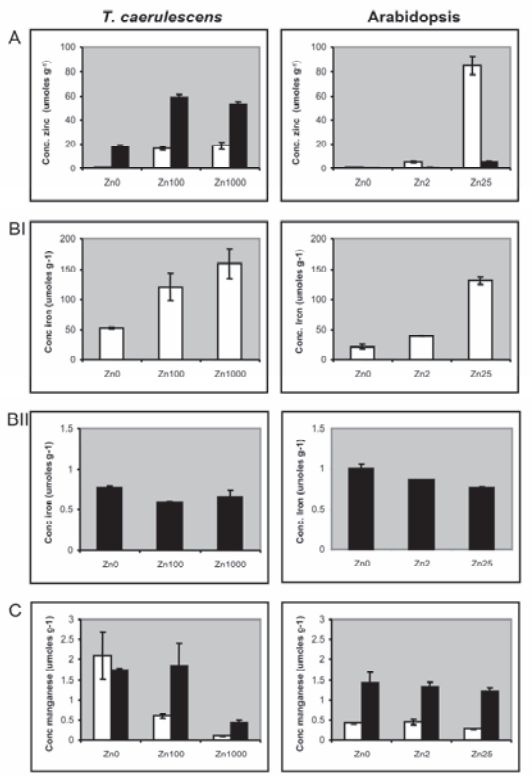

Figure 1. Zinc (A), iron (BI: roots; BII: leaves) and manganese (C) concentrations (µmoles g^{-1}; mean ± SE) in Arabidopsis and *T. caerulescens* roots (white bar) and leaves (black bar). Plants were grown for 3 weeks on nutrient solution containing sufficient zinc before exposure to zinc deficiency (0 µM $ZnSO_4$: Zn0), zinc sufficiency (2 or 100 µM $ZnSO_4$: Zn2/Zn100) and excess zinc (25 or 1000 µM $ZnSO_4$: Zn25/Zn1000).

For manganese the situation is the opposite in roots compared to iron. In *T. caerulescens* the manganese concentration decreases with increasing zinc supply and in Arabidopsis this only occurs upon excess zinc supply. The manganese concentration in *T. caerulescens* roots is about 5-fold higher under zinc deficiency than in Arabidopsis, but at sufficient zinc supply there is hardly any difference between the species. There are only few differences for the manganese concentration in leaves between the two species. Only at excess zinc, the manganese concentration in *T. caerulescens* decreases drastically by about 4-fold. Both *T. caerulescens* and

Arabidopsis accumulate manganese to a higher concentration in leaves than in roots, with the exception of *T. caerulescens* grown under zinc deficiency.

Zinc response in *Arabidopsis thaliana*

Genes responding to changes in zinc exposure conditions in Arabidopsis were identified using Agilent Arabidopsis 3 60-mer oligonucleotide microarrays containing 37,683 probes representing more than 27,000 annotated genes and over 10,000 non-annotated genomic regions for which there is transcriptional evidence. When analyzing the data, we only considered the hybridization data of probes with $p<0.05$. Only expression differences of $\geq$ 3-fold (between any of the three treatments) were considered to be relevant, even though lower expression differences were statistically significant at $p<0.05$. According to these criteria we identified 608 zinc-responsive genes when comparing the deficient, sufficient and excess zinc treatments. As expected, most differences were found between the most distant conditions, zinc deficiency and excess zinc. Many genes that were differentially expressed between zinc deficiency and sufficiency or between zinc sufficiency and excess zinc were also differentially expressed between deficiency and excess zinc, while few genes were found to be only differentially expressed between both zinc deficiency and sufficiency, and sufficiency and excess zinc.

After hierarchical clustering (average linkage hierarchical clustering with uncentered correlation) (Eisen et al., 1998) of all differentially expressed genes, four major clusters were distinguished. Cluster I (Supplemental Table I) consists of 98 genes that are lower expressed under zinc deficiency compared to sufficient and excess zinc. Within this group we find many genes with a function related to stress response, but also several with metabolism associated functions. Among the ten genes most differentially expressed between the zinc deficiency and sufficiency exposures are three genes encoding sHSPs (small heat shock proteins). Other genes found in this cluster are genes encoding Cu-Zn superoxide dismutases, a nodulin-like protein, a nitrate-responsive protein, an expansin-like protein and a universal stress protein. The last one is the highest expressed gene at sufficient zinc found in this cluster. Fifteen genes in this cluster encode proteins with an unknown function. Twenty probes correspond to transcripts that were not annotated as such in the Arabidopsis genome.

Table I: Arabidopsis genes higher expressed under excess (Zn25) zinc conditions when compared to sufficient (Zn2) or deficient (Zn0) zinc supply. [a]AGI gene code (At..) or chromosome position of non-annotated transcript (CHRX:..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 3) expressed genes between two zinc exposure conditions. Zn0 = 0 µM $ZnSO_4$; Zn2 = 2 µM $ZnSO_4$; Zn25 = 25 µM $ZnSO_4$; [d]Normalized spot intensity at 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. The ten probes with highest hybridization intensity at 2 µM $ZnSO_4$ are indicated with grey background. Ten probes with highest ratios for Zn0/Zn25 and Zn2/Zn 25 comparisons are presented in **bold**.

Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Intensity[d]
SAM1	At1g02500	S-adenosylmethionine synthetase 1	other cellular, metabolic, physiological processes	0.98	0.31	0.31	62719
GLP5	At1g09560	germin-like protein	biological process unknown	0.64	0.11	0.18	27296
	At1g73120	expressed protein	biological process unknown	0.85	0.30	0.35	13274
FRO2	At1g01580	ferric-chelate reductase	transport	0.27	**0.02**	**0.06**	12522
PAL2	At3g53260	phenylalanine ammonia-lyase 2	response to abiotic or biotic stimulus	1.00	0.28	0.28	12379
	At1g74760	zinc finger (C3HC4-type RING finger) family protein	protein metabolism	0.70	0.20	0.29	10028
	At4g13860	glycine-rich RNA-binding protein	biological process unknown	0.39	0.15	0.38	7988
	At1g63090	F-box family protein	protein metabolism	0.39	0.11	0.30	7941
	At5g45080	disease resistance protein-related	response to abiotic or biotic stimulus	0.62	0.24	0.39	7650
	At3g53480	ABC transporter family protein	biological process unknown	0.74	0.28	0.38	7391
NAS1	At5g04950	nicotianamine synthase	other cellular, metabolic, physiological processes	2.63	0.62	0.24	4791
IREG2	At5g03570	iron-responsive transporter	biological process unknown	1.25	0.27	0.22	3592
MTP3	At3g58810	zinc transporter	transport	0.68	0.04	0.06	3178
ATNRAMP4	At5g67330	NRAMP metal ion transporter 4	transport	0.69	0.32	0.46	2644
FRO3	At1g23020	ferric-chelate reductase	electron transport or energy pathways	0.87	0.18	0.21	2559
ZIP8	At5g45105	metal transporter	transport	0.33	**0.02**	**0.06**	2099
	At3g12900	oxidoreductase, 2OG-Fe(II) oxygenase family protein	biological process unknown	0.09	**0.00**	**0.04**	1937
FRO1	At1g01590	ferric-chelate reductase	electron transport or energy pathways	0.23	0.04	0.16	1659
IRT1	At4g19690	iron-responsive transporter	transport	0.35	**0.02**	**0.06**	1530
OPT3	At4g16370	oligopeptide transporter OPT family protein	transport	0.39	0.12	0.31	931
IRT2	At4g19680	iron-responsive transporter	transport	0.38	**0.01**	0.04	832
bHLH100	At2g41240	basic helix-loop-helix (bHLH) family protein	transcription	0.21	1.00	**0.02**	572
CYP82C3	At4g31950	cytochrome P450 family protein	electron transport or energy pathways	0.18	**0.01**	0.08	527
CYP82C2	At4g31970	cytochrome P450 family protein	electron transport or energy pathways	0.21	**0.01**	0.05	476
CYP82C4	At4g31940	cytochrome P450 family protein	electron transport or energy pathways	0.14	**0.00**	**0.03**	389
MYB72	At1g56160	myb family transcription factor	transcription	0.37	**0.01**	**0.03**	99
MTP8	At3g58060	cation efflux family protein	transport	0.45	0.05	0.11	87

Cluster II (Table I, Supplemental Table II) is a large cluster consisting of 128 genes. These genes are generally higher expressed under excess zinc conditions when compared to sufficient or deficient zinc supply. This cluster contains several metal homeostasis related genes associated with iron rather than with zinc homeostasis. These genes encode metal transporters (*IRT1*, *IRT2, ZIP8*, *MTP3*, *MTP8, NRAMP4* and *IREG2*), a nicotianamine synthase gene (*NAS1*), a YS-like oligopeptide transporter (*OPT3*) and ferric-chelate reductases (*FRO1*, *2* and *3*). Not only metal homeostasis genes are found in this cluster but also some stress response genes like a disease resistance gene. In addition, metabolic genes like *PAL2* (which encodes a key enzyme acting early in the phenylpropanoid biosynthesis pathway leading to flavonoids, anthocyanins and lignins) and genes belonging to the cytochrome P450 family (*CYP98A3*, *CYP82C2*, *CYP82C3, CYP82C4*, *CYP71B5* and *CYP71B38*) are found in this cluster. This cluster also contains a small set of genes encoding transcription factors of the bHLH, myb and zinc finger families.

Cluster III (Table II, Supplemental Table III) consists of 347 genes, which are higher expressed under zinc deficiency compared to the other two treatments. Many genes in this cluster, especially the ones which show the largest difference in expression between zinc deficiency and excess zinc, belong to metal homeostasis gene families encoding *ZIP* metal transporters, a cation diffusion facilitator (CDF) (*MTP* gene family), a P_{IB}-type ATPase transporter (*HMA* gene family), two nicotianamine synthases (*NAS*), a MATE efflux protein (*FRD3*), two ferric-chelate reductase-like proteins (*FRO4* and *5*), ferritin (*FER1*) and two Yellow Stripe1-like proteins (*YSL2* and *YSL*3). A surprisingly large fraction of 164 genes encode proteins without a known function (83), or represent non-annotated transcripts (81). Three of the latter are among the ten most differentially expressed genes when comparing the three zinc exposure conditions. Other genes identified in this cluster encode proteins involved in protein stability (F-box proteins), signal transduction (calcineurin-like phosphoesterase, auxin response factor, calmodulin-binding proteins, calcium-binding protein, protein kinase), transcriptional regulation (MADS-box, zinc finger and bHLH proteins) and metabolism. Among the ten genes with the highest expression (at sufficient zinc), five encode proteins with an unknown function (At5g19380, At5g16870, At2g16990, At3g15630 and At4g29905), one encodes a non-annotated transcript and three are involved in transcriptional regulation (At1g72220, At3g01970 and At3g51080).

Table II: Arabidopsis genes higher expressed under zinc deficiency (Zn0) compared to sufficient (Zn2) and excess (Zn25) zinc supply.
[a]AGI gene code (At..) or chromosome position of non-annotated transcript (CHRX:..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $p < 0.05$) differential (≥ 3) expressed genes between two zinc exposure conditions. Zn0 = 0 µM $ZnSO_4$; Zn2 = 2 µM $ZnSO_4$; Zn25 = 25 µM $ZnSO_4$; [d]Normalized spot intensity at 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. The ten probes with highest hybridization intensity at 2 µM $ZnSO_4$ are indicated with grey background. Ten probes with highest ratios in Zn0/Zn2 and Zn0/Zn25 comparisons are presented in **bold**.

Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Intensity[d]
	At1g72220	zinc finger (C3HC4-type RING finger) family protein	protein metabolism	1.36	3.35	2.46	83438
	At5g19380	expressed protein	biological process unknown	2.41	3.23	1.34	48547
	At5g16870	expressed protein	biological process unknown	2.36	4.44	1.88	37115
	At2g16990	expressed protein	biological process unknown	2.46	3.21	1.30	34791
	At1g72500	inter-alpha-trypsin inhibitor heavy chain-related	biological process unknown	2.73	3.33	1.22	30230
	At3g15630	expressed protein	biological process unknown	3.05	4.46	1.46	30117
	At4g29905	expressed protein	biological process unknown	3.98	7.83	1.97	29854
WRKY45	At3g01970	WRKY family transcription factor	transcription	2.22	3.30	1.49	26487
	At3g51080	zinc finger (GATA type) family protein	transcription	3.31	2.70	0.82	26114
	CHR5:20218751-20219267	Unknown	biological process unknown	2.93	3.43	1.17	25354
FSD1	At4g25100	superoxide dismutase [Fe]	response to abiotic or biotic stimulus	5.31	**76.80**	14.47	22269
ATFER1	At5g01600	ferritin 1	response to abiotic or biotic stimulus, transport	1.82	5.18	2.85	14350
ZIP3	At2g32270	zinc transporter	transport	6.10	31.45	5.16	7477
ZIP2	At5g59520	zinc transporter	transport	1.23	13.45	10.95	6366
NAS2	At5g56080	nicotianamine synthase	other cellular, metabolic, physiological processes	4.56	1.77	0.39	4994
	At5g50400	calcineurin-like phosphoesterase family protein	biological process unknown	10.45	13.83	1.32	4460
FRO4	At5g23980	ferric-chelate reductase	electron transport or energy pathways	3.02	14.47	4.79	4331
YSL3	At5g53550	transporter	transport	1.88	4.10	2.18	3633
	CHR2:15208700-15208384	Unknown	biological process unknown	**75.17**	**616.09**	8.19	2897
ZIP5	At1g05300	metal transporter	transport	5.75	22.53	3.92	1484
	At1g20380	prolyl oligopeptidase	protein metabolism	**42.64**	**106.37**	2.49	1368
ZIP4	At1g10970	metal transporter	transport	15.03	36.71	2.44	1263
FRO5	At5g23990	ferric-chelate reductase	electron transport or energy pathways	9.75	**105.49**	10.82	867
ZIP11	At1g55910	metal transporter	transport	3.61	4.00	1.11	717
	CHR4:6931720-6932736	Unknown	biological process unknown	**135.58**	**917.14**	6.76	702
ZIP1	At3g12750	zinc transporter	transport	9.37	15.64	1.67	681
YSL2	At5g24380	transporter	transport, response to abiotic or biotic stimulus	1.95	3.35	1.72	635
NAS4	At1g56430	nicotianamine synthase	other cellular, metabolic, physiological processes	**44.32**	18.11	0.41	630

Continue Table II

Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Intensity[d]
HMA2	At4g30110	ATPase E1-E2 type family protein	transport	6.48	7.44	1.15	628
ZIP9	At4g33020	metal transporter	transport	35.88	8.94	0.25	617
	At2g36260	iron-sulfur cluster assembly complex protein	biological process unknown	22.63	**117.05**	5.17	533
FRD3	At3g08040	MATE efflux family protein	other cellular, metabolic, physiological processes	5.63	7.71	1.37	483
IRT3	At1g60960	metal transporter	transport	22.71	**90.01**	3.96	435
	At1g71200	basic helix-loop-helix (bHLH) family protein	transcription	4.37	8.60	1.97	431
MTP2	At3g61940	zinc transporter	transport	**175.34**	**214.38**	1.22	152
ZIP10	At1g31260	metal transporter	transport	7.46	9.47	1.27	136
	CHR2:15209769-15208753	Unknown	biological process unknown	**92.35**	**157.48**	1.70	66
ZIP12	At5g62160	metal transporter	transport	9.23	12.48	1.35	58

Of the ten most differentially expressed genes, *FRO5*, *MTP2, NAS4,* and *IRT3* have a supposed role in metal homeostasis based on their predicted function or their similarity to other genes previously implicated in metal homeostasis. Only the superoxide dismutase is remarkably differentially expressed between all three treatments, decreasing in expression upon increasing the zinc concentration in the medium. Cluster IV (Supplemental Table IV) consists of 35 genes, which show a lower expression under excess zinc exposure compared to deficient and sufficient zinc exposures. Genes in this cluster are involved in (secondary) metabolism, (a)biotic stress response and transcription. Five genes in this cluster encode proteins with an unknown function. Nine genes were not annotated.

When comparing all three zinc exposure conditions only genes encoding ferric-chelate reductases (*FRO1* and *FRO5*), two ZIP metal transporters (*ZIP3* and *ZIP9*), Fe-superoxide dismutase (*FSD1*), an oxidoreductase (At3g12900), an iron-sulfur cluster assembly complex protein (At2g36260), cytochrome P450 CYP82C4 and an expressed protein (At3g59930), are differentially expressed between all conditions (Tables I + II, Supplemental Table III).

Heterologous microarray hybridization

We used the same Arabidopsis array platform for heterologous hybridization with labelled *T. caerulescens* cDNA. From analysis of around 3500 ESTs we previously determined that *T. caerulescens* shares about 85-90% DNA identity in coding regions with Arabidopsis (Rigola et al., 2006). However, since most probes present on the arrays were designed to fit less conserved regions of the Arabidopsis transcripts, we verified the suitability of cross-species hybridization of these arrays. First, Agilent Arabidopsis 1 oligonucleotide arrays, representing around 13,500 putative genes, were hybridized with labelled cDNA from Arabidopsis and *T. caerulescens* roots grown under sufficient zinc conditions. The spot intensities of the *T. caerulescens* hybridizations were on average only 1.7-fold lower than the spot intensities of Arabidopsis hybridizations, which is sufficient for reliable expression analysis. In addition genomic DNA hybridization of *T. caerulescens* to the Agilent Arabidopsis 3 oligonucleotide array showed an average 2.0-fold lower signal intensity for *T. caerulescens* compared to the Arabidopsis signal intensities.

Table III: *T. caerulescens* genes higher expressed under deficient (Zn0) compared to sufficient (Zn100) and excess (Zn1000) zinc supply.
[a]AGI gene code (At..) or chromosome position of non-annotated transcript (CHRX:..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 3) expressed genes between two zinc exposure conditions. Zn0 = 0 µM $ZnSO_4$; Zn100 = 100 µM $ZnSO_4$; Zn 1000 = 1000 µM $ZnSO_4$. [d]Normalized spot intensity at 100 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity at 100 µM $ZnSO_4$. Ten probes with highest ratios for Zn0/Zn100 and Zn0/Zn1000 comparisons are presented in **bold**. A horizontal line separates clusters I and II.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Intensity[d]
I	ZIP4	AT1G10970.1	metal transporter	transport	2.47	4.73	1.91	47323
	FSD1	AT4G25100.1	iron superoxide dismutase	circadian rhythm	5.89	**30.04**	5.10	6430
	ZIP3	AT2G32270.1	zinc transporter	transport	2.22	5.38	2.43	2151
	ZIP9	AT4G33020.1	metal transporter	transport	1.95	4.43	2.27	1472
II	CYP83A1	AT4G13770.1	cytochrome P450 family protein	response to abiotic or biotic stimulus	**6.70**	4.33	0.65	7900
		AT1G20380.1	prolyl oligopeptidase, putative	protein metabolism	**7.63**	4.95	0.65	4007
	ZIP1	AT3G12750.1	zinc transporter	transport	**9.33**	**10.11**	1.08	1569
	ZIP2	AT5G59520.1	zinc transporter	transport	4.48	3.49	0.78	1539
	NAS4	AT1G56430.1	nicotianamine synthase	other cellular, metabolic, physiological processes	4.99	3.55	0.71	1432
	ERD9	AT1G10370.1	glutathione S-transferase	other cellular, metabolic, physiological processes	**18.83**	**8.17**	0.43	902
	FRO5	AT5G23990.1	ferric-chelate reductase	electron transport or energy pathways	**8.47**	**7.10**	0.84	391
		AT1G75260.1	isoflavone reductase family protein	biological process unknown	**9.42**	4.53	0.48	278
		AT5G06730.1	peroxidase	response to abiotic or biotic stimulus	**8.69**	5.41	0.62	276
	CA2	AT5G14740.1	carbonic anhydrase 2	other metabolic processes	**50.67**	**40.39**	0.80	93
	DIR5	AT1G64160.1	disease resistance-responsive family protein	response to abiotic or biotic stimulus	**124.24**	**67.00**	0.54	82

Overall, only probes corresponding to 220 genes did not hybridize with *T. caerulescens* genomic DNA (less than 3-fold less signal intensity). These 220 genes were excluded from the dataset.

Zinc response in *T. caerulescens*

When comparing the expression of genes in roots of *T. caerulescens* plants grown on deficient, sufficient and excess zinc media, we identified 350 genes that were significantly (FDR $p < 0.05$) differentially expressed ($\geq$ 3-fold) in any of the three possible comparisons. Only 50 of these are also differentially expressed in response to different zinc exposures in Arabidopsis. Six clusters were identified in this set upon cluster analysis (average linkage hierarchical clustering with uncentered correlation). Clusters I and II (Table III, Supplemental Table V) consist of 38 genes, which are higher expressed at zinc deficiency compared to sufficient or excess zinc treatments. ZIP-like genes *ZIP3, ZIP4* and *ZIP9* are co-expressed, showing higher expression at sufficient zinc compared to excess zinc. This in contrast to the *ZIP1* and *ZIP2* genes, which are expressed at similar levels under zinc sufficiency and excess zinc. Other known metal homeostasis genes found in this cluster are the *NAS4* and *FRO5* genes. These were also found to be higher expressed in zinc deficient Arabidopsis roots. The *FSD1* iron superoxide dismutase, which was also found to be differentially expressed in Arabidopsis, is found in this cluster. The most differentially expressed gene encodes a Dirigent protein (*DIR5*), involved in lignin biosynthesis. This gene is hardly expressed under sufficient zinc conditions, which explains the strong differential expression.

Clusters IIIA and IIIB (Supplemental Table VI) consist of 74 and 16 genes respectively, which are higher expressed under deficient and excess zinc conditions compared to sufficient zinc. Genes in cluster IIIA are predominantly higher expressed under zinc deficiency; genes in cluster IIIB are predominantly higher expressed under excess zinc. Many genes associated with oxidative stress response, senescence, ethylene biosynthesis and plant defence are found in these clusters, including genes encoding peroxidases and four Plant Defensin Fusion genes (*PDF1.1*, *PDF1.2b*, *PDF1.2c* and *PDF1.3*). There are eleven genes with an unknown function in this cluster of which one is not annotated.

Clusters IVA and IVB (Supplemental Table VII) consist of 19 and 14 genes respectively, which are all highest expressed under excess zinc compared to the other two conditions. Two of these genes (At5g05250 and At2g41240) are also found in a similar cluster for Arabidopsis roots (Table I, Supplemental Table II). Compared to Arabidopsis, the Thlaspi cluster is much smaller and lacks all of the iron homeostasis genes.

The remaining 189 genes fall into two additional clusters, generally higher expressed under sufficient than under deficient conditions (Supplemental Table VIII+IX). Almost half of these encode proteins with an unknown function. Many of the other genes are involved in general metabolism and stress response.

Difference in zinc response between Arabidopsis and *T. caerulescens*

To identify genes that may be crucial for the adaptive differences between Arabidopsis and *T. caerulescens*, we compared the gene expression profiles between the two species for each of the tested physiological conditions. Taking into account that we performed a heterologous hybridization and that probes generally did not hybridize as efficiently to *T. caerulescen*s cDNA as to Arabidopsis cDNA, we only considered significant probes with a more than 5-fold higher normalized hybridization signal in *T. caerulescens* compared to Arabidopsis in any of the three comparisons, to be of biological relevance.

According to these criteria in total 2272 genes were found to be at least five times significantly higher (FDR $p < 0.05$) expressed in *T. caerulescens* compared to Arabidopsis (Supplemental Table X). 420 of these genes (18.5%) were not found to be expressed in roots of Arabidopsis under comparable conditions (Supplemental Table XI). A large class of 1147 of the 2272 differentially expressed genes has an unknown biological function. Other classes represent genes encoding for proteins involved in cellular processes, transport processes, stress response and transcription. A total of 929 genes showed little variation in expression under the three tested conditions, suggesting a constitutive higher expression in *T. caerulescens* roots. To test if this expression is anyhow functionally related to metal stress adaptation, the functional distribution of this group was compared to that of all 2272 differentially expressed genes, but this did not show specific gene classes to be over- or underrepresented (data not shown). Only 121 of the 2272

genes are differentially expressed in *T. caerulescens* in response to different zinc exposures. The highest expressed genes among these are the *ZIP4* and *IRT3* metal transporters (Table III + IV). Remarkable is the large difference in expression between *T. caerulescens* and Arabidopsis of four members of the *PDF* gene family (Table IV, Supplemental Table XII). These genes are especially highly expressed in *T. caerulescens* under deficient and excess zinc conditions compared to Arabidopsis. To facilitate the further analysis of this large class of genes, a selection was made of 235 genes including the 50 most differentially expressed genes under zinc deficiency and genes of which the proposed function could be relevant to explain the metal adaptation differences between both species (Table IV, Supplemental Table XII).

Next to putative metal homeostasis genes and stress response genes, also several genes suggested to be involved in lignin biosynthesis (Ehlting et al., 2005) were much higher expressed in *T. caerulescens* compared to Arabidopsis. In addition to the higher expression of the lignin biosynthesis genes, also genes potentially involved in suberin biosynthesis (*CER3*, *CER6* and 11 *LTP*-genes), are higher expressed in *T. caerulescens* though not as high as the lignin biosynthesis genes (Table IV, Supplemental Table XII). Out of 24 genes putatively involved in lignin biosynthesis, 11 were generally more than 10-fold higher differentially expressed and four were among the 15 highest expressors when absolute expression levels at sufficient zinc were considered (Table IV, Supplemental Table XII). This higher expression was expected to cause visible differences in lignification between *T. caerulescens* and Arabidopsis roots. To identify such differences, transverse sections of four-, six- and nine-week-old roots from *T. caerulescens* and four- and six-week-old roots from Arabidopsis plants grown hydroponically at sufficient zinc supply were made and examined by UV microscopy at wavelengths that induce lignin and suberin auto-fluorescence. After four weeks, Arabidopsis roots only showed auto-fluorescence of the xylem and the outer wall of epidermis cells in sections taken at two cm from the root tip (Figure 2A). At this age *T. caerulescens* roots also showed autofluorescence of the xylem vessels, but in addition also the inner wall of the endodermis cells lighted up (Figure 2D, E). Epidermal fluorescence was not seen in *T. caerulescens*.

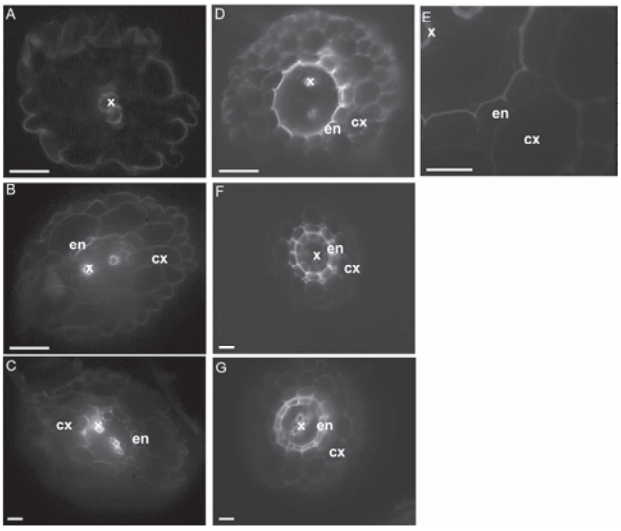

Figure 2. UV-autofluorescence of lignin and suberin deposition in comparable cross sections of roots of hydroponically grown Arabidopsis (A, B, C) and *T. caerulescens* (D, E, F, G). (A) Cross-section made two cm from the tip of a four-week-old Arabidopsis root showing blue UV fluorescence of the xylem vessels and the epidermis outer cell walls. (B) Cross-section made two cm from the root tip of a six-week-old Arabidopsis root showing strong UV fluorescence of the xylem vessels and light fluorescence of the inner wall of endodermis cells. (C) Cross-section made six cm from the root tip of a six-week-old Arabidopsis root showing strong UV fluorescence of the xylem and faint fluorescence of the inner wall of the endodermis cells. (D) Cross-section made at two cm from the tip of a four-week-old *T. caerulescens* root showing strong UV fluorescence of the xylem vessels and the inner walls of the endodermis cells. (E) Close up of a cross-section made one cm from the root tip of a four-week-old *T. caerulescens* root showing the UV fluorescence of the xylem vessels and the inner walls of the endodermis cells. (F) Cross-section made six cm from the root tip of a six-week-old *T. caerulescens* root showing UV fluorescence of the xylem vessels and the inner or outer walls of endodermis cells. Remarkable is the apparent formation of a second layer of endodermis cells, of which the inner walls are also fluorescent. (G) Cross-section of a nine-week-old *T. caerulescens* root made six cm from the root tip showing two layers of endodermis, both of which show UV fluorescence. cx, cortex; en, endodermis; x, xylem. Bar indicates 20 µm.

Sections of older *T. caerulescens* root parts, more distant from the tip, showed stronger fluorescence of especially the endodermis cells compared to the younger parts. In Arabidopsis this staining was much weaker (Figure 2B, C). The formation of a second layer with endodermis-like fluorescent staining was observed in the older Thlaspi roots (Figure 2F, G), but never in Arabidopsis roots (Figure 2B, C).

Table IV: A selection of 109 genes higher expressed in *T. caerulescens* (Tc) compared to Arabidopsis (At).
Included are the 50 most differentially expressed genes under zinc deficiency and genes encoding putative metal homeostasis related transporters, lignin biosynthesis proteins, transcription factors and signal transduction proteins. [a]AGI gene code (At..) or chromosome position of non-annotated transcript (CHRX:..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR $p < 0.05$) differential (≥ 3 or 5) expressed genes between two zinc exposure conditions. Values are ratios based on the average logratios of two biological replicates. Zn0 = 0 µM $ZnSO_4$; Zn2 = 2 µM $ZnSO_4$; Zn25 = 25 µM $ZnSO_4$; Zn100 = 100 µM $ZnSO_4$; Zn1000 = 1000 µM ZnSO4; [d]Normalized spot intensity at 100 µM $ZnSO_4$.15 genes with highest normalized spot intensity at 100 µM $ZnSO_4$ are indicated with gray background. Genes not significant differential expressed are indicated with **bold** ($p \geq 0.05$).

				Tc/At			Tc			At			
Name	Code[a]	Putative function	GO Annotation[b]	0/ 0[c]	100/ 2[c]	1000/ 25[c]	0/ 100[c]	0/ 1000[c]	100/ 1000[c]	0/ 2[c]	0/ 25[c]	2/ 25[c]	Intensity[d]
PDF1.1	At1g75830	plant defensin-fusion protein	response to stress	948.50	10.17	717.66	8.38	**0.65**	0.08	0.09	**0.49**	5.49	12363
	At4g02280	sucrose synthase	metabolism; sucrose biosynthesis	597.18	131.02	1369.28	**2.48**	**0.46**	**0.18**	**0.54**	**1.05**	**1.93**	20639
RPL13C	At3g48960	60S ribosomal protein L13	protein biosynthesis	365.86	167.78	202.29	**1.04**	**0.99**	**0.96**	**0.47**	**0.55**	**1.16**	47849
	At5g43935	flavonol synthase	metabolism; flavonol synthesis	330.48	112.84	211.01	**1.38**	**1.31**	**0.95**	0.47	**0.84**	**1.78**	5242
	At4g28005	expressed protein	electron transport; iron ion binding	280.19	123.06	216.14	**1.01**	**1.07**	**1.05**	**0.45**	**0.82**	**1.84**	10503
PDF1.2b	At2g26020	plant defensin-fusion protein	response to stress	260.94	18.41	522.12	7.22	**0.59**	0.08	**0.51**	**1.19**	**2.33**	3264
	At5g28510	ferredoxin hydrogenase protein	carbohydrate metabolism	219.05	103.40	180.24	**1.19**	**1.34**	**1.13**	**0.56**	**1.10**	**1.97**	60950
	CHR4:11881512-11882528	Unknown	biological process unknown	201.76	144.61	315.46	**0.56**	**0.43**	**0.76**	**0.40**	**0.67**	**1.66**	25676
	At1g17710	expressed protein	metabolism; thiamine biosynthesis	180.12	26.33	115.19	**2.99**	**0.71**	**0.24**	**0.44**	**0.46**	**1.04**	1583
PDF1.3	At2g26010	plant defensin-fusion protein	response to stress	166.01	7.64	284.86	6.34	**0.50**	0.08	0.29	**0.86**	**2.95**	11977
	At2g16200	hypothetical protein	biological process unknown	161.28	87.95	178.28	**1.07**	**1.23**	**1.15**	**0.58**	**1.35**	**2.33**	10725
	At3g50270	transferase family protein	response to stress	153.69	30.87	113.21	**2.83**	**2.07**	**0.73**	**0.57**	**1.53**	**2.69**	3598
DIR13	At4g11190	dis. res.-responsive family protein	metabolism; lignin biosynthesis	149.04	114.62	124.81	**1.16**	**1.16**	**1.00**	**0.89**	**0.97**	**1.09**	135715
PDF1.2c	At5g44430	plant defensin-fusion protein	response to stress	139.17	8.83	323.60	6.48	**0.50**	0.08	**0.41**	**1.17**	**2.85**	11917
APX2	At3g09640	L-ascorbate peroxidase 1b	response to stress	137.11	54.53	169.21	**1.42**	**0.87**	**0.61**	**0.57**	**1.08**	**1.91**	33325
HAK5	At4g13420	potassium transporter	cation transport	121.37	36.31	124.20	**1.16**	**0.83**	**0.72**	0.35	**0.85**	2.47	14613
	CHR4:9566304-9565288	Unknown	biological process unknown	121.07	138.45	117.38	**0.52**	**0.50**	**0.95**	**0.60**	**0.48**	**0.81**	4470
	At5g41820	geranylgeranyl transferase	protein metabolism	120.48	104.47	72.61	**0.51**	**0.45**	**0.88**	**0.44**	0.27	**0.61**	9054
	At5g55430	hypothetical protein	biological process unknown	116.78	52.60	27.51	**1.52**	**1.32**	**0.87**	**0.68**	**0.31**	**0.45**	7203
	At1g26360	hypothetical protein	metabolism; biotin biosynthesis	111.19	98.83	166.66	**1.13**	**1.04**	**0.92**	**1.00**	**1.56**	**1.55**	5549
	At3g13784	beta-fructosidase	carbohydrate metabolism	104.90	84.25	31.02	**1.18**	**1.06**	**0.89**	**0.95**	**0.31**	**0.33**	2545
	CHR2:5739298-5739397	Unknown	biological process unknown	103.32	111.21	176.21	**0.58**	**0.44**	**0.76**	**0.62**	**0.75**	**1.21**	22083
	At1g06030	pfkB-type carbohydrate kin. prot.	D-ribose metabolism	101.24	46.43	129.15	**1.42**	**0.95**	**0.67**	**0.65**	**1.22**	**1.87**	26682

Continue Table IV

				Tc/At			Tc			At			
Name	Code[a]	Putative function	GO Annotation[b]	0/ 0[c]	100/ 2[c]	1000/ 25[c]	0/ 100[c]	0/ 1000[c]	100/ 1000[c]	0/ 2[c]	0/ 25[c]	2/ 25[c]	Intensity[d]
	At1g14960	major latex protein-related	unknown; defense related	98.69	66.29	71.91	**1.06**	**1.08**	**1.02**	**0.71**	**0.78**	**1.11**	89031
	At4g21950	hypothetical protein	biological process unknown	98.10	50.35	30.94	**1.12**	**0.79**	**0.71**	**0.57**	**0.25**	**0.43**	1744
	At3g59590	jacalin lectin family protein	biological process unknown	96.47	76.25	48.03	**0.88**	**1.12**	**1.27**	**0.70**	**0.56**	**0.80**	14688
	At2g16490	XH domain-containing protein	biological process unknown	96.25	98.11	220.05	**0.47**	**0.38**	**0.80**	**0.48**	**0.86**	**1.80**	20789
	At4g23150	protein kinase family protein	protein metabolism	94.01	88.21	220.21	**0.53**	**0.46**	**0.87**	**0.50**	**1.08**	**2.17**	8278
PHT3	At5g43360	inorganic phosphate transporter	phosphate transport	93.26	38.32	78.99	**1.77**	**1.23**	**0.70**	**0.73**	**1.04**	**1.44**	19836
	At1g49250	ATP dependent DNA ligase protein	DNA repair	91.87	50.56	31.91	**1.34**	**1.19**	**0.89**	**0.74**	**0.41**	**0.56**	2284
	CHR5:19326437-19325921	Unknown	biological process unknown	91.69	106.55	132.71	**0.48**	**0.41**	**0.84**	**0.56**	**0.59**	**1.05**	10162
	CHR5:26539519-26539835	Unknown	biological process unknown	91.58	128.49	148.44	**0.51**	**0.42**	**0.82**	**0.71**	**0.67**	**0.95**	15584
	At1g62690	expressed protein	biological process unknown	91.31	46.75	68.49	**1.01**	**0.89**	**0.89**	**0.51**	**0.67**	**1.30**	2325
	CHR3:23273840-23274356	Unknown	biological process unknown	87.80	96.98	208.78	**0.60**	**0.43**	**0.71**	**0.66**	**1.01**	**1.53**	14196
	At1g40087	hypothetical protein	biological process unknown	87.50	41.06	56.51	**1.41**	**1.20**	**0.85**	**0.66**	**0.77**	**1.17**	1915
	CHR2:7453552-7452536	Unknown	biological process unknown	87.17	84.55	441.91	**0.52**	**0.40**	**0.76**	**0.51**	**2.01**	3.97	28488
	At2g38250	DNA-binding protein	transcription	84.05	57.36	120.09	**0.54**	**0.40**	**0.76**	0.37	**0.58**	**1.58**	23033
	At2g21330	fructose-bisphosphate aldolase	metabolism	78.66	32.23	73.24	**1.77**	**1.04**	**0.59**	**0.72**	**0.96**	**1.33**	91786
	At5g58330	malate dehydrogenase	metabolism	77.13	54.94	107.84	**0.62**	**0.48**	**0.77**	**0.44**	**0.67**	**1.52**	7882
	At2g35740	sugar transporter family protein	transport	75.23	81.52	143.47	**0.56**	**0.43**	**0.76**	**0.60**	**0.81**	**1.34**	11976
	CHR4:6561567-6560402	Unknown	biological process unknown	73.91	119.35	209.56	**0.51**	**0.42**	**0.83**	**0.83**	**1.20**	**1.45**	7686
	At2g30830	2-oxoglutarate-dependent dioxygenase	metabolism	71.27	17.04	19.01	2.49	**2.14**	**0.86**	**0.60**	**0.57**	**0.96**	5756
	CHR1:13243304-13243548	Unknown	biological process unknown	70.40	35.47	141.84	**1.61**	**0.41**	**0.26**	**0.81**	**0.83**	**1.02**	1853
	CHR1:11026453-11026996	Unknown	biological process unknown	70.20	101.45	231.20	**1.29**	**1.31**	**1.02**	**1.38**	**2.50**	**1.81**	199
	At4g28750	photosystem I reaction center subunit IV	photosynthesis	69.01	41.98	227.92	**0.55**	**0.42**	**0.77**	**0.33**	**1.39**	4.19	15894
	CHR3:7075276-7075592	Unknown	biological process unknown	68.68	66.19	26.01	**0.95**	**1.07**	**1.12**	**0.92**	**0.40**	**0.44**	2637
4CLL8	At5g38120	4-coumarate--CoA ligase family protein	metabolism; lignin biosynthesis	68.48	60.18	53.69	**0.69**	**0.96**	**1.39**	**0.61**	**0.75**	**1.24**	5574
	At5g37980	NADP-dependent oxidoreductase	response to abiotic or biotic stimulus	68.30	63.11	71.61	**0.68**	**0.55**	**0.81**	**0.63**	**0.58**	**0.92**	12423
GA4H	At1g80340	gibberellin 3-beta-dioxygenase	metabolism; gibberellin biosynthesis	67.35	16.56	23.69	4.79	4.08	**0.85**	**1.18**	**1.44**	**1.22**	1155
	At3g50940	AAA-type ATPase family protein	biological process unknown	66.42	46.58	54.31	**1.01**	**0.83**	**0.82**	**0.71**	**0.68**	**0.95**	2803

Continue Table IV

				Tc/At			Tc			At			
Name	Code[a]	Putative function	GO Annotation[b]	0/ 0[c]	100/ 2[c]	1000/ 25[c]	0/ 100[c]	0/ 1000[c]	100/ 1000[c]	0/ 2[c]	0/ 25[c]	2/ 25[c]	Intensity[d]
LTP4	At5g59310	lipid transfer protein 4	transport	47.97	48.95	52.56	**0.51**	**1.02**	**1.98**	**0.52**	**1.12**	**2.13**	5979
4CLL3	At1g20500	4-coumarate--CoA ligase family	metabolism; lignin biosynthesis	47.67	45.86	53.39	**0.65**	**0.90**	**1.39**	**0.62**	**1.01**	**1.62**	3956
MTP8	At3g58060	cation efflux family protein	cation transport	43.33	29.35	3.05	**0.67**	**0.71**	**1.06**	**0.45**	0.05	0.11	2693
KAT1	At5g46240	inward rectifying potassium channel	cation transport	40.06	34.09	40.41	**1.10**	**1.15**	**1.05**	**0.93**	**1.16**	**1.24**	4106
MT2A	At3g09390	metallothionein protein	response to stress; copper binding	32.47	20.25	84.39	**1.40**	**0.51**	0.37	**0.87**	**1.33**	**1.52**	131218
4CLL2	At1g20490	AMP-dependent synthetase/ligase	metabolism; lignin biosynthesis	27.24	37.52	26.07	**0.79**	**0.94**	**1.19**	**1.08**	**0.90**	**0.83**	15214
	At1g73550	lipid transfer protein (LTP) family protein	transport	24.68	13.12	10.12	**1.09**	**0.96**	**0.88**	**0.58**	0.39	**0.68**	13278
NRAMP3	At2g23150	NRAMP metal ion transporter 3	cation transport	23.97	7.86	18.56	2.07	**0.89**	0.43	**0.68**	**0.69**	**1.02**	18082
DIR11	At1g22900	disease resistance-responsive fam. prot.	metabolism; lignin biosynthesis	23.47	17.99	14.33	**1.10**	**1.44**	**1.31**	**0.84**	**0.88**	**1.04**	3974
CCR2	At1g80820	cinnamoyl-CoA reductase	metabolism; lignin biosynthesis	22.03	10.81	10.86	**1.24**	**1.60**	**1.29**	**0.61**	**0.79**	**1.30**	22963
DIR23	At3g13660	dis. res. response protein-related	metabolism; lignin biosynthesis	17.36	52.11	16.87	**0.52**	**1.32**	**2.56**	**1.55**	**1.29**	**0.83**	2860
CCRL14	At5g19440	cinnamyl-alcohol dehydrogenase	metabolism; lignin biosynthesis	17.20	9.07	11.55	**1.03**	**1.07**	**1.04**	0.54	**0.72**	**1.32**	98965
HMA4	At2g19110	ATPase E1-E2 type family protein	cation transport	16.41	11.15	15.36	**1.73**	**0.96**	**0.56**	**1.18**	**0.90**	**0.77**	105390
IRT3	At1g60960	metal transporter	cation transport	13.82	123.65	281.56	**2.54**	**4.42**	**1.74**	22.71	90.04	**3.96**	42981
bHLH100	At2g41240	basic helix-loop-helix (bHLH) fam. prot.	transcription	13.62	**3.24**	**0.59**	**0.89**	0.09	0.10	**0.21**	0.00	0.02	2195
CAX7	At5g17860	cation exchanger	cation transport	12.79	7.33	13.12	**1.69**	**0.90**	**0.53**	**0.97**	**0.92**	**0.95**	850
DIR20	At1g58170	dis. res.-responsive protein-related	metabolism; lignin biosynthesis	11.93	7.22	6.05	**2.15**	**2.30**	**1.07**	**1.30**	**1.17**	**0.90**	2774
	At1g62500	lipid transfer protein (LTP) family protein	transport	11.85	6.77	12.80	**1.22**	**1.13**	**0.93**	**0.70**	**1.22**	**1.75**	7639
CADL5	At4g39330	mannitol dehydrogenase	metabolism; lignin biosynthesis	10.12	15.81	11.44	0.46	**0.82**	**1.79**	**0.72**	**0.93**	**1.30**	14903
KUP3	At3g02050	potassium transporter	cation transport	9.69	9.38	9.58	**1.12**	**1.11**	**0.99**	**1.08**	**1.09**	**1.01**	3395
CER3	At5g02310	eceriferum3 protein	transcription; zinc binding	9.54	9.31	11.15	**1.20**	**1.03**	**0.86**	**1.17**	**1.20**	**1.03**	5275
FAH1	At4g36220	cyt. P450 84A1 ferulate-5 hydroxylase	metabolism; lignin biosynthesis	9.27	22.78	18.70	**0.63**	**0.77**	**1.23**	**1.54**	**1.56**	**1.01**	95951
CCRL3	At1g09500	cinnamyl-alcohol dehydrogenase family	metabolism; lignin biosynthesis	8.79	3.65	8.81	2.60	**1.25**	**0.48**	**1.08**	**1.25**	**1.15**	1147
GLP11c	At3g04180	germin-like protein	metabolism; lignin biosynthesis	8.78	6.75	8.16	**1.13**	**0.51**	**0.46**	**0.87**	**0.48**	**0.55**	355
COMTL8	At1g63140	O-methyltransferase	metabolism; lignin biosynthesis	8.15	14.35	6.42	**0.62**	**1.05**	**1.71**	**1.09**	**0.83**	**0.76**	636
CAX9	At3g14070	cation exchanger	cation transport	8.03	3.53	4.89	**1.17**	**0.96**	**0.83**	0.51	**0.59**	**1.15**	1454
ZIP10	At1g31260	metal transporter	cation transport	7.66	33.02	17.24	**1.73**	**4.20**	**2.43**	**7.46**	9.47	**1.27**	5104

Continue Table IV

				Tc/At			Tc			At			
Name	Code[a]	Putative function	GO Annotation[b]	0/ 0[c]	100/ 2[c]	1000/ 25[c]	0/ 100[c]	0/ 1000[c]	100/ 1000[c]	0/ 2[c]	0/ 25[c]	2/ 25[c]	Intensity[d]
CCRL13	At5g14700	cinnamoyl-CoA reductase-related	metabolism; lignin biosynthesis	7.63	**1.33**	**1.15**	4.84	5.01	**1.04**	**0.84**	**0.75**	**0.89**	531
	At3g22120	lipid transfer protein (LTP) family prot	transport	7.17	6.07	8.20	**1.02**	**1.59**	**1.56**	**0.86**	**1.82**	**2.11**	16632
CCOMTL1	At1g67980	caffeoyl-CoA 3-O-methyltransferase	metabolism; lignin biosynthesis	7.10	**0.83**	8.45	3.64	**1.01**	0.28	**0.42**	**1.20**	**2.83**	1191
LTP3	At5g59320	lipid transfer protein 3	other physiological processes	7.01	18.79	15.18	0.52	**1.08**	2.09	**1.39**	2.35	**1.69**	11235
	At4g00165	lipid transfer protein (LTP) family protein	transport	6.98	13.36	7.89	**0.50**	**0.93**	**1.86**	**0.95**	**1.05**	**1.10**	9474
	At5g48490	lipid transfer protein (LTP) family protein	transport	6.31	4.75	7.23	**1.29**	**0.93**	**0.72**	**0.97**	**1.07**	**1.10**	108087
CAD1	At4g34230	cinnamyl-alcohol dehydrogenase	metabolism; lignin biosynthesis	6.22	5.96	5.05	**1.27**	**1.19**	**0.94**	**1.22**	**0.97**	**0.79**	64332
HMA3	At4g30120	ATPase E1-E2 type family protein	cation transport	6.05	5.70	1.98	**0.98**	**1.20**	**1.23**	**0.92**	0.39	**0.43**	5882
CADL1	At1g72680	cinnamyl-alcohol dehydrogenase	metabolism; lignin biosynthesis	5.85	5.17	6.06	**0.82**	**0.79**	**0.97**	**0.72**	**0.82**	**1.14**	13341
COMTL4	At1g21130	O-methyltransferase	metabolism; lignin biosynthesis	5.79	**3.27**	8.12	**1.76**	**1.36**	**0.78**	**0.99**	**1.91**	**1.93**	7465
YSL7	At1g65730	oligopeptide transporter OPT fam. prot.	cation transport	5.76	3.43	6.95	**1.43**	**0.81**	**0.57**	**0.85**	**0.98**	**1.15**	1121
IRT2	At4g19680	iron-responsive transporter	cation transport	5.72	**1.17**	0.06	**1.83**	**1.37**	**0.75**	**0.38**	0.01	0.04	962
CLA3	At2g27250	CLAVATA3 CLAVATA3/ESR-Related	signal transduction; development	5.56	14.73	6.84	**0.40**	**1.27**	**3.20**	**1.05**	**1.56**	**1.49**	48437
	At1g73780	lipid transfer protein (LTP) family protein	transport	5.48	7.98	4.49	**0.65**	**1.31**	2.00	**0.95**	**1.07**	**1.12**	14883
ZIP4	At1g10970	metal transporter	cation transport	4.88	29.66	37.86	2.47	4.73	**1.91**	15.03	36.71	2.44	47323
CER3	At5g02300	eceriferum3 protein	transcription; zinc binding	4.87	15.64	24.65	**0.57**	**0.40**	**0.70**	**1.83**	**2.02**	**1.10**	29407
CCRL9	At2g23910	cinnamoyl-CoA reductase-related	metabolism; lignin biosynthesis	4.83	6.46	5.13	**0.53**	**0.71**	**1.34**	**0.71**	**0.76**	**1.07**	2244
4CLL9	At5g63380	4-coumarate--CoA ligase family protein	metabolism; lignin biosynthesis	4.73	3.83	5.73	**1.06**	**0.80**	**0.75**	**0.86**	**0.97**	**1.12**	4488
LTP2	At2g38530	nonspecific lipid transfer protein 2	other physiological processes	4.58	6.23	5.44	**0.72**	**1.05**	**1.46**	**0.98**	**1.24**	**1.27**	39419
MT-2B	At5g02380	metallothionein protein 2B	response to stress; copper binding	4.38	2.30	6.50	**1.72**	**0.66**	0.38	**0.90**	**0.97**	**1.08**	164846
	At4g22460	lipid transfer protein (LTP) family protein	transport	4.25	16.23	4.82	**0.46**	**0.92**	**1.99**	**1.77**	**1.05**	**0.59**	21693
LAC07	At3g09220	laccase family protein	metabolism; lignin biosynthesis	3.85	4.20	5.20	**0.95**	**0.96**	**1.01**	**1.04**	**1.30**	**1.26**	27502
CER6	At1g68530	very-long-chain fatty acid cond. enzyme	metabolism	3.58	11.07	5.34	**0.43**	**1.26**	2.92	**1.33**	**1.88**	**1.41**	2808
FER4	At2g40300	ferritin	iron ion homeostasis	3.50	6.72	5.20	**0.92**	**1.45**	**1.58**	**1.77**	2.16	**1.22**	3330
MTP1	At2g46800	zinc transporter	cation transport	3.33	4.66	5.62	**0.64**	**0.60**	**0.94**	**0.89**	**1.01**	**1.13**	67213
FRD3	At3g08040	MATE efflux family protein	iron ion homeostasis	3.08	11.86	23.52	**1.46**	**1.01**	**0.69**	5.63	7.71	**1.37**	6335
4CL1	At1g51680	4-coumarate--CoA ligase 1	metabolism; lignin biosynthesis	3.05	5.23	**0.99**	**1.01**	**1.29**	**1.28**	**1.73**	0.42	0.24	28495

Continue Table IV

				Tc/At			Tc			At			
Name	Code[a]	Putative function	GO Annotation[b]	0/ 0[c]	100/ 2[c]	1000/ 25[c]	0/ 100[c]	0/ 1000[c]	100/ 1000[c]	0/ 2[c]	0/ 25[c]	2/ 25[c]	Intensity[d]
	At3g43720	lipid transfer protein (LTP) family protein	transport	2.84	6.24	6.13	0.53	**0.86**	**1.61**	**1.17**	**1.86**	**1.58**	4113
AE6	At5g16340	AMP-binding protein	metabolism; lignin biosynthesis	2.70	3.66	5.55	**0.70**	**0.60**	**0.85**	**0.95**	**1.23**	**1.29**	5392
NAS2	At5g56080	nicotianamine synthase	electron transport or energy pathways	**1.22**	6.47	2.96	**0.86**	**0.73**	**0.85**	4.56	**1.77**	0.39	41042
ZIP5	At1g05300	metal transporter	cation transport	**0.93**	**2.34**	6.50	**2.29**	**3.23**	**1.41**	5.75	22.54	**3.92**	4438
FRO5	At5g23990	ferric-chelate reductase	electron transport or energy pathways	**0.43**	**0.49**	6.32	8.47	**7.10**	**0.84**	9.75	105.46	10.82	391

Semi-quantitative RT-PCR

For confirmation of the microarray expression profiling data, a small subset consisting of differentially expressed genes and random genes was subjected to semi-quantitative RT-PCR. In the absence of *T. caerulescens* DNA sequences suitable for designing species-specific PCR primers, orthologous *T. caerulescens* gene fragments were first amplified by low stringency PCR using Arabidopsis-specific primers and confirmed by DNA sequencing. This sequence was used to design primers for semi-quantitative RT-PCR hybridizing at comparable positions of *T. caerulescens* and Arabidopsis gene sequences. Expression of the target genes was studied in both root and leaf tissues of plants grown hydroponically at different zinc supply conditions (Figure 3A+B). In general the root expression levels determined by semi-quantitative RT-PCR were comparable to those determined by microarray analysis, confirming the significance of the heterologous microarray hybridization results.

When considering the expression in leaves, there are some striking differences between Arabidopsis and *T. caerulescens* that could not be observed in the root microarray comparison. First of all, the expression of three of the four nicotianamine synthase (*NAS*) genes is different between the two species. *AtNAS1* is predominantly expressed in roots in Arabidopsis. In contrast, *TcNAS1* only shows detectable expression in leaves of *T. caerulescens*. *AtNAS3* is mainly expressed under zinc deficiency in both roots and leaves of Arabidopsis. In *T. caerulescens*, the *TcNAS3* gene is much stronger expressed in leaves than in roots, with only a slightly higher expression at lower zinc supply levels. In Arabidopsis, *AtNAS4* is induced by zinc deficiency in roots and zinc excess in leaves. The *TcNAS4* in *T. caerulescens* does not show zinc responsive expression and is constitutively expressed in the roots and leaves. Of four *NAS* genes, only *AtNAS2* and *TcNAS2* show comparable expression in both species.

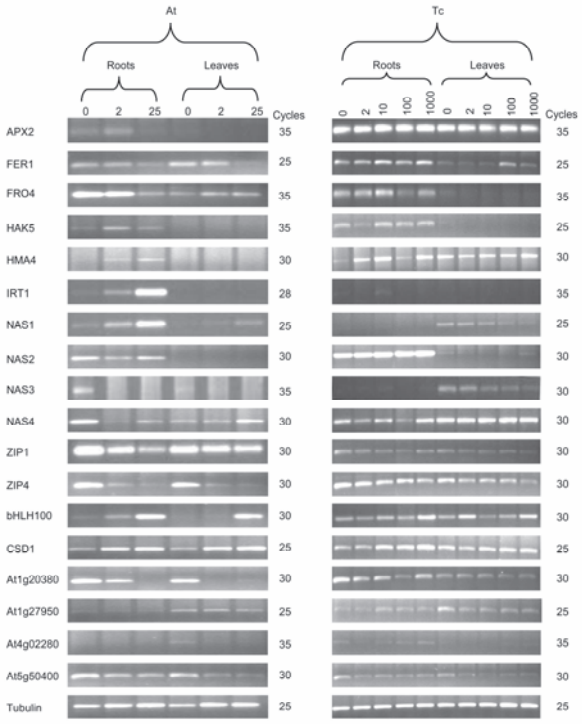

Figure 3. Comparative semi-quantitative RT-PCR of selected putative metal homeostasis related differentially expressed genes (APX2 to At1g20380) and three randomly picked genes (At1g27950 to At5g50400) in Arabidopsis (A) and *T. caerulescens* (B). For amplification, species specific primers were designed at comparable locations in each orthologous gene pair. Roots and leaves were harvested separately after one week of exposure of three-week-old plants to 0, 2 and 25 µM $ZnSO_4$ for Arabidopsis and 0, 2, 10, 100 and 1000 µM $ZnSO_4$ for *T. caerulescens*. APX2, L-ascorbate peroxidase, At3g09640; FER1, ferritin, At5g01600; FRO4, ferric chelate reductase-like, At5g23980; HAK5, potassium transporter, At4g13420; HMA4, Zn ATPase E1-E2 type, At2g19110; IRT1, Fe (II) transporter, At4g19690; NAS1, nicotianamine synthase, At5g04950; NAS2, nicotianamine synthase, At5g56080; NAS3, nicotianamine synthase, At1g09240; NAS4, nicotianamine synthase, At1g56430; ZIP1, zinc transporter, At3g12750; ZIP4, zinc transporter, At1g10970; bHLH100, basic helix-loop-helix transcription factor, At2g41240; CSD1, copper/zinc superoxide dismutase, At1g08830; putative prolyl oligopeptidase, At1g20380; lipid transfer protein related, At1g27950; sucrose synthase, At4g02280; calcineurin-like phosphoesterase, At5g50400. Tubulin (At1g04820) was used as a control for equal cDNA use.

When comparing the other differentially expressed genes, *TcAPX2*, *TcHMA4* and *TcZIP4 (ZNT1*) are all constitutively expressed in *T. caerulescens* leaves. Expression of these genes was either not detected in Arabidopsis leaves (*APX2* and *HMA4*) or expression is only detected at zinc deficiency conditions (*ZIP4*). Also the expression of *FER1* and *FRO4* in leaves differs between

the two species: *TcFER1* is induced under zinc excess conditions whereas *AtFER1* is induced under low zinc concentrations and *TcFRO4* is not expressed in the leaves while *AtFRO4* is slightly induced at sufficient and excess zinc conditions. The *HAK5* potassium transporter and the *CSD1* copper/zinc superoxide dismutase genes show a similar expression in leaves and roots of both species. In Arabidopsis the At1g20380 gene (encoding a putative prolyl oligopeptidase) is strongly induced at low zinc conditions, whereas the *T. caerulescens* orthologue is more or less constitutively expressed at low levels in roots and leaves. *ZIP1* is also constitutively low expressed in *T. caerulescens* roots and leaves, but induced in Arabidopsis roots at zinc deficiency. Furthermore we found the *IRT1* iron transporter gene to be very differently expressed in Arabidopsis compared to *T. caerulescens*. *AtIRT1* is induced by excess zinc in Arabidopsis roots. For *TcIRT1* this induction is much weaker in *T. caerulescens* roots, and also the overall expression levels are much lower than for the *AtIRT1* gene, confirming the observed absence of this gene from Thlaspi Cluster IV (Supplemental Table VII).

DISCUSSION

In this study, we investigated the expression of genes in roots of the non-accumulator species *Arabidopsis thaliana* (Arabidopsis) in response to exposure to three very different zinc concentrations in hydroponic culture. We postulate that genes that show differential expression under different zinc exposures are most likely to be involved in metal homeostasis. Most of these will be differentially expressed as a consequence of downstream changes in the physiological status of plants due to changes in metal homeostasis, but a few genes will be directly involved in regulating metal homeostasis. In trying to identify the latter ones, we also examined the differential expression of genes in the zinc hyperaccumulator *Thlaspi caerulescens.* Of the three metals we tested, only zinc homeostasis is clearly different between the two species. While Arabidopsis is not able to maintain non-toxic zinc levels in roots upon exposure to excess zinc levels in the nutrient solution (Figure 1A; Becher et al., 2004; Talke et al., 2006), *T. caerulescens* is perfectly able to do this while even translocating high amounts of zinc to the leaves (Figure 1A) (Assunção et al., 2003a). After only one week, the zinc content in *T. caerulescens* is not as

high as previously found by Assunção et al. (2003b), who measured after several weeks of exposure. Unexpectedly, iron accumulates in the roots of both *T. caerulescens* and Arabidopsis at increasing zinc concentrations (Figure 1BI). Based on absence of an effect of iron status on zinc uptake in *T. caerulescens* (Lombi et al. 2002) and the antagonistic effect found for Arabidopsis seedlings (Thomine et al., 2003) we expected no effect or an antagonistic effect of the zinc status on iron uptake. The synergistic effect we found suggests that both species may increase their iron uptake as a response to a possible risk of iron deficiency in leaves. If so, this strategy is effective since no actual decrease is seen in iron concentration in *T. caerulescens* leaves and only a slight decrease in Arabidopsis leaves (Figure 1BII). For manganese there is an antagonistic response of decreased uptake upon increased zinc uptake in the roots (Figure 1C).

When examining gene expression in the same material of both species, we expected that genes that are differentially expressed between the two species, and especially those that show a difference in response to changes in the external zinc concentration, may be crucial to the adaptive difference between a zinc accumulator and a non-accumulator. Previously, aspects of the metal accumulator vs. non-accumulator gene expression comparison have been studied for *Arabidopsis thaliana* and *Arabidopsis halleri* (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006). Additional to those studies, we now compared and verified root gene expression under three different zinc exposure conditions (deficient, sufficient and excess zinc), which clearly lead to different zinc concentrations in roots and leaves of both species. Another important addition to previous studies is that the Agilent 3 oligo microarray we used contains ~40,000 probes representing more than 27,000 annotated genes and more than 10,000 non-annotated transcripts (http://www.chem.agilent.com), and is thus an almost complete representation of the Arabidopsis transcriptome. We propose that genes, which are induced in expression upon transfer to zinc deficiency or upon transfer to excess zinc, are most interesting for further understanding of zinc homeostasis in Arabidopsis. Among the first class are some genes already known to be involved in zinc homeostasis like *ZIP2, 4, 5* and *9*, *NAS2* and *HMA2* genes (Grotz et al., 1998; Wintz et al., 2003; Talke et al., 2006). In addition we confirmed previous suggestions of genes to be involved in zinc homeostasis, such as *ZIP1*, *3* and *10*, *IRT3*, *MTP2* and *NAS4*, to be higher expressed under zinc deficiency. *ZIP1*, *NAS2* and *NAS4* were also induced in *Arabidopsis halleri* in response to

low zinc supply (Becher et al., 2004). Our results now suggest there are at least ten different members of the ZIP-gene family (Guerinot and Eide, 1999), that play a role in zinc uptake in roots (*ZIP1, 2, 3, 4, 5, 9, 10, 11* and *12* and *IRT3*). Hypothesizing that these transporters are involved in transport of cations across the plasma membrane, it is unlikely that all of them are involved in the uptake of zinc in the same tissue. Most likely, these transporters exert a similar function in different parts of the root, or are located in intracellular membranes.

Of the other two known zinc transporters induced by zinc deficiency, *HMA2* has been implicated in transport of zinc into the vasculature, either to promote zinc export from root to shoot via the xylem or from shoot to root via the phloem (Eren and Argüello, 2004; Hussain et al., 2004). In the first case, higher expression under zinc deficiency could be a response to a higher zinc demand from the shoot; in the latter it could be to accommodate the higher zinc demand of the root by remobilizing zinc from the shoot. The very strong induction of *MTP2* is remarkable. Rather than *MTP1* (previously known as *ZAT*), which is constitutively expressed in Arabidopsis (van der Zaal et al., 1999; Kobae et al., 2004), the induction of *MTP2* by zinc deficiency suggests a specific role of this transporter in counteracting the effect of zinc deficiency.

Metals are often chelated *in planta* to nicotianamine (NA). The absence of NA has severe effects on metal homeostasis, as was observed in the *chloronerva* mutant of tomato (Ling et al., 1999) or in NA metabolizing NAAT-overexpressing tobacco plants (Takahashi et al., 2003). NA is formed by trimerization of S-adenosylmethionine (SAM) catalyzed by the enzyme NA synthase (NAS). Arabidopsis contains four *NAS* genes, at least three of which are able to catalyze the last step in the synthesis of NA (Suzuki et al., 1999; Becher et al., 2004; Weber et al., 2004). Only *NAS2* and *NAS4* are higher expressed in roots under zinc deficiency compared to sufficiency (Figure 3A), but the presence of several, apparently paralogous, *NAS* genes with different overlapping gene expression profiles suggests complementary and possibly redundant functions.

In addition to the higher expression of *NAS* genes, also some *YSL* genes are induced by zinc deficiency. These genes are implicated in transport of metal-NA chelates within the plant (Curie et al., 2001; Waters et al., 2006) and possibly the entry of metals into the phloem or xylem

(Didonato et al., 2004). We find expression of *YSL2* and *YSL3* to be only slightly affected by different zinc treatments and contrary to the observations by Schaaf et al. (2005) we find the genes to be slightly induced by lower zinc concentrations. Recently Waters et al. (2006) also showed there is an induction of *YSL3* in Arabidopsis grown under zinc deficiency conditions. Unexpected was the high zinc deficiency induced expression of *FRD3, FRO4* and *FRO5*. Although the *frd3* mutant has a zinc accumulation phenotype, *FRD3* has been mainly implicated in iron homeostasis (Lahner et al., 2003; Green and Rogers, 2004). *FRO4* and *FRO5* resemble the ferric chelate reductase gene *FRO2* (Robinson 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). The current results suggest a much broader role in general metal homeostasis of these genes than previously thought.

In addition to these genes we identified 328 other probes with a similar differential transcription profile (Table II, Supplemental Table III), indicating a similar involvement in zinc homeostasis for the corresponding genes (Eisen et al., 1998). For some of these probes the only indication of a corresponding gene came from Massive Parallel Sequence Signature (MPSS) analysis (Meyers et al., 2004), as no Arabidopsis gene was annotated at that position. Three of these are among the ten most differentially expressed when comparing zinc deficient and sufficient condition. So far we have not identified the corresponding gene. For three other genes in this cluster, a prolyl oligopeptidase (At1g20380), a calcineurin-like phosphoesterase (At5g50400) and a basic helix-loop-helix family protein (At1g71200), KO-mutants were examined but not found to display any aberrant phenotype under differential zinc exposure (data not shown). Prolyl oligopeptidase and calcineurin-like phosphoesterase need metals, possibly also zinc, to function properly. The same holds for the Fe-S cluster assembly protein and carbonic anhydrase 1. This can explain their zinc responsive expression profile.

Another large cluster of 128 differentially expressed genes is higher expressed upon exposure to zinc excess. Expression of many of these appears to be associated with the defense against oxidative stress caused by this treatment (e.g., peroxidase, respiratory burst oxidase proteins). This cluster also comprises genes of families that are associated with iron deficiency response, such as *ZIP*-genes (*IRT1*, *IRT2*, *ZIP8*), *FRO*-genes (*FRO1*, *FRO2*, *FRO3*), *MTP*s

(*MTP3*, *MTP8*), a *NAS*-gene (*NAS1*), an oligopeptide tranporter (*OPT3*) and *IREG2*. A large fraction of these were also found to be differentially expressed in the comparison between wild type Arabidopsis and the *fit1* mutant (Colangelo and Guerinot, 2004). The *fit1* mutant is defective in a bHLH transcription factor controlling several genes involved in iron deficiency response. When comparing the list of 72 genes of which the expression is (partially) dependent on *FIT1* (Colangelo and Guerinot, 2004) with the zinc excess induced cluster (Table I, Supplemental Table II) there are 30 genes in common. This apparent interaction between zinc and iron homeostasis in Arabidopsis, with zinc excess leading to iron deficiency, is not supported by a clear decrease in iron concentration in Arabidopsis leaves (Figure 1), suggesting that this change in gene expression is indeed effective in avoiding actual iron deficiency in leaves.

For *T. caerulescens* the zinc deficiency and zinc excess response is slightly different from Arabidopsis. This does not seem to be due to technical hybridization differences. The expression of the *T. caerulescens* genes confirmed by RT-PCR corresponded very well with the results obtained from the microarray analysis (Figure 3, Supplemental Table XIII). One cluster of co-regulated genes is clearly differently expressed in *T. caerulescens* compared to Arabidopsis (Table III, Supplemental Table V). Whereas in Arabidopsis the three ZIP family members *ZIP3*, *ZIP4*, and *ZIP9* are only higher expressed under zinc deficiency, their *T. caerulescens* orthologues are also relatively highly expressed under sufficient zinc conditions. Based on sequence similarity, the *T. caerulescens ZNT1* and *ZNT2* genes appear to be the orthologues of the Arabidopsis *ZIP4* and *IRT3* genes. Both were previously found to be very highly expressed in *T. caerulescens*, almost regardless of the zinc concentration in the medium (Pence et al., 2000; Assunção et al., 2001). Also under zinc deficient conditions, these two *T. caerulescens* genes are much higher expressed than their Arabidopsis orthologues (Table IV, Supplemental Table XII).

Comparable to Arabidopsis, *T. caerulescens* expresses a cluster of genes in response to zinc deficient conditions (Table III, Supplemental Table V), although this cluster is much smaller than in Arabidopsis. Such might be caused by differences in hybridization efficiency, but this is probably not the case, as the *T. caerulescens* orthologues of *FRD3*, *ZIP10* and *HMA4* are not significantly differentially expressed within *T. caerulescens*, even though they are much higher expressed in *T. caerulescens* than in Arabidopsis (Table IV, Supplemental Table XII). In a recent

microarray study *FRD3* and *HMA4* also appeared to be constitutively higher expressed in *A. halleri* compared to *A. thaliana* (Talke et al., 2006). Similarly, the expression of the *NAS2* and *FER1* orthologues in *T. caerulescens* is more or less constitutive rather than zinc-deficiency-induced as in Arabidopsis (Figure 3).

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*. The different expression profiles between Arabidopsis and *T. caerulescens* of the other three *NAS* genes (Figure 3) suggest a major function for these genes in the metal adaptation of *T. caerulescens*. The presence of at least four *NAS* gene copies in both species, which are apparently all functional and highly redundant (no visible phenotypes are observed in soil-grown single and double K.O. Arabidopsis mutants; data not shown), will have provided ample flexibility to sustain adaptive changes in *NAS* gene expression. Also in view of the observed effect on especially nickel tolerance upon NAS overexpression in non-accumulating, non-tolerant species (Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005), the *NAS* genes may be crucial to metal tolerance in *T. caerulescens*.

Most interesting for the identification of genes that contribute to the adaptation of *T. caerulescens* to high zinc exposure are the genes that are differentially expressed when comparing *T. caerulescens* and Arabidopsis at comparable zinc exposures (Table IV, Supplemental Table X). More than 2200 genes are significantly ($p<0.05$) differentially expressed ($\geq$ 5-fold) at any of the three zinc exposure treatments. This compares well with the recent transcript profile comparison of *T. caerulescens* and *T. arvense* shoot tissue, in which close to 3500 genes were found to be more than 2-fold differentially expressed, at $p<0.05$ (Hammond et al., 2006). More than 50% of the genes we find higher expressed in *T. caerulescens* are of unknown function. In a recent *T. caerulescens* EST analysis (Rigola et al., 2006) especially genes of unknown function were overrepresented while genes involved in general and protein metabolism were underrepresented when compared to Arabidopsis. Even though the fraction of genes involved in (a)biotic stress response is comparable in both species, the stress response genes expressed in *T. caerulescens* are generally different from those expressed in Arabidopsis.

We further analyzed this large set of species-specific differentially expressed genes in different ways. When sorting them according to the highest differential expression under zinc

deficiency, which we consider to be most informative, there are several genes that are more than 100-fold higher expressed in *T. caerulescens*. Among the 15 most differentially expressed genes are four *PDF* or defensin genes, of which *PDF1.1* is close to 1000-fold higher expressed under zinc deficient and excess conditions. The biological role of defensins is not very clear. These small cysteine-rich peptides are generally induced by fungal infections and implicated in pathogen defense, hence their name (Thomma et al., 2002). Mirouze et al., (2006) recently showed that the *A. halleri PDF* family confers zinc tolerance, and they hypothesize that defensins interfere with divalent metal cation trafficking to confer the Zn tolerance phenotype. In Arabidopsis expression of *PDF1.2* is induced by the stress hormone jasmonic acid (JA) (Penninckx et al., 1998). Maksymiec et al., (2005) recently showed that also heavy metal stress induces JA accumulation in plants. Armengaud et al., (2004) found that *PDF1.2a, b, c* and *PDF1.3* are also among the most induced genes upon potassium starvation in Arabidopsis and they suggested a relation between potassium starvation and JA signaling. Of the in total 415 genes they found to be differentially expressed under potassium starvation, we found 46 genes to be higher expressed in *T. caerulescens* compared to Arabidopsis. How, or if, JA, potassium starvation and zinc response are correlated remains still elusive. However, in this context it is also interesting to note that *HAK5, KUP3* and *KAT*, three potassium transporters, are much higher expressed in *T. caerulescens* roots compared to Arabidopsis.

Zinc hyperaccumulation is a constitutive trait in *T. caerulescens* and thus we expect it requires a constitutive expression of metal hyperaccumulation genes and no specific induction at zinc deficiency or excess. It is complicated to identify such zinc accumulation genes from the large set of more or less constitutively higher expressed *T. caerulescens* genes, as many genes in this large set will be involved in general species differences. However, when considering the sixteen highest expressed genes at 100 μM $ZnSO_4$ already six metal homeostasis genes are among them, four of which are known zinc transporters: *HMA4*, *MTP1* and the already discussed *ZIP4* and *IRT3*. *HMA4* was previously identified by Papoyan and Kochian (2004) as a zinc transporting P-type ATPase possibly involved in zinc hyperaccumulation, particularly in loading of zinc into the xylem. The *T. caerulescens* orthologue of the Arabidopsis *MTP1* gene is previously described as the *ZTP1* gene (Assunção et al., 2001) and has already been suggested to

play a role in metal tolerance of *T. caerulescens* (Assunção et al., 2001) and *T. goesingense* (Persans et al., 2001; Kim et al., 2004). Paralogues of the *AtMTP1* gene in *A. halleri* also cosegregate with zinc tolerance in a segregating population (Dräger et al., 2004). Other zinc transporter genes that are higher expressed in *T. caerulescens* are *HMA3*, *MTP8* and *NRAMP3*. *HMA3* is a P-type ATPase, similar to *HMA4*. When expressed in yeast it is able to transport cadmium, but zinc transport could not be proven and in Arabidopsis the expression of the gene is not affected by exposure to zinc (Gravot et al., 2004). The *MTP8* gene is another member of the CDF-family. Especially at zinc deficient and sufficient conditions the gene is higher expressed in *T. caerulescens* compared to Arabidopsis, suggesting a function in zinc uptake, although the regulation in Arabidopsis by *FIT1* (Colangelo and Guerinot, 2004) also indicates a role in iron homeostasis. An *mtp8* K.O. mutant was examined but not found to display any aberrant phenotype under differential zinc exposure (data not shown). *AtNRAMP3* is a vacuolar transporter, which is able to transport iron and cadmium, but no zinc (Thomine et al., 2000). The specific induction of *TcNRAMP3* gene expression by zinc deficiency and excess zinc, suggests it to play an important role in mobilization of zinc and iron in *T. caerulescens* (Lanquar et al., 2005).

Unexpected was that in contrast to Arabidopsis, expression of the iron homeostasis genes *IRT1*, *IRT2* and *FRO2* is not induced in *T. caerulescens* upon excess zinc treatment. When tested by RT-PCR we could not detect the expression of *TcIRT1* except for roots at lower zinc exposure levels (Figure 3B, supplemental table XIII). This suggests that *T. caerulescens* is either able to regulate zinc and iron homeostasis independently, unlike Arabidopsis, or that the continued expression of zinc transporters at high zinc exposure levels ensures low efficiency, but sufficient, iron uptake in *T. caerulescens*. The *IRT1* gene plays an interesting role in metal homeostasis in *T. caerulescens*, since it is very highly expressed upon iron deficiency in the cadmium hyperaccumulating accession Ganges, but much lower in the cadmium excluding accession Prayon (Lombi et al., 2002). The latter is physiologically and geographically very close to La Calamine.

Two metallothionein genes, *MT2B* and especially *MT2A*, are very highly expressed in *T. caerulescens*. MT2 expression is generally associated with copper stress tolerance (Zhou and

Goldsbrough, 1995; van Hoof et al., 2001), but overexpression in *Vicia faba* also induced cadmium tolerance (Lee et al., 2004). Why these two, more copper-associated, genes are so highly expressed in *T. caerulescens* is as yet unclear. High zinc uptake due to high expression of zinc transporters with a low affinity for copper could of course cause some copper stress, but high copper levels have not been reported for *T. caerulescens*. It is more likely that these genes have a function in general stress response, or alternatively serve to maintain copper-homeostasis, as was also suggested for the *MT3* gene of *T. caerulescens* (Roosens et al., 2004).

Very surprising was the relatively high expression of 24 genes with a suggested function in lignin biosynthesis (Ehlting et al., 2005) and 13 genes implicated in suberin biosynthesis (*CER3, CER6* and 11 LTP genes) (Costaglioli et al., 2005) in *T. caerulescens* (Table IV). *CER3* is known to be expressed in Arabidopsis roots, but the expression of *CER6* in *T. caerulescens* roots is very different from the expression in Arabidopsis (Hannoufa et al., 1996; Hooker et al., 2002). The high expression of lignin/suberin biosynthesis genes coincides well with the progressed U-shaped lignification/suberinization of the endodermis cells and the occasional presence of a second endodermis layer found in *T. caerulescens* roots, but not in Arabidopsis roots (Figure 2). Casparian strip development and lignification in cortical cells was recently also observed by Zelko et al. (2005) in *T. caerulescens* but not in the closely related non-accumulator *Thlaspi arvense*. Comparable endodermis wall thickenings were also observed in the salt adapted crucifer *Thelungiella halophila* (Inan et al., 2004). Strong deposition of lignin and suberin on the radial and inner tangential walls resulting in a U-like appearance of the endodermal cells is not uncommon for plants (Zeier and Schreiber, 1998). Since this cell wall deposition occurs most prominently at older parts of the root where root hairs are no longer active, we hypothesize that this layer acts to prevent excess efflux of metals from the vascular cylinder rather than to prevent uncontrolled influx.

With so many genes differentially expressed, one expects also alterations in the transcript levels of transcription factors. In the *T. caerulescens*-Arabidopsis comparison we found 131 transcriptional regulators with over 5-fold higher expression (FRD $p < 0.05$) in *T. caerulescens* (Supplemental Table XIV). Of 19 genes that are more than 10-fold higher expressed under zinc sufficient conditions, two genes (*INO*, *SPL*) are associated with flower development in

Arabidopsis (Villanueva et al., 1999; Yang et al., 1999) and expression is very atypical. However, in line with this atypical expression, we also find the *FIS2* gene higher expressed in *T. caerulescens* roots. In *A. thaliana* this gene is predominantly expressed in developing seeds (Luo et al., 2000), but also in *A. halleri* and *A. lyrata* this gene is induced in roots in response to zinc exposure (www.genevestigator.ethz.ch).

In conclusion, the comparative transcriptional analysis of the hyperaccumulator *T. caerulescens* and the non-accumulator *A. thaliana* emphasizes the role of previously implicated zinc homeostasis genes in adaptation to high zinc exposure, but also suggest a similar role for many more, as yet uncharacterized genes, often without any known function. While some of these genes were also differentially expressed when comparing *A. halleri* with *A. thaliana*, many more were not or at very different levels, suggesting that there will be overlap in the mechanisms of metal accumulation and metal tolerance, but also many differences between metal hyperaccumulator species.

MATERIALS AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Col-0 (Arabidopsis) and *Thlaspi caerulescens* J. & C. Presl accession La Calamine seeds were germinated on garden peat soil (Jongkind BV, Aalsmeer, The Netherlands). Three-week-old seedlings were transferred to 600-ml polyethylene pots (three plants per pot) containing a modified half-strength Hoagland's nutrient solution (Schat et al., 1996): 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 μM KCl, 25 μM H_3BO_3, 2 μM $ZnSO_4$, 2 μM $MnSO_4$, 0.1 μM $CuSO_4$, 0.1 μM $(NH_4)_6Mo_7O_{24}$, 20 μM Fe(Na)EDTA. The pH buffer MES was added at a 2-mM concentration and the pH was set at 5.5 using KOH. Each polyethylene pot contained three seedlings of *T. caerulescens* or Arabidopsis. Three weeks after growing on this solution, the *T. caerulescens* plants were transferred for seven days to the same modified half-strength Hoagland's nutrient solution with a deficient (0 μM), sufficient (100 μM) or excess (1000 μM) $ZnSO_4$ concentration. The Arabidopsis plants were

transferred to the same nutrient solution with deficient (0 μM), sufficient (2 μM) or excess (25 μM) $ZnSO_4$. During the first three weeks, the nutrient solution was replaced once a week and thereafter twice a week. Germination and plant culture were performed in a climate chamber (20/15°C day/night temperatures; 250 μmoles light m^{-2} s^{-1} at plant level during 14 h/d (*T. caerulescens*) or 12 h/d (Arabidopsis); 75% RH) (Assunção et al., 2001).

Root and shoot metal accumulation assay

Two pools of three plants, grown as described before, were used per treatment. After four weeks of growth the plants were harvested, after desorbing the root system with ice-cold 5 mM $PbNO_3$ for 30 min. Roots and shoots were dried overnight at 65°C, wet-ashed in a 4:1 mixture of HNO_3 (65%) and HCl (37%) in Teflon bombs at 140°C for 7 hours and analyzed for zinc, iron and manganese using flame atomic absorption spectrometry (Perkin Elmer 1100B). Metal concentrations in roots and shoots were calculated as μmoles per g dry weight.

Microarray experiment

A common reference model was used to design the microarray experiment (Yang and Speed, 2002), in which cDNA from *T. caerulescens* roots exposed to 100 μM (sufficient) zinc was used as the common reference. Every slide was always hybridized with the common reference sample and with a sample from one of the treatments (Arabidopsis or *T. caerulescens* exposed to deficient, sufficient or excess zinc). The common reference was labeled with the fluorescent dye Cyanine 3 (Cy3) and the treatment samples were labeled with Cyanine 5 (Cy5). As a quality control step we performed a dye-swap hybridization for one sample (from *T. caerulescens* roots exposed to sufficient zinc).

Roots of one pot containing three Arabidopsis or three *T. caerulescens* plants per treatment were pooled and homogenized in liquid nitrogen. Each pool was considered as one biological replicate and two biological replicates were used. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Approximately 300 mg tissue was used for the RNA extraction performed according to the manufacturer's instructions. After the RNA extraction, total RNA was purified with the RNeasy spin columns (Qiagen Benelux B.V., Venlo, Netherlands) and genomic

DNA was digested with the RNase-free DNase set (Qiagen Benelux B.V., Venlo, Netherlands). Ten µg of total RNA was used to synthesize cDNA with MMLV reverse transcriptase and DNA primer. The cDNA was labeled with Cyanine 3-dCTP or Cyanine 5-dCTP and hybridized to Agilent Arabidopsis 3 60-mer oligonucleotide microarrays (Agilent Technologies Inc., Palo Alto, CA, USA) representing ~40,000 putative genes. The microarrays contain all Arabidopsis genes of known function and genes with a high degree of similarity to genes of known function from heterologous organisms - this includes more than 27,000 annotated genes and more than 10,000 non-annotated transcripts based on MPSS data (http://www.chem.agilent.com), and is thus an almost complete representation of the Arabidopsis transcriptome.

After hybridization the slides were scanned, analyzed and normalized with the Agilent Feature Extraction Software (Agilent Technologies Inc., Palo Alto, CA, USA). The arrays were first normalized using Agilent's standard normalization within each array. The remaining statistical analysis was done using the limma package (Smyth, 2005a) in R/BioConductor (Gentleman, 2004). Between-array quantile normalization was performed on the common reference channel while leaving the log-ratios unchanged (Yang and Thorne, 2003). To find differentially expressed genes we performed a separate channel analysis (Smyth, 2005b) between the pairs of interest using a moderated t-test (Smyth, 2004). This test is similar to a standard t-test for each probe except that the standard errors are moderated across genes to ensure more stable inference for each gene. This prevents a gene from being judged as differentially expressed with a very small fold change merely because of an accidentally small residual standard deviation. The resulting p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate (FDR) adjustment (Benjamini and Hochberg, 1995). Genes were considered to be significantly differentially expressed if both the FDR p-values were < 0.05 (controlling the expected false discovery rate to no more than 5%) and the fold change was ≥ 3 (within species) or ≥ 5 (between species). Genes found to be significantly differentially expressed were clustered using Cluster/Treeview (Eisen et al., 1998). Average linkage hierarchical clustering with uncentered correlation was used within Cluster to perform the clustering analysis. Primary microarray data are available in Supplemental Table XV.

Genomic DNA hybridizations were performed using 1 µg random primed genomic DNA. As a quality control step we performed a dye-swap hybridization. After hybridization the slides were scanned, analyzed and normalized with the Agilent Feature Extraction Software (Agilent Technologies Inc., Palo Alto, CA, USA). The arrays were further normalized using linear Lowess analysis. The features which hybridized with Arabidopsis genomic DNA (both polarities of the dye swap) and did not hybridize with *T. caerulescens* genomic DNA were extracted from the dataset by Spotfire using a fold change ≥ 3 as cut-off value.

Semi-quantitative RT-PCR

Total RNA of leaves and roots of a third Arabidopsis or *T. caerulescens* biological replica was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Selected *T. caerulescens* genomic and cDNA fragments were PCR-amplified using primers designed for the orthologous Arabidopsis gene and cloned into the pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA). Clones were sequenced and new primers were designed for semi-quantitative RT-PCR to ensure amplification of the correct *T. caerulescens* gene. Five µg of total RNA was used to synthesize cDNA with MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) as a primer (Invitrogen, Carlsbad, CA, USA). The PCR-amplification was performed with a cDNA aliquot (1 µl) and gene-specific primers (Supplemental Table XIII). Care was taken to design Arabidopsis primers at comparable positions and with comparable length and T_m as for *T. caerulescens* primers to allow proper comparison of the expression data. Primers for Tubulin (Supplemental Table XIII) were used as a control for similar cDNA quantity between the samples. Between 25- to 35-cycle PCRs (30s at 94°C, 30s at 50°C, and 60s at 68°C) were performed in a 50-µl volume, preferably with the same number of cycles for Arabidopsis and *T. caerulescens* samples. 20 µl of the reaction was separated on an ethidium bromide stained 1% agarose gel. Gel-image analysis using QuantityOne® Software (BioRad, Hercules, CA, USA), was used to quantify the DNA fragment intensities (Supplemental Table XIII). The DNA fragment intensities were corrected for background signal and corrected for cDNA quantity using the intensities of Tubulin.

Microscopic analysis of lignification in *T. caerulescens*

T. caerulescens La Calamine seeds were germinated and plants were grown as described before on sufficient zinc medium. After four, six and nine weeks, roots of two plants were collected and hand sections were made by repeatedly chopping roots on a microscope slide using a razorblade. These sections were analyzed with a Nikon Labophot Brightfield Microscope.

T. caerulescens sequences used for semi-quantitative RT-PCR analysis have been deposited with the EMBL/Genbank data libraries under accession numbers DQ384055 (*TcAPX2*), DQ384057 (*TcbHLH100*), DQ923700 (*TcCSD1*), DQ384056 (*TcFER1*), DQ384058 (*TcFRO4*), DQ923702 (*TcHAK5*), DQ384059 (*TcIRT1*), DQ384060 (*TcZIP1*), DQ384061 (*T. caerulescens* orthologue of prolyl oligopeptidase; At1g20380), DQ923699 (*T. caerulescens* orthologue of lipid transfer protein related; At1g27950), DQ923701 (*T. caerulescens* orthologue of sucrose synthase; At4g02280), DQ923703 (*T. caerulescens* orthologue of calcineurin-like phosphoesterase; At5g50400).

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SUPPLEMENTARY MATERIAL

(For supplementary material visit http://www.plantphysiol.org/cgi/content/full/pp.106.082073/DC1)

Supplemental Table I:	Arabidopsis genes lower expressed under zinc deficient conditions compared to sufficient and excess zinc
Supplemental Table II:	Arabidopsis genes higher expressed under excess zinc conditions compared to sufficient or deficient zinc supply.
Supplemental Table III:	Arabidopsis genes higher expressed under zinc deficiency compared to sufficient and excess zinc supply.
Supplemental Table IV:	Arabidopsis genes lower expressed under excess zinc compared to deficient and sufficient zinc conditions.
Supplemental Table V:	*T. caerulescens* genes higher expressed under deficient compared to sufficient and excess zinc supply.
Supplemental Table VI:	*T. caerulescens* genes higher expressed under deficient and excess zinc conditions compared to sufficient zinc
Supplemental Table VII:	*T. caerulescens* genes higher expressed under excess zinc conditions compared to deficient and sufficient zinc.
Supplemental Table VIII:	*T. caerulescens* genes lower expressed under zinc deficient conditions compared to sufficient and excess zinc.
Supplemental Table IX:	*T. caerulescens* genes lower expressed under deficient and excess zinc conditions compared to sufficient zinc.
Supplemental Table X:	*T. caerulescens* genes higher expressed under zinc deficiency, sufficiency or excess compared to Arabidopsis.
Supplemental Table XI:	Expressed genes in *T. caerulescens* for which no expression was detected in the roots of Arabidopsis under all three conditions tested.
Supplemental Table XII:	A selection of genes higher expressed in *T. caerulescens* compared to Arabidopsis.
Supplemental Table XIII:	Differentially expressed genes verified by semi-quantitative RT-PCR.
Supplemental Table XIV:	Transcription factor genes higher expressed in *T. caerulescens* compared to Arabidopsis.
Supplemental Table XV:	Microarray primary data.

Chapter 3

Expression differences for genes involved in lignin, glutathione and sulfate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*

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ABSTRACT

Cadmium (Cd) is a widespread, naturally occurring element present in soil, rock, water, plants and animals. Cd is a non-essential element for plants and toxic at higher concentrations. Transcript profiles of roots of *Arabidopsis thaliana* (Arabidopsis) and *Thlaspi caerulescens* plants exposed to Cd and zinc (Zn) are examined, with the main aim to determine the differences in gene expression between the Cd tolerant Zn hyperaccumulator *T. caerulescens* and the Cd sensitive non-accumulator Arabidopsis. This comparative transcriptional analysis emphasized the role of genes involved in lignin, glutathione and sulfate metabolism. Furthermore the transcription factors *MYB72* and *bHLH100* were studied for their involvement in metal homeostasis, as they showed an altered expression after exposure to Cd. The Arabidopsis *myb72* knock-out mutant was more sensitive to excess zinc or iron deficiency than wild type, while Arabidopsis transformants overexpressing *bHLH100* showed increased tolerance to high zinc and nickel compared to wild type plants, confirming their role in metal homeostasis in Arabidopsis.

INTRODUCTION

Cadmium (Cd) is a widespread, naturally occurring element present in soil, rock, water, plants and animals. It occurs naturally in deposits of zinc (Zn) and phosphorus (P), but unlike these nutrients, it is not essential for life. Instead Cd is a health-threatening heavy metal pollutant. High Cd concentrations can result in bone disease and kidney damage in mammals, including humans. Cd uptake by humans occurs through food consumption, smoking and environmental exposure associated with zinc smelting, coal burning and the use of mineral phosphate fertilizers (Pinot et al., 2000).

Uptake of Cd by crop plants is the main entry pathway into the human food chain. Although plants generally try to prevent the uptake of Cd, the uptake depends on the availability of Cd to the plant, which largely depends on the Cd concentration in the soil, the pH and organic matter content of the soil. Cd is thought to be transported into the roots via Zn and Fe transporters belonging to the ZIP family or via Ca channels (Perfus-Barbeoch et al., 2002). Within the cell Cd is bound to sulfur ligands (Salt et al., 1995a) like glutathione (GSH), GSH-derived oligopeptides called phytochelatins (PCs) (Clemens, 2006), or to organic acid ligands (Lugon-Moulin et al., 2004). The sulfur ligand-Cd complexes are actively sequestered into the vacuoles of plant cells (Vögeli-Lange and Wagner, 1990), probably through ABC transporters (Salt and Rauser, 1995b). In uncomplexed form, Cd can also be transported by CAX cation/proton antiporters (Hirschi et al., 1996). The further distribution of Cd to the shoots depends on the transport of ligand-Cd complexes and free Cd through the xylem and the movement of Cd across the different membranes before xylem loading and after xylem unloading. Cd accumulation in plants affects water and nutrient uptake and photosynthesis and it results in growth inhibition, browning of the root tips, leaf chlorosis and finally death (Kahle, 1993).

Although Cd is generally toxic to most plants, there are species that can accumulate high amounts of Cd without any sign of toxicity. Species accumulating metals in the above ground tissues are called hyperaccumulators (Baker and Brooks, 1989). Over 400 hyperaccumulator species from a wide range of unrelated families have been described. About 75% are nickel hyperaccumulators, fifteen are zinc hyperaccumulators and currently only four species are known

to hyperaccumulate Cd: *Thlaspi caerulescens*, *Thlaspi praecox*, *Arabidopsis halleri* and *Sedum alfredii* (Baker et al., 1992; Brooks, 1994; Vogel-Mikuš et al., 2005; Deng et al., 2006). They are mainly, though not exclusively, found on calamine soils contaminated with Pb, Zn or Cd (Meerts and Van Isacker, 1997; Schat et al., 2000; Bert et al., 2002). *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) is a self-compatible species, which is closely related to *Arabidopsis thaliana* (L.) Heynh. (Arabidopsis), with on average 88.5 % DNA identity in coding regions (Rigola et al., 2006) and 87 % DNA identity in the intergenic transcribed spacer regions (Peer et al., 2003). *T. caerulescens* accessions La Calamine (LC) and Ganges (GA), both from Zn/Cd/Pb-enriched calamine soil, are more tolerant to Zn and Cd compared to the accessions Lellingen (LE) and Monte Prinzera (MP), originating from non-metalliferous and serpentine soil, respectively (Assunção et al., 2003b). Especially the *T. caerulescens* accessions from Southern France, like GA (Lombi et al., 2000) or St. Felix de Pallières (Peer et al., 2003) are strong Cd hyperaccumulators, with the ability to survive accumulation of more than 10,000 ppm Cd (d.w.) in the leaves.

The complex network of homeostatic mechanisms that evolved in plants to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001) also operates in metal hyperaccumulators. Previously published transcript profiling studies on the response to Zn or Cd in Arabidopsis (Wintz et al., 2003; Kovalchuk et al., 2005; Herbette et al., 2006; Weber et al., 2006) and comparative analysis of Arabidopsis and the Zn and Cd hyperaccumulating species *A. halleri* and *T. caerulescens* (Becher et al., 2004; Weber et al., 2004, 2006; van de Mortel et al., 2006) already identified several genes for which expression changes in response to Zn or Cd exposure in these species. The analyses also revealed that the transcriptional regulation of many genes is strikingly different in *A. halleri* and *T. caerulescens* compared to Arabidopsis.

In this paper we perform a between and within species comparison of transcript profiles of Arabidopsis and *T. caerulescens* plants exposed to different Cd concentrations, aiming to identify genes that are important for the adaptation of *T. caerulescens* to high Cd exposure. Therefore, we examined the response of roots of Arabidopsis and *T. caerulescens* to Cd excess and compared the transcript profiles with those caused by alterations in Zn supply.

RESULTS

Experimental design

To analyze the response of Arabidopsis and *T. caerulescens* to different Cd exposures, we compared the transcript profiles of roots of plants grown under sufficient nutrient supply without Cd with those of plants grown under excess Cd conditions. To minimize variation in the bioavailability of micronutrients, we used a hydroponic culturing system. For excess Zn or Cd conditions the induction of severe stress to the plants was avoided by exposing them only for one week to these conditions. Arabidopsis plants (accession Columbia) were grown on a nutrient solution containing 2 µM $ZnSO_4$, which yields healthy and robust plants with normal seed set even after prolonged cultivation. After three weeks the plants were transferred to fresh solutions for exposure to Zn deficiency (0 µM $ZnSO_4$), excess Zn (25 µM $ZnSO_4$) or excess Cd (15 µM $CdSO_4$, 2 µM $ZnSO_4$). One quarter of the plants remained as a control at sufficient Zn (2 µM $ZnSO_4$). Upon root harvesting, the plants growing on Zn deficient and sufficient medium did not show any visible phenotypic differences, while roots of plants growing on excess Zn or excess Cd showed a slight growth inhibition (data not shown). At this stage, plants were not yet flowering.

For *T. caerulescens* a similar approach was followed. However, in order to properly compare the results between the two species we aimed at maintaining comparable physiological conditions. *T. caerulescens* accession La Calamine is Zn and Cd tolerant as well as Zn hyperaccumulating and requires more Zn than Arabidopsis for normal growth. Therefore a hydroponic solution containing 100 rather than 2 µM $ZnSO_4$ was used as Zn sufficient condition. To avoid any problems with possible precipitation of Zn or other minerals at very high Zn concentrations, 1000 µM $ZnSO_4$ was used for excess Zn exposure and 0.5 or 50 µM $CdSO_4$ for respectively low excess and high excess Cd treatment, respectively. To avoid severe competition of Cd by Zn upon Cd exposure, which occurs in *T. caerulescens* (Zhao et al., 2002), we used 2 instead of 100 µM $ZnSO_4$ in the Cd excess solutions. When root tissues were harvested after a one-week exposure, *T. caerulescens* plants showed no phenotype that could be attributed to Zn deficiency, Zn excess or Cd excess exposure.

Table I: Cadmium-responsive genes in Arabidopsis.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn2 = 2 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn25 = 25 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd15 = 2 µM $ZnSO_4$, 15 µM $CdSO_4$. [d]Normalized spot intensity at 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity[d]
I	UPM1	AT5G40850	urophorphyrin III methylase	porphyrin biosynthesis	0.70	1.27	1.82	**2.07**	16975
		AT5G64260	phosphate-responsive protein	biological process unknown	0.99	1.42	1.43	**2.32**	7342
		AT3G25190	nodulin, putative, similar to nodulin 21	biological process unknown	1.02	1.12	1.09	**2.29**	4528
		AT3G48390	MA3 domain-containing protein	biological process unknown	0.86	1.11	1.28	**2.65**	227
II		AT5G39150	germin - like protein	biological process unknown	0.92	0.45	0.49	**0.32**	12783
		AT5G09530	surface protein PspC-related	biological process unknown	1.27	0.89	0.70	**0.45**	8612
		AT2G23030	putative protein kinase	protein amino acid phosphorylation	1.37	1.24	0.90	**0.45**	5480
		AT4G19760	glycosyl hydrolase family 18	carbohydrate metabolism	1.03	0.47	0.45	**0.33**	4870
	PCNA1	AT1G07370	proliferating cellular nuclear antigen	regulation of DNA replication	1.46	1.04	0.71	**0.49**	2537
		AT1G28640	lipase, putative	lipid metabolism	1.30	1.00	0.76	**0.48**	2210
		AT2G18370	putative lipid transfer protein	lipid transport	1.12	0.90	0.81	**0.48**	2197
		AT5G15110	polysaccharide lyase family 1	biological process unknown	1.62	1.17	0.72	**0.49**	2005
	GLP6	AT5G39100	germin - like protein GLP6	biological process unknown	1.28	0.60	0.47	**0.15**	1898
		AT4G16563	aspartyl protease family protein	proteolysis	1.40	0.72	0.52	**0.26**	1168
	AtEP3	AT3G54420	glycosyl hydrolase family 19	hypersensitive response	0.96	0.53	0.55	**0.49**	981
		AT1G56320	hypothetical protein	biological process unknown	0.97	0.75	0.78	**0.45**	778
	PER20	AT2G35380	peroxidase family	response to oxidative stress	0.97	0.73	0.75	**0.50**	628
		AT2G18150	peroxidase, putative	response to stress	0.92	0.53	0.58	**0.39**	559
		AT3G51970	wax synthase-like protein	biological process unknown	1.18	1.47	1.25	**0.38**	295
		AT3G21690	MATE efflux family protein	multidrug transport	1.00	0.56	0.55	**0.44**	291
		AT2G26480	putative glucosyltransferase	metabolism	1.32	1.35	1.02	**0.49**	288
		AT4G33710	pathogenesis-related protein 1 precursor	biological process unknown	0.79	0.36	0.46	**0.11**	267
		AT5G57610	protein kinase family protein	protein amino acid phosphorylation	1.20	1.13	0.94	**0.49**	264
	MYB107	AT3G02940	myb family transcription factor	transcription	1.04	0.84	0.81	**0.46**	222
	CAX3	AT3G51860	Ca2+/H+-exchanging protein-like	cation transport	1.46	0.58	0.40	**0.25**	170
		AT3G46110	putative protein	biological process unknown	1.34	0.56	0.42	**0.27**	91
		AT1G77730	pleckstrin homology domain-containing protein	biological process unknown	1.72	0.86	0.50	**0.40**	73
		AT2G18660	expansin family protein	biological process unknown	2.49	1.11	0.45	**0.11**	51
		AT1G14150	PsbQ domain protein family	photosynthesis	1.90	1.61	0.85	**0.46**	38
		AT3G49580	putative protein	biological process unknown	1.55	1.05	0.68	**0.20**	37

Cadmium-regulated genes in Arabidopsis

Genes responding to changes in Cd exposure conditions in roots of Arabidopsis were identified by microarray hybridization using Agilent Arabidopsis2 60-mer oligonucleotide microarrays containing 21,500 probes representing over 80 % of the Arabidopsis genome. We only considered the hybridization data of probes with an FDR < 0.05 to be significant. Furthermore, only expression differences of ≥ 2-fold (between any of the four treatments; 0 µM $ZnSO_4$, 2 µM $ZnSO_4$, 25 µM $ZnSO_4$; 15 µM $CdSO_4$) were considered to be relevant, even though lower expression differences were statistically significant at FDR < 0.05. According to these criteria we identified 30 genes with a response to Cd only. After hierarchical clustering of these Cd-responsive genes (average linkage hierarchical clustering with uncentered correlation; Eisen et al., 1998), two major clusters were distinguished (Table I). Cluster I consists of four genes, which are lower expressed upon exposure to Cd conditions. One of these genes encodes an urophorphyrin III methylase (*UPM1*), which is involved in porphyrin biosynthesis. The other three genes encode a phosphate-responsive protein, a nodulin-like protein and a MA3 domain-containing protein. Cluster II (Table I) consists of 26 genes, which are higher expressed due to Cd exposure. Genes in this cluster are involved in transport (*CAX3*, MATE efflux family protein), transcription (*MYB107*) or stress-response (peroxidases). Other genes in this cluster encode pathogenesis-related proteins and genes involved in cell wall biosynthesis.

Cadmium- and zinc-regulated genes in *Arabidopsis thaliana*

In total 48 genes are responding to both Zn and Cd in Arabidopsis when comparing Zn deficiency, Zn sufficiency, Zn excess and Cd exposure. After hierarchical clustering of these genes, five major clusters were distinguished (Table II). Cluster I contains 25 genes, which are higher expressed under excess Zn and Cd exposure. This cluster contains *FRO2*, which encodes a ferric-chelate reductase. Other genes in this cluster are involved in transcription (*MYB4*, *MYB10*, *MYB72*, *ORG2*, *ORG3*, *bHLH100*), lignin biosynthesis (*4CL2*, *CYP71A20*, *CYP91A2*), stress response and general (secondary) metabolism. Cluster II (Table II) contains five genes, which are higher expressed under Zn deficient, Zn excess and Cd exposure, encoding for two germin-like proteins, the transcription factor *WRKY59* and a protein kinase.

Table II: Cadmium- and zinc-responsive genes in Arabidopsis.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn2 = 2 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn25 = 25 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd15 = 2 µM $ZnSO_4$, 15 µM $CdSO_4$. [d]Normalized spot intensity at 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity[d]
I	FRO2	AT1G01580	ferric-chelate reductase	iron chelate transport	**0.27**	**0.02**	**0.08**	**0.27**	14321
		AT5G05250	expressed protein	biological process unknown	0.61	**0.17**	**0.27**	**0.41**	3932
		AT5G39160	germin-like protein (GLP2a) copy1	biological process unknown	**0.46**	**0.18**	**0.40**	**0.41**	2422
		AT5G02780	In2-1 protein, putative	biological process unknown	**0.31**	**0.04**	**0.12**	**0.38**	2177
		AT4G19750	glycosyl hydrolase family 18	carbohydrate metabolism	0.94	**0.42**	**0.44**	**0.39**	2078
	MYB4	AT4G38620	putative transcription factor (MYB4)	regulation of transcription	1.05	**0.45**	**0.43**	**0.44**	1530
		AT4G10500	2-oxoglutarate-dependent dioxygenase family	secondary metabolism	1.21	**0.35**	**0.29**	**0.40**	1291
	4CL2	AT3G21240	putative 4-coumarate:CoA ligase 2	lignin metabolism	1.15	**0.19**	**0.17**	**0.41**	1230
	ORG1	AT5G53450	protein kinase family protein	protein amino acid phosphorylation	0.79	**0.12**	**0.16**	**0.30**	1085
	bHLH100	AT2G41240	bHLH protein	regulation of transcription	0.26	**0.01**	**0.02**	**0.08**	804
		AT3G61930	hypothetical protein	N-terminal protein myristoylation	0.39	**0.04**	**0.10**	**0.18**	780
	CYP71A20	AT4G13310	cytochrome p450 family	lignin metabolism	1.44	**0.25**	**0.17**	**0.23**	729
		AT4G13280	terpene synthase/cyclase family	response to wounding	0.71	**0.20**	**0.29**	**0.31**	630
		AT5G16570	glutamine synthetase	nitrate assimilation	0.97	**0.24**	**0.25**	**0.35**	599
	ORG3	AT3G56980	bHLH protein	regulation of transcription	1.03	**0.06**	**0.05**	**0.10**	590
		AT5G36890	glycosyl hydrolase family 1	carbohydrate metabolism	0.74	**0.07**	**0.09**	**0.44**	487
		AT2G30670	putative tropinone reductase	metabolism	0.73	**0.16**	**0.21**	**0.26**	444
	ORG2	AT3G56970	bHLH protein	regulation of transcription	**0.37**	**0.02**	**0.05**	**0.10**	379
		AT2G13810	putative aspartate aminotransferase	glutamate catabolism to oxaloacetate	0.64	**0.37**	0.58	**0.41**	329
	MYB10	AT3G12820	myb family transcription factor	regulation of transcription	0.53	**0.05**	**0.10**	**0.30**	316
		AT2G20030	putative RING zinc finger protein	biological process unknown	0.97	**0.33**	**0.34**	**0.49**	279
	MYB72	AT1G56160	myb family transcription factor	regulation of transcription	0.42	**0.03**	**0.06**	**0.27**	177
		AT1G52120	similar to jacalin lectin family protein	biological process unknown	0.90	**0.06**	**0.07**	**0.26**	166
		AT5G04150	basic helix-loop-helix (bHLH) family protein	regulation of transcription	0.85	**0.16**	**0.19**	**0.21**	151
	CYP91A2	AT4G37430	cytochrome p450 family	lignin metabolism	0.87	**0.34**	**0.39**	**0.32**	111

Continue Table II

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity[d]
II		AT5G39180	germin-like protein	biological process unknown	1.26	0.42	**0.34**	**0.23**	6785
		AT5G39110	germin -like protein	biological process unknown	1.56	0.76	**0.49**	**0.38**	6665
	WRKY59	AT2G21900	WRKY family transcription factor	regulation of transcription	**2.95**	1.28	0.43	**0.26**	973
		AT2G40580	putative protein kinase	protein amino acid phosphorylation	2.58	0.76	**0.29**	**0.23**	257
	RSU1	AT3G48130	ribosomal protein L13 homolog	biological process unknown	**4.06**	**4.00**	0.99	**0.16**	55
III		AT1G52820	2OG-Fe(II) oxygenase family protein	biological process unknown	0.98	**7.46**	**7.59**	**4.03**	12394
	CYP712A1	AT2G42250	cytochrome p450, putative	electron transport	0.86	**4.53**	**5.24**	**3.77**	12367
	ZIP2	AT5G59520	putative zinc transporter ZIP2 - like	zinc ion transport	1.46	**8.01**	**5.47**	**3.28**	10400
		AT5G50590	11-beta-hydroxysteroid dehydrogenase-like	metabolism	1.29	**10.96**	**8.50**	**4.35**	5550
		AT1G52800	2OG-Fe(II) oxygenase family protein	biological process unknown	0.63	1.56	**2.46**	**2.24**	3599
		AT1G15540	2OG-Fe(II) oxygenase family protein	N-terminal protein myristoylation	0.57	**5.56**	**9.68**	**5.82**	3209
		AT1G80320	2OG-Fe(II) oxygenase family protein	biological process unknown	0.62	**4.84**	**7.75**	**7.05**	3197
		AT1G52790	2OG-Fe(II) oxygenase family protein	biological process unknown	0.57	**4.04**	**7.08**	**5.38**	2023
	CYP93D1	AT5G06900	cytochrome p450 family	electron transport	0.82	**4.59**	**5.58**	**5.62**	1481
		AT1G24420	acyltransferase family	biological process unknown	1.15	**5.69**	**4.94**	**3.03**	1335
	CYP76G1	AT3G52970	cytochrome p450 family	lignin metabolism	0.70	**11.51**	**16.37**	**11.46**	1227
	LAS1	AT3G45130	cycloartenol synthase	pentacyclic triterpenoid biosynthesis	0.73	**2.63**	**3.61**	**3.49**	1172
	CYP712A2	AT5G06905	similar to cytochrome p450, putative	electron transport	0.82	**4.26**	**5.17**	**4.65**	1089
		AT3G26040	acyltransferase family	biological process unknown	0.55	**3.76**	**6.81**	**4.74**	670
IV	FRD3	AT3G08040	MATE efflux family protein, putative	iron ion homeostasis	**4.15**	**7.06**	1.70	**2.20**	802
	NAS4	AT1G56430	nicotianamine synthase, putative	nicotianamine biosynthesis	**30.58**	**13.08**	0.43	**0.32**	461
V		AT3G13950	hypothetical protein	biological process unknown	**0.37**	0.77	2.12	**2.53**	1355
		AT5G43935	flavonol synthase	biological process unknown	0.50	1.05	**2.09**	**3.22**	102

The ribosomal protein *RSU1* is higher expressed under Zn deficient and Cd excess exposure but not under Zn excess exposure. Cluster III (Table II) consists of 14 genes, which show decreased expression upon exposure to excess Zn and Cd. Five genes in this cluster encode members of the 2OG-Fe(II) oxygenase protein family, two encode members of the acyltransferase family, and four genes belong to the cytochrome P450 family (*CYP76G1, CYP93D1*, *CYP712A1* and *CYP712A2*). This cluster also contains the *ZIP2* gene, involved in Zn transport. The two genes in cluster IV (Table II) encode the metal homeostasis related genes *FRD3* (citrate efflux; Puig et al., 2007) and a nicotianamine synthase, *NAS4*, needed for biosynthesis of the metal chelating compound nicotianamine (Shojima et al., 1989a). Both genes show a similar change in expression profile in response to Zn, but in response to Cd the change in expression is different. *FRD3* is repressed under Cd exposure, whereas *NAS4* is higher expressed under Cd exposure. Cluster V (Table II) consists of two genes, encoding a hypothetical protein (At3g13950) and a flavonol synthase, which are lower expressed under Zn deficiency, Zn excess and Cd exposure compared to Zn sufficiency.

Heterologous microarray hybridization

We used the same Arabidopsis array platform for profiling of *T. caerulescens* gene expression through heterologous hybridization with labelled *T. caerulescens* cDNA. From EST analysis we previously learned that *T. caerulescens* shares about 85-90% DNA identity in coding regions with Arabidopsis (Rigola et al., 2006), which should be sufficient for proper heterologous hybridization. Additionally, we recently hybridized Agilent Arabidopsis3 oligonucleotide microarrays with *T. caerulescens* cDNA, which showed reliable heterologous hybridization (van de Mortel et al., 2006). As an additional confirmation, we performed genomic DNA hybridization of *T. caerulescens* to the Agilent Arabidopsis2 oligonucleotide array, which showed on average a 2.0-fold lower signal intensity for *T. caerulescens* compared to the Arabidopsis signal intensities. Overall, only 94 probes in the Arabidopsis2 oligonucleotide array hybridized with less than 3-fold lower signal intensity with *T. caerulescens* genomic DNA.

Table III:Genes repressed by cadmium in *T. caerulescens*

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 = 2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; [d]Normalized spotintensity at 100 µM $ZnSO_4$. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
I	AHB1	AT2G16060	class 1 non-symbiotic hemoglobin	response to stress	0.55	0.50	0.92	1.58	2.79	**4.39**	117037
	NIA1	AT1G77760	nitrate reductase 1	nitrate assimilation	0.81	1.33	1.64	1.04	**2.52**	**2.62**	41451
	PDC2	AT5G54960	pyruvate decarboxylase	response to stress	1.02	0.97	0.95	1.28	**3.12**	**3.98**	32271
		AT4G10270	probable wound-induced protein	response to stress	0.82	1.01	1.23	1.37	2.60	**3.58**	31740
		AT5G39890	expressed protein	other biological processes	0.55	0.45	0.82	1.13	3.40	**3.83**	31612
		AT4G27450	expressed protein	other biological processes	0.70	0.61	0.88	1.02	**3.09**	**3.16**	31058
		AT2G17850	senescence-associated protein	aging	0.53	0.48	0.91	1.04	**4.65**	**4.83**	29402
		AT3G29970	germination protein-related	other biological processes	0.43	0.60	1.41	0.91	**8.80**	**8.03**	23771
	ALAAT2	AT1G72330	putative alanine aminotransferase	L-alanine biosynthetic process from pyruvate	0.79	0.75	0.96	1.00	**3.14**	**3.15**	14772
		AT2G27830	expressed protein	other biological processes	1.01	0.79	0.78	1.09	**2.30**	**2.51**	12380
		AT4G34950	nodulin family protein	other biological processes	1.12	1.14	1.02	0.78	**4.18**	**3.26**	11571
		AT1G72360	member of the ERFB-2 transcription factor family	signal transduction	0.66	0.82	1.24	1.31	**2.29**	**3.00**	10526
		AT5G19140	auxin/aluminum-responsive protein	response to auxin stimulus	0.68	0.72	1.06	0.86	**3.66**	**3.16**	10238
		AT3G10040	expressed protein	transcription	0.76	0.95	1.25	1.03	3.26	**3.37**	10042
		AT1G76640	calmodulin-related protein	other biological processes	0.57	0.68	1.19	0.94	**4.16**	**3.90**	9407
		AT4G17260	L-lactate dehydrogenase	response to abscisic acid stimulus	0.77	0.81	1.06	0.94	**2.06**	**1.94**	9337
		AT1G43800	acyl-(acyl-carrier-protein) desaturase, putative	other cellular processes	0.70	0.75	1.07	1.33	**3.61**	**4.81**	9211
		AT1G47840	hexokinase, putative	electron transport or energy pathways	0.75	0.96	1.28	1.37	1.59	**2.19**	7653
		AT1G62770	invertase/pectin methylesterase inhibitor family protein	other biological processes	0.67	**0.52**	0.77	0.87	**2.87**	**2.50**	7521
	C/VIF1	AT1G47960	invertase/pectin methylesterase inhibitor family protein	other biological processes	0.82	0.63	0.77	0.94	**3.19**	**2.99**	5637
		AT1G20700	homeobox-leucine zipper family protein	transcription	0.70	**0.56**	0.80	0.94	**2.07**	**1.95**	5503
		AT3G27220	kelch repeat-containing protein	other biological processes	0.84	0.95	1.12	1.24	**3.04**	**3.78**	4945

Continue Table III

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT4G32460	expressed protein	other biological processes	**0.52**	0.79	1.52	1.42	1.71	**2.42**	4284
		AT5G47240	mutT domain protein-like	other biological processes	0.59	**0.51**	0.86	0.82	**2.24**	**1.84**	3769
		AT1G31330	photosystem I subunit III precursor, putative	photosynthesis	0.68	0.93	1.36	1.46	1.67	**2.43**	3758
		AT1G07160	protein phosphatase 2C, putative	protein metabolism	0.56	0.70	1.26	1.01	**2.81**	**2.83**	3431
		AT4G36850	PQ-loop repeat family protein	other biological processes	0.58	0.94	1.62	1.22	2.12	**2.58**	3397
		AT5G07580	member of the ERF subfamily B-3 transcription factor family	transcription	0.92	1.00	1.08	0.97	**2.38**	**2.30**	2940
		AT5G15120	expressed protein	other biological processes	0.82	1.08	1.33	1.17	2.52	**2.94**	2766
		AT5G54130	calcium-binding EF hand family protein	other biological processes	0.87	0.68	0.78	0.98	**2.10**	**2.06**	2382
		AT4G26270	phosphofructokinase family protein	electron transport or energy pathways	0.84	0.81	0.96	1.13	3.00	**3.38**	2182
	bHLH096	AT1G72210	basic helix-loop-helix (bHLH) family protein	transcription	1.39	1.27	0.91	1.08	**2.86**	**3.09**	1941
		AT4G21390	S-locus lectin protein kinase family protein	protein metabolism	1.01	0.94	0.93	1.42	**2.69**	**3.81**	1886
	ATL6	AT3G05200	zinc finger (C3HC4-type RING finger) family protein	other biological processes	0.92	0.90	0.97	1.09	**1.91**	**2.08**	1778
		AT1G15210	ABC transporter family protein	multidrug transport	0.53	0.65	1.22	1.20	1.75	**2.11**	1727
		AT3G12150	expressed protein	other biological processes	0.64	0.80	1.25	1.21	**1.86**	**2.25**	1698
	At-GTL1	AT1G33240	trihelix DNA-binding protein	other biological processes	0.64	0.89	1.40	1.22	**2.01**	**2.44**	1654
	APL3	AT4G39210	glucose-1-phosphate adenylyltransferase	starch biosynthetic process	1.01	1.01	1.00	1.08	**2.34**	**2.52**	1380
	ACD32.1	AT1G06460	heat shock protein, putative	response to abiotic or biotic stimulus	**0.64**	**0.55**	0.86	0.89	**2.15**	**1.90**	1307
	RHA3B	AT4G35480	zinc finger (C3HC4-type RING finger) family protein	other biological processes	0.99	1.72	1.74	1.12	**3.62**	**4.05**	1032
	APL4	AT2G21590	glucose-1-phosphate adenylyltransferase	starch biosynthetic process	0.98	0.96	0.98	0.91	**2.87**	**2.62**	857
	CLE6	AT2G31085	Clavata3 / ESR-Related-6	signal transduction	0.38	0.35	0.92	1.06	4.47	**4.75**	778
		AT1G80280	hydrolase	other biological processes	**0.59**	0.88	1.50	1.14	**1.86**	**2.13**	711
	EXGT-A4	AT5G13870	xyloglucan endotransglycosylase	carbohydrate metabolic process	0.52	0.85	1.63	1.02	**2.31**	**2.35**	678
	CLE4	AT2G31081	Clavata3 / ESR-Related-4	signal transduction	0.62	0.75	1.20	1.30	**1.93**	**2.51**	648
		AT1G45230	similar to defective chloroplasts and leaves (DCL) protein	other biological processes	**0.51**	0.97	1.90	1.04	**2.40**	**2.48**	616
		AT5G42900	expressed protein	other biological processes	0.60	**0.55**	0.93	1.13	1.81	**2.04**	482
		AT1G66045	hypothetical protein	other biological processes	0.95	2.13	2.25	0.83	**3.28**	**2.71**	225

Continue Table III

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
II	ATFER1	AT5G01600	ferritin 1 precursor	transport	0.70	1.46	2.07	2.20	1.32	**2.91**	24439
	FLA9	AT1G03870	fasciclin-like arabinogalactan-protein	cell adhesion	**0.53**	0.91	1.71	1.92	1.36	**2.62**	24419
		AT3G62270	anion exchange family protein	transport	0.63	0.62	0.98	1.40	1.44	**2.02**	9313
	PATL1	AT1G72150	SEC14 cytosolic factor family protein	transport	1.38	1.66	1.20	1.55	1.33	**2.07**	8784
		AT3G55230	disease resistance response protein-related	defense response	0.43	0.51	1.19	2.37	1.24	**2.93**	6790
		AT2G26190	calmodulin-binding family protein	protein metabolism	1.09	0.99	0.90	1.77	1.60	**2.82**	5199
	IRX3	AT5G17420	cellulose synthase catalytic subunit	cell organization and biogenesis	**0.61**	0.90	1.48	1.54	1.37	**2.11**	5191
		AT1G74500	bHLH family protein	transcription	0.61	1.09	1.80	1.73	1.21	**2.09**	4208
		AT5G55050	GDSL-motif lipase/hydrolase family protein	lipid metabolic process	0.53	0.71	1.35	**3.40**	1.02	**3.46**	3767
		AT4G22380	ribosomal protein L7Ae - like	cell organization and biogenesis	0.70	1.12	1.59	1.36	1.58	**2.15**	3057
	ATFER3	AT3G56090	ferritin	iron ion transport	0.80	1.34	1.67	2.01	1.12	**2.25**	2726
		AT5G60760	2-phosphoglycerate kinase-related	other biological processes	0.69	1.33	**1.93**	1.72	1.38	**2.36**	2643
		AT1G14290	acid phosphatase, putative	metabolic process	0.66	0.69	1.06	**1.79**	1.32	**2.36**	2544
		AT3G05150	sugar transporter family protein	transport	0.60	1.13	1.87	1.72	1.31	**2.24**	2393
		AT2G16630	proline-rich family protein	other biological processes	0.65	1.21	1.88	1.83	1.32	**2.41**	2190
		AT1G04610	flavin-containing monooxygenase	auxin biosynthetic process	0.66	0.69	1.04	**2.53**	0.88	**2.22**	2149
		AT5G18460	expressed protein	other biological processes	0.71	1.08	1.52	1.79	1.18	**2.12**	1349
	ULT1	AT4G28190	expressed protein	developmental processes	0.63	1.04	1.65	1.68	1.23	**2.07**	1305
		AT5G48250	zinc finger (B-box type) family protein	transcription	0.66	0.66	0.99	1.49	1.37	**2.03**	1150
		AT2G23050	phototropic-responsive NPH3 family protein	response to abiotic or biotic stimulus	0.73	1.03	1.40	1.84	1.16	**2.13**	1049
		AT1G22335	expressed protein	other biological processes	0.63	1.02	1.62	1.52	1.34	**2.03**	1038
		AT4G25110	latex-abundant family protein	protein metabolism	0.63	0.78	1.23	1.69	1.23	**2.09**	595
		AT5G59080	expressed protein	other biological processes	0.49	0.68	1.38	2.20	1.76	**3.87**	554
		AT2G43050	pectinesterase family protein	cell organization and biogenesis	0.67	1.16	1.74	1.58	1.61	**2.55**	493
		AT5G41900	hydrolase	aromatic compound metabolic process	0.59	1.12	1.90	1.70	1.22	**2.09**	382
		AT5G23870	pectinacetylesterase family protein	other biological processes	0.62	1.05	1.70	1.36	1.57	**2.15**	346
		AT1G78520	glycosyl hydrolase family protein 17	other biological processes	0.70	1.05	1.50	1.45	1.44	**2.08**	274

Previously we performed a similar genomic DNA hybridization to the Agilent Arabidopsis3 oligonucleotide array containing more probes (van de Mortel et al., 2006), which showed considerable overlap in both sets of poorly hybridizing probes. Based on both experiments, we excluded a total of 252 poorly hybridizing probes from the analysis.

Cadmium-regulated genes in *T. caerulescens*

To identify genes that are regulated by Cd in *T. caerulescens* we compared the expression of genes in roots of *T. caerulescens* plants grown on low and high Cd containing medium with those exposed to different Zn concentrations. Thus we identified 171 genes that were significantly (FDR < 0.05) differentially expressed (≥ 2-fold) in response to Cd only (Supplemental Table I). Only one of these genes (At2g18370) was also found to be differentially expressed in Arabidopsis in response to Cd (Table I). Seven major clusters were identified upon cluster analysis of the Cd-responsive genes (Supplemental Table I).

Cluster I (Table III) consists of 49 genes, which are repressed by both low and high Cd exposure compared to the Zn sufficient condition. Many genes in this cluster are involved in (a)biotic stress response, such as genes encoding the pyruvate decarboxylase-like protein family (*PDC2*), a wound-induced protein and *ACD32.1*, which encodes a heat shock protein. There are 10 genes with an unknown function in this cluster. Other genes identified in this cluster encode proteins involved in root development and signal transduction (*CLE4* and *CLE6*; Fiers et al., 2007), transcriptional regulation (ERF subfamily transcription factor, zinc finger family protein and a bHLH protein), protein metabolism and two genes involved in starch biosynthesis (*APL3* and *APL4*). Cluster II (Table III) consists of 27 genes, which are repressed only by high Cd exposure compared to the sufficient Zn exposure. These genes are involved in iron storage (*AtFER1*, *AtFER3*), transport (the membrane-trafficking protein *PATL1*, a sugar transporter and an anion exchange family protein), transcription (bHLH protein and zinc finger protein), lignin biosynthesis and protein metabolism. Cluster III consist of 13 genes, which are higher expressed under low Cd exposure compared to high Cd exposure (Supplemental Table I). These genes are involved in (a)biotic stress response, transcription, and transport. Cluster IV (Supplemental Table I) consists of 4 genes, which are higher expressed under low Cd exposure compared to sufficient

Table IV: Genes higher expressed under high cadmium exposure in *T. caerulescens*.
[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 = 2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; [d]Normalized spotintensity at 100 µM $ZnSO_4$. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
V		AT3G18290	zinc finger protein, putative	embryonic development	1.47	0.89	0.60	**0.34**	1.32	0.44	2381
		AT1G23200	pectinesterase family protein	cell organization and biogenesis	1.42	0.87	**0.61**	**0.40**	1.04	**0.41**	2145
	CYP71B22	AT3G26200	cytochrome p450 family	electron transport or energy pathways	2.04	1.61	0.79	**0.30**	1.38	**0.42**	1233
	CYP81D5	AT4G37320	cytochrome p450 family	electron transport or energy pathways	1.43	0.96	0.67	**0.41**	1.18	**0.48**	1206
	ATGSTU27	AT3G43800	glutathione S-transferase	toxin catabolic process	1.29	0.94	0.73	**0.36**	1.52	0.55	573
		AT1G03050	epsin N-terminal homology (ENTH) domain-containing protein	protein metabolism	1.11	1.09	0.99	**0.38**	0.94	**0.36**	453
		AT1G27740	basic helix-loop-helix (bHLH) family protein	transcription	0.77	0.81	1.05	0.49	0.62	**0.31**	406
		AT2G39490	F-box protein family	other biological processes	1.44	1.00	0.69	**0.44**	1.13	**0.50**	298
	XIF	AT2G31900	myosin family protein	transport	1.06	0.79	0.75	**0.33**	1.53	0.50	249
		AT3G10450	serine carboxypeptidase S10 family protein	protein metabolism	1.81	0.67	0.37	**0.19**	1.32	**0.24**	187
		AT3G25420	serine carboxypeptidase S10 family protein	protein metabolism	0.78	0.77	0.98	**0.26**	1.00	**0.26**	186
VI		AT3G16800	protein phosphatase 2C	other biological processes	1.73	1.62	0.93	0.91	**0.44**	**0.40**	9076
		AT2G43590	chitinase, putative	response to abiotic or biotic stimulus	1.32	0.77	0.58	0.84	0.46	**0.39**	8770
		AT5G42500	disease resistance response protein-related	defense response	1.48	1.14	0.77	0.87	**0.40**	**0.35**	7352
		AT5G28010	Bet v I allergen family protein	other biological processes	1.04	0.97	0.94	0.81	0.56	**0.45**	4373
		AT3G24500	ethylene-responsive transcriptional coactivator, putative	response to abiotic or biotic stimulus	1.39	1.01	0.73	0.89	**0.36**	**0.32**	4042
		AT3G24290	ammonium transporter, putative	transport	1.28	1.30	1.02	1.24	**0.48**	**0.59**	3330
		AT2G41660	expressed protein	other biological processes	1.59	1.33	0.83	0.87	**0.46**	**0.40**	1684
		AT2G37450	nodulin MtN21 family protein	other biological processes	1.32	1.38	1.04	0.75	0.60	**0.45**	1562
		AT1G26790	Dof-type zinc finger domain-containing protein	transcription	**2.00**	1.60	0.80	0.86	**0.45**	**0.38**	1496
	AHA9	AT1G80660	plasma membrane ATPase, putative	transport	2.20	1.08	0.49	0.97	0.18	**0.18**	1299
		AT3G12320	expressed protein	other biological processes	**1.92**	1.91	1.00	0.82	**0.46**	**0.38**	1096
		AT2G43580	chitinase, putative	response to abiotic or biotic stimulus	2.83	0.73	0.26	0.76	**0.19**	**0.15**	1081

Continue Table IV

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
	ZIP8	AT5G45105	metal transporter, putative	transport	1.19	1.06	0.89	0.65	0.49	**0.32**	991
		AT4G32190	centromeric protein-related	other biological processes	**1.85**	1.62	0.88	0.88	**0.43**	**0.38**	898
	EPR1	AT1G18330	myb family transcription factor	transcription	1.22	1.11	0.91	0.89	0.54	**0.48**	832
	RHA2A	AT1G15100	zinc finger (C3HC4-type RING finger) family protein	other biological processes	1.59	1.54	0.97	0.91	0.53	**0.48**	824
		AT3G47540	chitinase, putative	response to abiotic or biotic stimulus	1.19	0.73	0.61	0.83	0.50	**0.41**	822
		AT2G27180	expressed protein	other biological processes	**1.85**	1.35	0.73	0.88	**0.44**	**0.39**	516
		AT2G26975	copper transporter	transport	1.56	1.69	1.08	0.89	**0.49**	**0.44**	345
		AT1G29840	esterase/lipase/thioesterase family protein	other biological processes	1.06	0.93	0.88	0.87	0.36	**0.31**	225
		AT3G10585	myb family transcription factor	transcription	1.22	0.92	0.76	0.82	**0.30**	**0.24**	209
		AT4G16850	expressed protein	other biological processes	1.24	0.98	0.78	0.80	0.28	**0.22**	129
		AT3G17760	glutamate decarboxylase	carboxylic acid metabolic process	1.15	0.90	0.78	1.05	**0.32**	**0.34**	63
VII	ATPPC3	AT3G14940	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.94**	1.30	0.67	0.82	**0.53**	**0.43**	24481
	ATPPC1	AT1G53310	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.57**	1.06	0.68	0.84	**0.58**	**0.48**	21568
		AT3G54600	DJ-1 family protein	other biological processes	**1.81**	1.13	0.63	0.56	0.83	**0.47**	9632
		AT4G36610	hydrolase	aromatic compound metabolic process	1.23	0.90	0.73	0.66	0.68	**0.45**	6523
		AT2G31940	expressed protein	other biological processes	**1.62**	1.09	0.67	0.66	0.63	**0.41**	4432
	GLP9	AT4G14630	germin-like protein	other biological processes	2.17	1.39	0.64	0.45	0.57	**0.26**	4350
		AT2G18370	protease inhibitor/seed storage/lipid transfer family protein	transport	1.56	0.96	0.62	0.54	0.68	**0.37**	4247
		AT2G30660	3-hydroxyisobutyryl-coenzyme A hydrolase	metabolic process	1.59	0.97	0.61	0.72	0.59	**0.42**	3497
		AT1G23730	putative carbonic anhydrase	other metabolic processes	2.18	1.73	0.79	0.33	0.69	**0.23**	3213
	ATPPC2	AT2G42600	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.87**	1.41	0.75	0.64	0.73	**0.46**	3024
		AT3G44540	acyl CoA reductase - protein	developmental processes	1.09	0.73	0.66	**0.52**	0.88	**0.46**	2815
		AT5G57150	basic helix-loop-helix (bHLH) family protein	transcription	1.16	0.91	0.78	0.65	0.75	**0.49**	2813
		AT2G23960	defense-related protein	defense response	1.17	0.72	0.62	0.48	0.63	**0.30**	2205
		AT1G20823	zinc finger (C3HC4-type RING finger) family protein	other biological processes	**1.97**	1.52	0.77	0.70	0.70	**0.49**	1705
	ATPAP22	AT3G52820	purple acid phosphatase	other biological processes	1.40	0.79	0.56	0.69	0.64	**0.44**	1599

Continue Table IV

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT2G24430	NAM (no apical meristem)-like protein	multicellular organismal development	1.54	1.16	0.76	**0.56**	0.74	**0.42**	1279
		AT3G44550	acyl CoA reductase -like protein	developmental processes	1.17	0.89	0.76	0.57	0.66	**0.37**	1197
	MYB7	AT2G16720	member of the R2R3 factor gene family	response to abiotic or biotic stimulus	1.24	0.92	0.74	0.67	0.71	**0.47**	1094
		AT5G13330	member of the ERFsubfamily B-4 transcription factor family	transcription	2.07	1.17	0.57	0.55	0.61	**0.34**	1010
	SS3	AT1G74000	strictosidine synthase family	biosynthetic process	1.26	0.77	0.61	0.59	0.79	**0.47**	973
	HAP5B	AT1G56170	transcription factor, putative	transcription	1.13	0.67	0.59	**0.40**	0.68	**0.27**	962
	PGPS1	AT2G39290	phosphatidylglycerolphosphate synthase	phospholipid biosynthetic process	1.44	0.90	0.62	0.69	0.72	**0.49**	960
		AT5G56840	DNA-binding family protein	transcription	**1.73**	1.59	0.92	0.60	0.71	**0.43**	956
		AT4G26860	alanine racemase family protein	other biological processes	1.60	1.17	0.73	0.52	0.93	**0.49**	955
		AT1G35910	trehalose-6-phosphate phosphatase	trehalose biosynthetic process	1.64	0.94	0.57	0.64	0.54	**0.35**	892
		AT5G06720	peroxidase, putative	response to stress	2.64	2.44	0.93	0.43	0.69	**0.30**	775
		AT3G62190	DNAJ heat shock N-terminal domain-containing protein	protein metabolism	**1.70**	1.51	0.89	0.60	0.70	**0.42**	729
		AT2G29650	inorganic phosphate transporter	response to abiotic or biotic stimulus	**1.93**	**1.63**	0.84	0.68	**0.58**	**0.40**	507
		AT5G59490	haloacid dehalogenase-like hydrolase family protein	other metabolic processes	1.69	0.92	0.54	0.50	0.84	**0.42**	383
		AT1G34040	alliinase family protein	other biological processes	1.86	1.22	0.66	0.55	0.79	**0.44**	375
		AT5G15240	amino acid transporter family protein	transport	1.37	1.10	0.80	0.56	0.85	**0.47**	350
		AT3G45680	proton-dependent oligopeptide transport family protein	response to abiotic or biotic stimulus	1.26	0.51	0.41	0.48	0.62	**0.30**	350
	SOS3	AT5G24270	calcineurin B-like protein, putative	response to abiotic or biotic stimulus	1.15	1.07	0.93	0.48	0.63	**0.30**	348
		AT1G25460	oxidoreductase family protein	flavonoid biosynthetic process	1.51	1.12	0.74	0.54	0.79	**0.43**	337
	WNK6	AT3G18750	protein kinase family protein	protein metabolism	1.63	1.59	0.98	0.50	0.71	**0.36**	271
		AT5G63710	leucine-rich repeat transmembrane protein kinase	signal transduction	1.77	1.43	0.80	0.70	0.70	**0.49**	224
	IGPD	AT4G14910	imidazoleglycerol-phosphate dehydratase	histidine biosynthetic process	1.44	1.01	0.70	0.74	0.59	**0.44**	208
	ATSEH	AT2G26740	epoxide hydrolase	aromatic compound metabolic process	1.71	1.98	1.16	0.50	0.91	**0.45**	203
		AT3G25833	similar to myrcene/ocimene synthase, putative	other biological processes	2.13	1.05	0.49	0.40	0.68	**0.27**	196
		AT4G37970	mannitol dehydrogenase	other biological processes	1.88	1.53	0.81	0.54	0.87	**0.47**	153
		AT3G28450	leucine-rich repeat transmembrane protein kinase, putative	protein metabolism	1.39	1.05	0.76	0.56	0.66	**0.37**	151

Continue Table IV

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT3G17610	bZIP family transcription factor	other biological processes	1.89	1.70	0.90	0.57	0.65	**0.37**	126
		AT4G05470	F-box protein family	protein metabolism	1.16	0.92	0.79	0.56	0.71	**0.39**	124
		AT3G46680	UDP-glucoronosyl/UDP-glucosyl transferase family protein	metabolic process	2.20	1.42	0.64	0.43	0.64	**0.28**	67

Zn. These genes (*AtEXPA17*) are involved in cell wall loosening and metabolism. Clusters V, VI and VII (Table IV) consist of 11, 23 and 44 genes respectively, which are higher expressed under high Cd exposure compared to low Cd exposure and sometimes higher expressed under low Cd exposure compared to Zn sufficiency. Within these clusters several metal homeostasis related genes are found, such as genes encoding the transporters *AHA9* and *ZIP8*, but also a copper transporter and an oligopeptide transporter. Other genes in this cluster are involved in transcription (*HAP5B*, a bHLH protein and a myb family transcription factor), (a)biotic stress response and metabolism.

Cadmium- and zinc-regulated genes in *T. caerulescens*

In total 109 genes were significantly (FDR < 0.05) differentially expressed (≥ 2-fold) in response to both Cd and Zn. Of these genes, three were also found to be differentially expressed in Arabidopsis in response to Cd (*bHLH100*, *ZIP2* and At5g02780; Table II). After hierarchical clustering seven clusters were identified (Supplemental Table II). Cluster I (Supplemental Table II) consists of only two genes, which are higher expressed under Zn deficiency compared to sufficient and excess Zn and low Cd exposure. These genes encode a legume lectin family protein and a NWMU3 - 2S albumin 3 precursor. The seven genes in cluster II (Supplemental Table II) are higher expressed under Zn deficiency, Zn excess and high Cd exposure compared to Zn sufficiency and low Cd exposure. The genes in this cluster are involved in (a)biotic stress response (*P5CS1*), defence response (*ATTI2*) and senescence. Three genes in this cluster have no known function. In cluster III (Table V) 38 genes are co-expressed. These genes are higher expressed under Zn deficiency and high Cd exposure compared to sufficient and excess Zn exposure. Among these genes are *APS3*, *APK*, *AKN1*, *AKN2* and the low-affinity sulphate transport gene *SULTR2;1*, which are all involved in sulphate assimilation (Takahashi et al., 1997). Also the genes encoding *MYB28* and *CYP83A1*, which are involved in glucosinolate biosynthesis (Hirai et al., 2007) are identified in this cluster. Other genes co-expressed in this cluster are genes involved in (a)biotic stress response (*ATGSTF3*, *ATGSTU17, CYP79C2*, *CYP81D1*, *SAL1*, and peroxidases), transport (major intrinsic protein *NIP6;1*, carbohydrate transporter *ZIFL*, a phosphate transporter, a mitochondrial substrate carrier family protein

Table V: Genes higher expressed under zinc deficieny and high cadmium exposure in *T. caerulescens*

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 =2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; [d]Normalized spotintensity at 100 µM $ZnSO_4$. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
III		AT1G74100	sulfotransferase family protein	other biological processes	**3.05**	**2.08**	0.68	0.73	**2.01**	1.48	0.66	**0.48**	0.97	0.71	13733
	SUR1	AT2G20610	aminotransferase, putative	signal transduction	**3.25**	**2.05**	0.63	0.66	**2.29**	1.51	0.71	**0.46**	1.12	0.74	13163
		AT5G43180	expressed protein	other biological processes	**2.17**	**1.85**	0.85	0.80	1.10	0.88	**0.51**	**0.41**	**0.60**	**0.48**	12528
		AT1G18590	sulfotransferase family protein	other biological processes	**3.12**	**2.26**	0.73	0.69	**1.87**	1.30	0.60	**0.42**	0.83	0.57	9570
		AT4G33420	peroxidase, putative	response to oxidative stress	**2.61**	**2.15**	0.82	0.68	**1.80**	1.23	0.69	**0.47**	0.84	**0.57**	9299
		AT1G74090	sulfotransferase family protein	other biological processes	**3.72**	**2.30**	0.62	0.78	**1.91**	1.49	0.51	**0.40**	0.83	0.65	9052
		AT2G41480	peroxidase, putative	response to oxidative stress	**2.03**	**1.64**	0.81	0.60	**1.67**	1.01	0.82	**0.50**	1.02	0.61	7578
	CYP83A1	AT4G13770	cytochrome p450 family	glucosinolate catabolic process	**4.51**	**2.91**	0.65	0.54	**2.78**	1.51	0.62	**0.34**	0.95	**0.52**	6314
	ATGSTF3	AT2G02930	glutathione S-transferase, putative	toxin catabolic process	**3.69**	1.80	0.49	0.59	2.13	1.25	0.58	**0.34**	1.18	0.70	5705
		AT1G20160	subtilisin-like serine protease	protein metabolism	**3.89**	**2.05**	0.53	0.58	**2.16**	1.25	0.56	**0.32**	1.05	0.61	4143
		AT4G20070	Allantoate Amidohydrolase	protein metabolism	**2.18**	**1.95**	0.89	0.84	2.28	**1.91**	1.04	**0.38**	1.17	**0.39**	3168
		AT4G28940	nucleosidase-related	other biological processes	**2.60**	1.75	0.68	0.57	**2.33**	1.33	0.90	0.51	1.33	0.76	1983
	ATHB5	AT5G65310	homeobox-leucine zipper protein	transcription	**2.05**	**1.73**	0.84	0.72	1.27	0.91	**0.62**	**0.44**	0.73	**0.53**	1802
		AT1G22190	AP2 domain transcription factor RAP2, putative	transcription	**2.06**	1.38	0.67	0.72	1.14	0.83	0.55	**0.40**	0.83	0.60	1771
	AKN2	AT4G39940	adenylylsulfate kinase 2	sulfate assimilation	**3.74**	**2.36**	0.63	0.59	**2.63**	1.54	0.70	**0.41**	1.12	0.65	1757
	ZIFL	AT5G13750	transporter-like protein	transport	**4.14**	**2.44**	0.59	0.62	**2.49**	1.55	0.60	**0.37**	1.02	0.64	1693
		AT4G23920	UDPglucose 4-epimerase - like protein	galactose metabolic process	**2.17**	**2.12**	0.98	0.74	1.44	1.06	0.66	**0.49**	0.68	**0.50**	1691
	SAL1	AT5G63980	3(2),5-bisphosphate nucleotidase	response to abiotic or biotic stimulus	**3.04**	**2.32**	0.76	0.69	**1.97**	1.35	0.65	**0.44**	0.85	0.58	1469
	CYP79C2	AT1G58260	cytochrome p450 family	electron transport or energy pathways	**3.99**	2.38	0.60	0.48	**2.60**	1.25	0.65	**0.31**	1.09	0.53	1370
	APS3	AT4G14680	ATP-sulfurylase	sulfate assimilation	**4.30**	**3.58**	0.83	0.66	**2.69**	1.78	0.63	**0.41**	0.75	0.50	1191
	ATGSTU17	AT1G10370	glutathione transferase, putative	toxin catabolic process	**9.61**	**8.35**	0.87	0.62	**3.86**	2.39	0.40	**0.25**	0.46	**0.29**	1174
		AT4G11960	expressed protein	other biological processes	**2.08**	1.79	0.86	0.77	1.09	0.84	**0.52**	**0.40**	0.61	**0.47**	1060
		AT5G10770	chloroplast nucleoid DNA-binding protein	protein metabolism	**2.54**	2.22	0.87	0.52	**2.30**	1.21	0.91	0.47	1.04	0.54	953

Continue Table V

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
		AT2G30830	2-oxoglutarate-dependent dioxygenase	other biological processes	**2.61**	**2.28**	0.88	0.63	1.78	1.13	0.68	**0.43**	0.78	0.49	908
	SULTR2;1	AT5G10180	low-affinity sulfate transporter	sulfate transport	**3.34**	**2.12**	0.63	0.51	**2.04**	1.04	**0.61**	**0.31**	0.96	0.49	811
		AT5G05600	2OG-Fe(II) oxygenase family protein	other biological processes	**2.86**	1.71	0.60	0.71	1.51	1.08	0.53	**0.38**	0.89	0.63	696
		AT1G76430	phosphate transporter family protein	transport	**2.28**	**1.99**	0.87	0.96	1.16	1.11	0.51	**0.49**	0.58	0.56	679
		AT5G64700	nodulin MtN21 family protein	other biological processes	**3.25**	**2.62**	0.81	0.76	**2.00**	1.52	**0.61**	**0.47**	0.76	**0.58**	631
	AKN1	AT2G14750	adenylylsulfate kinase 1	sulfate assimilation	**3.09**	**2.28**	0.74	0.83	1.78	1.47	0.58	**0.48**	0.78	0.64	622
	NIP6;1	AT1G80760	major intrinsic family protein	transport	**2.03**	1.85	0.91	0.70	1.39	0.98	0.69	**0.48**	0.75	0.53	545
		AT1G52700	phospholipase/carboxylesterase family protein	other biological processes	**3.45**	**2.80**	0.81	0.58	**1.90**	1.10	0.55	**0.32**	0.68	**0.39**	489
		AT3G20015	aspartyl protease family protein	protein metabolism	**2.21**	1.64	0.74	0.52	**2.12**	1.09	0.96	0.49	1.30	0.67	435
		AT3G15650	phospholipase/carboxylesterase family protein	other biological processes	**2.58**	**2.51**	0.97	0.58	**2.03**	1.19	0.79	**0.46**	0.81	**0.47**	240
		AT1G68830	STN7 protein kinase	electron transport or energy pathways	**2.10**	1.72	0.82	0.61	1.49	0.92	0.71	**0.44**	0.87	0.53	237
	CYP81D1	AT5G36220	cytochrome p450 family	electron transport or energy pathways	**8.17**	3.00	0.37	0.82	**3.07**	2.50	0.38	0.31	1.02	0.84	233
		AT1G11080	serine carboxypeptidase S10 family protein	protein metabolism	**2.95**	1.77	0.60	0.54	**2.29**	1.23	0.78	**0.42**	1.29	0.69	232
	MYB28	AT5G61420	myb-related transcription factor	transcription	**2.17**	**2.53**	1.16	0.51	1.74	0.88	0.80	**0.41**	0.69	**0.35**	208
		AT2G17270	mitochondrial substrate carrier family protein	transport	**2.14**	**2.17**	1.01	0.67	1.47	0.98	0.69	**0.46**	0.68	**0.45**	163

(At2g17270)), transcription (*ATHB5*, involved in abscisic acid signalling) and metabolism. Cluster IV (Supplemental Table II) consists of 11 genes, which are higher expressed under Zn deficiency and low or high Cd exposure compared to sufficient and excess Zn. Two genes in this cluster encode the metal transporters *ZIP2* and *ZIP9*. Other genes in this cluster encode the high-affinity nitrate transporter *WR3,* a proton-dependent oligopeptide transporter and a wound-inducible protein. The 16 genes in cluster V (Supplemental Table II) are higher expressed under excess Zn and low Cd exposure compared to Zn sufficiency and Zn deficiency. Of these 16 genes, one is involved in transcription (*MYB111*), three genes have an unknown function and three genes are involved in abiotic stress response (*ATHSP22.0*, *CYP78A8*, *LEA14*). Cluster VI (Table VI) consists of 27 genes, which are higher expressed under Zn excess and high Cd exposure compared to Zn deficiency. The gene encoding *bHLH100* is also co-expressed in this cluster and the expression profile is similar to that in Arabidopsis with expression under excess Zn and high Cd exposure. Other genes in this cluster are involved in (a)biotic stress response, cell organization and signal transduction. The genes in cluster VII (Supplemental Table II) are higher expressed under excess Zn and low Cd exposure. Two of these six genes are involved in protein metabolism and two genes have an unknown function.

Difference in cadmium and zinc response between Arabidopsis and *T. caerulescens*

To identify genes that may be crucial for the adaptive differences between Arabidopsis and *T. caerulescens*, we compared the gene expression profiles between the two species for each of the tested physiological conditions. To avoid the large group of genes that are constitutively differentially expressed between both species (van de Mortel et al., 2006), we only focussed on genes that show a different expression profile between the two species. In addition, we only considered probes to be of biological relevance if they differed significantly (FDR <0.05) with a more than 3-fold in expression level between *T. caerulescens* and Arabidopsis. According to these criteria 186 Zn-regulated genes were found to be significantly differentially expressed between both species (data not shown) and in total 42 Cd-regulated genes (Table VII). Of the latter, 34 genes were differentially expressed between different Zn/Cd exposures in *T. caerulescens* but constitutively expressed in Arabidopsis. Ten of these genes are involved in

transcription. Other classes represent genes encoding for proteins involved in (a)biotic stress response and metabolism. Of the ZIP metal transporter genes, only *ZIP1* is higher expressed under high Cd exposure compared to sufficient Zn condition while this was not observed in Arabidopsis. Six of the Cd-regulated genes were found to be differentially expressed between treatments in Arabidopsis but constitutively in *T. caerulescens*. Of these six genes, two are involved in protein metabolism.

Next to differences in Cd-regulated genes in both species there is also a group of genes of which the response to both Cd and Zn exposures is different in *T. caerulescens* compared to Arabidopsis. This group consists of 33 genes (Table VIII), of which 17 are differentially expressed in *T. caerulescens* and 14 are differentially expressed in Arabidopsis. One of these genes is the zinc transporter gene *ZIP2*, which is higher expressed under Zn sufficiency compared to Zn excess and Cd excess conditions in Arabidopsis while this profile was not found in *T. caerulescens*. Also the ABC transporter *ATATH13* is regulated in a different way in the two species. This gene is higher expressed under excess Cd in *T. caerulescens* while in Arabidopsis this gene is not differentially expressed. Other genes differentially regulated between *T. caerulescens* and Arabidopsis are the transcription factors *bHLH100, CCA1*, *LHY1* and *WRKY59*; (a)biotic stress responsive genes encoding the cytochrome P450 genes *CYP83A1*, *CYP712A1*, *CYP76G1*, the trypsin inhibitor *ATTI2*, and the glutathione transferases *ATGSTF11* and *ERD9*; and genes involved in metabolism. *CYP78A8* and *FRO4* respond both in Arabidopsis and in *T. caerulescens* to Zn and Cd exposure, but in a different way. *CYP78A8* is higher expressed under Zn sufficient and Zn excess conditions in *T. caerulescens* while this gene is only induced under Zn deficient conditions in Arabidopsis and *FRO4* is higher expressed under low and high Cd in *T. caerulescens* whereas this gene is higher expressed under Zn sufficient conditions in Arabidopsis.

Semi-quantitative RT-PCR

For confirmation of the microarray expression profiling data, the expression of a small set of differentially expressed genes was determined by semi-quantitative RT-PCR. In the absence of *T. caerulescens* DNA sequences for designing species-specific PCR primers, orthologous *T. caerulescens* gene fragments were first amplified by low stringency PCR using Arabidopsis-

specific primers and their identity was confirmed by DNA sequencing. This sequence was used to design species-specific primers annealing to comparable positions in the *T. caerulescens* and Arabidopsis cDNA sequences, preferably flanking a predicted intron, for semi-quantitative RT-PCR. Expression of the target genes was studied in both root and leaf tissues of plants grown hydroponically at different Zn and Cd supply conditions (Figure 1A+B). The root expression levels determined by semi-quantitative RT-PCR were comparable to those determined by microarray analysis, confirming the significance of the heterologous microarray hybridization results.

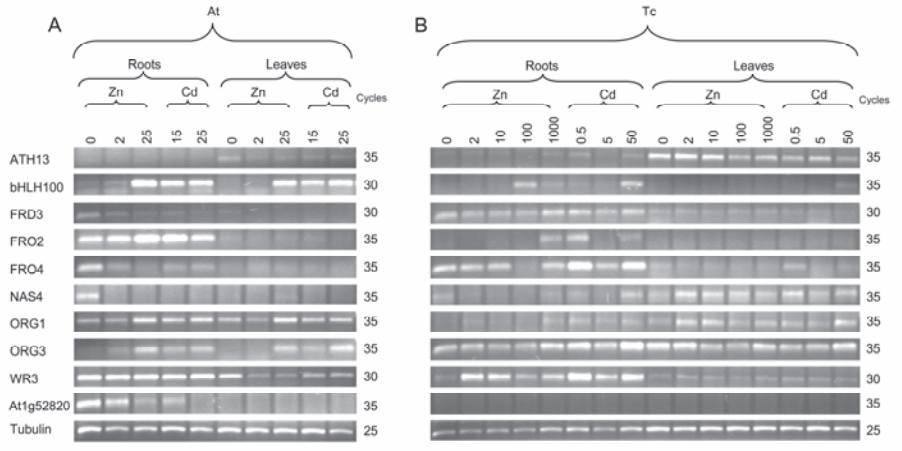

Figure 1. Comparative semi-quantitative RT-PCR of differentially expressed genes in Arabidopsis (A) and *T. caerulescens* (B) in response to different zinc and cadmium exposures. For amplification, species-specific primers were designed at comparable locations in each orthologous gene pair. Roots and leaves were harvested separately after one week of exposure of three-week-old plants to 0 µM $ZnSO_4$, 2 µM $ZnSO_4$, 25 µM $ZnSO_4$, 15 µM CdSO4 and 25 µM $CdSO_4$ for Arabidopsis and 0µM $ZnSO_4$, 2 µM $ZnSO_4$, 10 µM $ZnSO_4$, 100 µM $ZnSO_4$, 1000 µM $ZnSO_4$, 0.5 µM $CdSO_4$, 5 µM $CdSO_4$ and 50 µM $CdSO_4$ for *T. caerulescens*. *ATH13*, ABC transporter, At5g64940; *bHLH100*, basic helix-loop-helix transcription factor, At2g41240; *FRD3*, MATE efflux family protein, At3g08040; *FRO2*, ferric chelate reductase-like, At1g01580; *FRO4*, ferric chelate reductase-like, At5g23980; *NAS4*, nicotianamine synthase, At1g56430; *ORG1*, protein kinase family protein, At5g53450; *ORG3*, basic helix-loop-helix transcription factor, At3g56980; *WR3*, wound-responsive gene 3, At5g50200; 2-oxoglutarate-dependent dioxygenase, At1g52820; Tubulin (At1g04820) was used as a control for equal cDNA use.

When considering the expression in leaves, there were some striking differences between Arabidopsis and *T. caerulescens* that were not observed in roots. *FRD3* and *NAS4* are more or

less constitutively expressed in *T. caerulescens* leaves while *NAS4* is not expressed in Arabidopsis leaves and expression of *FRD3* is limited to Zn deficiency. Also *ORG3* is constitutively expressed in *T. caerulescens* while this gene is induced under excess Zn and Cd conditions in Arabidopsis leaves. *ORG1* is induced under excess Zn and Cd conditions in both roots and leaves in Arabidopsis but in *T. caerulescens* this gene is constitutively expressed in the leaves and induced to higher levels under excess Zn and Cd in the roots. The ferric-chelate reductase gene *FRO4* is not expressed in leaves of Arabidopsis, while in *T. caerulescens* this gene is induced under low and high Cd exposure in both tissues. Remarkable is also that the nitrate transporter gene *WR3*, which is more or less constitutively expressed in Arabidopsis roots and Zn deficient leaves, is hardly expressed in *T. caerulescens* leaves and repressed in Zn deficient roots. Finally, the transcription factor gene *bHLH100* is differentially expressed when comparing the two species. In Arabidopsis roots and leaves this gene is highly expressed under stress-inducing Zn excess and Cd exposure conditions, while the expression in *T. caerulescens* is lower and only detectable under high Cd exposure in both roots and leaves and under sufficient (100 μM $ZnSO_4$) but not high Zn in roots.

Transgenic Arabidopsis lines overexpressing *bHLH100* exhibit increased tolerance to zinc and nickel

The expression of *bHLH100* suggests a function related to stress tolerance in Arabidopsis and if this is true, lower expression in *T. caerulescens* at high Zn and Cd could mean this species experiences less stress at these conditions, in line with the relatively little adverse effects on *T. caerulescens* plant phenotype at longer exposures to high Zn and Cd. To establish if increased expression indeed enhances the stress tolerance, transgenic *A. thaliana* plants were generated overexpressing *AtbHLH100* under the control of the constitutive Cauliflower Mosaic Virus 35S promoter. Overexpression plants did not show any obvious morphological alterations when they were grown in soil (data not shown). However, when seedlings of homozygous lines were grown for five days on vertical plates containing different heavy metals, roots of *bHLH100* overexpression plants were longer compared to wild-type roots especially when exposed to

excess zinc and excess nickel (Figure 2), suggesting that there is indeed a positive effect on stress tolerance under these conditions.

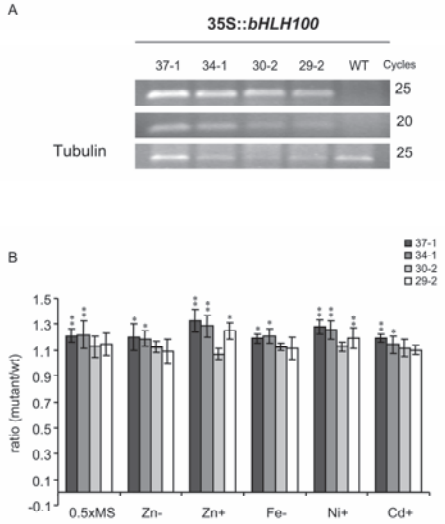

Figure 2. (A) Semi-quantitative RT-PCR analysis of *bHLH100* expression in seedlings of homozygous transgenic *A. thaliana* lines transformed with a CaMV 35S::*bHLH100* construct. *Tubulin* amplification was used as control of the total RNA levels. RT-PCR was performed with 25 and 20 cycles. (B)Root length ratio (mean ± SE) of 5 days old 35S::*bHLH100* seedlings grown on 0.5 x MS medium supplement with different Zn, Fe, Ni and Cd levels. Zn- = 0 µM $ZnSO_4$; Zn+ = 400 µM $ZnSO_4$; Fe- = 0.15 µM FeEDTA; Ni+ = 75 µM $NiSO_4$; Cd+ = 30 µM $CdSO_4$; * significantly different from WT at $P<0.05$; ** significantly different from WT at $P<0.01$. Significance is determined by one-way ANOVA. Five replicates with 15 to 20 seedlings were measured for each treatment. Numbers indicate plant lines from two independent transformants.

The *myb72* loss of function mutant exhibits increased sensitivity to zinc and iron

MYB72 is another gene that was especially induced by excess Zn and Cd in Arabidopsis. Expression in *T. caerulescens* could not be established by microarray, nor by semi-quantitative RT-PCR (data not shown). To further examine the function of this transcription factor the expression was first confirmed by semi-quantitative RT-PCR in Arabidopsis roots and shoots upon exposure to different Zn and Cd concentrations (Figure 3A). This confirmed the expression in roots and showed that *MYB72* is not expressed in Arabidopsis leaves. Two different *myb72* T-DNA insertion lines, which were kindly obtained from Corné Pieterse and Sjoerd van Ent (Utrecht University), were examined for any effect on metal tolerance when grown on vertical

plates containing different heavy metals (Figure 3B). Although in general the *myb72* mutants had shorter roots than the wild-type plants, this reduction in growth was especially prominent under excess Zn and Fe deficiency conditions, suggesting that this gene is involved in iron acquisition rather than general metal stress tolerance. When grown in soil, the *myb72* mutants showed no visibly different morphological phenotype compared to wild type (data not shown).

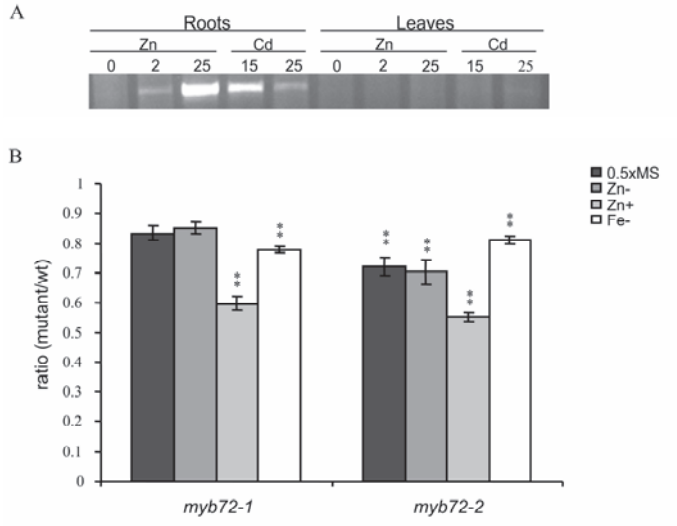

Figure 3. (A) Semi-quantitative RT-PCR of *MYB72* in Arabidopsis. Roots and leaves were harvested separately after one week of exposure of three-week-old plants to 0 µM $ZnSO_4$, 2 µM $ZnSO_4$, 25 µM $ZnSO_4$, 15 µM CdSO4 and 25 µM $CdSO_4$. (B) Root length ratio (mean ± SE) of 5 days old *myb72* seedlings grown on 0.5 x MS medium supplement with different Zn, and Fe levels. Zn- = 0 µM $ZnSO_4$; Zn+ = 400 µM $ZnSO_4$; Fe- = 0.15 µM FeEDTA; * significantly different from WT at $P<0.05$; ** significantly different from WT at $P<0.01$. Significance is determined by one-way ANOVA. Six replicates with 15 to 20 seedlings were measured for each treatment.

DISCUSSION

Recently we described the gene expression profiles in roots of Arabidopsis and *T. caerulescens* in response to different Zn exposures, in order to identify genes involved in Zn tolerance and/or accumulation (van de Mortel et al., 2006). In the present study we specifically investigated the response of Arabidopsis and *T. caerulescens* to Cd exposure. We anticipated

identifying genes that are primarily involved in Cd tolerance. As Cd resembles Zn in chemical interactions, we also expected that genes normally involved in Zn homeostasis would transcriptionally respond to Cd exposure. When comparing gene expression in both species, we assumed that some of the genes differentially expressed between the two species, and especially those that show a difference in response to changes in the external Zn and/or Cd concentration, would be crucial to the adaptive difference between a hyperaccumulator and a non-accumulator.

The 21,500 probes on the Agilent Arabidopsis2 microarray that we used in this study are also present on the Agilent Arabidopsis3 oligonucleotide microarray that we used previously (van de Mortel et al., 2006). Of the 561 genes, which were found to be significantly differentially expressed in Arabidopsis in response to different Zn exposures in the present experiment, 170 genes were also previously found to be significantly differentially expressed in response to Zn (van de Mortel et al., 2006). For *T. caerulescens* this is 93 of the 409 genes. When comparing Arabidopsis with *T. caerulescens,* 118 of the 186 genes we found to be differentially expressed in response to Zn in the present study, were also found previously. So, although there is not a full overlap between both experiments, simply due to unavoidable differences in plant growth conditions, tissue sampling and probe hybridization, the two experiments compare well.

Weber et al. (2006) recently published the transcriptome analysis of *A. thaliana* and the hyperaccumulator *A. halleri* in response to a short two-hour Cd exposure. Instead, we compared the root transcriptome of Arabidopsis and *T. caerulescens* plants exposed for one week to Cd, which avoids detection of the acute stress response that is expected after short Cd exposures and which allows adaptation of the plants to the new conditions. Knowing that *T. caerulescens* survives a long term Cd exposure, whereas Arabidopsis does not, we assumed that a long-term exposure to low concentrations of Cd is more realistic to identify genes involved in Cd tolerance and accumulation than a short exposure to much higher concentrations. Weber et al. (2006) found many genes involved in stress-response, like heat shock proteins and ethylene-responsive element-binding proteins, to be higher expressed in *A. thaliana* after a two-hour Cd exposure. These genes were not found to be differentially expressed after one week of exposure to 15 µM Cd.

The results of our analysis prompt us to conclude that: (1) Cd has little effect on the metal homeostasis mechanism in the plant; (2) Cd appears to induce Fe deficiency in Arabidopsis; (3) Cd has an effect on lignin biosynthesis in both species (4) Cd has an effect on sulfate assimilation in *T. caerulescens*; (5). A number of genes that are induced by Cd exposure in Arabidopsis are constitutively highly expressed in *T. caerulescens*. These conclusions will be discussed in more detail below.

Cd effects on metal homeostasis related genes in *T. caerulescens* and Arabidopsis

Unlike the Zn response, analysis of the Cd response indicated relatively few genes that were differentially expressed in Arabidopsis and *T. caerulescens* and there was no clear process that is most affected by the Cd exposure. Since the LC accession of *T. caerulescens*, which we used, is not accumulating Cd, our analyses again underline previous observations that Zn and Cd uptake are mediated at least in part by different molecular mechanisms (Zhao et al., 2002; Deniau et al., 2006). Cd exposure also has much less effect on Zn and Fe homeostasis genes than was previously observed upon alterations in Zn exposure. In part this can be explained by the fact that Cd is non-essential and that the molecular machinery to prevent deficiency is lacking. A complicating factor for Zn and Fe homeostasis is that high affinity Zn transporters have often low affinity for Fe and the other way around, which necessitates the activity of a whole set of Zn/Fe metal transporters to compensate for the inadvertent uptake of one element and deficiency of the other. Even though Cd probably enters the plant cell through Zn/Fe transporters, the affinity is generally low, which causes only little competition of Cd uptake with Zn/Fe uptake. In addition, Herbette et al. (2006) found that gene expression in response to Cd was more time-regulated than dose-regulated in Arabidopsis, which could explain why fewer genes were differentially expressed in response to Cd.

Still, among the genes differentially expressed in Arabidopsis in response to Cd exposure are some genes known to be involved in iron homeostasis, such as the *FRO2* ferric-chelate reductase gene, a citrate efflux protein *FRD3* (Durrett et al., 2007), and a nicotianamine synthase, *NAS4* (Table II). The ferric chelate-reductase *FRO2* is higher expressed both under excess Zn and excess Cd conditions, suggesting that the plants are experiencing Fe deficiency (Colangelo and

Table VI: Genes higher expressed under zinc excess and high cadmium exposure in *T. caerulescens*.
[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 =2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; [d]Normalized spotintensity at 100 µM $ZnSO_4$. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
VI		AT4G23230	protein kinase family protein	protein metabolism	0.53	**0.35**	0.67	0.69	0.64	**0.44**	1.21	0.83	1.79	1.24	41597
		AT4G17010	expressed protein	other biological processes	0.55	**0.36**	0.66	0.65	0.70	**0.45**	1.28	0.83	**1.93**	1.25	33680
		AT1G48330	expressed protein	other biological processes	0.53	**0.35**	0.67	0.70	0.64	**0.45**	1.22	0.86	1.81	1.27	32183
		AT1G05900	endonuclease-related	response to stress	0.50	**0.33**	0.67	0.66	0.62	**0.41**	1.25	0.83	1.87	1.23	28638
	APRR5	AT5G24470	pseudo-response regulator 5	developmental processes	**0.49**	**0.33**	0.68	0.67	0.67	**0.45**	1.36	0.92	**2.00**	1.34	27140
		AT1G34340	esterase/lipase/thioesterase family protein	other biological processes	**0.52**	**0.34**	0.66	0.66	0.64	**0.42**	1.23	0.81	**1.87**	1.23	26699
		AT4G34730	ribosome-binding factor A family protein	cell organization and biogenesis	**0.50**	**0.31**	0.62	0.65	0.61	**0.40**	1.22	0.79	**1.96**	1.27	21865
		AT4G25370	Clp amino terminal domain-containing protein	protein metabolism	**0.52**	**0.33**	0.64	0.62	0.66	**0.41**	1.29	0.79	**2.01**	1.24	21167
		AT2G16490	XH domain-containing protein	other biological processes	0.50	**0.31**	0.61	0.63	0.61	**0.39**	1.22	0.77	2.00	1.26	20672
		AT5G28894	copia-like retrotransposon family	other biological processes	**0.49**	**0.32**	0.65	0.68	0.58	**0.39**	1.19	0.81	1.83	1.24	19524
		AT3G54880	expressed protein	other biological processes	**0.54**	**0.42**	0.77	0.90	**0.53**	**0.48**	0.98	0.89	1.28	1.15	15676
		AT1G74200	leucine-rich repeat family protein	signal transduction	0.53	**0.33**	0.63	0.65	0.60	**0.39**	1.14	0.73	1.81	1.17	15492
	CLE2	AT4G18510	Clavata3 / ESR-Related-2	signal transduction	0.55	**0.36**	0.66	0.64	0.64	**0.41**	1.18	0.75	1.79	1.14	15028
	ATINT3	AT2G35740	sugar transporter family protein	transport	0.54	**0.36**	0.66	0.63	0.66	**0.42**	1.24	0.78	1.86	1.17	15005
		AT1G32580	plastid developmental protein DAG	other biological processes	0.53	**0.35**	0.66	0.68	0.62	**0.42**	1.17	0.80	1.78	1.22	13762
	CAM9	AT3G51920	calmodulin-9	signal transduction	0.60	**0.47**	0.79	0.76	0.59	**0.45**	0.99	0.75	1.26	0.96	13079
		AT5G55060	expressed protein	other biological processes	0.59	**0.35**	0.60	0.63	0.65	**0.41**	1.11	0.70	1.86	1.18	13077
		AT1G48650	helicase domain-containing protein	other biological processes	0.56	**0.35**	0.62	0.68	0.65	**0.44**	1.17	0.79	1.88	1.27	12580
		AT3G04560	expressed protein	other biological processes	0.58	**0.38**	0.65	0.67	0.68	**0.46**	1.19	0.80	1.82	1.22	12502
		AT4G23430	short-chain dehydrogenase/reductase fam. prot.	metabolic process	0.67	**0.44**	0.65	0.68	0.69	**0.47**	1.03	0.70	1.58	1.08	10511

Continue Table VI

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0-Cd0.5[c]	Zn0-Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
		AT4G02170	expressed protein	other biological processes	0.53	**0.35**	0.65	0.62	0.67	**0.41**	1.25	0.77	1.92	1.18	9417
		AT1G24320	alpha-glucosidase	oligosaccharide metabolic process	**0.61**	**0.28**	**0.46**	**0.59**	**0.41**	**0.24**	0.67	**0.40**	**1.46**	0.86	7182
	bHLH100	AT2G41240	basic helix-loop-helix (bHLH) family protein	transcription	0.69	**0.20**	0.29	0.24	0.75	**0.18**	1.08	**0.26**	**3.78**	0.90	2251
		AT5G13910	member of the ERFB-1 transcription factor family	transcription	0.61	**0.44**	0.73	0.70	**0.50**	**0.36**	0.83	**0.58**	1.14	0.80	2126
	ARPN	AT2G02850	plastocyanin-like domain-containing protein	developmental processes	**0.45**	**0.28**	0.61	0.39	0.64	**0.25**	1.42	0.55	**2.33**	0.91	895
		AT3G29190	terpene synthase/cyclase family	metabolic process	**0.33**	**0.18**	0.54	0.80	**0.29**	**0.23**	0.86	0.69	1.59	1.27	859
		AT2G39410	hydrolase, alpha/beta fold family protein	aromatic compound metabolic process	**0.56**	**0.43**	0.77	0.91	**0.45**	**0.41**	0.80	0.73	1.04	0.95	464

Guerinot, 2004; Mukherjee et al., 2005; Wu et al., 2005; Sarry et al., 2006) although another Fe deficiency marker, the *IRT1* Fe uptake transporter, is not induced by Cd. In line with the latter is that the *FRD3* expression is negatively affected by Cd exposure. Although this can be caused by a general downregulation of metabolic processes by Cd exposure (Herbette et al., 2006), *FRD3* was recently found to be involved in effluxing the metal chelator citrate into the root vasculature, enhancing root to shoot metal transport (Durrett et al., 2007). This suggests that plants are able to recognize Cd-uptake through *IRT1* (Rogers et al., 2000) and Cd root-to-shoot movement through *FRD3* and respond accordingly. The downregulation of the Zn-uptake transporter *ZIP2* (Grotz et al., 1998), as we found, can be because of the same reason. Like citrate, nicotianamine (NA) is a chelator of metals. NA is made by nicotianamine synthases (NAS) for which Arabidopsis contains four genes. Although we can not exclude that NA also chelates cadmium, the predominant expression of *NAS4* under Zn deficiency in roots suggest that the induced expression under Cd exposure is related to a modest response to Zn or Fe deficiency at high Cd exposure.

For *T. caerulescens* the Cd response is slightly different from Arabidopsis, with more genes differentially expressed in *T. caerulescens*. Of the 280 genes that were differentially expressed in response to Cd (Supplemental tables I and II), 109 genes also responded to the Zn exposures (Supplemental table II). Only three of these genes were also significantly differentially expressed in response to Cd in Arabidopsis. The little overlap in expression between the two species is in line with the differences in Cd tolerance of both species. Like in Arabidopsis, also in *T. caerulescens* we identified metal homeostasis genes among the differentially expressed genes responding to Cd but the expression profile of metal homeostasis related genes in the Zn/Cd hyperaccumulator *T. caerulescens* was less affected by Cd exposure than by alterations in Zn exposure. *T. caerulescens* acc. La Calamine is known to be more tolerant to Cd but contrary to the Cd hyperaccumulator accession GA, it is a poor Cd accumulator (Assunção et al., 2003b). Cd tolerance in higher plants is mainly studied in non-tolerant species and there is not much information on the mechanisms of metal tolerance in hypertolerant species (Schat et al., 2002). However, Cd tolerance is likely to be caused by changes in uptake, detoxification, bioavailability, cell wall properties and general stress tolerance.

Table VII: Genes differentially expressed between *T. caerulescens* and Arabidopsis in response to cadmium.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 3) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 = 2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; Zn2 = 2 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn25 = 25 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd15 = 2 µM $ZnSO_4$, 15 µM $CdSO_4$. [d]Normalized spotintensity at 100 µM or 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. Significant values are presented in grey (FDR < 0.05). The interaction of the gene expression profiles between the two species is presented in **bold**.

				T. caerulescens					Arabidopsis					
Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity Zn100[d]	Intensity Zn2[d]
RCI2B	AT3G05890	low temperature and salt responsive protein	response to cold	0.48	0.51	1.05	1.84	**2.85**	1.33	1.77	1.33	**0.87**	29594	36614
FLA9	AT1G03870	fasciclin-like arabinogalactan-protein	cell adhesion	0.53	0.91	1.71	1.36	**2.62**	1.54	1.66	1.08	**0.79**	24419	23381
	AT2G47550	pectinesterase family	cell wall modification	0.40	0.58	1.46	1.42	**2.24**	0.75	0.76	1.01	**0.69**	16152	3858
	AT4G34950	nodulin family protein	biological process unknown	1.12	1.14	1.02	**4.18**	**3.26**	0.61	0.15	0.25	**0.55**	11571	1876
	AT1G72360	putative AP2 domain transcription factor	regulation of transcription	0.66	0.82	1.24	2.29	**3.00**	0.77	0.55	0.71	**0.86**	10526	24078
	AT3G16800	protein phosphatase 2C (PP2C)	biological process unknown	1.73	1.62	0.93	**0.44**	**0.40**	1.02	0.83	0.82	**1.48**	9076	5693
FMO1	AT1G19250	flavin-dependent monooxygenase	defense response	0.32	0.29	0.92	**1.89**	1.55	0.66	0.45	0.68	**0.57**	6196	1348
	AT2G31120	unknown protein	biological process unknown	0.59	1.35	2.29	**2.51**	**2.62**	1.71	1.60	0.94	**0.79**	4930	1213
	AT3G24500	multiprotein bridging factor 1	regulation of transcription	1.39	1.01	0.73	**0.36**	**0.32**	0.50	0.56	1.12	**1.60**	4042	8463
	AT3G49630	oxidoreductase (din11), putative	cellular response to starvation	0.45	0.78	1.71	1.24	**3.19**	0.97	0.87	0.90	**0.93**	2542	545
	AT4G17215	Expressed protein	biological process unknown	0.42	0.49	1.14	1.49	**2.09**	0.80	0.73	0.90	**0.64**	2290	2313
	AT4G21390	serine/threonine kinase - like protein	protein amino acid phosphorylation	1.01	0.94	0.93	2.69	**3.81**	1.16	1.77	1.52	**1.13**	1886	800
	AT1G22190	AP2 domain transcription factor RAP2	regulation of transcription	2.06	1.38	0.67	0.55	**0.40**	1.28	1.08	0.85	**1.36**	1771	2444
ZIP1	AT3G12750	putative zinc transporter	zinc ion transport	6.20	8.54	1.38	0.53	**0.26**	8.42	20.62	2.45	**0.87**	1565	549
	AT3G12320	expressed protein	biological process unknown	1.92	1.91	1.00	**0.46**	**0.38**	1.07	1.02	0.96	**1.59**	1096	2434
RHA3B	AT4G35480	RING-H2 finger protein RHA3b	regulation of transcription	0.99	1.72	1.74	**3.62**	**4.05**	1.00	1.05	1.05	**0.87**	1032	394
HAP5B	AT1G56170	transcription factor, putative	regulation of transcription	1.13	0.67	0.59	0.68	**0.27**	0.84	0.86	1.02	**1.25**	962	425
	AT4G32190	putative protein	biological process unknown	1.85	1.62	0.88	0.43	**0.38**	1.08	1.39	1.29	**1.17**	898	244
EPR1	AT1G18330	myb family transcription factor	regulation of transcription	1.22	1.11	0.91	0.54	**0.48**	0.91	1.13	1.24	**1.49**	832	1363
	AT1G68740	putative receptor protein	biological process unknown	1.73	2.15	1.24	0.67	**0.44**	1.64	2.64	1.61	**1.31**	698	476

Continue Table VII

				T. caerulescens					Arabidopsis					
Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity Zn100[d]	Intensity Zn2[d]
	AT1G77700	thaumatin-like protein	response to other organism	1.31	1.35	1.03	**0.29**	1.05	0.84	0.57	0.68	**1.21**	601	533
	AT5G59080	putative protein	biological process unknown	0.49	0.68	1.38	1.76	**3.87**	1.58	0.87	0.55	**0.84**	554	367
	AT2G43050	pectinesterase family	cell wall modification	0.67	1.16	1.74	1.61	**2.55**	1.06	1.27	1.21	**0.68**	493	471
	AT1G03050	putative protein destination factor	N-terminal protein myristoylation	1.11	1.09	0.99	0.94	**0.36**	1.24	1.39	1.12	**1.26**	453	222
	AT1G27740	bHLH protein	regulation of transcription	0.77	0.81	1.05	0.62	**0.31**	1.48	1.80	1.21	**1.20**	406	478
SOS3	AT5G24270	calcium sensor homolog	potassium ion homeostasis	1.15	1.07	0.93	0.63	**0.30**	0.90	1.14	1.27	**1.03**	348	2333
	AT2G36870	xyloglucan endotransglycosylase, putative	carbohydrate metabolism	3.29	3.22	0.98	0.59	**0.38**	1.13	1.32	1.17	**1.35**	335	73
	AT1G29840	hypothetical protein	biological process unknown	1.06	0.93	0.88	0.36	**0.31**	0.68	0.90	1.32	**1.54**	225	562
MSBP1	AT5G52240	progesterone-binding protein-like	electron transport	1.45	1.31	0.91	**0.23**	1.27	0.82	0.92	1.12	**1.08**	173	11869
	AT1G26280	hypothetical protein	biological process unknown	1.52	1.42	0.93	0.35	**0.27**	0.84	1.01	1.20	**1.01**	152	2183
SIGE	AT5G24120	sigma-like factor	regulation of transcription	1.51	2.10	1.39	0.53	**0.20**	1.18	1.51	1.29	**1.32**	137	148
	AT3G17610	bZIP family transcription factor	regulation of transcription	1.89	1.70	0.90	0.65	**0.37**	0.82	0.84	1.02	**1.96**	126	263
	AT2G40620	bZip DNA binding protein	regulation of transcription	1.31	1.25	0.95	**0.18**	0.83	0.74	0.63	0.86	**2.34**	83	545
	AT3G17760	glutamate decarboxylase	carboxylic acid metabolism	1.15	0.90	0.78	**0.32**	**0.34**	0.98	0.89	0.91	**1.48**	63	34
	AT2G23030	putative protein kinase	protein amino acid phosphorylation	0.59	0.52	0.89	**1.48**	0.97	1.37	1.24	0.90	**0.45**	6124	5480
LAS1	AT3G45130	cycloartenol synthase	pentacyclic triterpenoid biosynthesis	0.80	1.13	1.40	**1.00**	**1.16**	0.73	2.63	3.61	**3.49**	2589	1172
	AT4G16563	aspartyl protease family protein	proteolysis	0.87	0.99	1.14	**1.60**	0.97	1.40	0.72	0.52	**0.26**	66	1168
	AT5G43935	1-aminocyclopropane-1-carboxylic acid oxidase-like protein	biological process unknown	1.26	1.02	0.81	**0.78**	**0.61**	0.50	1.05	2.09	**3.22**	3405	102
RSU1	AT3G48130	ribosomal protein L13 homolog	biological process unknown	0.88	0.96	1.09	**1.12**	**1.14**	4.06	4.00	0.99	**0.16**	59	55
	AT3G49580	putative protein	biological process unknown	1.24	1.14	0.92	**1.51**	**1.10**	1.55	1.05	0.68	**0.20**	52	37
	AT3G50980	dehydrin-like protein	response to stress	0.86	0.78	0.91	**0.53**	1.02	0.99	1.37	1.39	**1.79**	3008	10528
	AT5G59110	subtilisin-like serine protease-related	biological process unknown	1.09	1.30	1.19	**1.84**	1.44	1.16	0.63	0.54	**0.47**	58	38

Although the role of metal transporters may be limited in controlling Cd tolerance, two members of the *ZIP* gene family were differentially expressed in *T. caerulescens* in response to Cd. *ZIP8* is induced under excess Cd in *T. caerulescens* (Supplemental Table I) whereas this gene is only induced under excess Zn conditions in Arabidopsis. *ZIP8* was found before to be differentially expressed in Arabidopsis after exposure to excess Zn (van de Mortel et al., 2006) and as Zn excess induced more Fe acquisition genes due to Zn-Fe uptake competition, it was suggested that this gene is involved in iron homeostasis. However, *ZIP8* may also be involved in the transport of Mn or another transition metal that is typically transported by ZIPs and for which uptake may suffer from Zn/Cd competition. *ZIP1* is another ZIP family relative (Table VII) that is differentially expressed between Arabidopsis and *T. caerulescens*. *ZIP1* is induced by high Cd exposure in *T. caerulescens* whereas this gene is not responding to Cd in Arabidopsis. As *ZIP1* is also one of the major Zn uptake transporters (Grotz et al., 1998), this suggests that *T. caerulescens* may be more sensitive to competition of Zn uptake by Cd, especially under relatively low Zn supply of 2 μM Zn, or alternatively that the Zn deficiency signaling is disturbed due to Cd exposure, as was also described for Arabidopsis (Weber et al., 2006) and *Schizosaccharomyces pombe* (Chen et al., 2003).

Cd effects on lignin biosynthesis in Arabidopsis

The root cell wall is the structure, which is directly exposed to Cd. Genes involved in lignin biosynthesis such as a 4-coumarate: CoA ligase 2 (*4CL2*), peroxidases (At2g35380 (*PER20*), At2g18150) and cytochrome P450 (*CYP71A20*, *CYP76G1*, *CYP91A2*) and also the *EXPR3* gene (At2g18660) involved in cell expansion, are higher expressed under excess Cd in Arabidopsis roots (Tables I and II) suggesting that components to strengthen the cell wall are modified to protect the plants from Cd stress. Previously (van de Mortel et al., 2006) we found genes involved in lignin biosynthesis to be constitutively higher expressed in *T. caerulescens* compared to Arabidopsis roots exposed to Zn, but not in Arabidopsis in response to high Zn. We hypothesized that lignin biosynthesis prevents excess efflux of metals from the vascular cylinder in *T. caerulescens* by formation of an extra endodermal layer (van de Mortel et al., 2006). Although it is tempting to conclude the same from this study, the induction only in Arabidopsis,

which does not have a preferential metal transport to the shoot as is found in *T. caerulescens*, could also indicate the activation of a Cd specific detoxification mechanism that limits the entry of toxic metals in Arabidopsis roots, similar to what has been observed for *Phragmites australis* (Ederli et al., 2004).

Cd effect on sulfate assimilation in *T. caerulescens*

Genes involved in sulfate assimilation were found to be differentially expressed in *T. caerulescens* in response to Cd exposure (Table V). Sulfate is transported by high-affinity sulfate transporters from the soil into the root cells. Low-affinity transporters like *SULTR2;1* (*AST68*) are required for translocation of sulfate within the plant. Intracellular sulfate can be metabolized into primary and secondary metabolites. ATP sulfurylase (APS), the first enzyme in sulfate assimilation, catalyzes the formation of adenosine 5'-phosphosulfate (APS) from ATP and sulfate. APS is reduced to sulfite by APS reductase (Figure 4). Sulfite is further reduced by a ferredoxin-dependent sulfite reductase to sulfide, which is incorporated by O-acetylserine (thiol)lyase into the amino acid skeleton of O-acetylserine to form cysteine (Figure 4). Cysteine can be directly incorporated into proteins or peptides, such as glutathione (GSH) (Kopriva, 2006).

Sulfur is present in plant metabolites in the oxidized state as a sulfo-group, which play various roles in plant defense against biotic and abiotic stress, like Cd exposure.

The transfer of the sulfo-group, i.e. sulfation, is catalyzed by sulfotransferases (SOT) (Klein and Papenbrock, 2004). The SOT reaction requires 3'-phosphoadenosyl 5'-phospho-sulfate (PAPS), which is synthesized by phosphorylation of APS by APS kinase. Our results show an induction of SULTR2;1 under Zn deficiency and high Cd exposure compared to sufficient and excess Zn (Table V). A recent study by Herbette et al. (2006) reported that *SULTR2;1* is also induced in response to short-term Cd exposure in Arabidopsis. *SULTR2;1* is specifically expressed in the central cylinder of roots and in the vascular tissues of leaves in *Arabidopsis* during sulfate starvation (Takahashi et al., 1997), suggesting that *SULTR2;1* makes a significant contribution to the enhancement of the sulfate transport capacity in the vascular tissues of roots. This response could imply the first step of an adaptive process required to ensure an adequate supply in sulfur-containing compounds during Cd exposure.

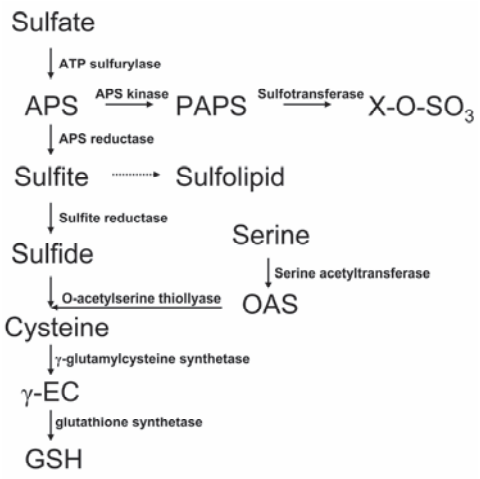

Figure 4. Sulfur assimilation pathway. Dashed line represents multiple reaction steps. APS, adenosine 5'phosphosulfate; PAPS, 3'-phosphoadenosyl 5'-phosphosulfate; OAS, O-acetylserine; γ-EC, γ-glutamylcysteine; GSH, glutathione.

In Arabidopsis three ATP sulfurylases, *APS1*, *APS2*, and *APS3* are identified (Murillo and Leustek, 1995). In our study *APS3* is higher expressed in *T. caerulescens* under Zn deficient and high Cd exposure compared to sufficient and excess Zn (Table V). *APS3* was also identified as being expressed differentially between *A. thaliana* and *A. halleri* when exposed to Cd (Weber et al., 2006). Two genes encoding APS kinases (*AKN1* and *AKN2*) are also differentially expressed in *T. caerulescens*. Both are higher expressed under Zn deficient and high Cd exposure compared to sufficient and excess Zn (Table V). In addition, four *T. caerulescens* genes encoding glutathione S-transferase (GST) (*ATGSTF3*, *ATGSTF10*, *ATGSTU17*, *ATGSTU28*) (TableV) are higher expressed under Zn deficient and Cd excess conditions. GST conjugates endogenous and xenobiotic compounds like Cd with glutathione (GSH) and it is suggested that these complexes are transported to the vacuole via a family of GS-X pumps (Rea et al., 1998). GSH is one of the end-products of the sulfate assimilation pathway (Figure 4). *T. caerulescens* clearly shows an activation of the sulfate assimilation pathway upon Cd exposure, as was also observed in other metal accumulating *Thlaspi* species (Freeman et al., 2004). Our results suggest the existence of a transcriptional regulation mechanism of the sulfur metabolism pathway involved in response to Cd. The rate limiting step for GSH biosynthesis is considered to be the availability of reduced sulfur for cysteine synthesis that occurs at the last step of the sulfur reduction pathway (Cobbett, 2000). Herbette et al. (2006) suggest the importance of the availability of reduced sulfur as an essential factor for Cd detoxification. In Arabidopsis it was shown that GSH decreases when exposed to Cd (Herbette et al., 2006). The importance of reduced glutathione in PC synthesis and,

consequently, in metal detoxification, is well illustrated by the Cd-sensitive *cad2-1* mutant of Arabidopsis, which is deficient in the first enzyme of the GSH biosynthesis pathway, γ-glutamylcysteine synthetase (γ-ECS) and as a result hypersensitive to Cd (Cobbett et al., 1998). A GSH decrease in *T. caerulescens* due to Cd exposure may therefore result in an induction of genes involved in sulfur metabolism. In *Saccharomyces cerevisiae*, exposure of cells to Cd led to a global drop in sulfur-containing protein synthesis and in a re-direction of sulfur metabolite fluxes towards the glutathione pathway (Lafaye et al., 2005). Thus, at least in yeast, heavy metal exposure and sulfur homeostasis are clearly linked and the same seems to be the case for Arabidopsis and *T. caerulescens*. Future reverse-genetic studies will be useful to determine if modified sulfate assimilation, compared to Arabidopsis, is actually required for Cd tolerance in *T. caerulescens*.

Another interesting observation in this respect was that in *T. caerulescens* the R2R3-Myb transcription factor *MYB28* was found to be higher expressed upon high Cd exposure (Table V). Recently *MYB28* was identified as being a transcription factor gene involved in the regulation of aliphatic glucosinolate production (Hirai et al., 2007). Glucosinolates are sulfur-rich, anionic natural products and degradation of glucosinolates provides an additional sulfur source under stress (Kliebenstein et al., 2001). Glutathione (gamma-glu-cys-gly; GSH) is the major reservoir of non-protein reduced sulfur and serves as an important line of defense against reactive oxygen species, xenobiotics and heavy metals. Recently Herbette et al. (2006) showed a down-regulation of genes involved in the glucosinolate biosynthesis pathway in Arabidopsis after exposure of Cd and they suggested that this diverted the sulfate demand for PC biosynthesis by decreasing the glucosinolate level in the plant. The higher expression of *MYB28* in *T. caerulescens* suggests an increase in glucosinolate level in the plant after exposure to Cd but no other genes involved in glucosinolate biosynthesis were differentially expressed in *T. caerulescens*. These results suggest an additional function of *MYB28* in the hyperacumulator *T. caerulescens* that might be linked to sulfate assimilation.

Table VIII: Genes differentially expressed between *T. caerulescens* and Arabidopsis in response to cadmium and zinc.
[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 3) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn100 = 100 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn1000 = 1000 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd0.5 = 2 µM $ZnSO_4$, 0.5 µM $CdSO_4$; Cd50 = 2 µM $ZnSO_4$, 50 µM $CdSO_4$; Zn2 = 2 µM $ZnSO_4$, 0 µM $CdSO_4$; Zn25 = 25 µM $ZnSO_4$, 0 µM $CdSO_4$; Cd15 = 2 µM $ZnSO_4$, 15 µM $CdSO_4$. [d]Normalized spotintensity at 100 µM or 2 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. Significant values are presented in grey (FDR < 0.05). The interaction of the gene expression profiles between the two species is presented in **bold**.

				T. caerulescens					Arabidopsis					
Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity Zn100[d]	Intensity Zn2[d]
ATTI2	AT2G43520	putative trypsin inhibitor	defense response	**3.62**	1.17	**0.32**	0.42	**0.28**	**1.01**	1.48	**1.46**	**1.32**	7192	1779
CYP83A1	AT4G13770	cytochrome p450 family	response to stress	**4.51**	2.91	0.65	0.62	**0.34**	**0.93**	1.39	1.49	**1.29**	6314	5042
	AT4G16000	expressed protein	biological process unknown	**0.49**	0.46	0.93	1.58	**2.03**	**2.16**	1.65	0.76	**0.56**	3137	919
	AT1G02590	aldehyde oxidase, putative	biological process unknown	0.45	1.50	**3.36**	0.85	**4.14**	0.94	0.90	**0.95**	**1.31**	2300	2942
ERD9	AT1G10370	glutathione transferase, putative	response to stress	**9.61**	8.35	0.87	0.40	**0.25**	**1.08**	1.02	0.94	**1.45**	1174	349
ATGSTF11	AT3G03190	glutathione transferase, putative	response to stress	**3.54**	2.42	0.68	0.46	**0.27**	**1.10**	1.25	1.13	**1.12**	959	1259
	AT2G38600	putative acid phosphatase	biological process unknown	**2.51**	2.02	0.80	0.39	**0.21**	**0.70**	0.62	0.88	**1.16**	751	417
LHY1	AT1G01060	myb family transcription factor	regulation of transcription	**8.41**	7.55	0.90	**0.12**	**0.11**	**1.62**	1.16	0.71	**1.20**	577	390
	AT1G19720	DYW7 protein	biological process unknown	**4.04**	3.48	0.86	**0.30**	**0.20**	**0.88**	0.70	0.79	**1.06**	545	136
	AT1G52700	phospholipase/carboxylesterase family protein	biological process unknown	**3.45**	2.80	0.81	0.55	**0.32**	**0.38**	0.29	0.75	**1.62**	489	302
	AT2G29320	putative tropinone reductase	metabolism	**2.88**	1.63	0.57	**0.23**	**0.17**	**0.77**	0.68	0.87	**1.11**	460	448
	AT2G29300	putative tropinone reductase	metabolism	**2.80**	1.14	0.41	0.28	**0.20**	**0.64**	0.33	0.51	**0.75**	315	349
SPA3	AT3G15354	similar to phytochrome A suppressor spa1	protein amino acid phosphorylation	**5.04**	4.00	0.79	0.47	**0.35**	**1.38**	1.52	1.10	**1.22**	307	93
	AT2G31790	UDP-glycosyltransferase family	metabolism	**5.55**	3.57	0.64	0.32	**0.18**	**1.03**	1.08	1.04	**0.80**	247	897
	AT3G15650	putative lysophospholipase	biological process unknown	**2.58**	2.51	0.97	0.79	**0.46**	**0.55**	0.39	0.71	**1.44**	240	1572
CCA1	AT2G46830	MYB-related transcription factor (CCA1)	regulation of transcription	**11.02**	13.21	1.20	**0.10**	**0.09**	**0.89**	0.96	1.08	**1.73**	237	1014
ATATH13	AT5G64940	ABC transporter-like	transport	**4.13**	3.58	0.87	**0.25**	**0.25**	**0.95**	1.00	1.05	**1.44**	123	77
CYP712A1	AT2G42250	cytochrome p450, putative	electron transport	0.62	0.95	**1.54**	**1.08**	1.65	0.86	4.53	**5.24**	**3.77**	19034	12367
ZIP2	AT5G59520	putative zinc transporter ZIP2 - like	zinc ion transport	2.56	2.67	**1.04**	**0.42**	**0.54**	1.46	8.01	**5.47**	**3.28**	3915	10400
	AT5G50590	11-beta-hydroxysteroid dehydrogenase-like	metabolism	0.85	1.24	**1.46**	**0.88**	**1.13**	1.29	10.96	**8.50**	**4.35**	10996	5550

Continue Table VIII

				T. caerulescens					Arabidopsis					
Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn0/Zn2[c]	Zn0/Zn25[c]	Zn2/Zn25[c]	Zn2/Cd15[c]	Intensity Zn100[d]	Intensity Zn2[d]
RHA2A	AT1G15100	putative RING-H2 zinc finger protein	biological process unknown	**1.59**	1.54	0.97	**0.53**	0.48	**0.50**	0.52	1.05	**1.63**	824	3517
	AT1G80320	2OG-Fe(II) oxygenase family protein	biological process unknown	0.76	1.41	**1.85**	**1.23**	**1.53**	0.62	4.84	**7.75**	**7.05**	7790	3209
	AT1G15540	2OG-Fe(II) oxygenase family protein	N-terminal protein myristoylation	0.70	1.40	**2.02**	**1.00**	**1.20**	0.57	5.56	**9.68**	**5.82**	5977	3197
	AT1G52790	2OG-Fe(II) oxygenase family protein	biological process unknown	0.40	0.75	**1.88**	**1.52**	2.41	0.57	4.04	**7.08**	**5.38**	21075	2023
CYP76G1	AT3G52970	cytochrome p450 family	response to stress	0.77	1.01	**1.31**	**1.23**	**1.24**	0.70	11.51	**16.37**	**11.46**	1386	1227
ORG1	AT5G53450	protein kinase family protein	protein amino acid phosphorylation	1.04	0.73	**0.70**	**1.15**	0.72	0.79	0.12	**0.16**	**0.30**	2415	1085
WRKY59	AT2G21900	WRKY family transcription factor	regulation of transcription	**0.79**	0.83	1.05	**1.23**	**1.20**	**2.95**	1.28	0.43	**0.26**	202	973
bHLH100	AT2G41240	bHLH protein	regulation of transcription	0.69	0.20	**0.29**	**1.08**	0.26	0.26	0.01	**0.02**	**0.08**	2251	804
	AT4G13280	terpene synthase/cyclase family	response to wounding	1.05	1.10	**1.04**	1.00	**1.02**	0.71	0.20	**0.29**	**0.31**	168	630
	AT2G30670	putative tropinone reductase	metabolism	1.01	1.22	**1.21**	**1.04**	1.04	0.73	0.16	**0.21**	**0.26**	306	444
	AT1G52120	similar to jacalin lectin family protein	biological process unknown	1.03	0.88	**0.86**	**1.03**	**1.13**	0.90	0.06	**0.07**	**0.26**	303	166
CYP78A8	AT1G01190	cytochrome P450, putative	electron transport	**0.60**	0.47	0.79	**2.43**	1.46	**2.65**	2.65	1.00	**0.53**	1262	360
FRO4	AT5G23980	FRO2 homolog	electron transport	6.02	7.23	**1.20**	**0.08**	**0.13**	2.37	17.24	**7.27**	**2.26**	606	2509

Regulation of Cd response in *T. caerulescens* and Arabidopsis

In addition to studying the structural genes involved in Cd response we are also interested in the regulatory genes that control expression of the structural genes. As there is almost no overlap in genes differentially expressed in response to Cd exposure between *T. caerulescens* and Arabidopsis it appears as if there is a strong difference in signaling of Cd exposure response in both species.

The *OBP3*-responsive genes (*ORG1*, *ORG2* and *ORG3*) are higher expressed under excess Zn and Cd exposure in Arabidopsis (Table II). *OBP3* is a Dof (DNA binding with one finger) protein, which plays an important role in plant growth and development (Kang and Singh, 2000). *ORG2* and *ORG3* encode basic helix-loop-helix domain (bHLH) containing transcription factors (Kang et al., 2003), while *ORG1* encodes a protein kinase. *ORG1*, *ORG2* and *ORG3* have been found to be higher expressed in response to salicylic-acid (SA) (Kang et al., 2003). SA is known to have a signaling role in a wide range of oxidative stresses (Rao and Davis, 1999; Borsani et al., 2001) and since Cd induces oxidative stress in plants SA may play a role in enhancing Cd tolerance by inducing the antioxidant defense mechanism (Choudhury and Panda, 2004). Freeman et al. (2005) showed that elevation of free SA levels in Arabidopsis, both genetically and by exogenous feeding, enhances the specific activity of serine acetyltransferase, leading to elevated glutathione and increased Ni resistance. *ORG1*, *ORG2* and *ORG3* were not as differentially expressed in *T. caerulescens* as in Arabidopsis, and the semi-quantitative RT-PCR showed only for *ORG3* an induced expression in the roots when exposed to Cd (Figure 1B). This is in line with the previous observation by Freeman et al. (2005) that Ni-hyperaccumulating *Thlaspi* species have a constitutively elevated level of SA compared to Arabidopsis.

Other transcription factors that are higher expressed under Cd exposure in Arabidopsis are *MYB4*, *MYB10*, *MYB72* and *bHLH100*. (Table I+II). *AtMYB4* is a key regulator of phenylpropanoid pathway gene expression, and is an example of a MYB protein that functions as a transcriptional repressor (Hemm et al., 2001). Its role in metal homeostasis or tolerance is not directly clear. *MYB10* and *MYB72* were identified as iron-regulated transcription factors and their regulation partly depends on *FIT1*, which encodes a putative bHLH transcription factor (Colangelo and Guerinot, 2004). The *myb72* mutant was more sensitive to growth under Fe

deficient conditions and Zn excess conditions (Figure 3B), which also appears to induce a Fe deficiency response in Arabidopsis (van de Mortel et al., 2006). Thus Arabidopsis exposed to excess Zn and Cd may become somewhat Fe deficient, which also explains the induction of other iron homeostasis related genes by Cd exposure. In both Arabidopsis and *T. caerulescens* the *bHLH100* transcription factor was higher expressed under high Cd exposure (Table II, Table VI, Figure 1). The 35S::*bHLH100* lines showed enhanced tolerance to excess Zn and Ni compared to wild type (Figure 2), but not a very clear enhancement of Cd tolerance, meaning that the function of this gene may be related to general abiotic stress response, but remains still largely elusive.

Differences in Cd response between *T. caerulescens* and Arabidopsis

Probably most interesting for the identification of genes that contribute to the Cd tolerance of *T. caerulescens* are the genes that are differentially expressed between *T. caerulescens* and Arabidopsis at comparable Cd and/or Cd/Zn exposures (Tables VII and VIII). This is a very large set of genes though, most of them not involved in metal homeostasis (van de Mortel et al., 2006). Therefore we only considered genes which showed a different expression profile when comparing Arabidopsis and *T. caerulescens*. Many of these genes are involved in transcriptional regulation (Table VII+VIII). *CCA1* (Circadian Clock Associated 1) and *LHY1*(Late Elongated Hypocotyl), which encode closely related MYB transcription factors regulating circadian rhythms in Arabidopsis (Green and Tobin, 2002) and the *Early-Phytochrome Responsive 1* (*EPR1*) gene, encoding another MYB transcription factor and highly similar to *CCA1* and *LHY1* (Kuna et al., 2003), are all higher expressed in *T. caerulescens* in response to Zn deficiency and particularly Cd exposure, while these genes are not responding to Zn or Cd in Arabidopsis. *CCA1* was also higher expressed in *A. halleri* under low Zn supply (Becher et al., 2004) suggesting some specific role of these circadian rhythm controlling factors in metal hyperaccumulators.

The similarities and differences in gene expression between the hyperaccumulator species *T. caerulescens* and *A. halleri* could not be established, because of differences in experimental setup. *A. halleri* was exposed for a short period to high Cd whereas *T. caerulescens* was exposed for a longer period to Cd. These results suggest that Cd response is time-regulated as well as dose-regulated, which was also suggested by Herbette et al. (2006).

CONCLUSION

In conclusion, the comparative transcriptional analysis of the Cd response of the Zn/Cd-hyperaccumulator *T. caerulescens* and the non-accumulator Arabidopsis emphasizes the role of genes involved in lignin-, glutathione- and sulfate metabolism but also suggests a similar role for many more genes, with unknown function. Genes found to be differentially expressed within this study are expected to be involved in Cd tolerance, rather than Cd hyperaccumulation, because *T. caerulescens* acc. La Calamine is not a very efficient hyperaccumulator of Cd. This indicates that there are specific responses to Cd exposure in Arabidopsis as well as in *T. caerulescens*.

MATERIAL AND METHODS

Plant material and growth conditions

Arabidopsis thaliana Col-0 (Arabidopsis) and *Thlaspi caerulescens* J. & C. Presl accession La Calamine were grown as described by van de Mortel et al. (2006). After three weeks of growth on a modified half strength Hoagland's nutrient solution, the *T. caerulescens* plants were transferred for seven days to the same modified half-strength Hoagland's nutrient solution (Schat et al., 1996) with a deficient (0 μM), sufficient (100 μM) or excess (1000 μM) $ZnSO_4$ concentration or a high (0.5 or 50 μM) $CdSO_4$ concentration. The Arabidopsis plants were transferred to the same nutrient solution with deficient (0 μM), sufficient (2 μM) or excess (25 μM) $ZnSO_4$ concentration or a high (15 μM) $CdSO_4$. The Cd-containing modified half-strength Hoagland's solutions with high $CdSO_4$ concentration contained a sufficient (2 μM) $ZnSO_4$ concentration. During the first three weeks, the nutrient solution was replaced once a week and thereafter twice a week. Germination and plant culture were performed in a climate chamber (20/15°C day/night; 250 μmoles m^{-2} s^{-1} at plant level during 14 h/d (*T. caerulescens*) or 12 h/d (Arabidopsis); 75% RH).

Microarray experiment

The microarray experiment was performed as described by van de Mortel et al. (2006) using the Agilent Arabidopsis2 60-mer oligonucleotide microarrays (Agilent Technologies Inc., Palo Alto, CA, USA) representing ~80% of the Arabidopsis transcriptome. Roots of one pot containing three Arabidopsis or three *T. caerulescens* plants per treatment were pooled. Each pool was considered as one biological replicate and two biological replicates were used for the microarray experiment. After hybridization the slides were scanned, analyzed and normalized with the Agilent Feature Extraction Software (Agilent Technologies Inc., Palo Alto, CA, USA) using Agilent's standard normalization within each array. The remaining statistical analysis was performed using the Limma package (Smyth, 2005a) in R/BioConductor (Gentleman et al., 2004). Between-array quantile normalization was performed on the common reference channel while leaving the log-ratios unchanged (Yang and Thorne, 2003). To find differentially expressed genes we performed a separate channel analysis (Smyth, 2005b) using a moderated t-test (Smyth, 2004) using a moderated t-test (Smyth, 2004). Within species all Zn and Cd treatments were compared. For the between species comparison each probe was tested for differences in the pattern of response to Zn or Cd in Arabidopsis as compared with *T. caerulescens*. The resulting p-values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate adjustment (Benjamini and Hochberg, 1995). A gene was considered to be significantly differentially expressed if FDR<0.05 (controlling the expected false discovery rate to no more than 5%) and the expression difference was ≥ 2 (within species) or ≥ 3 (between species) fold. Genes found to be significantly differentially expressed in all comparisons were clustered using Cluster/Treeview (Eisen et al., 1998). Average linkage clustering with uncentered correlation was used within Cluster to perform the clustering analysis.

Genomic DNA hybridizations were performed using 1 µg random primed genomic DNA. As a quality control step we performed a dye-swap hybridization. After hybridization the slides were scanned, analyzed and normalized with the Agilent Feature Extraction Software (Agilent Technologies Inc., Palo Alto, CA, USA) using Agilent's Linear & Lowess normalization. The features that hybridized with Arabidopsis genomic DNA (both polarities of the dye swap) and not

with *T. caerulescens* genomic DNA were left out from the dataset by Spotfire (Spotfire, Sommerville, MA, USA) using a ≥ 3-fold change as cut-off value.

Semi-quantitative RT-PCR

The semi-quantitative RT-PCR was performed as described by van de Mortel et al. (2006). Selected *T. caerulescens* genomic and cDNA fragments were PCR-amplified using primers designed for the orthologous Arabidopsis gene. PCR products were sequenced and new primers were designed for semi-quantitative RT-PCR to ensure amplification of the correct *T. caerulescens* gene. The PCR-amplification was performed with a cDNA aliquot (2 µl) and gene-specific primers (Supplemental Table III). Between 25- to 35-cycle PCRs (30s at 94°C, 30s at 50°C, and 60s at 68°C) were performed in a 50-µl reaction and 50 µl of the reaction was separated on an ethidium bromide stained 1% agarose gel. Gel-image analysis using QuantityOne® Software (BioRad, Hercules, CA, USA), was used to quantify the DNA fragment intensities (Supplemental Table III). The DNA fragment intensities were corrected for background signal and corrected for cDNA quantity using the intensities of Tubulin.

Construction of expression vectors

The full-length cDNA of *bHLH100* was amplified from Arabidopsis cDNA by PCR. Gateway primers were used for PCR, the forward was 5'-GGGGACAAGTTTGTACAAA AAAGCAGGCTCTCAAAATGTGTGCACTTGT-3' containing attB1 sequence (underlined) and the reversed primer was 5'-GGGGACCACTTTGTACAAGAAAGCTGGTACGAGAGA CAAAACAGAAAA-3' containing attB2 sequence (underlined). The PCRs were performed with the Pfx polymerase (Fermentas, http://www.fermentas.com) at 94°C for 2 min., followed by 40 cycles 94 °C for 45 sec., 50 °C for 45 sec. and 68 °C for 5 min, and finished by an extension at 68 °C for 5 min. PCR products were recombined to pDONOR201 (Invitrogen, http://www.invitrogen.com) in a 10-µl BP Clonase (Invitrogen) reaction following the manufacturer's instruction. The fragments were transferred from their donor constructs to the binary over-expression vector pH7WG2 (Karimi et al., 2002), under the control of the double 35S

CaMV enhancer, in 10 µl LR Clonase (Invitrogen) reaction following the manufacturer's instruction.

Plant transformation

The *bHLH100* overexpression construct was used to transform *Agrobacterium tumefaciens* strain AGL0, and transformants were selected on Luria-Bertani medium containing spectinomycin (100 µg/ml). These overexpression constructs were used to transform *A. thaliana* Columbia-0 (Col) by the standard flower dip method (Clough and Bent, 1998). T1 seeds obtained from self-fertilization of the primary transformants were surface-sterilized and sown on half-strength Murashige Skoog´s medium supplemented with hygromycin (20 µg/ml). Hygromycin-resistant plants were transferred to soil, and the T2 seeds resulting from self-fertilization were collected. The T2 seeds were surface sterilized, plated on the same selection medium and scored for resistance to hygromycin. Transgenic lines that displayed 3:1 segregation ratio for hygromycin resistance to hygromycin sensitivity in the T2 generation and that were 100% hygromycin resistant in the T3 generation were selected for further analysis. All further experiments were performed with T4 seeds.

Metal tolerance screening

Seeds of the Arabidopsis *myb72* T-DNA insertion lines and 35S::bHLH100 transgenic lines were sterilized and sown on the half-strength Murashige Skoog's medium containing no zinc (0 µM $ZnSO_4$), excess zinc (400 µM $ZnSO_4$), low iron (0.15 µM FeEDTA), excess nickel (75 µM $NiSO_4$) or cadmium (30 µM $CdSO_4$). Seeds grown on half-strength Murashige Skoog's medium were used as control. For each treatment, five to six replicates were performed and for each line 20 seeds were sown on each replicate plate. After sowing seeds were vernalized at 4 °C for 3 days. Germination and plant culture were performed in a climate chamber (20/20°C day/night temperatures; 250 µmoles light m^{-2} s^{-1} at plant level during 12 h/d; 75% RH). The tolerance phenotypes were studied on 5-day-old plants by measuring root length using Image J software (National Institute of Mental Health, Bethesda, Maryland, USA).

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Supplemental Table I: Cadmium-responsive genes in *T. caerulescens*

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$; Zn100 = 100 µM $ZnSO_4$; Zn1000 = 1000 µM $ZnSO_4$; Cd0 = 0 µM $CdSO_4$, 100 µM $ZnSO_4$; Cd0.5 = 0.5 µM $CdSO_4$, 2 µM $ZnSO_4$; Cd50 = 50 µM $CdSO_4$, 2 µM $ZnSO_4$. [d]Normalized spotintensity at 100 µM $ZnSO_4$. Genes are ordered according to decreasing spot intensity. Significant values are presented in **bold (FDR < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
I	AHB1	AT2G16060	class 1 non-symbiotic hemoglobin	response to stress	0.55	0.50	0.92	1.58	2.79	**4.39**	117037
	NIA1	AT1G77760	nitrate reductase 1	nitrate assimilation	0.81	1.33	1.64	1.04	**2.52**	**2.62**	41451
	PDC2	AT5G54960	pyruvate decarboxylase	response to stress	1.02	0.97	0.95	1.28	**3.12**	**3.98**	32271
		AT4G10270	probable wound-induced protein	response to stress	0.82	1.01	1.23	1.37	2.60	**3.58**	31740
		AT5G39890	expressed protein	other biological processes	0.55	0.45	0.82	1.13	3.40	**3.83**	31612
		AT4G27450	expressed protein	other biological processes	0.70	0.61	0.88	1.02	**3.09**	**3.16**	31058
		AT2G17850	senescence-associated protein	aging	0.53	0.48	0.91	1.04	**4.65**	**4.83**	29402
		AT3G29970	germination protein-related	other biological processes	0.43	0.60	1.41	0.91	**8.80**	**8.03**	23771
	ALAAT2	AT1G72330	putative alanine aminotransferase	L-alanine biosynthetic process from pyruvate	0.79	0.75	0.96	1.00	**3.14**	**3.15**	14772
		AT2G27830	expressed protein	other biological processes	1.01	0.79	0.78	1.09	**2.30**	**2.51**	12380
		AT4G34950	nodulin family protein	other biological processes	1.12	1.14	1.02	0.78	**4.18**	**3.26**	11571
		AT1G72360	member of the ERFB-2 transcription factor family	signal transduction	0.66	0.82	1.24	1.31	**2.29**	**3.00**	10526
		AT5G19140	auxin/aluminum-responsive protein	response to auxin stimulus	0.68	0.72	1.06	0.86	**3.66**	**3.16**	10238
		AT3G10040	expressed protein	transcription	0.76	0.95	1.25	1.03	3.26	**3.37**	10042
		AT1G76640	calmodulin-related protein	other biological processes	0.57	0.68	1.19	0.94	**4.16**	**3.90**	9407
		AT4G17260	L-lactate dehydrogenase	response to abscisic acid stimulus	0.77	0.81	1.06	0.94	**2.06**	**1.94**	9337
		AT1G43800	acyl-(acyl-carrier-protein) desaturase, putative	other cellular processes	0.70	0.75	1.07	1.33	**3.61**	**4.81**	9211
		AT1G47840	hexokinase, putative	electron transport or energy pathways	0.75	0.96	1.28	1.37	1.59	**2.19**	7653
		AT1G62770	invertase/pectin methylesterase inhibitor family protein	other biological processes	0.67	**0.52**	0.77	0.87	**2.87**	**2.50**	7521
	C/VIF1	AT1G47960	invertase/pectin methylesterase inhibitor family protein	other biological processes	0.82	0.63	0.77	0.94	**3.19**	**2.99**	5637
		AT1G20700	homeobox-leucine zipper family protein	transcription	0.70	**0.56**	0.80	0.94	**2.07**	**1.95**	5503
		AT3G27220	kelch repeat-containing protein	other biological processes	0.84	0.95	1.12	1.24	**3.04**	**3.78**	4945

Continue Supplemental Table I

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT4G32460	expressed protein	other biological processes	**0.52**	0.79	1.52	1.42	1.71	**2.42**	4284
		AT5G47240	mutT domain protein-like	other biological processes	0.59	**0.51**	0.86	0.82	**2.24**	**1.84**	3769
		AT1G31330	photosystem I subunit III precursor, putative	photosynthesis	0.68	0.93	1.36	1.46	1.67	**2.43**	3758
		AT1G07160	protein phosphatase 2C, putative	protein metabolism	0.56	0.70	1.26	1.01	**2.81**	**2.83**	3431
		AT4G36850	PQ-loop repeat family protein	other biological processes	0.58	0.94	1.62	1.22	2.12	**2.58**	3397
		AT5G07580	member of the ERF subfamily B-3 transcription factor family	transcription	0.92	1.00	1.08	0.97	**2.38**	**2.30**	2940
		AT5G15120	expressed protein	other biological processes	0.82	1.08	1.33	1.17	2.52	**2.94**	2766
		AT5G54130	calcium-binding EF hand family protein	other biological processes	0.87	0.68	0.78	0.98	**2.10**	**2.06**	2382
		AT4G26270	phosphofructokinase family protein	electron transport or energy pathways	0.84	0.81	0.96	1.13	3.00	**3.38**	2182
	bHLH096	AT1G72210	basic helix-loop-helix (bHLH) family protein	transcription	1.39	1.27	0.91	1.08	**2.86**	**3.09**	1941
		AT4G21390	S-locus lectin protein kinase family protein	protein metabolism	1.01	0.94	0.93	1.42	**2.69**	**3.81**	1886
	ATL6	AT3G05200	zinc finger (C3HC4-type RING finger) family protein	other biological processes	0.92	0.90	0.97	1.09	**1.91**	**2.08**	1778
		AT1G15210	ABC transporter family protein	multidrug transport	0.53	0.65	1.22	1.20	1.75	**2.11**	1727
		AT3G12150	expressed protein	other biological processes	0.64	0.80	1.25	1.21	**1.86**	**2.25**	1698
	At-GTL1	AT1G33240	trihelix DNA-binding protein	other biological processes	0.64	0.89	1.40	1.22	**2.01**	**2.44**	1654
	APL3	AT4G39210	glucose-1-phosphate adenylyltransferase	starch biosynthetic process	1.01	1.01	1.00	1.08	**2.34**	**2.52**	1380
	ACD32.1	AT1G06460	heat shock protein, putative	response to abiotic or biotic stimulus	**0.64**	**0.55**	0.86	0.89	**2.15**	**1.90**	1307
	RHA3B	AT4G35480	zinc finger (C3HC4-type RING finger) family protein	other biological processes	0.99	1.72	1.74	1.12	**3.62**	**4.05**	1032
	APL4	AT2G21590	glucose-1-phosphate adenylyltransferase	starch biosynthetic process	0.98	0.96	0.98	0.91	**2.87**	**2.62**	857
	CLE6	AT2G31085	Clavata3 / ESR-Related-6	signal transduction	0.38	0.35	0.92	1.06	4.47	**4.75**	778
		AT1G80280	hydrolase	other biological processes	**0.59**	0.88	1.50	1.14	**1.86**	**2.13**	711
	EXGT-A4	AT5G13870	xyloglucan endotransglycosylase	carbohydrate metabolic process	0.52	0.85	1.63	1.02	**2.31**	**2.35**	678
	CLE4	AT2G31081	Clavata3 / ESR-Related-4	signal transduction	0.62	0.75	1.20	1.30	**1.93**	**2.51**	648
		AT1G45230	similar to defective chloroplasts and leaves (DCL) protein	other biological processes	**0.51**	0.97	1.90	1.04	**2.40**	**2.48**	616
		AT5G42900	expressed protein	other biological processes	0.60	**0.55**	0.93	1.13	1.81	**2.04**	482
		AT1G66045	hypothetical protein	other biological processes	0.95	2.13	2.25	0.83	**3.28**	**2.71**	225

Continue Supplemental Table I

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
III		AT5G38930	germin - like protein	other biological processes	**0.50**	1.04	2.07	**2.83**	0.60	1.71	23306
		AT3G10530	transducin family protein	other biological processes	0.89	1.18	1.33	**2.25**	0.86	**1.94**	3191
		AT2G04460	expressed protein	other biological processes	0.73	1.17	1.61	**2.50**	0.54	1.36	1282
		AT1G30560	similar to glycerol-3-phosphate transporter	transport	0.79	0.53	0.67	**3.68**	0.56	2.08	811
		AT4G01925	DC1 domain-containing protein	other biological processes	0.79	0.79	1.00	**2.83**	**0.36**	1.00	703
		AT5G57390	ovule development protein, putative	transcription	1.73	1.52	0.88	0.78	**2.11**	1.64	637
		AT1G77700	pathogenesis-related thaumatin family protein	response to abiotic or biotic stimulus	1.31	1.35	1.03	**3.68**	**0.29**	1.05	601
	TUA1	AT1G64740	tubulin alpha-1 chain	cell organization and biogenesis	1.16	**1.89**	1.63	**2.21**	0.71	1.57	252
		AT4G17080	MORN repeat-containing protein	other biological processes	0.62	0.51	0.81	**2.76**	0.67	1.86	228
		AT5G43850	acireductone dioxygenase (ARD/ARD') family protein	other biological processes	1.52	1.04	0.69	**2.05**	**0.55**	1.12	206
	MSBP1	AT5G52240	progesterone-binding protein-like	cell organization and biogenesis	1.45	1.31	0.91	**5.51**	**0.23**	1.27	173
		AT2G40620	bZIP transcription factor family protein	transcription	1.31	1.25	0.95	4.67	**0.18**	0.83	83
		AT1G04870	protein arginine N-methyltransferase family protein	other biological processes	0.88	0.86	0.97	**10.06**	**0.11**	1.13	53
IV		AT3G20020	protein arginine N-methyltransferase family protein	other biological processes	0.86	0.75	0.86	1.43	**0.48**	0.68	59046
	ATEXPA17	AT4G01630	expansin, putative	cell organization and biogenesis	0.79	1.22	1.55	1.64	**0.39**	0.64	1709
		AT2G46860	putative inorganic pyrophosphatase	metabolic process	0.49	0.86	1.75	1.51	**0.36**	0.55	1428
		AT5G38710	proline oxidase, mitochondrial precursor -like protein	glutamate biosynthetic process	0.88	0.75	0.85	1.22	**0.39**	**0.48**	1338
V		AT3G18290	zinc finger protein, putative	embryonic development	1.47	0.89	0.60	**0.34**	1.32	0.44	2381
		AT1G23200	pectinesterase family protein	cell organization and biogenesis	1.42	0.87	**0.61**	**0.40**	1.04	**0.41**	2145
	CYP71B22	AT3G26200	cytochrome p450 family	electron transport or energy pathways	2.04	1.61	0.79	**0.30**	1.38	**0.42**	1233
	CYP81D5	AT4G37320	cytochrome p450 family	electron transport or energy pathways	1.43	0.96	0.67	**0.41**	1.18	**0.48**	1206
	ATGSTU27	AT3G43800	glutathione S-transferase	toxin catabolic process	1.29	0.94	0.73	**0.36**	1.52	0.55	573
		AT1G03050	epsin N-terminal homology (ENTH) domain-containing protein	protein metabolism	1.11	1.09	0.99	**0.38**	0.94	**0.36**	453
		AT1G27740	basic helix-loop-helix (bHLH) family protein	transcription	0.77	0.81	1.05	0.49	0.62	**0.31**	406
		AT2G39490	F-box protein family	other biological processes	1.44	1.00	0.69	**0.44**	1.13	**0.50**	298
	XIF	AT2G31900	myosin family protein	transport	1.06	0.79	0.75	**0.33**	1.53	0.50	249
		AT3G10450	serine carboxypeptidase S10 family protein	protein metabolism	1.81	0.67	0.37	**0.19**	1.32	**0.24**	187

Continue Supplemental Table I

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT3G25420	serine carboxypeptidase S10 family protein	protein metabolism	0.78	0.77	0.98	**0.26**	1.00	**0.26**	186
VI		AT3G16800	protein phosphatase 2C	other biological processes	1.73	1.62	0.93	0.91	**0.44**	**0.40**	9076
		AT2G43590	chitinase, putative	response to abiotic or biotic stimulus	1.32	0.77	0.58	0.84	0.46	**0.39**	8770
		AT5G42500	disease resistance response protein-related	defense response	1.48	1.14	0.77	0.87	**0.40**	**0.35**	7352
		AT5G28010	Bet v I allergen family protein	other biological processes	1.04	0.97	0.94	0.81	0.56	**0.45**	4373
		AT3G24500	ethylene-responsive transcriptional coactivator, putative	response to abiotic or biotic stimulus	1.39	1.01	0.73	0.89	**0.36**	**0.32**	4042
		AT3G24290	ammonium transporter, putative	transport	1.28	1.30	1.02	1.24	**0.48**	**0.59**	3330
		AT2G41660	expressed protein	other biological processes	1.59	1.33	0.83	0.87	**0.46**	**0.40**	1684
		AT2G37450	nodulin MtN21 family protein	other biological processes	1.32	1.38	1.04	0.75	0.60	**0.45**	1562
		AT1G26790	Dof-type zinc finger domain-containing protein	transcription	**2.00**	1.60	0.80	0.86	**0.45**	**0.38**	1496
	AHA9	AT1G80660	plasma membrane ATPase, putative	transport	2.20	1.08	0.49	0.97	0.18	**0.18**	1299
		AT3G12320	expressed protein	other biological processes	**1.92**	1.91	1.00	0.82	**0.46**	**0.38**	1096
		AT2G43580	chitinase, putative	response to abiotic or biotic stimulus	2.83	0.73	0.26	0.76	**0.19**	**0.15**	1081
	ZIP8	AT5G45105	metal transporter, putative	transport	1.19	1.06	0.89	0.65	0.49	**0.32**	991
		AT4G32190	centromeric protein-related	other biological processes	**1.85**	1.62	0.88	0.88	**0.43**	**0.38**	898
	EPR1	AT1G18330	myb family transcription factor	transcription	1.22	1.11	0.91	0.89	0.54	**0.48**	832
	RHA2A	AT1G15100	zinc finger (C3HC4-type RING finger) family protein	other biological processes	1.59	1.54	0.97	0.91	0.53	**0.48**	824
		AT3G47540	chitinase, putative	response to abiotic or biotic stimulus	1.19	0.73	0.61	0.83	0.50	**0.41**	822
		AT2G27180	expressed protein	other biological processes	**1.85**	1.35	0.73	0.88	**0.44**	**0.39**	516
		AT2G26975	copper transporter	transport	1.56	1.69	1.08	0.89	**0.49**	**0.44**	345
		AT1G29840	esterase/lipase/thioesterase family protein	other biological processes	1.06	0.93	0.88	0.87	0.36	**0.31**	225
		AT3G10585	myb family transcription factor	transcription	1.22	0.92	0.76	0.82	**0.30**	**0.24**	209
		AT4G16850	expressed protein	other biological processes	1.24	0.98	0.78	0.80	0.28	**0.22**	129
		AT3G17760	glutamate decarboxylase	carboxylic acid metabolic process	1.15	0.90	0.78	1.05	**0.32**	**0.34**	63
VII	ATPPC3	AT3G14940	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.94**	1.30	0.67	0.82	**0.53**	**0.43**	24481
	ATPPC1	AT1G53310	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.57**	1.06	0.68	0.84	**0.58**	**0.48**	21568

Continue Supplemental Table I

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT3G54600	DJ-1 family protein	other biological processes	**1.81**	1.13	0.63	0.56	0.83	**0.47**	9632
		AT4G36610	hydrolase	aromatic compound metabolic process	1.23	0.90	0.73	0.66	0.68	**0.45**	6523
		AT2G31940	expressed protein	other biological processes	**1.62**	1.09	0.67	0.66	0.63	**0.41**	4432
	GLP9	AT4G14630	germin-like protein	other biological processes	2.17	1.39	0.64	0.45	0.57	**0.26**	4350
		AT2G18370	protease inhibitor/seed storage/lipid transfer family protein	transport	1.56	0.96	0.62	0.54	0.68	**0.37**	4247
		AT2G30660	3-hydroxyisobutyryl-coenzyme A hydrolase	metabolic process	1.59	0.97	0.61	0.72	0.59	**0.42**	3497
		AT1G23730	putative carbonic anhydrase	other metabolic processes	2.18	1.73	0.79	0.33	0.69	**0.23**	3213
	ATPPC2	AT2G42600	phosphoenolpyruvate carboxylase	response to abiotic or biotic stimulus	**1.87**	1.41	0.75	0.64	0.73	**0.46**	3024
		AT3G44540	acyl CoA reductase - protein	developmental processes	1.09	0.73	0.66	**0.52**	0.88	**0.46**	2815
		AT5G57150	basic helix-loop-helix (bHLH) family protein	transcription	1.16	0.91	0.78	0.65	0.75	**0.49**	2813
		AT2G23960	defense-related protein	defense response	1.17	0.72	0.62	0.48	0.63	**0.30**	2205
		AT1G20823	zinc finger (C3HC4-type RING finger) family protein	other biological processes	**1.97**	1.52	0.77	0.70	0.70	**0.49**	1705
	ATPAP22	AT3G52820	purple acid phosphatase	other biological processes	1.40	0.79	0.56	0.69	0.64	**0.44**	1599
		AT2G24430	NAM (no apical meristem)-like protein	multicellular organismal development	1.54	1.16	0.76	**0.56**	0.74	**0.42**	1279
		AT3G44550	acyl CoA reductase -like protein	developmental processes	1.17	0.89	0.76	0.57	0.66	**0.37**	1197
	MYB7	AT2G16720	member of the R2R3 factor gene family	response to abiotic or biotic stimulus	1.24	0.92	0.74	0.67	0.71	**0.47**	1094
		AT5G13330	member of the ERF subfamily B-4 transcription factor family	transcription	2.07	1.17	0.57	0.55	0.61	**0.34**	1010
	SS3	AT1G74000	strictosidine synthase family	biosynthetic process	1.26	0.77	0.61	0.59	0.79	**0.47**	973
	HAP5B	AT1G56170	transcription factor, putative	transcription	1.13	0.67	0.59	**0.40**	0.68	**0.27**	962
	PGPS1	AT2G39290	phosphatidylglycerolphosphate synthase	phospholipid biosynthetic process	1.44	0.90	0.62	0.69	0.72	**0.49**	960
		AT5G56840	DNA-binding family protein	transcription	**1.73**	1.59	0.92	0.60	0.71	**0.43**	956
		AT4G26860	alanine racemase family protein	other biological processes	1.60	1.17	0.73	0.52	0.93	**0.49**	955
		AT1G35910	trehalose-6-phosphate phosphatase	trehalose biosynthetic process	1.64	0.94	0.57	0.64	0.54	**0.35**	892
		AT5G06720	peroxidase, putative	response to stress	2.64	2.44	0.93	0.43	0.69	**0.30**	775
		AT3G62190	DNAJ heat shock N-terminal domain-containing protein	protein metabolism	**1.70**	1.51	0.89	0.60	0.70	**0.42**	729
		AT2G29650	inorganic phosphate transporter	response to abiotic or biotic stimulus	**1.93**	**1.63**	0.84	0.68	**0.58**	**0.40**	507

Continue Supplemental Table I

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Intensity Zn100[d]
		AT5G59490	haloacid dehalogenase-like hydrolase family protein	other metabolic processes	1.69	0.92	0.54	0.50	0.84	**0.42**	383
		AT1G34040	alliinase family protein	other biological processes	1.86	1.22	0.66	0.55	0.79	**0.44**	375
		AT5G15240	amino acid transporter family protein	transport	1.37	1.10	0.80	0.56	0.85	**0.47**	350
		AT3G45680	proton-dependent oligopeptide transport family protein	response to abiotic or biotic stimulus	1.26	0.51	0.41	0.48	0.62	**0.30**	350
	SOS3	AT5G24270	calcineurin B-like protein, putative	response to abiotic or biotic stimulus	1.15	1.07	0.93	0.48	0.63	**0.30**	348
		AT1G25460	oxidoreductase family protein	flavonoid biosynthetic process	1.51	1.12	0.74	0.54	0.79	**0.43**	337
	WNK6	AT3G18750	protein kinase family protein	protein metabolism	1.63	1.59	0.98	0.50	0.71	**0.36**	271
		AT5G63710	leucine-rich repeat transmembrane protein kinase	signal transduction	1.77	1.43	0.80	0.70	0.70	**0.49**	224
	IGPD	AT4G14910	imidazoleglycerol-phosphate dehydratase	histidine biosynthetic process	1.44	1.01	0.70	0.74	0.59	**0.44**	208
	ATSEH	AT2G26740	epoxide hydrolase	aromatic compound metabolic process	1.71	1.98	1.16	0.50	0.91	**0.45**	203
		AT3G25833	similar to myrcene/ocimene synthase, putative	other biological processes	2.13	1.05	0.49	0.40	0.68	**0.27**	196
		AT4G37970	mannitol dehydrogenase	other biological processes	1.88	1.53	0.81	0.54	0.87	**0.47**	153
		AT3G28450	leucine-rich repeat transmembrane protein kinase, putative	protein metabolism	1.39	1.05	0.76	0.56	0.66	**0.37**	151
		AT3G17610	bZIP family transcription factor	other biological processes	1.89	1.70	0.90	0.57	0.65	**0.37**	126
		AT4G05470	F-box protein family	protein metabolism	1.16	0.92	0.79	0.56	0.71	**0.39**	124
		AT3G46680	UDP-glucoronosyl/UDP-glucosyl transferase family protein	metabolic process	2.20	1.42	0.64	0.43	0.64	**0.28**	67

Supplemental Table II: Cadmium- and zinc-responsive genes in *T. caerulescens*

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR < 0.05) differential (≥ 2) expressed genes between two zinc/cadmium exposure conditions. Zn0 = 0 µM $ZnSO_4$; Zn100 = 100 µM $ZnSO_4$; Zn1000 = 1000 µM $ZnSO_4$; Cd0 = 0 µM $CdSO_4$, 100 µM $ZnSO_4$; Cd0.5 = 0.5 µM $CdSO_4$, 2 µM $ZnSO_4$; Cd50 = 50 µM $CdSO_4$, 2 µM $ZnSO_4$. [d]Normalized spotintensity at 100 µM $ZnSO_4$. Significant values are presented in **bold (FRD < 0.05)**.

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
I		AT5G03350	legume lectin family protein	other biological processes	1.91	**2.36**	1.24	1.35	**0.40**	2.33	0.91	1.22	0.73	0.99	571
		AT4G27160	NWMU3 - 2S albumin 3 precursor	transport	**2.18**	1.35	0.62	1.10	**0.45**	2.05	0.85	0.94	1.37	1.51	126
II		AT5G44040	expressed protein	other biological processes	**2.11**	1.13	0.54	0.51	1.72	0.88	0.81	**0.42**	1.52	0.78	44574
		AT5G45020	expressed protein	other biological processes	**3.01**	1.22	0.41	0.38	2.38	0.90	0.79	**0.30**	1.95	0.74	29390
	GLIP3	AT1G53990	GDSL-motif lipase/hydrolase family protein	electron transport or energy pathways	**1.82**	0.86	**0.47**	0.60	**1.78**	1.06	0.98	0.58	**2.08**	1.24	10807
	ATTI2	AT2G43520	putative trypsin inhibitor	defense response	**3.62**	1.17	**0.32**	0.68	1.52	1.03	0.42	**0.28**	1.30	0.88	7192
		AT5G61820	expressed protein	other biological processes	**2.48**	1.16	**0.47**	0.57	**2.22**	1.27	0.90	**0.51**	**1.91**	1.09	1854
		AT5G66170	senescence-associated protein	aging	**2.16**	0.99	0.46	0.47	1.55	0.73	0.72	**0.34**	1.56	0.74	630
	P5CS1	AT2G39800	delta-1-pyrroline 5-carboxylase synthetase	response to abiotic or biotic stimulus	**2.84**	1.07	**0.38**	**0.46**	**2.08**	0.96	0.73	**0.34**	**1.94**	0.90	555
III		AT1G74100	sulfotransferase family protein	other biological processes	**3.05**	**2.08**	0.68	0.73	**2.01**	1.48	0.66	**0.48**	0.97	0.71	13733
	SUR1	AT2G20610	aminotransferase, putative	signal transduction	**3.25**	**2.05**	0.63	0.66	**2.29**	1.51	0.71	**0.46**	1.12	0.74	13163
		AT5G43180	expressed protein	other biological processes	**2.17**	**1.85**	0.85	0.80	1.10	0.88	**0.51**	**0.41**	**0.60**	**0.48**	12528
		AT1G18590	sulfotransferase family protein	other biological processes	**3.12**	**2.26**	0.73	0.69	**1.87**	1.30	0.60	**0.42**	0.83	0.57	9570
		AT4G33420	peroxidase, putative	response to oxidative stress	**2.61**	**2.15**	0.82	0.68	**1.80**	1.23	0.69	**0.47**	0.84	**0.57**	9299
		AT1G74090	sulfotransferase family protein	other biological processes	**3.72**	**2.30**	0.62	0.78	**1.91**	1.49	0.51	**0.40**	0.83	0.65	9052
		AT2G41480	peroxidase, putative	response to oxidative stress	**2.03**	**1.64**	0.81	0.60	**1.67**	1.01	0.82	**0.50**	1.02	0.61	7578
	CYP83A1	AT4G13770	cytochrome p450 family	glucosinolate catabolic process	**4.51**	**2.91**	0.65	0.54	**2.78**	1.51	0.62	**0.34**	0.95	**0.52**	6314
	ATGSTF3	AT2G02930	glutathione S-transferase, putative	toxin catabolic process	**3.69**	1.80	0.49	0.59	2.13	1.25	0.58	**0.34**	1.18	0.70	5705
		AT1G20160	subtilisin-like serine protease	protein metabolism	**3.89**	**2.05**	0.53	0.58	**2.16**	1.25	0.56	**0.32**	1.05	0.61	4143
		AT4G20070	Allantoate Amidohydrolase	protein metabolism	**2.18**	**1.95**	0.89	0.84	2.28	**1.91**	1.04	**0.38**	1.17	**0.39**	3168
		AT4G28940	nucleosidase-related	other biological processes	**2.60**	1.75	0.68	0.57	**2.33**	1.33	0.90	0.51	1.33	0.76	1983
	ATHB5	AT5G65310	homeobox-leucine zipper protein	transcription	**2.05**	**1.73**	0.84	0.72	1.27	0.91	**0.62**	**0.44**	0.73	**0.53**	1802
		AT1G22190	AP2 domain transcription factor RAP2	transcription	**2.06**	1.38	0.67	0.72	1.14	0.83	0.55	**0.40**	0.83	0.60	1771

Continue Supplemental Table II

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
	AKN2	AT4G39940	adenylylsulfate kinase 2	sulfate assimilation	**3.74**	**2.36**	0.63	0.59	**2.63**	1.54	0.70	**0.41**	1.12	0.65	1757
	ZIFL	AT5G13750	transporter-like protein	transport	**4.14**	**2.44**	0.59	0.62	**2.49**	1.55	0.60	**0.37**	1.02	0.64	1693
		AT4G23920	UDPglucose 4-epimerase - like protein	galactose metabolic process	**2.17**	**2.12**	0.98	0.74	1.44	1.06	0.66	**0.49**	0.68	**0.50**	1691
	SAL1	AT5G63980	3(2),5-bisphosphate nucleotidase	response to abiotic or biotic stimulus	**3.04**	**2.32**	0.76	0.69	**1.97**	1.35	0.65	**0.44**	0.85	0.58	1469
	CYP79C2	AT1G58260	cytochrome p450 family	electron transport or energy pathways	**3.99**	2.38	0.60	0.48	**2.60**	1.25	0.65	**0.31**	1.09	0.53	1370
	APS3	AT4G14680	ATP-sulfurylase	sulfate assimilation	**4.30**	**3.58**	0.83	0.66	**2.69**	1.78	0.63	**0.41**	0.75	0.50	1191
	ATGSTU17	AT1G10370	glutathione transferase, putative	toxin catabolic process	**9.61**	**8.35**	0.87	0.62	**3.86**	2.39	0.40	**0.25**	0.46	**0.29**	1174
		AT4G11960	expressed protein	other biological processes	**2.08**	1.79	0.86	0.77	1.09	0.84	**0.52**	**0.40**	0.61	**0.47**	1060
		AT5G10770	chloroplast nucleoid DNA-binding protein	protein metabolism	**2.54**	2.22	0.87	0.52	**2.30**	1.21	0.91	0.47	1.04	0.54	953
		AT2G30830	2-oxoglutarate-dependent dioxygenase	other biological processes	**2.61**	**2.28**	0.88	0.63	1.78	1.13	0.68	**0.43**	0.78	0.49	908
	SULTR2;1	AT5G10180	low-affinity sulfate transporter	sulfate transport	**3.34**	**2.12**	0.63	0.51	**2.04**	1.04	**0.61**	**0.31**	0.96	0.49	811
		AT5G05600	2OG-Fe(II) oxygenase family protein	other biological processes	**2.86**	1.71	0.60	0.71	1.51	1.08	0.53	**0.38**	0.89	0.63	696
		AT1G76430	phosphate transporter family protein	transport	**2.28**	**1.99**	0.87	0.96	1.16	1.11	0.51	**0.49**	0.58	0.56	679
		AT5G64700	nodulin MtN21 family protein	other biological processes	**3.25**	**2.62**	0.81	0.76	**2.00**	1.52	**0.61**	**0.47**	0.76	**0.58**	631
	AKN1	AT2G14750	adenylylsulfate kinase 1	sulfate assimilation	**3.09**	**2.28**	0.74	0.83	1.78	1.47	0.58	**0.48**	0.78	0.64	622
	NIP6;1	AT1G80760	major intrinsic family protein	transport	**2.03**	1.85	0.91	0.70	1.39	0.98	0.69	**0.48**	0.75	0.53	545
		AT1G52700	phospholipase/carboxylesterase fam. prot.	other biological processes	**3.45**	**2.80**	0.81	0.58	**1.90**	1.10	0.55	**0.32**	0.68	**0.39**	489
		AT3G20015	aspartyl protease family protein	protein metabolism	**2.21**	1.64	0.74	0.52	**2.12**	1.09	0.96	0.49	1.30	0.67	435
		AT3G15650	phospholipase/carboxylesterase fam. prot.	other biological processes	**2.58**	**2.51**	0.97	0.58	**2.03**	1.19	0.79	**0.46**	0.81	**0.47**	240
		AT1G68830	STN7 protein kinase	electron transport or energy pathways	**2.10**	1.72	0.82	0.61	1.49	0.92	0.71	**0.44**	0.87	0.53	237
	CYP81D1	AT5G36220	cytochrome p450 family	electron transport or energy pathways	**8.17**	3.00	0.37	0.82	**3.07**	2.50	0.38	0.31	1.02	0.84	233
		AT1G11080	serine carboxypeptidase S10 fam. prot.	protein metabolism	**2.95**	1.77	0.60	0.54	**2.29**	1.23	0.78	**0.42**	1.29	0.69	232
	MYB28	AT5G61420	myb-related transcription factor	transcription	**2.17**	**2.53**	1.16	0.51	1.74	0.88	0.80	**0.41**	0.69	**0.35**	208
		AT2G17270	mitochondrial substrate carrier fam. prot.	transport	**2.14**	**2.17**	1.01	0.67	1.47	0.98	0.69	**0.46**	0.68	**0.45**	163
IV	WR3	AT5G50200	wound-responsive gene 3	nitrate transport	1.39	**2.50**	1.79	1.51	0.69	1.04	**0.49**	0.75	**0.28**	**0.42**	34841

Continue Supplemental Table II

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
	ZIP2	AT5G59520	putative zinc transporter	transport	**2.56**	**2.67**	1.04	1.30	1.07	1.39	**0.42**	0.54	**0.40**	0.52	3915
	ZIP9	AT4G33020	metal transporter, putative	transport	1.90	**3.45**	1.81	1.22	1.46	1.78	0.77	0.94	**0.42**	0.52	2123
		AT3G53960	proton-dependent oligopeptide transporter	transport	**1.99**	**2.53**	1.27	0.84	1.43	1.20	0.72	**0.60**	**0.56**	**0.47**	760
		AT1G68740	EXS family protein	other biological processes	1.73	**2.15**	1.24	0.65	1.16	0.75	0.67	**0.44**	**0.54**	**0.35**	698
		AT2G29300	tropinone reductase, putative	metabolic process	**2.80**	1.14	**0.41**	0.74	0.77	0.57	**0.28**	**0.20**	0.68	**0.50**	315
		AT4G15430	early-responsive to dehydration protein	other biological processes	2.24	**2.75**	1.23	0.62	1.30	0.81	0.58	**0.36**	0.47	**0.29**	236
	SIGE	AT5G24120	RNA polymerase sigma subunit SigE	transcription	1.51	**2.10**	1.39	**0.38**	0.80	**0.30**	0.53	**0.20**	**0.38**	**0.14**	137
		AT1G22730	MA3 domain-containing protein	other biological processes	**3.58**	**2.51**	0.70	1.84	1.31	**2.41**	**0.36**	0.36	0.67	0.52	120
		AT1G76570	chlorophyll A-B binding family protein	other metabolic processes	2.20	**2.45**	1.12	1.24	0.92	1.14	0.42	0.52	**0.37**	0.46	119
V	AIR1	AT4G12550	protease inh./seed storage/lipid trans. prot.	developmental processes	0.65	**0.46**	0.70	0.73	0.92	0.67	1.42	1.03	**2.01**	1.47	80711
	LEA14	AT1G01470	late embryogenesis abundant protein	response to abiotic or biotic stress	**0.59**	**0.49**	0.84	0.88	1.04	0.92	**1.77**	1.56	**2.12**	**1.87**	11042
		AT3G45160	expressed protein	other biological processes	0.88	**0.45**	**0.51**	0.61	1.32	0.81	1.50	0.92	**2.93**	**1.80**	8665
		AT5G02780	In2-1 protein, putative	other biological processes	1.58	0.54	**0.34**	**0.30**	**2.28**	0.69	1.45	**0.43**	**4.23**	1.27	4359
		AT4G10720	ankyrin repeat family protein	other biological processes	**0.51**	**0.46**	0.91	0.72	0.99	0.71	**1.94**	1.39	**2.13**	1.53	3404
	MYB111	AT3G46130	Myb family transcription factor	transcription	**0.48**	0.60	1.25	0.62	1.02	0.64	**2.11**	1.32	1.69	1.06	2712
		AT5G02620	ankyrin repeat family protein	other biological processes	**0.63**	**0.48**	0.76	0.73	1.05	0.76	**1.66**	1.21	**2.18**	**1.58**	2354
		AT3G29035	protein with transcription factor activity	developmental processes	0.59	**0.39**	0.66	0.67	1.12	0.75	1.91	1.28	**2.91**	1.95	1732
	CYP78A8	AT1G01190	cytochrome P450, putative	electron transport or energy pathways	0.60	**0.47**	0.79	0.60	1.46	0.88	**2.43**	1.46	**3.09**	1.86	1262
		AT1G54310	expressed protein	other biological processes	1.07	**0.45**	0.42	0.91	1.02	0.92	0.95	0.86	**2.26**	2.05	893
		AT4G00910	expressed protein	other biological processes	0.86	**0.46**	0.53	**0.40**	1.70	0.69	1.97	0.80	**3.73**	1.51	890
		AT1G36560	gypsy-like retrotransposon family	other biological processes	**2.11**	1.13	0.54	1.35	1.54	**2.08**	0.73	0.99	1.36	**1.84**	279
	ATHSP22.0	AT4G10250	heat shock protein 22.0	response to abiotic or biotic stress	0.69	**0.12**	0.17	0.54	0.94	0.51	1.36	0.73	**8.02**	4.32	243
		AT4G25720	glutamine cyclotransferase family protein	other biological processes	**2.42**	1.57	0.65	1.29	**1.89**	**2.43**	0.78	1.00	1.21	1.55	164
		AT5G63250	glycosyl hydrolase family 17	other biological processes	**2.61**	0.78	**0.30**	1.70	1.54	**2.62**	0.59	1.00	**1.97**	**3.34**	137
	TTN1	AT3G60740	tubulin folding cofactor D	protein metabolism	1.73	**0.13**	**0.08**	0.38	**0.45**	**0.46**	1.31	0.50	**17.30**	**6.62**	128

Continue Supplemental Table II

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
		AT4G17010	expressed protein	other biological processes	0.55	**0.36**	0.66	0.65	0.70	**0.45**	1.28	0.83	**1.93**	1.25	33680
		AT1G48330	expressed protein	other biological processes	0.53	**0.35**	0.67	0.70	0.64	**0.45**	1.22	0.86	1.81	1.27	32183
		AT1G05900	endonuclease-related	response to stress	0.50	**0.33**	0.67	0.66	0.62	**0.41**	1.25	0.83	1.87	1.23	28638
	APRR5	AT5G24470	pseudo-response regulator 5	developmental processes	**0.49**	**0.33**	0.68	0.67	0.67	**0.45**	1.36	0.92	**2.00**	1.34	27140
		AT1G34340	esterase/lipase/thioesterase family protein	other biological processes	**0.52**	**0.34**	0.66	0.66	0.64	**0.42**	1.23	0.81	**1.87**	1.23	26699
		AT4G34730	ribosome-binding factor A family protein	cell organization and biogenesis	**0.50**	**0.31**	0.62	0.65	0.61	**0.40**	1.22	0.79	**1.96**	1.27	21865
		AT4G25370	Clp amino terminal domain-containing protein	protein metabolism	**0.52**	**0.33**	0.64	0.62	0.66	**0.41**	1.29	0.79	**2.01**	1.24	21167
		AT2G16490	XH domain-containing protein	other biological processes	0.50	**0.31**	0.61	0.63	0.61	**0.39**	1.22	0.77	2.00	1.26	20672
		AT5G28894	copia-like retrotransposon family	other biological processes	**0.49**	**0.32**	0.65	0.68	0.58	**0.39**	1.19	0.81	1.83	1.24	19524
		AT3G54880	expressed protein	other biological processes	**0.54**	**0.42**	0.77	0.90	**0.53**	**0.48**	0.98	0.89	1.28	1.15	15676
		AT1G74200	leucine-rich repeat family protein	signal transduction	0.53	**0.33**	0.63	0.65	0.60	**0.39**	1.14	0.73	1.81	1.17	15492
	CLE2	AT4G18510	Clavata3 / ESR-Related-2	signal transduction	0.55	**0.36**	0.66	0.64	0.64	**0.41**	1.18	0.75	1.79	1.14	15028
	ATINT3	AT2G35740	sugar transporter family protein	transport	0.54	**0.36**	0.66	0.63	0.66	**0.42**	1.24	0.78	1.86	1.17	15005
		AT1G32580	plastid developmental protein DAG	other biological processes	0.53	**0.35**	0.66	0.68	0.62	**0.42**	1.17	0.80	1.78	1.22	13762
	CAM9	AT3G51920	calmodulin-9	signal transduction	0.60	**0.47**	0.79	0.76	0.59	**0.45**	0.99	0.75	1.26	0.96	13079
		AT5G55060	expressed protein	other biological processes	0.59	**0.35**	0.60	0.63	0.65	**0.41**	1.11	0.70	1.86	1.18	13077
		AT1G48650	helicase domain-containing protein	other biological processes	0.56	**0.35**	0.62	0.68	0.65	**0.44**	1.17	0.79	1.88	1.27	12580
		AT3G04560	expressed protein	other biological processes	0.58	**0.38**	0.65	0.67	0.68	**0.46**	1.19	0.80	1.82	1.22	12502
		AT4G23430	short-chain dehydrogenase/reductase fam. prot.	metabolic process	0.67	**0.44**	0.65	0.68	0.69	**0.47**	1.03	0.70	1.58	1.08	10511
		AT4G02170	expressed protein	other biological processes	0.53	**0.35**	0.65	0.62	0.67	**0.41**	1.25	0.77	1.92	1.18	9417
		AT1G24320	alpha-glucosidase	oligosaccharide metabolic process	**0.61**	**0.28**	**0.46**	**0.59**	**0.41**	**0.24**	0.67	**0.40**	**1.46**	0.86	7182
	bHLH100	AT2G41240	basic helix-loop-helix (bHLH) family protein	transcription	0.69	**0.20**	0.29	0.24	0.75	**0.18**	1.08	**0.26**	**3.78**	0.90	2251
		AT5G13910	member of the ERF transcript. factor fam.	transcription	0.61	**0.44**	0.73	0.70	**0.50**	**0.36**	0.83	**0.58**	1.14	0.80	2126
	ARPN	AT2G02850	plastocyanin-like domain-containing protein	developmental processes	**0.45**	**0.28**	0.61	0.39	0.64	**0.25**	1.42	0.55	**2.33**	0.91	895
		AT3G29190	terpene synthase/cyclase family	metabolic process	**0.33**	**0.18**	0.54	0.80	**0.29**	**0.23**	0.86	0.69	1.59	1.27	859

Continue Supplemental Table II

Cluster	Name	Code[a]	Putative function	GO Annotation[b]	Zn0/Zn100[c]	Zn0/Zn1000[c]	Zn100/Zn1000[c]	Cd0.5/Cd50[c]	Zn0/Cd0.5[c]	Zn0/Cd50[c]	Zn100/Cd0.5[c]	Zn100/Cd50[c]	Zn1000/Cd0.5[c]	Zn1000/Cd50[c]	Intensity Zn100[d]
		AT1G71050	heavy-metal-associated domain-containing protein	transport	**0.32**	**0.37**	1.16	0.73	**0.26**	**0.19**	0.81	0.60	0.70	0.51	977
		AT5G14360	ubiquitin family protein	protein metabolism	0.62	**0.47**	0.75	0.82	**0.34**	**0.28**	0.55	**0.45**	0.73	0.60	358
VII		AT3G18880	ribosomal protein S17 family protein	protein metabolism	0.71	**0.48**	0.68	1.92	**0.58**	1.11	0.81	1.56	1.20	**2.30**	1220
		AT1G15620	expressed protein	other biological processes	0.44	**0.18**	0.42	2.41	**0.25**	0.60	0.57	1.37	1.36	3.28	782
		AT1G72110	expressed protein	other biological processes	0.62	**0.32**	0.52	2.27	0.40	0.90	0.64	1.45	1.24	**2.82**	644
		AT5G09560	KH domain-containing protein	other biological processes	0.88	**0.49**	0.55	**2.38**	**0.60**	1.43	0.68	1.62	1.23	**2.93**	552
		AT1G72290	trypsin and protease inhibitor family protein	other biological processes	0.57	**0.40**	0.71	1.32	0.68	0.90	1.20	1.58	1.69	**2.24**	344
		AT1G73080	leucine-rich repeat receptor kinase	protein metabolism	0.73	**0.41**	0.56	1.81	**0.55**	0.99	0.75	1.36	1.35	**2.43**	255

Supplemental Table III: Differentially expressed genes verified by semi-quantitative RT-PCR

[a]Ratio of differential expression between two zinc or cadmium exposure conditions.

Arabidopsis					*T. caerulescens*				Microarray results								Semi-quantitative RT-PCR									
Primer pair for:	Source	PCR (bp)	Direction	Sequence	Source	PCR (bp)	Direction	Sequence	Tc0/Tc100[a]	Tc0/Tc1000[a]	Tc100/Tc1000[a]	TcCd0.5/TcCd50[a]	TcZn100/TcCd0.5[a]	TcZn100/TcCd50[a]	At0/At2[a]	At0/At25[a]	At2/At25[a]	Cd0/Cd15[a]	Tc0/Tc100[a]	Tc0/Tc1000[a]	Tc100/Tc1000[a]	TcCd0.5/TcCd50[a]	Zn100/Cd0.5[a]	Zn100/Cd50[a]	At0/At2[a]	At0/At25[a]	At2/At25[a]	Cd0/Cd15[a]
ATH13	At5g64940	430	F	TCGTCTCTGCTTTTACCAAACATC		430	F	TCGTCTCCTCCTGTTACCGAACAT	**4.1**	3.6	0.9	1.0	**0.2**	**0.2**	**0.9**	1.0	1.1	**1.4**	0.6	0.4	0.6	1.0	0.4	0.4	1.8	1.4	0.8	0.6
			R	TGTTGTTCTTAAACCAAGCATCCT			R	TGGCTCTTCTTAAACCAAGCATCC																			
bHLH100	At2g41240	170	F	GACGGTGACTCATGAGAATGTGTCG	DQ384057	170	F	GACGGTGACTCATGAGAATGTGTCG	0.7	0.2	**0.3**	0.2	**1.1**	0.3	0.3	0.0	**0.0**	**0.1**	0.3	0.2	0.9	0.7	0.8	0.6	1.1	0.1	0.1	0.1
			R	TCTTCGTTTGATTGGTGGGAGG			R	TCTTCGTTTGATTGGTGGGAGG																			
FRD3	At3g08040	218	F	AAGGGCATTTCTTCACCTACA		218	F	AGTAACAACACCAACCAGCC	1.3	1.2	0.9	0.9	0.9	0.8	**4.2**	**7.1**	1.7	**2.2**	0.8	0.4	0.5	0.9	0.5	0.4	2.4	1.8	0.7	0.6
			R	TTCACTCCCATGACGCCTA			R	GCACCTTCGAACTGAAAATC																			
FRO2	At1g01580	212	F	CTGCAGGAGAAGAATTTAAGGATA		212	F	CTGCGGGAGAGAGTTTAAGGCTAT	0.9	0.4	0.5	0.9	0.6	0.6	**0.3**	**0.0**	**0.1**	**0.3**	0.6	0.2	0.3	1.5	0.2	0.3	1.7	1.1	0.7	0.6
			R	GACCATCATTGGGAACATATACAT			R	GGCCATCATTGGGAACATATACAT																			
FRO4	At5g23980	104	F	CCAGAAGAAACCAACCCAACCT	DQ384058	197	F	CCAGAAGAAACCCACCCAGA	6.0	7.2	**1.2**	1.6	**0.1**	**0.1**	2.4	17.2	**7.3**	**2.3**	1.2	0.5	0.4	1.1	0.2	0.2	4.3	5.4	1.2	0.5
			R	ACTCCGTGGCTGTCACTATCCC			R	ATTCTGTGGCTGTCACTATCCCCA																			
MYB72	At1g56160	509	F	ATGGGGAAAGGAAGAGCACCATGC			F		1.0	0.7	0.7	0.7	0.8	0.6	0.4	**0.0**	**0.1**	**0.3**										
			R	CACTCGCCTTGGTGATGTGACGAA			R																				
NAS4	At1g56430	475	F	ACCAACTCGTAAACAAGATCTGCGA		475	F	ACCAGCTAGTGAGCACGATCT	**4.9**	**4.2**	0.9	0.6	1.0	0.6	30.6	13.1	0.4	0.3	1.1	0.5	0.5	0.8	0.4	0.3	2.4	2.2	0.9	1.4
			R	ACCATATTCGCTGATGGGTCGATGT			R	ACCGAATTCGCTGTTGAGTC																			
ORG1	At5g53450	389	F	CCAAATCTGCCGAATTTACAAGTGT		389	F	CCAAACTCGGAACTGGAAGTGTTG	1.0	0.7	**0.7**	0.6	**1.1**	0.7	0.8	**0.1**	**0.2**	**0.3**	0.4	0.2	0.5	1.1	0.4	0.4	1.6	0.6	0.4	0.4
			R	CTTCTTCCTCGTCTTTGAGCTCGA			R	CTTCTTCCTCGTCTTTGAGCGTGA																			
ORG3	At3g56980	160	F	TGAAATAGACAACAATCCGGTGGTCGT		160	F	TGAGATAGGCAGCAATCCCGTCGT	2.0	1.2	0.6	0.9	0.7	0.6	1.0	**0.1**	**0.1**	**0.1**	0.8	0.4	0.5	0.9	0.4	0.3	6.5	1.4	0.2	0.3
			R	TCGAGAAACCGTCGCAGGAATGCT			R	CCTTGAAACCGTCGCAGGAATACT																			
WR3	At5g50200	285	F	GCTTCACTTCTCATATGCTCACTGAT		285	F	GCTTCACTTCTCGTTTGCTTACTG	1.4	**2.5**	1.8	1.5	**0.5**	0.7	1.2	0.9	0.8	0.8	0.3	0.2	0.5	1.2	0.3	0.3	1.7	1.4	0.8	0.6
			R	GAAGAGCTCGTCATGGGTTTTG			R	TTGAAGAGCTCATTATGGGTCTTG																			
	At1g52820	440	F	CCTTCGCCTTCCGGTCATCGATTT		440	F	CCTCTTCACCTCCCACTCATCGAT	0.4	0.7	1.7	1.8	1.3	2.4	1.0	7.5	7.6	4.0	0.7	0.4	0.6	1.0	0.5	0.5	2.1	2.8	1.4	0.8
			R	CGAGTCCGAAGCTCTCCATGATCA			R	CACGAGTCCGAAGCTCTCCATGTA																			
Tubulin	At1g04820	405	F	AAGCTTGCTGATAACTGTACTGGT		270	F	CCTACGCACCAGTCATCTCT	1.1	1.0	0.9	0.9	1.2	1.1	0.9	1.0	1.1	0.9	0.8	0.5	0.7	1.0	0.6	0.6	1.7	1.4	0.8	0.6
			R	GGTTTGGAACTCAGTGACATCA			R	CGAGATCACCTCCTGGAACA																			

F = Forward primer; R = Reversed primer

Chapter 4

Expression differences in genes for cell wall modification and stress response in the Zn/Cd-hyperaccumulator *Thlaspi caerulescens* acc. Ganges and La Calamine in response to cadmium

Judith E. van de Mortel, Diederik Wehkamp and Mark G.M. Aarts

ABSTRACT

Cadmium is a health-threatening pollutant and cadmium accumulation in plants affects photosynthesis and water and nutrient uptake and results in chlorosis, growth inhibition, browning of the root tips and finally death. Although cadmium is very toxic to most plants, the metal hyperaccumulator *Thlaspi caerulescens* can accumulate high amounts of cadmium without any sign of toxicity. *T. caerulescens* accession GA has the capability to accumulate high amounts of cadmium and is extremely tolerant to cadmium, whereas the La Calamine accession is also tolerant but its capability to accumulate cadmium is much less compared to Ganges. We examined in detail the transcription profiles of leaves and roots of *T. caerulescens* accessions Ganges and La Calamine grown with and without cadmium using the Qiagen-Operon Arabidopsis Genome Array. A total of 161 genes with at least a two-fold difference in expression were found when comparing the two accessions of *T. caerulescens* in response to changes in cadmium supply and 38 genes were significantly differentially expressed in *T. caerulescens* accession GA leaves in response to Cd. The comparative transcriptional analysis emphasized that there are just minor differences between the two accessions but the genes that are differentially expressed could play an important role in the hyperaccumulation of cadmium in Ganges. The microarray data suggest that especially genes involved in cell wall modification and stress response cause the major difference between the two accessions in cadmium hyperaccumulation.

INTRODUCTION

Thlaspi caerulescens J. & C. Presl (*T. caerulescens*) is a metal hyperaccumulating species belonging to the Brassicaceae family, with on average 88.5 % DNA identity with the coding regions of the model plant *Arabidopsis thaliana* (L.) Heynh. (Rigola et al., 2006), and one of the model species used to study plant heavy metal hyperaccumulation (Assunção et al., 2003a). *T. caerulescens* is a widespread but uncommon species of Western and Central Europe, growing on metalliferous and nonmetalliferous soil from Poland and the Czech Republic in the east, to Great Britain in the west and as south as to northern Spain (Reeves et al., 2001). This species has the ability to hyperaccumulate zinc (Zn) but occasionally also nickel (Ni) and cadmium (Cd) (Baker and Brooks, 1989; Lombi et al., 2000). While some heavy metals, such as Zn, Cu and Fe are essential minerals for the growth of plants, others are toxic to plants especially when available at high concentrations. Hyperaccumulators accumulate these heavy metals to extremely high amounts in their aboveground tissues without showing any sign of toxicity. Among the different *T. caerulescens* accessions there is considerable variation for metal specificity, tolerance and accumulation (Meerts and van Isacker, 1997; Escarré et al., 2000; Lombi et al., 2000; Schat et al., 2000; Pollard et al., 2002; Assunção et al., 2003b; Meerts et al., 2003). *T. caerulescens* accessions La Calamine (LC) and Ganges (GA) are more tolerant to Zn and Cd compared to the accessions Lellingen (LE) and Monte Prinzera (MP) (Assunção et al., 2003a). In contrast to other accessions the accession GA has also been shown to be a Cd hyperaccumulator, accumulating more than 10,000 ppm Cd in the dry weight of its aboveground parts (Lombi et al., 2000). Recent transcript profiling studies of the hyperaccumulators *A. halleri* and *T. caerulescens* and the non-accumulator *T. arvense* (Becher et al., 2004; Hammond et al., 2006; van de Mortel et al., 2006; Weber et al., 2004, 2006) revealed that the transcriptional regulation of many genes is strikingly different in the hyperaccumulator species compared to *A. thaliana* in response to Zn or Cd.

Although genes involved in heavy metal homeostasis, such as those encoding transporters and heavy metal chelators, are good candidates for studying tolerance and accumulation, the knowledge about the transcriptional networks involved in these traits remains limited (Chiang et al., 2006). Therefore we compared the transcriptional profile of roots and leaves of *T.*

caerulescens accessions LC and GA exposed to a low Cd concentration (1µM $CdSO_4$) or grown on medium without Cd, using the Qiagen-Operon Arabidopsis Genome Array. The analysis is expected to identify genes that are differentially regulated between these two accessions, which may give a lead as to how the exceptional Cd accumulation of GA is controlled.

RESULTS

Experimental design

To analyse the response of *T. caerulescens* accessions GA and LC to different cadmium (Cd) exposures, we compared the transcript profiles of plants grown under sufficient nutrient supply without Cd (0 µM $CdSO_4$) with plants grown under the same conditions but with Cd (1 µM $CdSO_4$) added. To minimize variation in the bioavailability of micronutrients we used a hydroponic instead of a soil-based culture system.

Figure 1. A loop-design including dye swaps was used to set-up the microarray experiment. cDNA from four biological replicates of *T. caerulescens* (Tc) accession La Calamine (LC) and Ganges (GA) roots and leaves exposed to 100 µM (sufficient) zinc (Zn100) with (1 µM $CdSO_4$) or without (0 µM $CdSO_4$) cadmium (Cd) was labelled with fluorescent dye Cyanine 3 (Cy3, grey) or Cyanine 5 (Cy5, black). Two biological replicates were hybridised according to this set-up and two biological replicates were dye-swapped.

The *T. caerulescens* accessions GA and LC were cultivated on a nutrient solution containing 100 µM $ZnSO_4$, which yields healthy and robust plants with normal seed set. After three weeks the plants were transferred to fresh solutions containing 100 µM $ZnSO_4$ and 1 µM $CdSO_4$. Half of the plants remained at sufficient Zn (100 µM $ZnSO_4$) as a control. Upon harvesting root and shoot tissue, the plants growing on medium with Cd or without Cd did not show any visible phenotypic differences (data not shown). At this stage, plants did not flower. A loop design (Kerr and Churchill, 2001), including dye swaps, was used for the setup of the microarray experiment (Figure 1). The advantage of a loop design is that this setup collects twice as much data on the variables of interest (Kerr and Churchill, 2001). Furthermore, variables are balanced with respect to dyes because each variety is labeled once with the red and green dye (Kerr and Churchill, 2001). In total four biological replicates were used.

Cadmium-response within *T. caerulescens* acc. La Calamine and Ganges

Genes expressed in leaves and roots of *T. caerulescens* acc. GA and LC grown on normal medium and on medium containing Cd were identified using Qiagen-Operon Arabidopsis Genome Array (Microarray AtOligo2.3.2.y (http://www.ag.arizona.edu/microarray/)) containing 27,216 70-mer oligonucleotides representing ~80% of the Arabidopsis genome. When analyzing the data, we only considered the hybridization data of probes with a False Discovery Rate (FDR) of <0.05. Expression differences of ≥ 2-fold (between the treatments) were considered to be biologically relevant, even though lower expression differences were statistically significant.

At first we determined which genes were differentially expressed within each accession when comparing plants grown on medium with and medium without Cd. For the Cd tolerant and Cd hyperaccumulating accession GA, we only found significantly differentially expressed genes (38) when comparing leaf samples (Table I), but not for roots. Three of these genes are repressed by Cd, the others are induced. One of the repressed genes is a calcium binding protein, the others have no obvious relationship to metal homeostasis. Of the 35 genes that are induced upon Cd exposure in GA leaves eight genes encode proline-rich extensin-like family proteins, which are involved in cell wall organization. Another gene also affecting cell wall modification encodes for a pectin methylesterase.

Table I: Differentially expressed genes in leaves of *T. caerulescens* acc. Ganges at Cd exposure (1 µM $CdSO_4$) vs. no Cd exposure.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 2) expressed genes between two cadmium exposure conditions. L0 = 0 µM $CdSO_4$, 100 µM $ZnSO_4$; L1 = 1 µM $CdSO_4$, 100 µM $ZnSO_4$.

Name	Code[a]	Putative function	GO Annotation[b]	L0/L1[c]
	At4g08390	stromal ascorbate peroxidase, putative (sAPX)	response to oxidative stress	3.60
	At1g21550	calcium-binding protein	biological process unknown	2.75
	At3g24830	60S ribosomal protein L13A (RPL13aB)	protein metabolism	2.33
	At5g09350	phosphatidylinositol 4-kinase	phosphoinositide biosynthesis	0.50
	At4g09500	glycosyltransferase family protein	biological process unknown	0.50
CYP705A3	At4g15360	cytochrome p450 family	electron transport and energy pathways	0.50
	At4g20690	expressed protein	biological process unknown	0.50
PIP2B	At2g37170	aquaporin (plasma membrane intrinsic protein 2B)	water transport	0.49
	At4g03930	pectin methylesterase	cell wall modification	0.49
	At4g14390	ankyrin repeat family protein	biological process unknown	0.49
CTF2B	At2g29720	monooxygenase family	electron transport and energy pathways	0.49
	At4g27810	expressed protein	biological process unknown	0.48
ATEXT3	At1g21310	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.48
	At4g38940	kelch repeat-containing F-box family protein	biological process unknown	0.48
	At4g08370	proline-rich extensin-like family protein	cell organization and biogenesis	0.48
	At1g35350	similar to EXS family protein	biological process unknown	0.48
NSF	At4g04910	AAA-type ATPase family protein	biological process unknown	0.47
	At4g25300	oxidoreductase, 2OG-Fe(II) oxygenase family protein	flavonoid biosynthesis	0.47
	At2g02660	expressed protein	biological process unknown	0.47
	At1g42440	expressed protein	biological process unknown	0.47
ATDI19	At1g56280	drought-responsive family protein	response to water deprivation	0.47
SFP1	At5g27350	sugar-porter family protein 1	response to abiotic or biotic stimulus	0.47
MIF3	At1g18830	Zinc finger homeobox family protein	biological process unknown	0.46
	At3g47890	ubiquitin carboxyl-terminal hydrolase-related	ubiquitin-dependent protein catabolism	0.46
	At4g27830	glycosyl hydrolase family 1	carbohydrate metabolism	0.45
	At4g04310	zinc knuckle (CCHC type) protein family	biological process unknown	0.45
	At5g49080	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.45
ATEXT4	At1g76930	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.45
	At4g04760	putative sugar transporter	carbohydrate transport	0.44
	At3g45410	receptor-like protein kinase	biological process unknown	0.43
	At4g04420	putative transposon protein	biological process unknown	0.42
	At4g11410	short-chain dehydrogenase/reductase (SDR) family protein	metabolism	0.42
PAP2/MYB90	At1g66390	Myb-related transcription factor	regulation of transcription	0.41
	At4g08410	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.41
	At4g08400	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.41
	At3g28550	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.40
	At1g59030	similar to putative anter-specific proline-rich protein	lipid metabolism	0.39
AtEXT2	At3g54590	proline-rich extensin-like family protein	cell wall organization and biogenesis	0.39

In addition, two genes involved in the regulation of water transport or deprivation (*PIP2B* and *ATDI19*) are induced by Cd exposure. Other genes induced by Cd are related to stress-response (*CYP705A3*, *CTF2B*, *SFP1*), transcription (*MIF3*, *PAP2*, At5g57660) and transport (At4g04760). There were no differentially expressed genes identified when comparing LC roots and leaves grown with and without Cd. Overall, the transcriptional response of plants to Cd exposure is very modest, suggesting that plants hardly sense the used Cd exposure concentration, or that the response is not expressed at the transcript level at this time point.

Differences between *T. caerulescens* accessions Ganges and La Calamine under normal growth conditions

The higher Cd tolerance and accumulation of GA compared to LC, may be due to constitutive gene expression differences between both accessions and not be much affected by exposure to Cd. We therefore compared leaves and roots of both accessions exposed to the same Cd concentrations in the medium. When comparing leaves of GA and LC grown on normal medium (no Cd) 15 genes were found to be significantly differentially expressed (Table II, Figure 2). Of these genes, eight are higher expressed in GA and seven are higher expressed in LC. The GA-enhanced genes are involved in stress-response (*CYP709B3*, a peroxidase, citrate synthase-like protein) and transcription (bZIP family transcription factor). Two genes encode proteins with an unknown function of which one encodes a non-annotated protein. Another gene encodes a metallothionein gene (*MT3*), which has previously been implicated in metal homeostasis (Hassinen et al., 2007) and particularly in controlling Cu-homeostasis of a Cd-hyperaccumulating accession similar to GA (Roosens et al., 2004). The seven genes higher expressed in LC leaves encode for a heat shock protein 70 (*CPHSC70-2*), the stress-response protein *KIN2*, the phosphate transporter *HAK5*, a jacalin lectin family protein and three genes encoding glycin-rich proteins (*GRP14*, *GRP3S* and At2g05510).

When comparing the roots of both accessions, 10 genes were significantly differentially expressed (Table III, figure 2), five of which are higher expressed in GA and five in LC (Table III). Among the GA induced genes is a gene encoding an SPX domain-containing protein, which is almost 30 times higher expressed in GA. SPX domains are also found in the inorganic

phosphate transporter family PHO. Blast analysis showed that the predicted protein also shares similarities with major facilitator superfamily (MFS) proteins, involved in transporting small organic compounds. Also among the GA enhanced genes is again the metallothionein protein *MT3*, which was also higher expressed in GA leaves compared to LC leaves. The most dramatic difference between LC and GA roots was a glycosyl hydrolase gene (10-fold higher in LC vs GA), which in Arabidopsis is induced upon (a)biotic stresses (www.arabidopsis.org).

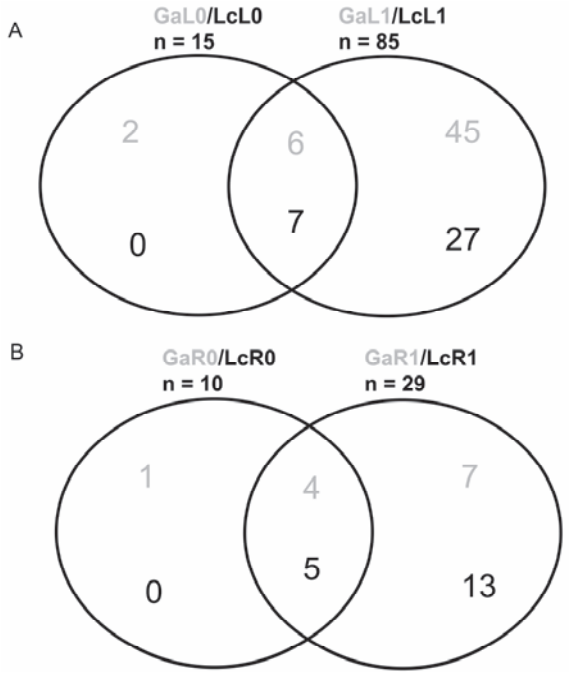

Figure 2. Venndiagram showing the number of genes differentially expressed between *T. caerulescens* accessions Ganges (GA) and La Calamine (LC) leaves (A) and roots (B) exposed to sufficient zinc (100 µM $ZnSO_4$) without cadmium (0 µM $CdSO_4$) or with cadmium (1 µM $CdSO_4$). Using the Qiagen-Operon Arabidopsis Genome Array, in total 161 genes with at least a two-fold difference in expression were identified in *T. caerulescens*. L = leaves; R = roots; 0, 1 = concentration $CdSO_4$ in µM.

Table II: Differentially expressed genes in leaves of *T. caerulescens* accessions GA and LC grown under normal Zn supply (100 µM $ZnSO_4$) in the absence of Cd.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 2) expressed genes between 0 and 1 µM cadmium exposure conditions. Ga = *T. caerulescens* acc. Ganges; Lc = *T. caerulescens* acc. La Calamine; L0 = leaves at 0 µM $CdSO_4$, 100 µM $ZnSO_4$. xxxx = probe between two annotated genes.

Name	Code[a]	Putative function	GO Annotation[b]	GaL0/LcL0[c]
CYP709B3	At4g27710	cytochrome P450 - like protein	electron transport and energy pathways	8.13
bZIP53	At3g62420	bZIP family transcription factor	regulation of transcription	4.44
MT3	At3g15353	metallothionein protein	copper ion homeostasis	4.19
	At5g59080	expressed protein	biological process unknown	3.37
	At3g28200	peroxidase	response to oxidative stress	3.17
	At3g52470	hairpin-induced protein	biological process unknown	2.68
	At3g58740	citrate synthase -like protein	electron transport and energy pathways	2.67
	xxxx	expressed protein (At1g65295.1) and MADS-box protein (At1g65300.1)	biological process unknown	2.34
HAK5	At4g13420	potassium transporter	transport	0.44
CPHSC70-2	At5g49910	heat shock protein 70	response to stress	0.23
GRP14	At5g07510	glycine-rich protein	nutrient reservoir activity	0.18
GRP3S	At2g05380	glycine-rich protein	biological process unknown	0.17
KIN2	At5g15970	stress-responsive protein	response to stress	0.14
	At3g16430	jacalin lectin family protein	biological process unknown	0.14
	At2g05510	glycine-rich protein	biological process unknown	0.12

Table III: Differentially expressed genes in roots of *T. caerulescens* accessions GA and LC grown under normal Zn supply (100 µM $ZnSO_4$) in the absence of Cd.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 2) expressed genes between two *T. caerulescens* accessions. Ga = Ganges; Lc = La Calamine; R0 = 0 µM $CdSO_4$, 100 µM $ZnSO_4$.

Name	Code[a]	Putative function	GO Annotation[b]	GaR0/LcR0[c]
	At4g22990	SPX (SYG1/Pho81/XPR1) domain-containing protein	biological process unknown	28.79
	At1g63450	exostosin family protein	biological process unknown	7.77
MT3	At3g15353	metallothionein protein	copper ion homeostasis	2.95
	At3g30390	amino acid transporter family protein	amino acid transport	2.70
TCTP	At3g16640	translationally controlled tumor protein-like protein	biological process unknown	2.29
	At5g19440	alcohol dehydrogenase	biological process unknown	0.26
TET8	At2g23810	Member of TETRASPANIN family	aging	0.23
	At4g27260	encodes an IAA-amido synthase	auxin homeostasis	0.19
	At4g25880	pumilio-like protein	biological process unknown	0.16
	At4g19810	glycosyl hydrolase family 18	carbohydrate metabolism	0.10

Differences between *T. caerulescens* accessions Ganges and La Calamine under cadmium exposure

Whereas the difference between the two accessions on normal medium was modest and often did not reach the threshold of >2-fold expression difference, the comparison of both accessions at Cd exposure revealed more genes, 85 and 29, to be differentially expressed when comparing leaves, resp. roots (Tables IV and V respectively). A summary of all comparisons is shown in Figure 2. Of the 85 genes that were differentially expressed in leaves, 51 genes were higher expressed in GA and 34 were higher in LC. Thirteen of the 85 genes were already found to be differentially expressed between GA and LC under normal growth conditions (Table II). Higher expressed in GA leaves are genes involved in transport (5 genes), transcription (5 genes), signalling (1 gene), stress-response (4 genes) and general metabolism (4 genes). Most differentially expressed in GA compared to LC (~40-fold) is the gene encoding the *AHA2* H^+-transporting ATPase, involved in external acidification and providing the proton motive force driving secondary transporters (Morsomme and Boutry, 2000). Another seven genes are >20-fold enhanced in GA, four of which are annotated as hypothetical or expressed proteins and two others appear to be involved in signal transduction. Five genes encoding transcription factors are induced in GA, two *MYB*-genes, two b/HD-ZIPs and one Zn-finger gene. Two genes have a supposed function in metal homeostasis, the metallothionein gene *MT3* and a copper homeostasis factor. Of the 34 genes higher expressed in LC leaves there are two cation transporter genes, one encodes *NRAMP4*, a member of the Natural Resistance Associated Macrophage protein (NRAMP) family, which is known to play a role in metal transport (Thomine et al., 2000), the other is *HAK5*, a potassium transporter. Other genes are involved in stress-response (3 genes), protein metabolism (5 genes) and transcription (1 gene).

Of the 29 genes that are differentially expressed between GA and LC in roots, 11 genes are higher expressed in GA and 18 in LC (Table V). Of the 11 GA-enhanced genes, four are also higher expressed in GA roots under normal growth conditions (Table III, Figure 2). The probe indicating the gene with the highest expression difference between GA and LC is associated with the intergenic region between At4g00340 and At4g00350 in the Arabidopsis genome. The second most differentially expressed gene is again encoding the SPX-domain protein, most differentially

Table IV: Differentially expressed genes in leaves of *T. caerulescens* accessions GA and LC grown under cadmium exposure conditions (1 µM $CdSO_4$.

[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 2) expressed genes between two *T. caerulescens* accessions. Ga = Ganges; Lc = La Calamine; L1 = 1 µM $CdSO_4$, 100 µM $ZnSO_4$. xxxx = probe between two annotated genes.

Name	Code[a]	Putative function	GO Annotation[b]	GaL1/LcL1[c]
AHA2	At4g30190	putative H^+-transporting ATPase	transport	39.12
	At1g36970	expressed protein	biological process unknown	29.81
	At3g43460	hypothetical protein	biological process unknown	27.67
	At4g02760	F-box family protein	biological process unknown	26.91
	At2g19700	expressed protein	biological process unknown	25.73
	At4g23060	similar to calmodulin-binding family protein	biological process unknown	25.17
	At1g05450	protease inhibitor/seed storage/lipid transfer protein (LTP)-related	biological process unknown	21.94
	At5g61300	expressed protein	biological process unknown	20.33
SQP1,2	At5g24160	squalene monooxygenase 1,2	sterol biosynthesis	15.27
	At1g18950	aminoacyl-tRNA synthetase family	biological process unknown	15.01
	At2g41390	expressed protein	biological process unknown	9.15
PAP1/MYB75	At1g56650	myb-related protein	regulation of transcription	8.59
	At3g62740	glycosyl hydrolase family 1	carbohydrate metabolism	8.57
	At2g18680	expressed protein	biological process unknown	8.49
	At5g11750	ribosomal protein L19 family protein	protein biosynthesis	7.99
GAE6	At3g23820	NAD dependent epimerase	nucleotide-sugar metabolism	7.40
	At5g44390	FAD-linked oxidoreductase family	electron transport and energy pathways	6.49
	At3g52470	hairpin-induced protein	biological process unknown	6.01
CYP709B3	At4g27710	cytochrome P450 - like protein	electron transport and energy pathways	5.27
	At4g34900	xanthine dehydrogenase	electron transport and energy pathways	4.88
MYB44	At5g67300	putative myb-related protein	regulation of transcription	4.66
	xxxx	lipid transfer protein (At3g18280) and zinc finger protein (At3g18290)	biological process unknown	4.31
MT3	At3g15353	metallothionein protein	copper ion homeostasis	4.03

Continue Table IV

Name	Code[a]	Putative function	GO Annotation[b]	GaL1/LcL1[c]
bZIP53	At3g62420	bZIP family transcription factor	regulation of transcription	3.60
	At2g18620	geranylgeranyl pyrophosphate synthase	isoprenoid biosynthesis	3.37
	At5g35830	ankyrin repeat family protein	biological process unknown	3.18
	At5g14490	no apical meristem (NAM) family protein	developmental processes	3.13
	At4g22770	DNA-binding family protein	biological process unknown	3.04
	At1g48640	lysine and histidine specific transporter	amino acid transport	2.88
	At3g22620	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	transport	2.87
ATX1	At1g66240	copper homeostasis factor	copper ion homeostasis	2.83
	At3g60810	expressed protein	biological process unknown	2.82
	At4g33690	similar to RNA recognition motif (RRM)-containing protein	biological process unknown	2.82
PMP	At3g24160	type 1 membrane protein	biological process unknown	2.82
RALFL32	At4g14010	rapid alkalinization factor (RALF) family protein	cell-cell signaling	2.81
	At5g59080	expressed protein	biological process unknown	2.78
	At5g55410	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	lipid transport	2.75
	At2g40070	expressed protein	biological process unknown	2.49
AMY1	At4g25000	alpha-amylase - like protein	response to gibberellic acid stimulus	2.42
	At1g13930	expressed protein	biological process unknown	2.34
	At2g31670	expressed protein	biological process unknown	2.32
	At2g05530	glycine-rich protein	biological process unknown	2.31
HB23	At1g26960	HD-Zip transcription factor	regulation of transcription	2.28
	At4g25750	ABC transporter family protein	transport	2.18
	At5g15060	expressed protein	biological process unknown	2.15
	At4g14370	disease resistance protein (TIR-NBS-LRR class)	defense response	2.14
	xxxx	disease resistance protein (At1g12290) and helicase domain-containing protein (At1g12300)	biological process unknown	2.12
UBC26	At1g53020	ubiquitin-conjugating enzyme	protein metabolism	2.10
	At4g09740	glycosyl hydrolase family 9	carbohydrate metabolism	2.09

Continue Table IV

Name	Code[a]	Putative function	GO Annotation[b]	GaL1/LcL1[c]
	At5g66270	zinc finger (CCCH-type) family protein	regulation of transcription	2.02
PER31	At3g28200	peroxidase	response to stress	2.00
ASK13	At3g60010	E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 (At13)	biological process unknown	0.47
	At3g12850	COP9 signalosome complex-related	biological process unknown	0.46
GDCH	At2g35370	glycine cleavage system H protein 1	photorespiration	0.44
CPHSC70-2	At5g49910	heat shock protein 70	response to stress	0.44
	At4g02710	kinase interacting family protein	biological process unknown	0.43
	At3g59540	60S RIBOSOMAL PROTEIN L38-like protein	protein metabolism	0.43
	At2g47640	small nuclear ribonucleoprotein D2	mRNA metabolism	0.43
SAP1	At1g12440	zinc finger (AN1-like) family protein	regulation of transcription	0.41
CPHSC70-2	At5g49910	heat shock protein 70	response to stress	0.41
	At3g46320	histone H4	protein metabolism	0.41
	At5g52930	expressed protein	biological process unknown	0.41
	At1g20140	E3 ubiquitin ligase SCF complex subunit	protein metabolism	0.40
BTI1	At4g23630	reticulon family protein (RTNLB1)	biological process unknown	0.40
CYP71B2	At1g13080	cytochrome p450 family	electron transport and energy pathways	0.39
	At5g46430	ribosomal protein L32	protein biosynthesis	0.38
CPHSC70-2	At5g49910	heat shock protein 70	response to stress	0.38
MTHFR2	At2g44160	methylenetetrahydrofolate reductase 2	methionine metabolism	0.37
GRXS15	At3g15660	glutaredoxin family protein	cell redox homeostasis	0.37
	At4g27020	expressed protein	biological process unknown	0.36
MTHFR2	At2g44160	methylenetetrahydrofolate reductase 2	methionine metabolism	0.32
PIP2A	At3g53420	plasma membrane intrinsic protein 2A	transport	0.31
GRP14	At5g07510	glycine-rich protein	nutrient reservoir activity	0.30
	At5g37690	GDSL-motif lipase/hydrolase family protein	lipid metabolism	0.30
GRP3S	At2g05380	glycine-rich protein	biological process unknown	0.27

Continue Table IV

Name	Code[a]	Putative function	GO Annotation[b]	GaL1/LcL1[c]
HAK5	At4g13420	potassium transporter	transport	0.27
NRAMP4	At5g67330	NRAMP metal ion transporter 4	metal ion homeostasis	0.23
	At2g05510	glycine-rich protein	biological process unknown	0.21
	At3g03150	expressed protein	biological process unknown	0.19
KIN2	At5g15970	stress-responsive protein	response to stress	0.15
	At2g34585	expressed protein	biological process unknown	0.15
RABA2d	At5g59150	Ras-related GTP-binding protein	protein transport	0.14
	At4g01590	expressed protein	biological process unknown	0.14
	At3g16430	jacalin lectin family protein	biological process unknown	0.11
	At1g72510	expressed protein	biological process unknown	0.07

expressed between GA and LC roots under normal conditions. Also the metallothionein gene *MT3* is higher expressed in GA than in LC roots under Cd exposure. In LC roots 18 genes are higher expressed than in GA roots when exposed to Cd. Five of these 18 genes were also found to be differentially expressed in the absence of Cd, including the most differentially expressed glycosyl hydrolase gene (Table III). Among the remaining 13 genes is another glycosyl hydrolase and genes involved in stress-response (2 genes), protein metabolism (2 genes) and signal transduction (2 proteins).

Semi-quantitative RT-PCR

For validation of the microarray expression profiling data, the expression of a subset of differentially expressed genes was determined by semi-quantitative RT-PCR. Expression of the target genes was determined for both accessions in root and leaf tissue of plants of an additional biological replica, including plants exposed to different Zn concentrations (Figure 3). In addition to the genes that were significantly expressed in the microarray experiment also the expression of *FRO2* and *NAS2* were determined by semi-quantitative RT-PCR because these genes were found to be differentially expressed in the root/leaf comparison (Figure 3). Microarray data for the genes selected for semi-quantitative RT-PCR analysis are shown in Supplemental Table I. In general the semi-quantitative RT-PCR confirmed the microarray analysis, although there were differences between the microarray data and the semi-quantitative RT-PCR for some genes. Most striking is the difference regarding the H^{+}-transporting ATPase *AHA2*. Whereas the microarray data indicated a strongly enhanced expression in GA vs. LC, this was not very clear in the RT-PCR analysis. This may be caused by differences in gene sequence, causing differences in probe hybridisation efficiency, or by the cross-hybridisation of the probe to another, closely related gene, which is differentially expressed. When considering the expression in response to zinc in the roots and leaves, the difference between the two accessions was not so striking. *FRO2* is constitutively higher expressed in the roots of both accessions and this gene is also slightly induced in the leaves of both accessions (Figure 3) in response to Zn. *NAS2* shows a similar expression pattern as *FRO2* but the expression in LC roots is slightly higher compared to GA roots (Figure 3) in response to Zn and Cd. *HAK5* is also constitutive higher expressed in the roots

compared to the leaves of GA and LC but in addition this gene is also higher expressed in LC leaves compared to GA leaves in response to Zn and Cd (Figure 3). The metallothionein *MT3* is constitutively expressed in the leaves of GA and LC but the expression in GA is higher compared to LC (Figure 3). In the roots of GA this gene is more induced under 10 µM $CdSO_4$, whereas in LC the expression in the roots is constitutive (Figure 3). The oligopeptide transporter *OPT3* is lower expressed in the roots of GA and LC under Zn deficient and 1 µM $CdSO_4$ conditions but in the leaves its expression is more or less constitutive (Figure 3). The genes encoding an expressed protein (At1g72510) and a glycosyl hydrolase 18 (At4g19810) are more or less constitutively expressed in GA and LC roots and leaves (Figure 3), whereas from the microarray data the expressed protein seemed more induced in *T. caerulescens* acc. LC compared to GA and the glycosyl hydrolase 18 is more induced in LC roots compared to GA roots.

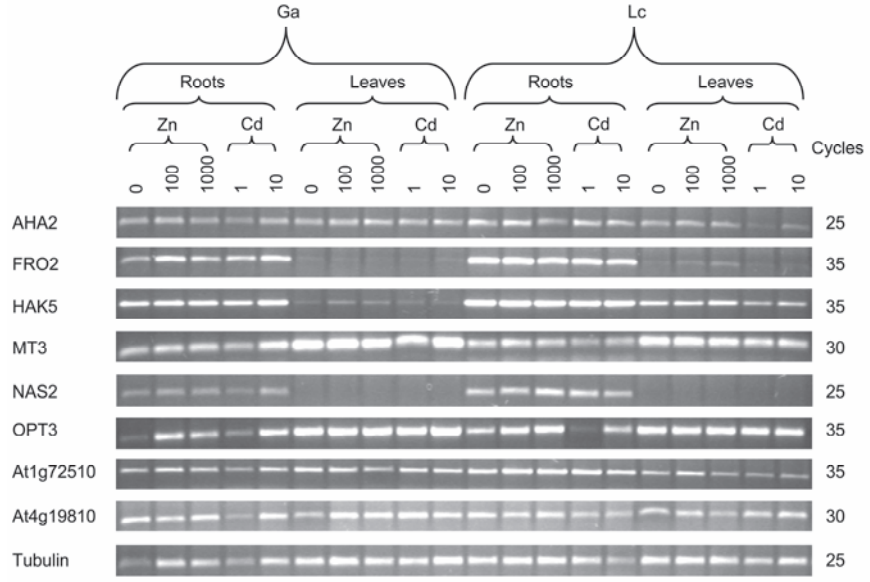

Figure 3. Semi-quantitative RT-PCR of differentially expressed genes in *T. caerulescens* accession Ganges (GA) and La Calamine (LC) in response to different Zn and Cd concentrations in the growing medium. Roots and leaves of four-week-old plants were harvested separately after one week of exposure to 0 µM $ZnSO_4$, 100 µM $ZnSO_4$, 1000 µM $ZnSO_4$, 1 µM $CdSO_4$ (100 µM $ZnSO_4$) and 10 µM $CdSO_4$ (100 µM $ZnSO_4$) for both accessions. *AHA2*, H^+-transporting ATPase, At4g30190; *FRO2*, ferric chelate reductase-like, At1g01580; *HAK5*, potassium transporter, At4g13420; *NAS2*, nicotianamine synthase, At5g56080; *OPT3*, oligopeptide transporter, At4g16370; Expressed protein, At1g72510; Glycosyl hydrolase 18, At4g19810; Tubulin (At1g04820) was used as a control for equal cDNA use.

Table V: Differentially expressed genes in roots of *T. caerulescens* accessions GA and LC grown under cadmium exposure conditions (1 µM $CdSO_4$).
[a]AGI gene code (At..). [b]GO annotations according to biological process. [c]Ratio of significant (FDR p < 0.05) differential (≥ 2) expressed genes between two *T. caerulescens* accessions.
Ga = Ganges; Lc = La Calamine; R1 = 1 µM $CdSO_4$, 100 µM $ZnSO_4$. xxxx = probe between two annotated genes

Name	Code[a]	Putative function	GO Annotation[b]	GaR1/LcR1[c]
	xxxx	Similar to receptor-like protein kinase precursor (At4g00340) and MATE efflux family protein (At4g00350)	biological process unknown	30.46
	At4g22990	SPX (SYG1/Pho81/XPR1) domain-containing protein	biological process unknown	13.14
	At5g48750	auxin-responsive protein	extracellular matrix organization and biogenesis	11.62
	At4g34900	xanthine dehydrogenase	allantoin biosynthesis	5.28
	At1g79600	ABC1 family protein	biological process unknown	4.73
	xxxx	cytochrome p450 (At5g38970) and expressed protein (At5g38980)	biological process unknown	4.51
	At1g10810	aldo/keto reductase family protein	biological process unknown	3.02
MT3	At3g15353	metallothionein protein	copper ion homeostasis	2.54
OPT3	At4g16370	oligopeptide transporter OPT family protein	oligopeptide transport	2.53
	At3g30390	amino acid transporter family protein	amino acid transport	2.27
TCTP	At3g16640	translationally controlled tumor protein-like protein	biological process unknown	2.03
	At3g28940	avirulence-responsive protein	response to abiotic or biotic stimulus	0.48
CAM9	At3g51920	calmodulin-9	signal transduction	0.46
TET8	At2g23810	Member of TETRASPANIN family	aging	0.38
	At5g35830	ankyrin repeat family protein	biological process unknown	0.33
	xxxx	Expressed protein (At5g38035) and glucosyltransferase-like protein (At5g38040)	biological process unknown	0.28
APUM5	At4g25880	pumilio-like protein	RNA binding	0.27
	At5g37290	armadillo/beta-catenin repeat family protein	biological process unknown	0.26
	At5g06520	SWAP (Suppressor-of-White-APricot)/surp domain-containing protein	DNA or RNA binding	0.25
	At5g19440	alcohol dehydrogenase	biological process unknown	0.24
	At4g21370	S-locus protein kinase	protein metabolism	0.22
	At3g23880	F-box protein family	biological process unknown	0.19
	At3g05300	cytidine/deoxycytidylate deaminase family protein	biological process unknown	0.18
	At5g50340	DNA repair protein-like	response to abiotic or biotic stimulus	0.15
	At4g27260	encodes an IAA-amido synthase	auxin homeostasis	0.14
	At2g36550	expressed protein	biological process unknown	0.12
	At4g19810	glycosyl hydrolase family 18	carbohydrate metabolism	0.08
	At1g66530	arginyl-tRNA synthetase	protein metabolism	0.07
	At4g19770	glycosyl hydrolase family 18	carbohydrate metabolism	0.04

DISCUSSION

In order to identify genes involved in Cd accumulation we investigated the response of *T. caerulescens* accessions GA and LC to Cd exposure. We expected that genes that are differentially expressed between the two accessions might be crucial for the cadmium hyperaccumulation trait, which differs between the accessions studied here (Assunção et al., 2003b). In this study the accessions GA and LC were used and these accessions are known to be more tolerant to Zn and Cd than non-metallicolous and serpentine ones. However, LC has a much lower Cd accumulation rate compared to GA (Assunção et al., 2003b). Because the study by Assunção et al. (2003b) showed that the difference in accumulation rate between the two accessions gave the biggest effect between 0.5 and 5.0 µM $CdSO_4$ (15-17 times lower in LC compared to GA), we decided to use a 1 µM $CdSO_4$ supply level to identify expression differences between the two accessions. To our knowledge not many expression-profiling experiments have been performed in which different accessions of the same species are compared. In Arabidopsis 60 genes of which the mRNA levels differed in different accession backgrounds in an organ-dependent manner were identified (Chen et al., 2005). Genes that showed a substantial genetic variation in mRNA level were those with a function in signal transduction, transcription and stress response, suggesting the existence of variations in the regulatory mechanisms for these genes among different accessions (Chen et al., 2005). Also for *T. caerulescens* a microarray experiment was performed in which two different accessions, LC and LE, were compared in response to zinc (Plessl et al., 2005) using a microarray containing ~ 1900 cDNAs.

In total 115 genes were found to be significantly differentially expressed between the two accessions (Table II-V) and only 38 genes were significantly differently expressed in GA leaves grown with or without Cd (Table I). This limited response to heavy metals was also found in other microarray studies in which hyperaccumulators were exposed to different concentrations of Zn (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006; van de Mortel et al., 2006) or Cd (Weber et al., 2006; van de Mortel et al., chapter 3). In general hyperaccumulators show a much more constitutive (high) expression of metal homeostasis genes, or at least much less

dependent on the concentration of metals in the roots than in non-accumulator species (Becher et al., 2004; Weber et al., 2004, 2006; Hammond et al., 2006; van de Mortel et al., 2006). Apparently the constitutive expression in the roots of LC could be a reason for the fact that there are no genes significantly differentially expressed in LC leaves grown with or without Cd and might correlate with the reduced accumulation of Cd in LC compared to other *T. caerulescens* accessions (van de Mortel et al., 2006).

However in the leaves of the GA accession, genes were differentially expressed in response to Cd exposure. Eight genes belong to the proline-rich extensin-like protein family (Table I), which are found in cell walls of higher plants (Showalter, 1993). The genes encoding proline-rich extensin-like proteins *AtEXT2*, *AtEXT3* and *AtEXT4* are known to be induced by various stresses (Showalter, 1993; Yoshiba et al., 2001). Another gene induced by Cd in GA, which is involved in cell wall modification encodes a pectin methylesterase. Pectin methylesterase is important in plant development and defense by influencing the susceptibility of the wall (Lionetti et al., 2007). Induction of these genes by Cd suggests that modifications in cell wall structure are important to cope with Cd exposure in the GA accession.

The MYB-related transcription factor *PAP2* is higher expressed in GA Cd treated leaves. *PAP2* is suggested to have a function in the regulation of the phenylpropanoid biosynthesis pathway (Borevitz et al., 2000) leading to flavonoids, anthocyanins and lignins, which putatively links this gene to changes in the cell wall structure. Also the general flavonoid pathway regulator *PAP1* was differentially expressed between GA and LC leaves in response to Cd, which could be related to a different response of the genes in both accessions to stress or due to their role in cell wall structure as suggested for *PAP2*. It is possible that *T. caerulescens* acc. GA has adapted to hyperaccumulation of Cd by inducing a stress-response mechanism different from that in LC.

It is known that Cd accumulation in plants affects photosynthesis, water- and nutrient uptake and results in chlorosis, growth inhibition, browning of the root tips and finally death (Kahle, 1993). Two genes involved in water transport (*PIP2*B) or deprivation (*ATDI19*) are higher expressed under Cd exposure in GA leaves suggesting that water uptake and transport mechanism are affected in the hyperaccumulating species and might require upregulating these genes.

Genes differentially expressed between the two accessions are thought to be more important for the hyperaccumulation trait, assuming that most genes will be constitutively expressed between the two accessions. The metallothionein *MT3* was higher expressed in GA roots and leaves compared to LC independent of the Cd exposure (Table II-V). Metallothioneins have been identified in plants, animals, eukaryotic microorganisms and certain prokaryotes (Rauser, 1999). While their exact function remains a matter of debate, they appear to play a role in metal tolerance and/or homeostasis. Metallothioneins are known to chelate metals like Zn, Cd and Cu (Romero-Isart and Vasak, 2002). An earlier study suggested that *TcMT3 is* more involved in ensuring adequate Cu homeostasis and that it seemed not responsible for Cd tolerance in *T. caerulescens* (Roosens et al., 2004). However, because it is more or less constitutively expressed both in GA and LC (Figure 3) it is suggested that this gene could be involved in hyperaccumulation of Cd in *T. caerulescens*.

Seven genes are higher expressed in leaves of *T. caerulescens* acc. LC compared to GA leaves independent of the growth condition (Table II and IV). These are *HAK5*, *CPHSC70-2*, *KIN2*, *GRP14*, *GRPS3*, At2g05510 and At3g16430. One of these genes is the potassium transporter *HAK5*, which is a member of the KUP/HAK family of K^+ transporters. Armengaud et al. (2004) found that *HAK5* was induced upon potassium starvation in Arabidopsis and van de Mortel et al. (2006) also identified *HAK5* as one of the genes that was much higher expressed in *T. caerulescens* LC roots compared to Arabidopsis. In this study *HAK5* is equally expressed in the roots of GA and LC (Figure 3) but higher expressed in LC leaves, *HAK5* might be more induced because of potassium starvation in the leaves of LC. This gene could also be involved in the general stress response, which might occur in LC leaves but not in GA leaves. Maksymiec et al. (2005) recently showed that heavy metal stress induces jasmonic acid (JA) accumulation in plants and Armengaud et al. (2004) suggested a relation between potassium starvation and JA signaling. Some of the other genes (*CPHSC70-2*, *KIN2*) that were more induced in LC leaves are also stress-related and also point in the same direction.

The number of genes differentially expressed between GA and LC is higher when both accessions are exposed to 1 µM $CdSO_4$ (Table IV, V). Considering the difference in Cd accumulation between the two accessions, some of these differentially expressed genes may be

functionally related to Cd accumulation. One obvious candidate is the plasma membrane H^+-ATPase gene *AHA2,* which is constitutively expressed in GA but repressed in LC leaves exposed to Cd (Figure 3). A plasma membrane H^+-ATPase actively pumps protons from the cytoplasm to the apoplasm and generates an electrochemical potential difference across the membrane. This, in turn, activates solute transport through a variety of transporters and channels (Moriau et al., 1999). This could suggest that *AHA2* is triggering the transport mechanism of Cd across membranes in GA. Another gene involved in transport is a lysine and histidine specific transporter. Although histidine is known to be a principal ligand for Zn, Cu and Ni (Rauser, 1999) it is not for Cd. This could mean that GA is disrupted in the Cu homeostasis mechanism in the leaves, which would also explain why the plant reacts with a higher expression of *MT3* and a copper homeostasis factor.

The metal transporter *NRAMP4* is higher expressed in LC leaves compared to GA leaves exposed to Cd. In Arabidopsis it was shown that both *NRAMP3* and *NRAMP4* are involved in the mobilization of vacuolar Fe stores during seed germination (Lanquar et al., 2005). Expression studies in *T. caerulescens* acc. GA and LC indicated that *TcNRAMP4* was more induced in *T. caerulescens* acc. LC leaves under excess Zn and 10 μM $CdSO_4$ and in the roots under Zn deficiency conditions whereas in *T. caerulescens* acc. GA this gene is constitutively expressed in leaves and roots (Jian Wu, personal communication). The specific induction of *TcNRAMP4* gene expression in LC leaves exposed to Cd, suggests, together with expression comparison data of *T. caerulescens* acc. GA and LC (Jian Wu, personal communication) that this gene is involved in mobilization of Zn or Fe but not of Cd in *T. caerulescens* acc. LC leaves.

When comparing the roots of *T. caerulescens* acc. GA and LC exposed to Cd (Table V), the oligopeptide transporter *OPT3* was more expressed in GA (Table V, Figure 3). The OPT3 protein of Arabidopsis was suggested to direct long-distance movement of metal-chelate complexes in the plant (Stacey et al., 2006) and we previously found that the gene was induced under excess Zn in Arabidopsis together with iron homeostasis genes (van de Mortel et al., 2006). Their slightly reduced expression both in GA and LC after exposure to Cd could imply that these plants are not Fe deficient. Recently, Plaza et al. (2007) showed that *TcIRT1* expression was enhanced by Fe deficiency or by exposure to Cd. The functions of *TcIRT1* from Ganges and

Prayon and the Arabidopsis homologue were analyzed by heterologous expression in yeast. All three *IRT1* genes were able to facilitate growth on low Fe concentrations. Cd sensitivity of yeast was conferred in the order *AtIRT1>TcIRT1-1GA>TcIRT1-2P*. Cd uptake after four hours was only detectable following complementation by *AtIRT1*. The results suggest that although *TcIRT1-GA* may be involved in Cd hyperaccumulation in the GA accession of *T. caerulescens*, the transporter expressed in yeast does not have an enhanced ability to transport Cd compared with *AtIRT1*.

In conclusion, the comparative transcriptional analysis of *T. caerulescens* acc. GA and LC emphasizes that there are minor differences between the two accessions but the genes which are differentially expressed could play an important role in the hyperaccumulation of Cd in GA. These data suggest that especially genes involved in cell wall modification and stress response cause the major difference between the two accessions in Cd hyperaccumulation. In addition we obtained indications that the two accessions respond differently in their expression of stress related genes, which could indicate differences in stress levels or stress types experienced by the two accessions or differences in the way they regulate their stress-related genes.

MATERIAL AND METODS

Plant material and growth conditions

Thlaspi caerulescens J. & C. Presl accessions La Calamine (LC) and Ganges (GA) seeds were sterilized, sown, germinated and grown on vertical 1% agar plates containing half-strength MS (Murashige and Skoog) medium (GibcoBRL, Life Technologies, Rockville, MD) in a climate chamber (25/25°C day/night temperatures; 250 µmoles light m^{-2} s^{-1} at plant level during 24 h (75% RH). Three-week-old seedlings were transferred to 600-ml polyethylene pots (three plants per pot) containing a modified half-strength Hoagland's nutrient solution (Schat et al., 1996): 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM H_3BO_3, 100 µM $ZnSO_4$, 2 µM $MnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA. The pH buffer MES was added at a 2-mM concentration and the pH was set at 5.5 using KOH. Each polyethylene pot contained three seedlings of *T. caerulescens* acc. LC or GA. Plants were grown

for three weeks on this medium, which was refreshed weekly. Then, the plants were transferred and grown for one week on the same modified half-strength Hoagland's nutrient solution containing sufficient Zn (100 μM $ZnSO_4$) with (1 μM) or without (0 μM) $CdSO_4$. During this week the nutrient solution was refreshed twice. Plants were grown in a climate chamber (20/15°C day/night temperatures; 250 μmoles light m^{-2} s^{-1} at plant level during 12 h/d (75% RH)).

Microarray experiment

A loop design (Kerr and Churchill, 2001), including dye swaps, was used for the setup of the microarray experiment (Figure 1). cDNA samples from *T. caerulescens* acc. LC or GA roots or leaves exposed to 100 μM (sufficient) Zn, with or without Cd, were labeled with fluorescent dye Cyanine 3 (Cy3) or Cyanine 5 (Cy5). Roots of two pots containing three *T. caerulescens* LC or three GA plants per treatment were pooled and homogenized in liquid nitrogen. These two pools of three plants were considered as one biological replicate and four biological replicates were used. Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Between 280 and 900 mg tissue was used for the RNA extraction performed according to the manufacturer's instructions. After the total RNA extraction, poly A^+ RNA was isolated with Dynabeads® Oligo $(dT)_{25}$ (Dynal Biotech, Hamburg, Germany). One to two μg of poly A^+ RNA was used to synthesize cDNA with Superscript Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and random primers. The cDNA was labeled with Cyanine 3-dCTP or Cyanine 5-dCTP and hybridized to the Qiagen-Operon Arabidopsis Genome Array (Microarray AtOligo2.3.2.y) containing 27,216 70-mer oligonucleotides representing ~80% of the Arabidopsis transcriptome (www.ag.arizona.edu/microarray). After hybridization the slides were scanned with a ScanArray 3000 (Packard BioChip Technologies, Billerica, MA, USA) and analyzed with ImaGene (BioDiscovery, El Segundo, CA, USA).

Statistical analysis of the microarray data was performed with the Microarray Analysis of Variance (MA ANOVA) software package (Kerr et al., 2000) (http://www.jax.org/staff/churchill/labsite/software/anova/index.html), implemented in the R programming language (http://www.jax.org/staff/churchill/labsite/software/Rmaanova/index.html). First the data were log-transformed and subjected to joint spatial and intensity based Lowess normalization. After

normalization, we have fitted a linear, fixed effect model, accounting for array and dye effects and a factor to capture the relevant experimental information. Our experimental design involves three factors with two levels each (Table VI). Because the current implementation of MA ANOVA does not allow for three-way interaction terms to be tested, we have constructed a single factor, in which each level corresponds to a specific combination of the three experimental factors in our study. Because we analyze a system with three factors of two levels each, our combined factor contains $2^3 = 8$ levels. Next, all pair wise combinations of the eight levels could be tested. For example, the first two levels of the constructed factor are:

1 - accession = LC tissue = Root and diet = **0 Cd.**

2 - accession = LC, tissue = Root and diet = **1 Cd.**

Table VI: The three experimental factors in the microarray experiment.
LC is *T. caerulescens* accession La Calamine, GA is accession Ganges.

Factor	Levels	
Accession	LC	GA
Tissue	Root	Leaves
Diet	0 µmol Cd	1 µmol Cd

The comparison of these two levels results in genes in *T. caerulescens* acc. LC roots with significant different levels of expression as a result of the different diet. All other 27 combinations of two levels are tested correspondingly. F-tests were performed to obtain gene-specific p-values for each test. In particular, the Fs statistics (Cui and Churchill, 2003) was employed, using random permutations (1000 iterations) to assess significance instead of relying on tabulated F values. The p-values were adjusted for the FDR and a cutoff of 0.05 was applied to select the significant genes for each comparison. To identify differentially expressed genes a fold change cut-off was taken of ≥ 2.

Semi-quantitative RT-PCR

Total RNA of leaves and roots of a fifth biological replica, each containing 12-18 plants, was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Selected *T. caerulescens* acc. LC

genomic and cDNA fragments were PCR-amplified using primers designed for the orthologous Arabidopsis gene and sequenced. New primers were designed for semi-quantitative RT-PCR to ensure amplification of the correct *T. caerulescens* gene (Supplemental Table I). Five µg of total RNA was used to synthesize cDNA with MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) as a primer (Invitrogen, Carlsbad, CA, USA). The PCR-amplification was performed with a cDNA aliquot (2 µl) and gene-specific primers (Supplemental Table I). Primers for Tubulin (Supplemental Table I) were used as a control for similar cDNA quantity between the samples (Hassinen et al., 2006). Between 25- to 35-cycle PCRs (30 s at 94 °C, 30 s at 50 °C, and 60 s at 68 °C) were performed in a 50-µl volume, with the same number of cycles for *T. caerulescens* acc. LC and GA samples. 50 µl of the reaction was separated on an ethidium-bromide-stained 1%-agarose gel.

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Supplemental Table I: Validation of differentially expressed *T. caerulescens* genes by semi-quantitative RT-PCR in roots and leaves of the accessions Ganges and La Calamine grown at different Zn and Cd exposure conditions.

[a]Ratio of differential expression between two zinc or cadmium exposure conditions. Ga =. Ganges; Lc = La Calamine; L = leaves; R = roots; 0 = 0 µM $CdSO_4$, 100 µM $ZnSO_4$; 1 = 1 µM $CdSO_4$, 100 µM $ZnSO_4$. Significantly differentially expressed data are presented in **bold** (FDR < 0.05).

Primer pair for:	Source	Putative function	PCR (bp)	Direction	Sequence	GaL0/GaL1[a]	LcL0/LcL1[a]	GaR0/GaR1[a]	LcR0/LcR1[a]	GaL0/LcL0[a]	GaR0/LcR0[a]	GaL1/LcL1[a]	GaR1/LcR1[a]	GaR0/GaL0[a]	LcR0/LcL0[a]	GaR1/GaL1[a]	LcR1/LcL1[a]
AHA2	At4g30190	putative H+-transporting ATPase	357	Forward	AATTCCGATTGAGGAAGTTTTCCAG	0.80	1.89	1.75	0.47	16.64	54.96	**39.12**	14.81	0.70	1.52	1.06	0.94
				Reversed	AGCACCTTGGTTTTAGGGGCAA												
FRO2	At1g01580	ferric-chelate reductase	212	Forward	CTGCGGGAGAGAGTTTAAGGCTAT	0.96	1.42	0.35	0.61	2.20	0.99	3.25	1.71	3.79	**8.42**	10.37	**19.70**
				Reversed	GGCCATCATTGGGAACATATACAT												
HAK5	At4g13420	potassium transporter	340	Forward	TGGGGCACTTCCATGGTCATAGGT	4.98	3.05	0.63	0.58	**0.44**	0.74	**0.27**	0.69	8.14	4.80	**64.92**	25.12
				Reversed	GGAAAACACCTCCGAGGGAGATCC												
MT3	At3g15353	metallothionein protein	150	Forward	CTGTTGTGACAAGACCCAGTGCG	1.20	1.15	0.90	0.78	**4.19**	**2.95**	**4.03**	**2.54**	0.18	0.26	0.24	0.39
				Reversed	CAGCTGCAGGTAGAGCCGCA												
NAS2	At5g56080	nicotianamine synthase	262	Forward	ATGGCTTGCGAAAACAACCT	0.65	0.73	0.68	1.05	0.73	0.43	0.82	0.66	**3.42**	**5.85**	3.24	4.03
				Reversed	GAGAGCCTATGATTGTGGAGAA												
OPT3	At4g16370	oligopeptide transporter OPT fam. prot.	440	Forward	GAAGTTGGCTCGGACTCTTCCC	0.57	0.67	1.04	1.74	1.58	1.51	1.85	**2.53**	0.50	0.52	0.27	**0.20**
				Reversed	ATAGATATCCTGGGAAGCGCGT												
	At1g72510	expressed protein	375	Forward	CCACGAAGCTATTCACACCAGA	0.55	0.79	0.96	0.69	0.05	0.46	**0.07**	0.33	0.66	0.07	0.37	0.08
				Reversed	GCATTCTTGGTGAATCTAAGCTTCT												
	At4g19810	glycosyl hydrolase family 18	270	Forward	GGGGTGCGGTTCTACTCCAACACT	0.70	1.08	1.30	1.16	1.27	**0.10**	1.94	0.08	0.90	11.97	0.48	11.13
				Reversed	GACGCCATGCGTAGCCGTAGTATG												
	At1g04820	Tubulin	270	Forward	CCTACGCACCAGTCATCTCT												
				Reversed	CGAGATCACCTCCTGGAACA												

Chapter 5

Nicotianamine synthases control metal distribution and enhance metal tolerance in Arabidopsis and the related metal hyperaccumulator *Thlaspi caerulescens*

Judith E. van de Mortel, Wu Jian, Marcel van Culemborg, Liana Tsiatsiani, Hetty Blankestijn, Henk Schat and Mark G.M. Aarts

ABSTRACT

Nicotianamine (NA) is a non-proteinogenic amino acid often found in plants to chelate heavy metals, like Fe, Mn, Ni or Zn. NA is formed by trimerization of S-adenosylmethionine, which is catalyzed by the enzyme NA synthase (NAS). Arabidopsis contains four *NAS* genes. Here we report the cloning of full-length cDNAs of four *NAS* genes of *T. caerulescens* accession La Calamine, which were named *TcNAS1, TcNAS2, TcNAS3* and *TcNAS4* based on their homology to *Arabidopsis thaliana* genes *AtNAS1, AtNAS2, AtNAS3* and *AtNAS4* respectively. Evidence for a functional role of NAS in metal homeostasis was sought in studying the *Arabidopsis thaliana* single, double, triple and a quadruple *nas* T-DNA insertion mutants. The combination of null mutations in three or four *AtNAS* genes, results in a severe phenotype that includes interveinal chlorosis and altered metal concentrations in leaves, roots and seeds. *Arabidopsis thaliana* wild-type and *nas* multiple-mutant plants over-expressing *TcNAS1, TcNAS2, TcNAS3* or *TcNAS4* were tested for their response to growth on media with different zinc, iron, nickel and/or cadmium supply. This provided evidence that all Thlaspi genes encode genuine NAS proteins, as they all complement the NAS deficiency in specific triple knock-out mutants. *Arabidopsis thaliana* wild-type plants overexpressing *TcNAS3* or *TcNAS4* showed an enhanced zinc and nickel tolerant phenotype compared to wild type plants.

INTRODUCTION

Micronutrients are elements which are essential for plant growth and play an important role in physiological and metabolic processes of the plant (Ramesh et al., 2004), but are required in much smaller amounts than the major nutrients, nitrogen, phosphorus and potassium. Micronutrient deficiencies in crops are wide-spread, as revealed by soil and plant analysis. However, some micronutrients can also be very toxic when available to the plant at excessive concentrations. Pollution of soil with metals is a global problem occurring through mining but also through industrial and agricultural processes.

Plants have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001), in which transmembrane metal transporters play a crucial role. A number of genes encoding potential metal transporters have been identified and characterized including heavy metal ATPases (HMAs), members of the natural resistance associated macrophage proteins (NRAMP), the cation diffusion facilitators (CDF) , ZRT- and IRT-like proteins (ZIPs) and the cation antiporters (Kim et al., 2005). These transport systems are thought to be involved in metal homeostasis.

One potential mechanism for heavy metal detoxification in plants is the chelation of metal ions to ligands like organic acids, amino acids, peptides and polypeptides. Chelation is important for the allocation of metals within the plant body, because it influences their mobility within the plant. Nicotianamine (NA) is known to act as a metal ligand. NA is a non-proteinogenic amino acid produced by the trimerization of S- adenolsylmethionine (SAM) catalyzed by the enzyme nicotianamine synthase (NAS) in both graminaceous (Shojima et al., 1989a; Shojima et al., 1989b) and non-graminaceous plants (Shojima et al., 1989a; Higuchi et al., 1995; Higuchi et al., 1996).

In contrast to most other plant species, graminaceous plants use a specific strategy for iron acquisition called Strategy II. Rather than reducing Fe(III) to Fe (II) by ferric reductases, which can be taken up through IRT-like transporters (Strategy I), these plants secrete phytosiderophores that chelate Fe (III), which can be taken up by the root cells through YS-like transporters (Didonato et al., 2004). These phytosiderophores are derived from NA. As a first step,

nicotianamine aminotransferase (NAAT) catalyzes the aminotransfer of NA, converting it to deoxymugeneic acid (DMA). DMA is further hydroxylated in subsequent steps to form other mugeneic acids (MAs) (Mori and Nishizawa, 1987; Shojima et al., 1990; Takahashi et al., 2003). MAs are the only examples of plant specific siderophores known so far (Takagi, 1976). Nongraminaceous plants do not produce MAs and here NA has been implicated in the internal transport of metal ions (Scholz et al., 1992; Stephan and Scholz, 1993; Stephan et al., 1994). Unlike MAs, NA is not secreted and forms in vitro stable complexes with Fe^{2+}, Fe^{3+}, Cu^{2+}, Zn^{2+}, Ni^{2+} and Mn^{2+} (Didonato et al., 2004).

The genes encoding NAS were first identified in tobacco (Noma et al., 1971) and were thereafter identified in other plants like *Hordeum vulgare* (Herbik et al., 1999; Higuchi et al., 1999; Kim et al., 2005), *Lycopersicon esculentum* (Ling et al., 1999), *Arabidopsis thaliana* (Suzuki et al., 2001), *Oryza sativum* (Higuchi et al., 2001; Inoue et al., 2003), *Zea mays* (Mizuno et al., 2003), *Arabidopsis halleri* (Becher et al., 2004; Weber et al., 2004), and *Thlaspi caerulescens* (Pianelli et al., 2005; van de Mortel et al., 2006). NAS was thought to be plant specific with only one very distant related gene in archeon *Methanobacterium thermoautotrophicum*. However, recently Trampczynska et al. (2006) identified a functional nicotianamine synthase in the filamentous fungus *Neurospora crassa*.

The tomato *chloronerva* (*chln*) mutant was the first plant mutant shown to be deficient in NA. It exhibits retarded growth of the whole plant, stunted roots, thickened root tips, interveinal chlorosis of the young leaves and defects in metal accumulation and translocation (Stephan and Scholz, 1993; Herbik et al., 1996). The absence of NA was caused by a single base pair change in the *CHLORONERVA* gene, which encodes nicotianamine synthase (Ling et al., 1999). The uptake mechanism in the *chln* mutant is constitutively expressed and the mutant accumulates more copper, iron, manganese and zinc in all tissues compared to the wild type (Scholz et al., 1987; Stephan and Grün, 1989; Pich and Scholz, 1996; Ling et al., 1999). Iron and manganese were accumulated in the leaves of the NA-less mutant *chln*, whereas the accumulation of copper and zinc was restricted to the roots and the axis (Pich et al., 1994) In strategy I plants such as tomato, failure of NA synthesis leads to the permanent activation of iron acquisition processes in roots including proton extrusion and increased ferric chelate reductase activity. Thus, in mutant leaves

iron is accumulated to nonphysiologically high concentrations leading to the precipitation of iron in different cell compartments and organelles (Becker et al., 1995; Liu et al., 1998)

NAS activity was found to be significantly induced by Fe-deficiency in five graminaceous plants (rice, sorghum, maize, wheat and barley) (Higuchi et al., 1996) but in contrast to induction in graminaceous plants, NAS activity is not induced in iron deficient tobacco, tomato and soybean (Higuchi et al., 1995; Higuchi et al., 1996). Barley, which is comparatively tolerant to iron deficiency, has an approximately five-fold increase of NAS activity in total root tissue when grown under iron deficient conditions (Herbik et al., 1999). The genome of barley contains a family of seven NAS encoding genes (Herbik et al., 1999; Higuchi et al., 1999). In rice, three genes encoding NAS have been identified (*OsNAS1*, *OsNAS2* and *OsNAS3)* (Higuchi et al., 2001). Rice is sensitive to iron deficiency and secretes only very small amounts of MAs in the roots compared to barley roots. Nevertheless, iron deficiency induces expression of the *NAS* genes in rice roots and leaves and the amount of endogenous NA is higher in both tissues compared to the NA concentrations in barley (Higuchi et al., 2001). Higuchi et al. (2001) hypothesize that in barley all NA is converted to MAs, whereas in rice some endogenous NA remains. Promoter-GUS analysis revealed that the expression of all three genes was found in cells participating in long-distance transport of iron (Inoue et al., 2003). Three genes encoding NAS were identified in maize (*Zea mays*) (Mizuno et al., 2003). *ZmNAS1* and *ZmNAS2* are only expressed in roots under iron deficient conditions and *ZmNAS3* is only expressed under iron sufficient conditions. Mizuno et al. (2003) revealed that maize has two types of NAS proteins based on their expression pattern and subcellular localization. Based on homology with the barley *NAS* genes, Suzuki et al. (1999) identified three NAS-like sequences in the DNA database of *Arabidopsis thaliana*, *AtNAS1*, *AtNAS2* and *AtNAS3*. Later on, a fourth gene was also identified (Bauer et al., 2004; Becher et al., 2004). The Arabidopsis *NAS* genes are divided into two subgroups, encoded by genes on chromosome 1 (*AtNAS3* (At1g09240) and *AtNAS4* (At1g56430)) and 5 (*AtNAS1* (At5g04950) and *AtNAS2* (At5g56080)), respectively (Bauer et al., 2004). Expression studies showed that the Arabidopsis *NAS* genes are iron and zinc regulated (Bauer et al., 2004; Becher et al., 2004; Weber et al., 2004; van de Mortel et al., 2006). In addition, promoter activities of all four *NAS* genes in *Arabidopsis thaliana* were increased in response to

excess nickel in the medium (Kim et al., 2005). Douchkov et al. (2005) recently showed that ectopic (over)expression of *AtNAS1* in tobacco increased the NA level which led to: (1) an increased iron level in leaves of adult plants, (2) the accumulation of zinc and manganese but not copper in young leaves, (3) an improvement of the iron use efficiency in adult plants grown under iron limitation and (4) an enhanced tolerance against nickel. In *Arabidopsis halleri* four *NAS* genes were identified and sequence analysis showed 94-98% similarity to the four *Arabidopsis thaliana* genes (Weber et al., 2004). Also in *Thlaspi caerulescens* four *NAS* genes were identified (van de Mortel et al., 2006). Comparative transcriptomic studies of *Arabidopsis thaliana* and the hyperaccumulator species *Arabidopsis halleri* (Becher et al., 2004; Weber et al., 2004) or *Thlaspi caerulescens* (van de Mortel et al., 2006) revealed that the *NAS* genes are differentially expressed in these hyperaccumulating plant species in response to zinc compared to expression in the non-accumulator. *Arabidopsis thaliana* overexpressing the *Thlaspi caerulescens NAS3* gene (previously named *NAS1* in de absence of other cloned *TcNAS* genes, but as this gene is most similar to *AtNAS3*, we propose to rename it to *TcNAS3*) over-accumulated nicotianamine and displayed a high resistance to nickel (Pianelli et al., 2005).

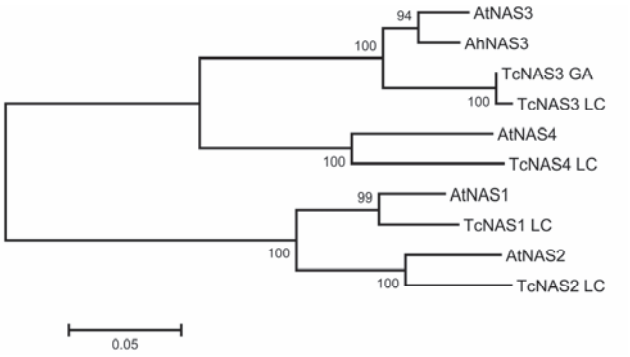
Here we report the cloning of full-length cDNAs of four nicotianamine synthase gene members of *T. caerulenscens* accession La Calamine, which were named *TcNAS1, TcNAS2, TcNAS3* and *TcNAS4* based on their homology to *Arabidopsis thaliana* genes *AtNAS1, AtNAS2, AtNAS3* and *AtNAS4* respectively. Evidence for a functional role in metal homeostasis was sought in studying single, double, triple and a quadruple T-DNA insertion knock-out mutants of the *Arabidopsis thaliana NAS* genes, and in *Arabidopsis thaliana* plants over-expressing *TcNAS1, TcNAS2, TcNAS3* or *TcNAS4*. The various knock-out and overexpression genotypes were tested for their growth response on media with different zinc, iron, nickel and/or cadmium supply and their ability to accumulate metals under various conditions. By means of these results we discuss a putative function for the *NAS* genes in metal homeostasis.

RESULTS

Cloning and sequence analyses of *T. caerulescens NAS* genes

A *T. caerulescens* root cDNA library (Assunção et al., 2001) was screened to clone the full-length cDNAs for *T. caerulescens* genes orthologous to *AtNAS1* (At5g04950), *AtNAS2* (At5g56080), *AtNAS3* (At1g09240) and *AtNAS4* (At1g56430). Four positive clones (1-4) were identified using as probes the partial cDNAs of *NAS1* and *NAS2*. The two longest clones for the *NAS1* probe and one clone identified with the *NAS2* probe were sequenced. Phylogenetic relationships were evaluated constructing a neighbour-joining tree (Figure 1A). The sequence of positive clone one had 91.3% DNA identity and 92.2% amino acid identity with *AtNAS1* (Figure 1A+B) and the sequence of positive clone two had 90.2% DNA identity and 88.3% amino acid identity with *AtNAS4* (Figure 1A+E). The sequence of clone three had 91.0% DNA identity and 91.3% amino acid identity to *AtNAS2* (Figure 1A+C). Positive clones one and two contain an open reading frame of 969 bp, encoding for a 322-amino acid polypeptide, covering the predicted *AtNAS1* and *AtNAS4* ORF respectively. The positive clone three was the full-length cDNA *TcNAS2* with an open reading frame of 963 bp, encoding a 320-amino acid polypeptide. The full-length cDNA sequence of *TcNAS3* was isolated by using primers made on the *T. caerulescens* acc. Ganges *NAS3* sequence (Mari et al., 2006) amplifying the *T. caerulescens* acc. La Calamine *NAS3* cDNA. This sequence had 87.6% DNA identity and 84.4% amino acid identity to *AtNAS3* (Figure 1A+D). *TcNAS3* of La Calamine had 99.4% DNA identity and 99.0% amino acid identity to *TcNAS3* of Ganges (Figure 1A+D). *T. caerulescens* La Calamine *NAS3* has also 90.6% DNA identity and 89.8% amino acid identity to *A. halleri NAS3* (Figure 1A+D). *TcNAS3* has an open reading frame of 945 bp, encoding a 314-amino acid polypeptide.

A



B

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AtNAS1          MACQNNLVVKQIIDLYDQISKLKSLKPSKNVDTLFGQLVSTCLPTDTNIDVTNMCEEVKD
TcNAS1 LC       MACNNNFVVKRIIDLYDQISKLESLKPSKNVDTLFGQLVSTCLPTDTNIDVTKMSEEVKD
                ***:**:***:***********:*****************************:*.*****

AtNAS1          MRANLIKLCGEAEGYLEQHFSTILGSLQEDQNPLDHLHIFPYYSNYLKLGKLEFDLLSQH
TcNAS1 LC       MRSNLIKLCGEAEGYLEQHFSTILGSLQEDGNPLDHLHIFPYYDNYLKLSKIEFDLLSQH
                **:************************** ************.*****.*:********

AtNAS1          SSHVPTKIAFVGSGPMPLTSIVLAKFHLPNTTFHNFDIDSHANTLASNLVSRDPDLSKRM
TcNAS1 LC       TTHVPTKIAFVGSGPMPLTSIVLAKFHLPNTTFHNFDIDSHANTLASSLVSRDPDLSKRM
                ::*********************************************.************

AtNAS1          IFHTTDVLNATEGLDQYDVVFLAALVGMDKESKVKAIEHLEKHMAPGAVLMLRSAHALRA
TcNAS1 LC       IFHTTDVLNANEGLDQYDVVFLAALVGMDKEAKVRAIEHLEKHMAPGAVLMLRSAHALRA
                **********.*********************:**:*************************

AtNAS1          FLYPIVDSSD-LKGFQLLTIYHPTDDVVNSVVIARKLGGPTTPGVNGTRGCMFMPCNCSK
TcNAS1 LC       FLYPIVDSSDDLKGFQLLTIYHPTDDVVNSVVIARKLGGSTTAGINGTRGCMFMPCNCSK
                ********** ****************************.**.*:***************

AtNAS1          IHAIMNNRGKKN-MIEEFSAIE
TcNAS1 LC       VHAIMNNRCKKKKMIEEFSAIE
                :******* **: *********
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C

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AtNAS2          MACENNLVVKQIMDLYNQISNLESLKPSKNVDTLFRQLVSTCLPTDTNIDVTEIHDEKVK
TcNAS2 LC       MACENNLVVKQIIDLYSQISNLENLKPSKNVDTLFGQLVSTCLPTDTNIDVTKIHDEKVK
                ************:***.******.********** ****************:*******

AtNAS2          DMRSHLIKLCGEAEGYLEQHFSAILGSFEDNPLNHLHIFPYYNNYLKLGKLEFDLLSQHT
TcNAS2 LC       DMRSHLIKLCGEAEGYLEEHFSTIIGSLEDNPLNHLHIFPYYNNYLKLSKLEFDLLAQHI
                ******************:***:*:**:********************.*******:**

AtNAS2          THVPTKVAFIGSGPMPLTSIVLAKFHLPNTTFHNFDIDSHANTLASNLVSRDSDLSKRMI
TcNAS2 LC       THVPTKIAFIGSGPMPLSSIVLAKFHLPNTTIHNFDIDSQANTLASNLVSRDPDLSKRMI
                ******:**********:*************:********:************.*******

AtNAS2          FHTTDVLNAKEGLDQYDVVFLAALVGMDKESKVKAIEHLEKHMAPGAVVMLRSAHGLRAF
TcNAS2 LC       FHTTDVLNAKEGLDQYEVVFLAALVGMDKESKVKAIEHLEKHMAPGAVLMLRSAHGLRAF
                ****************:*********************************:***********

AtNAS2          LYPIVDSCDLKGFEVLTIYHPSDDVVNSVVIARKLGGSNGARGSQIGRCVVMPCNCSKVH
TcNAS2 LC       LYPIVDSCDLKGFEVLTIYHPSDDVVNSVVIARKLGGSREARSSQIGRCVVMPCNCSKVH
                ************************************. **.*****************

AtNAS2          AILNNRGMEKNLIEEYSAIE
TcNAS2 LC       VIMNSRGMKKNMIEELSAIE
                .*:*.***:**:*** ****
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D

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TcNAS3 LC       --------MKTICDLYEKISKLESLKPSEDVNILFKQLVSTCIPPNPNINVTKMCDTVQE
TcNAS3 GA       MGCQDEQLVKTICDLYEKISKLESLKPSEDVNILFKQLVSTCIPPNPNINVTKMCDTVQE
AtNAS3          MGCQDEQLVQTICDLYEKISKLESLKPSEDVNILFKQLVSTCIPPNPNIDVTKMCDRVQE
AhNAS3          MGYQDEQLVQTICDLYEKISKLESLKPSEDVNILFKQLVSTCIPPNPNIDVTKMCETVQE
                        ::****************************************:*****: ***

TcNAS3 LC       IRQKLIKICGEAEGHLEHHFSTILTSFQDNPLLHLNIFPYYNNYIKLGKLEFDLLTENLN
TcNAS3 GA       IRQKLIKICGEAEGHLEHHFSTILTSFQDNPLLHLNIFPYYNNYIKLGKLEFDLLTENLN
AtNAS3          IRLNLIKICGLAEGHLENHFSSILTSYQDNPLHHLNIFPYYNNYLKLGKLEFDLLEQNLN
AhNAS3          IRLNLIKICGVAEGHLEHHFSSILTSFQDNPLHHLNIFPYYNNYLKLGKLEFDLLTQNLN
                ** :****** ******:***:****:***** ***********:********** :***

TcNAS3 LC       GFVPKTIAFIGSGPLPLTSIVLASSHLKDTIFHNFDIDPSANSLASLLVSSDPDISQRML
TcNAS3 GA       GFVPKTIAFIGSGPLPLTSIVLASSHLKDTIFHNFDIDPSANSLASLLVSSDPDISQRMF
AtNAS3          GFVPKSVAFIGSGPLPLTSIVLASFHLKDTIFHNFDIDPSANSLASLLVSSDPDISQRMF
AhNAS3          GFVPKSVAFIGSGPLPLTSIVLASLHLKDTIFHNFDIDPSANSLASLLVSSDPDISQRMF
                *****::***************** ********************************:

TcNAS3 LC       FHTVDIMDVTESLKSFDVVFLAALVGMNKEEKVRVIEHLQKHMAPGAVLMLRSAHGPRAF
TcNAS3 GA       FHTVDIMDVTESLKSFDVVFLAALVGMNKEEKVRVIEHLQKHMAPGAVLMLRSAHGPRAF
AtNAS3          FHTVDIMDVTESLKSFDVVFLAALVGMNKEEKVKVIEHLQKHMAPGAVLMLRSAHGPRAF
AhNAS3          FHTVDIMDVTESLRSFDVVFLAALVGMNKEEKVKVIEHLQKHMAPGAVLMLRSAHGPRAF
                *************:******************:*************************

TcNAS3 LC       LYPIVEPCDLQGFEVLSIYHPTDDVINSVVISKKLPVVSTENVGG-VSCLLMPCHCSKIP
TcNAS3 GA       LYPIVEPCDLQGFEVLSIYHPTDDVINSVVISKKLPVVSTENVGG-VSCLLMPCHCSKIP
AtNAS3          LYPIVEPCDLQGFEVLSIYHPTDDVINSVVISKKHPVVSIGNVGGPNSCLLKPCNCSKTH
AhNAS3          LYPIAEPCDLQGFEVLSIYHPTDDVINSVVISKKHPVVSTGNVGGPNSCLLKPCNCSKIH
                ****.***************************** ****  ****  **** **:***
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TcNAS3 LC       AIMNKKKNMQMIDEFGAREEHFS
TcNAS3 GA       AIMNKKK-NMMIDEFGAREEHFS
AtNAS3          AKMNKNM---MIEEFGAREEQLS
AhNAS3          AIMNKNM---MIEKFGAREEQLS
                * ***:    **::******::*
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E

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AtNAS4          MGYCQDDQLVNKICDLYEKISKLETLKPCEDVDTLFKQLVSTCIPPNPNIDVTKMSENIQ
TcNAS4 LC       MGYCQDDQLVSTICDLYEKISKLETLKPCQDVDTLFKQLVSTCIPPNPNIDVTKMSESIQ
                **********..*****************:**************************.**

AtNAS4          EMRSNLIKICGEAEGYLEHHFSSILTSFEDNPLHHLNLFPYYNNYLKLSKLEFDLLEQNL
TcNAS4 LC       EMRSNLIKLCGEAEGHLEHHFTSILNSFEDNPLHHLNLFPYYDNYLKLSKLEFDLLEQNL
                ********:******:*****:***.****************:*****************

AtNAS4          NGFVPRTVAFIGSGPLPLTSVVLASSHLKDSIFHNFDIDPSANMVAARLVSSDPDLSQRM
TcNAS4 LC       KGSVPKTVAFIGSGPLPLTSIVLASYHLKDSIFHNFDIDSTANSVAARLVSSDPDLSQRM
                :* **:**************:**** ************.:** ****************

AtNAS4          FFHTVDIMDVTESLKGFDVVFLAALVGMDKKEKVKVVEHLEKHMSPGALLMLRSAHGPRA
TcNAS4 LC       FFHTVDVMDVTESLKGFDVVFLAALVGMDKQEKIKVIEHLEKHMSPGALLMLRSAHGPRA
                ******:************************:**:**:**********************

AtNAS4          FLYPIVEPCDLEGFEVLSVYHPTDEVINSIVISRKLGEDANGVVHDHIDQASDLACNCSK
TcNAS4 LC       FLYPIVEPCDLQGFQVLSIYHPTDEVINSVVISRKLGEDGVEHEDHMDHQDSGLACNCSK
                **********:**:***:**********:*********.    ..  .* *.*******

AtNAS4          IHVIMNKKKSIIEEFAGANEE-
TcNAS4 LC       IHAIMNKKKSIIEEFAIEEQLS
                **.*************  ::
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Figure 1. (A) Phylogenetic comparison of *TcNAS1*, *TcNAS2*, *TcNAS3* and *TcNAS4* with the predicted protein sequences of six *NAS* family members from Brassicaceae. The neighbor joining phylogenetic tree was conducted using MEGA version 3.1 (Kumar et al., 2004). GenBank accession numbers are: *AtNAS1* (NM_120577), *AtNAS2* (NM_124990), *AtNAS3* (NM_100794), *AtNAS4* (NM_104521), *AhNAS3* (AJ580399), *TcNAS3* GA (AJ300446). Sequence alignment of *TcNAS1* from *T. caerulescens* and *A. thaliana AtNAS1* (At5g04950, NM_120577) (B), of *TcNAS2* from *T. caerulescens* and *A. thaliana AtNAS2* (At5g56080, NM_124990) (C), of *TcNAS3* from *T. caerulescens*, *A. thaliana AtNAS3* (At1g09240, NM_100794), *TcNAS3* isolated from accession Ganges (AJ300446) and *A. halleri AhNAS3* (AJ580399) (D) and of *TcNAS4* from *T. caerulescens* and *A. thaliana AtNAS4* (At1g56430, NM_104521) (E). Deduced amino acid sequences are shown aligned using ClustalW. Non-identical residues are shaded. "*" = residues or nucleotides in that column are identical in all sequences in the alignment; ":" = conserved substitutions have been observed; "." = semi-conserved substitutions are observed.

Differential transcriptional response of *NAS* genes from *A. thaliana* and *T. caerulescens* upon zinc exposure

The function of NAS in metal accumulation and homeostasis in *T. caerulescens* and Arabidopsis was first evaluated by their expression upon exposure to various zinc conditions (Figure 2). With the exception of *NAS2*, which is expressed in roots at all zinc exposure conditions, the expression of the *NAS* genes is different between the two species (Figure 2). *AtNAS1* is expressed in roots and leaves and induced under zinc excess conditions (25 µM $ZnSO_4$) in Arabidopsis, whereas *TcNAS1* shows constitutive expression in leaves of *T. caerulescens* at all tested Zn exposure conditions no expression in the roots (Figure 2). *AtNAS3* is mainly expressed under zinc deficiency in both roots and leaves of Arabidopsis whereas *TcNAS3* is constitutively expressed in leaves and roots of *T. caerulescens* (Figure 2). *AtNAS4* is induced by zinc deficiency in roots and zinc excess in leaves of Arabidopsis. The expression of *TcNAS4* in *T. caerulescens* resembles that of *TcNAS1* with constitutive expression in the leaves (Figure 2).

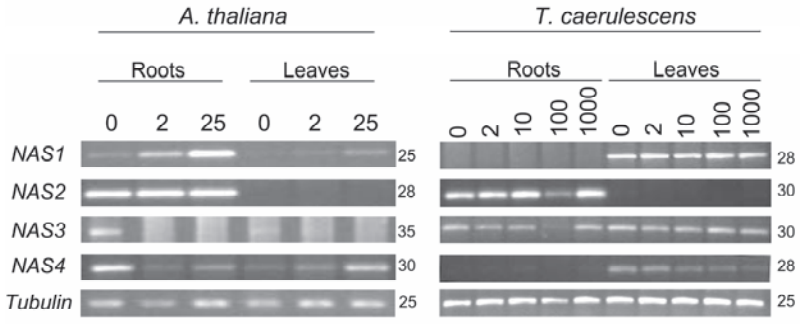

Figure 2. Comparative semi-quantitative RT-PCR of four *NAS* genes from *Arabidopsis thaliana* and *Thlaspi caerulescens*. For amplification, species specific primers were designed. Roots and leaves were harvested separately after one week of exposure of three-week-old plants to 0, 2 and 25 µM $ZnSO_4$ for Arabidopsis and 0, 2, 10, 100 and 1000 µM $ZnSO_4$ for *T. caerulescens*. For Arabidopsis: *NAS1*, nicotianamine synthase, At5g04950; *NAS2*, nicotianamine synthase, At5g56080; *NAS3*, nicotianamine synthase, At1g09240; *NAS4*, nicotianamine synthase, At1g56430. Tubulin (At1g04820) was used as a control for equal cDNA template use.

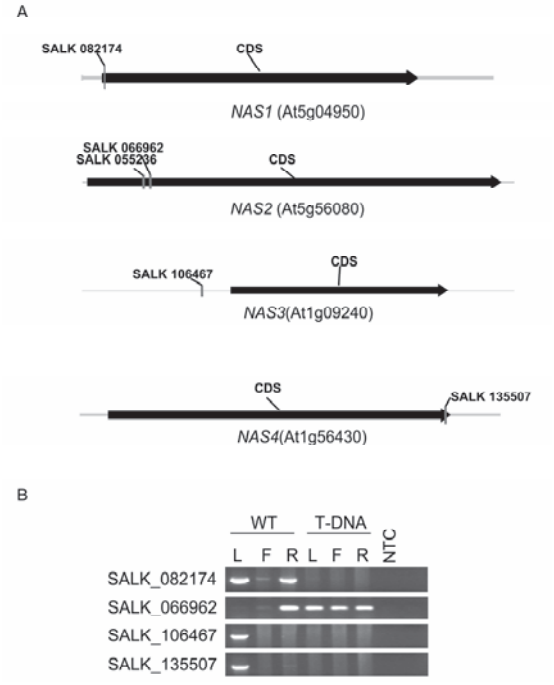

Figure 3. (A) The genomic DNA structure of the *Arabidopsis thaliana NAS1*, *NAS2*, *NAS3* and *NAS4* genes. The black boxes represent the coding sequences (CDS) and the lines represent the non-coding regions. The position of the T-DNA insertions in the respective genes is represented by a vertical bar. (B) Semi-quantitative RT-PCR of roots (R), leaves (L) and flowers (F) of the T-DNA insertion lines using gene specific primers amplifying a cDNA fragment downstream of the T-DNA insertion. Semi-quantitative RT-PCR was performed with 35 cycles and 20 µl of the reaction was separated on an ethidium bromide stained 1% agarose gel. NTC = No template control.

Arabidopsis loss of function mutants

The presence of four *NAS* copies with different expression patterns in Arabidopsis suggests non-redundant functions. To test this, T-DNA insertion knock-out mutants were identified and combined. Salk T-DNA insertion mutants *nas1* (SALK_082174), *nas2* (SALK_066962), *nas3* (SALK_106467) and *nas4* (SALK_135507) (Figure 3A) were confirmed to have T-DNA insertions by PCR and homozygous lines were isolated, which were confirmed to be null mutants by the absence of semi-quantitative RT-PCR detectable mRNA for *NAS1*, *NAS3*

and *NAS4* in roots, flowers and leaves (Figure 3B). The mRNA of *NAS2* was still detectable in the corresponding mutant (Figure 3B) and suggests there is no knock-out mutant for *NAS2*. According to the position of the T-DNA insertion, which is in the middle of the ORF *NAS2* is probably not functional and is expected to be a knock-out mutant (Figure 3A). Crosses between single homozygous T-DNA insertion lines were performed to generate double T-DNA insertion lines and homozygous double mutants were identified among the F2 individuals by PCR. With the homozygous double mutants additional crosses were made to generate the triple T-DNA insertion lines. Homozygous triple mutants were identified in the F2 individuals by PCR. A similar approach by inter-crossing the homozygous triple mutants was applied to generate the homozygous quadruple mutant.

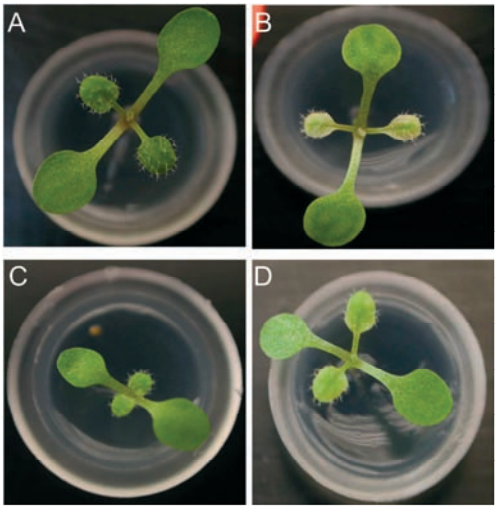

Figure 4. One-week-old *Arabidopsis thaliana* Col-0 and triple *nas* mutants grown hydroponically on a modified half-strength Hoagland's solution. Note the yellowing of the primary leaves, with veins remaining green, of especially the *nas1nas2nas4* triple mutant and to a lesser extent the *nas1nas3nas4* triple mutant. Col-0 control plant (A), *nas1nas2nas4* mutant (B), *nas1nas2nas3* mutant (C), and *nas1nas3nas4* mutant (D).

Growth of the single and double mutants on soil did not show any noticeable phenotypic difference with wild-type Col-0 plants (WT) (data not shown). However, after growing the triple *nas1nas2nas4* mutant and quadruple *nas1nas2nas3nas4* mutant plants for approximately one week on soil, they showed chlorotic leaves, with an interveinal chlorosis pattern resembling that of the *chloronerva* mutant (Stephan and Scholz, 1993; Herbik et al., 1996). Similar symptoms were identified when the triple *nas1nas2nas4* mutant (Figure 4B) was grown on hydroponics with a modified half-strength Hoagland's solution for one week. This was not identified for the WT plants and the other triple mutants (Figure 4A, C, D). Supplementation of sequestrene (Fe-EDTA) to soil-grown plants complemented the chlorotic phenotype of the triple and quadruple mutants, suggesting that iron deficiency was the main cause of chlorosis.

Mineral deficiencies normally affect plant growth. To investigate this further, seedlings of single, double, triple and quadruple plants were screened for root growth on vertical plates with different concentrations of Zn, Fe, Ni and Cd (Figure 5). Surprisingly, the single mutants had a longer root length under both zinc deficient and sufficient conditions compared to WT (Figure 5A), suggesting an enhanced sensitivity to these conditions. Under zinc excess and iron deficient conditions the *nas1* single mutant had shorter roots compared to the WT (Figure 5A) whereas the roots of all other single mutants were longer than WT (Figure 5A). The double mutants had a root length comparable to the WT under zinc deficient and sufficient conditions (Figure 5B). Under zinc excess (200 μM) and iron deficient conditions the double mutants had shorter roots compared to the WT (Figure 5B) suggesting that the double mutants are more sensitive to excess zinc concentrations and iron deficiency. Also the triple and quadruple mutants had shorter roots compared to the WT under normal growth conditions as well as under zinc deficiency, zinc excess, iron deficiency, nickel excess and cadmium excess conditions (Figure 5C). These results suggest that the *NAS* genes in Arabidopsis are only partially redundant.

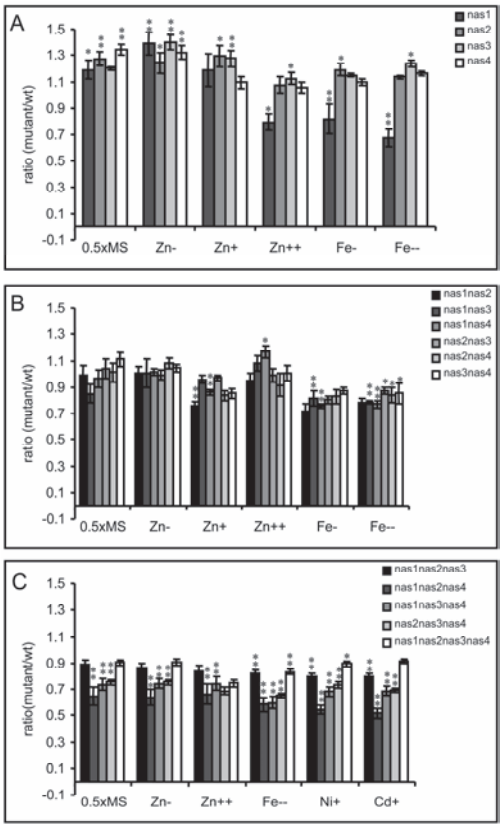

Figure 5. Root length (mean ± SE) of 5-day-old seedlings grown on vertical plates with half-strength MS medium containing different concentrations of Zn, Fe, Ni and Cd. Zn- = 0 µM $ZnSO_4$; Zn+ = 200 µM $ZnSO_4$; Zn++ = 400 µM $ZnSO_4$; Fe- = 0.5 µM FeEDTA; Fe-- = 0.15 µM FeEDTA; Ni+ = 75 µM $NiSO_4$; Cd+ = 30 µM $CdSO_4$; (A) *NAS* single mutants; (B) *NAS* double mutants; (C) NAS triple and quadruple mutants. * significantly different from wild type at $P<0.05$; ** significantly different from wild type at $P<0.01$. Significance is determined by one-way ANOVA. Four to ten replicate plates with 15 to 20 seedlings were measured for each treatment.

Mineral content analysis of the triple mutants

Changes in the NA content in the plant are expected to have an effect on mineral uptake and distribution within the plant**.** Mineral concentrations were measured (Figure 6 + 7) in roots and shoots of wild-type Col-0 and *nas1nas2nas3*, *nas1nas2nas4*, *nas1nas3nas4* and *nas2nas3nas4* triple mutant plants grown on a modified half-strength Hoagland's nutrient

solution. The triple mutant *nas1nas2nas4* exhibited interveinal chlorosis of the youngest leaves under all the conditions tested (Figure 4B), whereas the other three mutants as well as the WT remained green. Zinc, iron, manganese, magnesium and calcium concentrations were determined and compared to Col-0 (Figure 6A-F). The zinc concentration in the roots (Figure 6A) of all triple mutants grown under normal conditions was higher compared to the WT whereas in the leaves the concentration zinc was lower compared to the WT. The iron concentration in the roots differed between the triple mutants, only the triple mutant *nas2nas3nas4* had an increased iron concentration in the roots under normal growth conditions compared to the WT (Figure 6C). The triple mutant *nas1nas3nas4* had 25% less iron in the leaves compared to the WT (Figure 6D) whereas the other mutants were not differing in iron concentration from the WT. The manganese concentration in the roots was significantly lower for the triple mutant *nas1nas2nas4* compared to the WT but was not changed for the other mutants (Figure 6E) and the manganese concentration in the leaves was similar for the mutants and WT (Figure 6F). Under zinc deficient conditions the triple mutants *nas1nas2nas3* and *nas1nas3nas4* had a root iron concentration increased by 12% and 29% compared to the WT respectively (Figure 6C) but the leaves of the mutants were not different from the WT in their iron content (Figure 6D). In the roots of the triple mutant *nas1nas2nas4* the manganese concentration was decreased (66%) when grown under zinc deficient conditions compared to the WT (Figure 6E). The concentration manganese in the leaves of plants grown under zinc deficient conditions was not different between mutants and WT (Figure 6F). When the triple mutants were grown under zinc excess conditions the zinc concentration in the roots of the triple mutant *nas1nas3nas4* and *nas2nas3nas4* was lower compared to the WT (Figure 6A). The concentration zinc in the leaves of the triple mutants exposed to excess zinc was lower for *nas1nas2nas4* compared to the WT under all conditions tested (Figure 6B). The concentration iron in the roots of the triple mutants grown under excess zinc conditions did not differ from the WT (Figure 6C). The triple mutant *nas1nas2nas4* had a 16% decrease in iron content in the leaves grown under zinc excess conditions compared to the WT (Figure 6D). Manganese concentration in the roots of *nas1nas3nas4* was decreased in comparison to the WT under zinc excess conditions (Figure 6E).

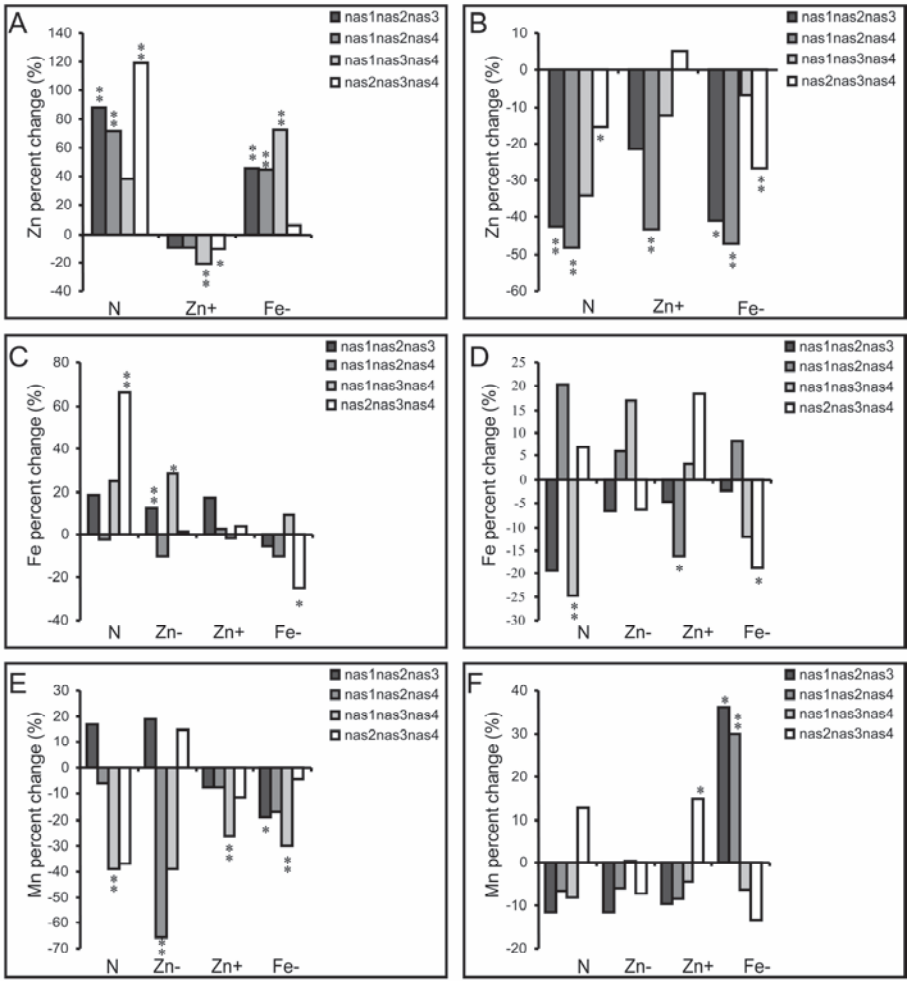

Figure 6. Metal concentrations of different organs of wild-type and *nas1nas2nas3*, *nas1nas2nas4*, *nas1nas3nas4* and *nas2nas3nas4* triple mutants grown under various mineral exposure conditions. Plants were grown for two weeks on half-strength Hoagland's solution containing sufficient zinc before exposure to zinc deficiency (0 µM $ZnSO_4$: Zn-), zinc sufficiency (2 µM $ZnSO_4$: N), excess zinc (35 µM $ZnSO_4$: Zn+) or iron deficiency (1 µM FeEDTA: Fe-). Results are given in percentage change in the mutants (n = 5) compared to wild-type plants (n = 4). (A) Zinc concentrations in roots. (B) Zinc concentrations in leaves. (C) Iron concentrations in roots. (D) Iron concentrations in leaves. (E) Manganese concentrations in roots. (F) Manganese concentrations in leaves. * significantly different from wild type at $P<0.05$; ** significantly different from wild type at $P<0.01$. Significance is determined by one-way ANOVA.

The triple mutant *nas2nas3nas4* had an increased (15%) manganese concentration under excess zinc conditions (Figure 6F). The concentration zinc in the roots of the triple mutants grown under iron deficient conditions was higher compared to the WT (Figure 6A) whereas in the leaves the zinc content was lower compared to the WT for three of the mutants (Figure 6B). The iron concentration in the roots of the *nas2nas3nas4* triple mutant under iron deficient conditions decreased by 25% (Figure 6C). Under iron deficient conditions only the triple mutant *nas2nas3nas4* had a 19% decrease in iron content compared to the WT (Figure 6D). The triple mutants *nas1nas2nas3* and *nas1nas3nas4* contained less manganese in the roots when grown under iron deficient conditions (Figure 6E). The manganese concentration in the leaves of the triple mutants *nas1nas2nas3* and *nas1nas2nas4* was significantly increased when grown under iron deficient conditions whereas the other two mutants did not differ from the WT (Figure 6F). No difference was observed for calcium and magnesium content in the roots and leaves between the mutants and WT (data not shown).

In conclusion, the triple mutants take up more zinc into the roots but zinc is not translocated to the leaves. The triple mutants differ in their iron and manganese uptake into the roots and translocation of iron and manganese to the shoots.

To follow up this experiment, the mineral content of the seeds was measured in the single, double and triple mutants. In the seeds of the single mutants the manganese concentration was 1.2-2.8 times higher compared to the WT (Figure 7C) whereas the zinc and iron content did not differ from the WT (Figure 7A+B). The zinc content in the seeds of the double and triple mutants was lower compared to the WT and single mutants (Figure 7A). Also the iron content in the seeds of the double and triple mutants was lower compared to the WT and the single mutants (Figure 7B). The manganese concentration was, like in the seeds of the single mutants, higher in the triple mutants compared to the WT (Figure 7C). Overall the Zn, Fe and Mn uptake and translocation mechanism in these triple mutants is disturbed in that the mutant plants take up and translocate less Zn and Fe but more Mn.

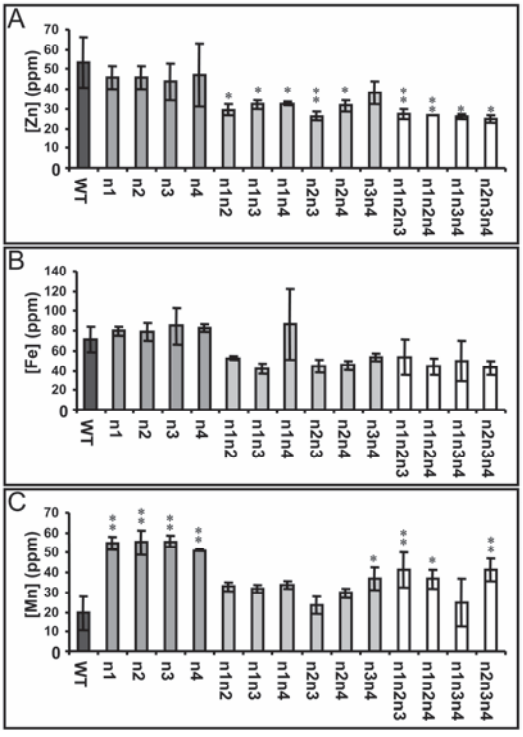

Figure 7. Metal concentrations in the seeds of wild-type plants and single, double and triple *nas* mutants grown on soil. Results are given in ppm (n = 3). (A) Zinc concentration in seeds. (B) Iron concentration in seeds. (C) Manganese concentration in seeds. * significantly different from wild type at P<0.05; ** significantly different from wild type at P<0.01. Significance is determined by one-way ANOVA. n1 = *nas1*; n2 = *nas2*; n3 = *nas3*; n4 = *nas4*.

Overexpression of *T. caerulescens NAS* genes rescues the *nas1nas2nas4* phenotype

From the results shown above we know that the *AtNAS* genes are highly redundant and probably do not show a difference in gene function. In this respect it would also be of interest to know if the *TcNAS* genes are functional *NAS* genes. To test whether the *T. caerulescens NAS* genes are functional *NAS* genes, full-length *T. caerulescens NAS1*, *NAS3* and *NAS4* were expressed in the *A. thaliana* triple mutant *nas1nas2nas4*, which showed severe chlorosis under normal growth conditions (Figure 8A). Transgenic plants carrying the *T. caerulescens NAS1* (Figure 8B), *NAS3* (Figure 8C) or *NAS4* (Figure 8D) gene had normal leaf pigmentation

compared to the triple mutant carrying only the vector pH7WG2 (Figure 8A). These results demonstrated that the *T. caerulescens NAS* genes encode NAS proteins.

Transgenic *A. thaliana* lines overexpressing *TcNAS3 and TcNAS4* exhibit increased tolerance to zinc and nickel

To characterize the function of the *T. caerulescens* NAS genes, transgenic *A. thaliana* plants overexpressing *TcNAS3* and *TcNAS4* were generated under the control of a constitutive cauliflower mosaic virus 35S promoter. Plants expressing *TcNAS3* and *TcNAS4* did not show obvious morphological alterations when they were grown in soil (data not shown). These transgenic lines were further examined for their tolerance to metal stresses. Plants were grown on vertical plates with different metal levels for five days before measuring root growth. Plants overexpressing *TcNAS1* and *TcNAS2* were not included in this analysis because the expectation is that similar results will be obtained as for *TcNAS3* and *TcNAS4*.

TcNAS3 overexpression seems to induce marginally longer roots compared to the WT under normal growth conditions, excess zinc, excess nickel and cadmium supply (Figure 9A). The *TcNAS3* overexpressing line 45-2 showed the longest roots compared to WT grown under excess zinc. This *TcNAS3* overexpressing line is also the highest overexpressor according to the semi-quantitative RT PCR data (data not shown). Overexpression of *TcNAS4* resulted in longer roots compared to the WT under zinc excess and nickel excess conditions (Figure 9B). In conclusion, overexpression of the *TcNAS3* and *TcNAS4* gene in Arabidopsis enhanced tolerance to zinc and nickel.

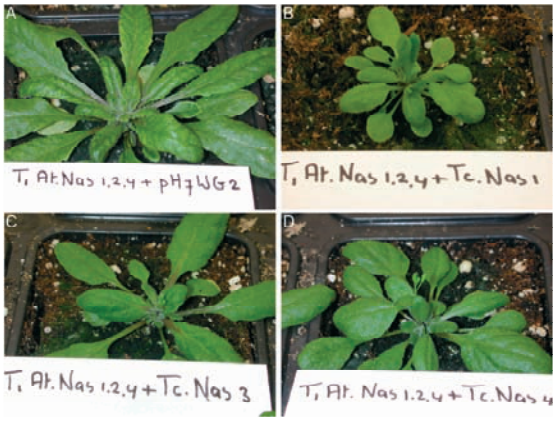

Figure 8. Complementation of the yellow-leaf-green-veins Arabidopsis *nas1nas2nas4* triple mutant phenotype by overexpression of three *TcNAS* genes. (A) Four-week-old soil-grown triple mutant *nas1nas2nas4* control plant transformed with empty vector. (B) Triple mutant *nas1nas2nas4* overexpressing *TcNAS1*. (C) Triple mutant *nas1nas2nas4* overexpressing *TcNAS3*. (D) Triple mutant *nas1nas2nas4* overexpressing *TcNAS4*.

DISCUSSION

Biochemical evidence and enzyme assays have previously shown that the tomato mutant *chloronerva* lacks NA (Ling et al., 1999). Specifically nicotianamine (NA) appears to be involved in the transport of iron throughout the plant. However, it is still not clear how NA is involved in transport of metals in the plant. NA has unique properties that make it an interesting candidate for other metal related roles in Strategy I plants. NA is able to bind metals other than iron and because of this it is likely to play an important role in the homeostasis of other metals. In this study we attempted to gather evidence for a function of nicotianamine synthase in metal homeostasis in the Strategy I plants *Arabidopsis thaliana* and *Thlaspi caerulescens*.

Four *NAS* genes have been identified in *Arabidopsis thaliana*, *NAS1*, *NAS2*, *NAS3* and *NAS4*. In *T. caerulescens* acc. La Calamine four *NAS* genes were revealed, corresponding to homologs of *AtNAS1-4*. Southern blot analysis should be performed to reveal if *T. caerulescens* contains four or more nicotianamine synthase genes. The four Arabidopsis *NAS* genes have

distinct spatial expression patterns and all four genes are differentially regulated by zinc and iron at the mRNA level (Figure 2; Bauer et al., 2004; van de Mortel et al., 2006). This suggests that each individual gene has its own function in the demand that the plant may have for NA. Based on the expression profiles some very general roles can be assigned to the *NAS* genes in Arabidopsis. *NAS1*, *NAS2* and *NAS4* are expressed in the roots, where it is likely that NA is involved in the lateral movement of minerals in the roots (Didonato et al., 2004). Plants use two methods to take up metals from the soil: (1) acidification of the rhizosphere through plasma membrane pumps and (2) secretion of ligands, which chelate the metal. The soluble metals can enter the root symplast by crossing the plasma membrane (PM) of the root endodermal cells or they enter the root apoplast through the space between cells. The solutes can subsequently enter the xylem from where it is translocated to aerial tissues. The transport of ions into the xylem generally is a tightly controlled process mediated by membrane transport proteins. Chelation with certain ligands such as histidine, nicotianamine, mugineic acid (Pich et al., 1994), citrate, malate or oxalate (Senden et al., 1995) appears to be required for transport within the xylem. *NAS1*, *NAS3* and *NAS4* are expressed in the leaves, where NA may be involved in transporting the minerals from the xylem into the leaves, stem and flowers, or loading the minerals into the vasculature (phloem) for transport to the shoot apex or back to the root. It will be necessary to perform in situ hybridization analysis or promoter-GUS analysis to determine the localization of each gene before making conclusive statements about the precise roles of the individual *NAS* genes in metal homeostasis.

The different expression profiles between Arabidopsis and *T. caerulescens* of three of the four *NAS* genes (Figure 2) suggest a function for these genes in the metal adaptation of *T. caerulescens*. Only *NAS2* showed a similar expression pattern for *T. caerulescens* and Arabidopsis. All four *NAS* genes are more or less constitutively expressed in roots or leaves of *T. caerulescens*, rather than induced or repressed by alterations in Zn supply (Figure 2). A similarly higher and constitutive expression compared to Arabidopsis was previously reported for more metal homeostasis related genes in the metal tolerant and hyperaccumulation species *T. caerulescens* (Pence et al., 2000; Assunção et al., 2001; Hammond et al., 2006; van de Mortel et al., 2006).

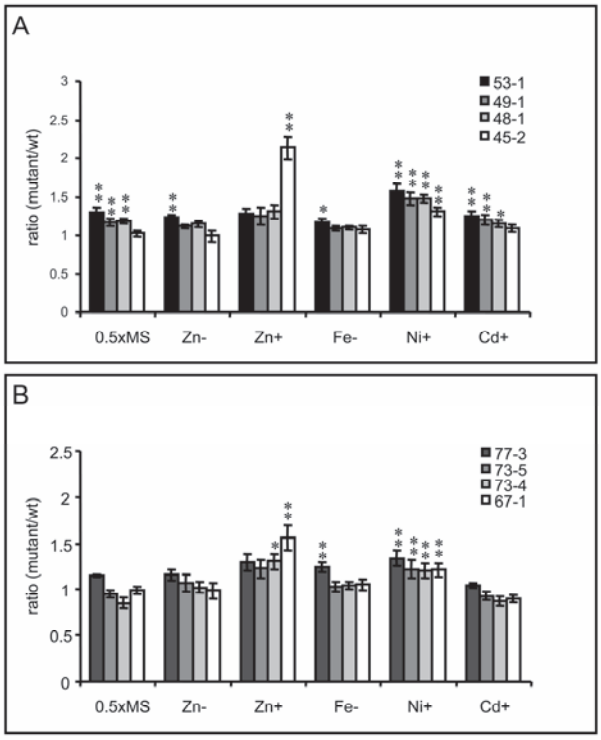

Figure 9. Metal tolerance screen for 35S::*TcNAS3* and 35::*TcNAS4* transgenic lines. Root length (mean ± SE) of 5-day-old seedlings grown on half-strength MS medium containing different concentrations of zinc, iron, nickel or cadmium. Zn- = 0 µM $ZnSO_4$; Zn+ = 400 µM $ZnSO_4$; Fe- = 0.15 µM FeEDTA; Ni+ = 75 µM $NiSO_4$; Cd+ = 30 µM $CdSO_4$; (A): 35S::*TcNAS3*; (B) 35S:: *TcNAS4*, * significantly different from wild type at P<0.05; ** significantly different from wild type at P<0.01 as determined by one-way ANOVA; Five replicates with 15 to 20 seedlings were measured for each treatment. Numbers indicate lines from two independent transformants.

As zinc hyperaccumulation in *T. caerulescens* is a constitutive trait involving strongly enhanced metal uptake and root to shoot translocation (Assunção et al., 2003b), higher transport capacity will be needed for taking up metals from soil or for intracellular storage and allocation (Lasat et al. 1996). *T. caerulescens* seems to have dealt with the demand for higher transport capacity in increasing the expression of the available metal transporters (Pence et al., 2000; Assunção et al., 2001, van de Mortel et al., 2006), but also in higher expression of the *NAS* genes.

In order to further identify the function of the *NAS* genes in metal homeostasis, the Arabidopsis *NAS* single, double, triple and quadruple knock-out mutants and 35S::*TcNAS3* and 35S::*TcNAS4* transgenic lines were studied. The single and double mutants did not show any discernible phenotype when grown in soil, suggesting that *NAS* genes are at least partially redundant. However, the triple mutant *nas1nas2nas4* and the quadruple mutant *nas1nas2nas3nas4* showed a phenotype comparable to the *chloronerva* mutant in tomato (Ling et al., 1999), when grown in soil. The *chloronerva* mutant exhibits interveinal chlorosis of the young leaves (Ling et al., 1999) and the yellowing between the veins is a classic symptom of iron deficiency. The phenotype of the *nas1nas2nas4* mutant and the quadruple mutant was corrected by the addition of iron by subirrigation with FeEDTA (0.5 mg/l). The other triple mutants did not show this phenotype probably because one of the *NAS* genes is still functional in the roots whereas there is no functional *NAS* gene in the roots of the triple mutant *nas1nas2nas4* because the remaining *NAS3* gene is not or at a very low level expressed in this organ. The interveinal chlorosis in the *nas1nas2nas4* triple mutant suggests that this mutant is more iron deficient than the other triple mutants. However, according to the mineral content analysis of the triple mutants the iron level was not changed in the *nas1nas2nas4* triple mutant. The chlorotic phenotype could be caused by a less efficient distribution of the metals within the plants and the transport of the metals from the roots to the leaves could be less efficient in the *nas1nas2nas4* triple mutant compared to the other triple mutants. The uptake of zinc into the roots was increased in all triple mutants compared to the WT but the translocation of zinc to the leaves was decreased compared to the WT (Figure 6A+B). This suggests an important function of the *NAS* genes in the translocation and distribution of zinc to the leaves. Zinc deficiency in the leaves results probably in a higher uptake rate in the roots. The low levels of zinc in the leaves of the triple mutants indicate an alteration in zinc transport during vegetative growth of these mutants. Similar observations were described for the *ysl1ysl3* mutant in Arabidopsis, which had an aberrant iron deficiency phenotype due to alteration in iron and copper homeostasis (Waters et al., 2006). Next to a low zinc level in the leaves also the zinc content in the seeds of the double and triple mutants was decreased compared to the zinc content in the seeds of the single mutants and WT (Figure 7). The manganese content in the triple mutants was also altered (Figure 6E+F). The triple mutants

contained less manganese in their roots while the concentration of manganese in the leaves was not different for the triple *nas1nas3nas4* and *nas2nas3nas4* mutants compared to the WT. The triple mutants *nas1nas2nas3* and *nas1nas2nas4* also had a higher manganese concentration in their leaves when grown under iron deficient conditions (Figure 6F). Also the manganese concentration in the seeds is enhanced (Figure 7C). These results suggest that manganese root to shoot translocation or manganese seed allocation is not governed by NA, but by another iron-deficiency-induced chelator like for example citrate. Quantitative PCR analysis should be performed to further confirm if the expression level of the functional *NAS* gene in the triple mutants is changing. Additional analysis of the triple and quadruple mutant lines for detection and quantification of NA by HPLC would confirm whether NA is indeed reduced or absent in these mutants.

Interveinal chlorosis also was observed in transgenic tobacco overexpressing the barley nicotianamine aminotransferase gene (*NAAT*) (Takahashi et al., 2003). Young leaves showed interveinal chlorosis and this chlorosis disappeared gradually as the leaves grew older (Takahashi et al., 2003). NAAT is a critical enzyme in the biosynthetic pathway of MAs that catalyzes the aminotransfer of NA and overexpression of NAAT results in less NA in the plant (Mori and Nishizawa, 1987; Shojima et al., 1990). Takahashi et al. (2003) identified a difference in the distribution of iron in the leaves of *NAAT* tobacco and control tobacco. In control tobacco, iron was present in both the veins and the interveinal area of young leaves. By contrast, *naat* tobacco leaves had only a small quantity of iron in the veins and an extremely low quantity in the interveinal area. These results indicate that the young *naat* tobacco leaves were iron deficient. Because the demand for iron is much higher in young leaves than in older leaves, Mori (1998) concluded that the chlorosis in young *naat* tobacco leaves was caused by insufficient iron transport to the leaves, particularly to the interveinal area. The interveinal chlorosis observed in the Arabidopsis *NAS* triple and quadruple mutants can be caused by a less efficient distribution of the micronutrients such as zinc, iron and manganese (Yoshimura et al., 2000) but probably because for the mineral content analysis in this study whole rosettes were taken, the differences in mineral content were not obvious. The mineral content analysis should be repeated by only making use of young leaves of the *NAS* triple and quadruple mutants.

The vertical plate experiment showed that the single mutants *nas2*, *nas3* and *nas4* had only a mild mutant phenotype of increased root growth under all conditions tested (Figure 5A) whereas the single mutant *nas1* was a little more sensitive to excess zinc and iron deficiency. These results together with the expression analysis (Figure 2; Bauer et al., 2004) suggest a function for *NAS1* in iron homeostasis in the roots. For *AtNAS2* and *AtNAS4* a similar function could be suggested as for *AtNAS1* but these two genes could also be involved in zinc homeostasis according to the expression level when plants are exposed to zinc (Figure 2). *AtNAS3* is thought to have a function in zinc homeostasis in the roots because this gene responds more to zinc deficient conditions, rather than to changes in iron availability (Bauer et al., 2004). *NAS1*, *NAS3* and *NAS4* could be involved in the distribution of iron and zinc in the leaves whereas *NAS2* is not, because it is barely in the leaves (Figure 2; Bauer et al., 2004). Some of the double mutants showed a more sensitive phenotype when grown under zinc excess or iron deficient conditions (Figure 5B), which indicates that two non-functional *NAS* genes can already influence plant development. The triple and quadruple mutants have a mild root length reduction under all conditions tested (Figure 5C) but the quadruple is performing better compared to the triple mutants. However, this may be misleading. One has to note that the quadruple mutant was supplied with extra iron to rescue the otherwise severe phenotype. This will have increased the seed iron levels compared to plants that had not been supplemented.

The 35S::*TcNAS3* and 35S::*TcNAS4* transgenic lines clearly outperformed the Arabidopsis wild-type Col-0 in terms of nickel tolerance (Figure 9A+B). Previous studies have shown that overexpression of nicotianamine synthase in the plant enhances tolerance to nickel (Douchkov et al., 2005; Kim et al., 2005; Pianelli et al., 2005). Nickel is chemically related to iron and cobalt and plants cannot complete their life cycle without an adequate nickel supply. In addition Ni is also required for the activity of several enzymes (Marschner, 1995; Douchkov et al., 2005). Nickel tolerance and hyperaccumulation have been shown to be correlated with the enhanced production of histidine in the hyperaccumulating Alyssum species (Krämer et al., 1996, 2000). The NA-dependent tolerance to high nickel concentrations described here could be due to enhanced chelation of nickel by NA in the xylem as suggested by Vacchina et al. (2003) but could also be related to the implication of NA in cell-to-cell transport of iron and possibly other

micronutrients as was suggested by Dochkov et al. (2005). Mineral content analysis of the transgenic lines should be performed to further confirm this. Some of the 35S::*TcNAS3* transgenic lines also performed better when grown under excess zinc or excess cadmium conditions (Figure 9A). These results indicate that ectopic expression of *TcNAS3* in Arabidopsis results in an induced tolerance to high zinc, high nickel and high cadmium concentrations due to probably an increase in concentration of NA (Pianelli et al., 2005). To confirm this, the NA concentration in these transgenic lines should be determined.

In conclusion, the presence of at least four *NAS* gene copies in both species, which are apparently all functional and partly redundant, probably has provided ample flexibility to sustain adaptive changes in *NAS* gene expression. Also in view of the observed effect on especially nickel tolerance of NAS overexpression in non-accumulating, non-tolerant species, the *NAS* genes may be crucial to metal tolerance in *T. caerulescens*.

MATERIAL AND METHODS

Library screening

A *Thlaspi caerulescens* roots and shoots cDNA library (Assunção et al., 2001) was used for full-length cDNA cloning. The shoots cDNA library was screened using a *TcNAS1* probe and roots cDNA library was screened using a *TcNAS2* probe. DNA fragments amplified from genomic DNA using primers based on *A. thaliana NAS* sequences were used as probes. For *TcNAS1* amplification, the forward primer was 5'-ACATGGGTTGCCAAGACG-3'and the reverse primer was 5'-ATGAGAAGGCACGAGACTCC-3'; for *TcNAS2* amplification the forward primer was 5'-ATGGCTTGCGAAAA-3', the reverse primer was 5'-CTTCTCCATACCACGATTGT-3'. The PCR was performed at 94 °C for 2 min, followed by 35 cycles 94 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s, and finished by an extension at 72 °C for 2 min. Single fragments were amplified for each primer pair with a length of about 900 bp for *TcNAS1* and 1 kb for *TcNAS2* respectively. One µl of each PCR product was labeled with [α-^{32}P]dATP using the Hexalabel™ DNA labeling kit (Fermentas, http://www.fermentas.com/).

Membranes were hybridized as described by Assunção et al. (2001). The isolated cDNA clones were sequenced by ABI PRISM BigDye terminator cycle sequencing technology v2.0 using an ABI3700 DNA analyzer, according to the manufacturer's instruction (Applied Biosystems; http://www.appliedbiosystems.com). Sequence comparison was performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignment was conducted by MegAlign (DNAstar, Madison WI). The phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al., 2004)

Plant material and growth conditions

Arabidopsis *NAS* T-DNA insertion lines were sown in Petri dishes on water-saturated filter paper followed by a 4-day vernalization treatment at 4 ºC. Plates were transferred to a climate room at 25 ºC and 16 h light for two days before planting germinating seeds in 7-cm pots with standard potting soil. The plants were grown in a climate chamber with 70 % relative humidity, 20 ºC, 12 h day length and 30 Wm^{-2} light intensity.

For the analysis of the mineral content and expression study the seeds were sown in Petri dishes on water-saturated filter paper followed by a 4-day vernalization treatment at 4 ºC, and then transferred to a climate room at 25 ºC and 16 h light for one day before transplanting in agar-filled tubes grown on hydroponics containing a modified half-strength Hoagland's nutrient solution (Schat et al., 1996): 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 1 µM KCl, 25 µM H_3BO_3, 2 µM $ZnSO_4$, 2 µM $MnSO_4$, 0.1 µM $CuSO_4$, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 20 µM Fe(Na)EDTA. The pH buffer MES was added at a 2-mM concentration and the pH was set at 5.5 using KOH. The agar-filled tubes were prepared by cutting a small part of the conical end of 0.5-ml eppendorf tubes (SARSTEDT) then filled by 0.55% DAISHIN agar (DUCHEFA BIOCHEMIE, D1004.1000) and left to solidify. The agar-filled tubes were placed in holes prepared in non-translucent plastic 3-mm thick board used as lids for the containers (46 x 31 x 8 cm, FAVORITE) containing 8 liters of the hydroponics solution. During the first two weeks, the nutrient solution was not replaced and thereafter once every two weeks. Germination and plant culture were performed in a climate chamber (20/15 °C day/night temperatures; 250 µmoles light m^{-2} s^{-1} at plant level during 12 h/d; 75% RH).

Root and shoot metal accumulation assay

Five pools of nine plants, grown as described before, were used per treatment. After four weeks of growth the plants were harvested, after desorbing the root system with ice-cold 5 mM $PbNO_3$ for 30 min. Roots and shoots were dried overnight at 65 °C, wet-ashed in a 4:1 mixture of HNO_3 (65%) and HCl (37%) in Teflon bombs at 140°C for 7 hours and analyzed for zinc, iron, manganese, calcium and magnesium using flame atomic absorption spectrometry (Perkin Elmer 1100B). Metal concentrations in roots and shoots were calculated as µmoles per gram dry weight.

Identification and Characterization of T-DNA Insertion Alleles

A T-DNA insertion event within each of the four *NAS* coding sequences (*NAS1* (SALK_082174), *NAS2* (SALK_066962), *NAS3* (SALK_106467) and *NAS4* (SALK_135507)) was found by a database search with the SIGnAL Arabidopsis Gene Mapping Tool (Alonso et al., 2003; http://signal.salk.edu/cgi-bin/tdnaexpress). Seeds were requested and obtained from ABRC (Columbus, OH, USA) and NASC (Loughborough, UK). A gene-specific primer set of LP and RP (Table I) and a T-DNA border primer LBa1 (5'-TGGTTCACGTAGTGGGCCATCG-3') were used to confirm the T-DNA insertion event and to select plants homozygous for each T-DNA insertion.

Table I. Primers used in this study

Primer name	Sequence (5' - 3')	Size (bp)
Gateway cloning primers		
TcNAS1 cDNA forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTATAGTGTCGATATGGCTTG	1035
TcNAS1 cDNA reversed	GGGGACCACTTTGTACAAGAAAGCTGGGTTTACTCGATGGCACTAAA	
TcNAS2 cDNA forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGACATGGCTTGCGAAA	1117
TcNAS2 cDNA reversed	GGGGACCACTTTGTACAAGAAAGCTGGGTCCACACGCATCACAACAAAG	
TcNAS3 cDNA forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGTCGACATGGGTTGCCAA	1100
TcNAS3 cDNA reversed	GGGGACCACTTTGTACAAGAAAGCTGGGTGCAACACACGAACAAGGACAC	
TcNAS4 cDNA forward	GGGGACAAGTTTGTACAAAAAAGCAGGCTCGACATGGGTTATTGCCA	1155
TcNAS4 cDNA reversed	GGGGACCACTTTGTACAAGAAAGCTGGGTGCTAAGAAACAGAGCAAAGAAG	
T-DNA insertion primers		
AtNAS1 forward	CCTCATAGTGTCGATATGGCTTGCC	883
AtNAS1 reversed	ATCCACGAGTACCATTAACCCCGG	
AtNAS2 forward	GACATGGCTTGCGAAAA	933
AtNAS2 reversed	CTTCTCCATACCACGATTGT	
AtNAS3 forward	CATGTAATCCATCAATTAATGGAGA	1556
AtNAS3 reversed	AGGAAACAAGAAACGTCCTCC	
AtNAS4 forward	AGTGTCGACATGGGTTATTGC	1002
AtNAS4 reversed	CAAGAACACAGCATTTTTCTAGG	
RT-PCR primers T-DNA insertions		
AtNAS1 forward	GTGCTTACCCACGGATACAA	726
AtNAS1 reversed	ATCCACGAGTACCATTAACCCCGG	
AtNAS2 forward	GATGCCACTTACTTCCATCGT	187
AtNAS2 reversed	GTATTGGTCTAACCCCTCCTTAG	
AtNAS3 forward	GTCGACATGGGTTGCCAA	969
AtNAS3 reversed	AGGAAACAAGAAACGTCCTCC	
AtNAS4 forward	AGTGTCGACATGGGTTATTGC	957
AtNAS4 reversed	GGTAAGTTGTTCTTCATTAGCACC	
RT-PCR primers Arabidopsis and *T. caerulescens NAS* genes		
AtNAS1 forward	CCTCATAGTGTCGATATGGCTTGCC	822
AtNAS1 reversed	ATCCACGAGTACCATTAACCCCGG	
AtNAS2 forward	GACATGGCTTGCGAAAA	933
AtNAS2 reversed	CTTCTCCATACCACGATTGT	
AtNAS3 forward	GTCGACATGGGTTGCCAA	989
AtNAS3 reversed	AGGAAACAAGAAACGTCCTCC	
AtNAS4 forward	AGTGTCGACATGGGTTATTGC	980
AtNAS4 reversed	GGTAAGTTGTTCTTCATTAGCACC	
At tubulin forward	AAGCTTGCTGATAACTGTACTGGT	405
At tubulin reversed	GGTTTGGAACTCAGTGACATCA	
TcNAS1 forward	CGACTTATACGGACCAAATCTC	307
TcNAS1 reversed	ACTCGATCTTGCTTAGCTTGAG	
TcNAS2 forward	ATTATCCCCAAACTCTAACTCTCTC	304
TcNAS2 reversed	GAGAGCCTATGATTGTGGAGAA	
TcNAS3 forward	ACATGGGTTGCCAAGACG	873
TcNAS3 reversed	ATGAGAAGGCACGAGACTCC	
TcNAS3 forward	CTCACTGAAAACCTAAACGG	529
TcNAS3 reversed	ATGAGAAGGCACGAGACTCC	
TcNAS4 forward	ACCAGCTAGTGAGCACGATCT	475
TcNAS4 reversed	ACCGAATTCGCTGTTGAGTC	
Tc tubulin forward	CCTACGCACCAGTCATCTCT	270
Tc tubulin reversed	CGAGATCACCTCCTGGAACA	

Tc = *Thlaspi caerulescens*; At = *Arabidopsis thaliana*.

Construction of expression vectors

The full-length coding sequences of *TcNAS1*, *TcNAS2*, *TcNAS3* and *TcNAS4* were amplified from *T. caerulescens* genomic DNA by PCR. Primers suitable for Gateway cloning (Invitrogen, http://www.invitrogen.com) were used for PCR (Table I) and the PCRs were performed with proof-reading Pfx DNA polymerase (Fermentas, http://www.fermentas.com) at 93 °C for 5 min, followed by 30 cycles 93 °C for 1 min, 55 °C for 1 min and 68 °C for 3 min, and finished by an extension at 68 °C for 10 min. PCR products were recombined into pDONOR201 (Invitrogen, http://www.invitrogen.com) in a 10-µl BP Clonase (Invitrogen, http://www.invitrogen.com) reaction following the manufacture's instruction. The fragments were transferred from the donor constructs to the binary over-expression vector pH7WG2 (Karimi et al., 2002) in 10-µl LR Clonase (Invitrogen, http://www.invitrogen.com) reaction following the manufacture's instruction. In this plasmid the cloned genes are controlled by the double CaMV 35S promoter.

Plant transformation

The *TcNAS1*, *TcNAS2*, *TcNAS3* and *TcNAS4* overexpression constructs were used to transform *Agrobacterium tumefaciens* strain AGL0 and transformants were selected on LB-medium containing spectinomycin (100 µg/ml). These overexpression constructs were used to transform *A. thaliana* accession Columbia-0 (Col) or the *nas1nas2nas4* triple mutant by the standard flower dip method (Clough and Bent, 1998). T1 seeds obtained upon self-fertilization of the primary transformants were surface-sterilized and sown on half-strength MS medium supplemented with hygromycin (20 µg/ml). Hygromycin-resistant plants were transferred to soil, left to self-fertilize and the T2 seeds were collected. The T2 seeds were surface sterilized, plated on the same selection medium and scored for resistance to hygromycin. Transgenic lines that displayed 3:1 segregation ratio for hygromycin resistance to hygromycin sensitivity in the T2 generation and that were 100% hygromycin resistant in the T3 generation were selected for further analysis. All further experiments were performed with T4 seeds.

Semi-quantitative RT-PCR

Total RNA of leaves and roots of Arabidopsis and *T. caerulescens* was extracted with Trizol (Invitrogen, Carlsbad, CA, USA). Five µg of total RNA was used to synthesize cDNA with MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) as a primer (Invitrogen, Carlsbad, CA, USA). The PCR-amplification was performed with a cDNA aliquot (2 µl) and gene-specific primers (Table I). Primers for Tubulin (Table I) were used as a control for similar cDNA quantities used for each sample. Between 25- to 35-cycle PCRs (30s at 94°C, 30s at 50°C, and 60s at 68°C) were performed in a 50-µl volume. 20 µl of the reaction was separated on an ethidium-bromide-stained 1% agarose gel.

Metal tolerance screening

Seeds of *Arabidopsis thaliana* Col-0, Arabidopsis *NAS* single, double, triple mutants and 35S::*TcNAS3* and 35S::*TcNAS4* transgenic lines were sterilized and sown on vertical plates containing half-strength MS medium, 1 % agar and normal zinc (17 µM $ZnSO_4$), no zinc (0 µM $ZnSO_4$), excess zinc (200 or 400 µM $ZnSO_4$), low iron (0.15 or 0.5 µM FeEDTA), excess nickel (75 µM $NiSO_4$) or cadmium (30 µM $CdSO_4$). For each treatment, four to ten replicate plates were performed and for each line 20 seeds were sown on each replicate plate. Each replicate plate contained 20 seeds of *Arabidopsis thaliana* Col-0 wild type. After sowing, seeds were vernalized at 4 °C for 3 days. Germination and plant culture were in a climate chamber (20/20°C day/night temperatures; 250 µmoles light m^{-2} s^{-1} at plant level during 12 h/d; 75% RH). The root length of individual plants was measured on 5-day-old plants and compared to wild type using Image J software (National Institute of Mental Health, Bethesda, Maryland, USA), to determine tolerance to the applied condition.

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Chapter 6

General Discussion

Macro- and micronutrients are essential for plant growth. Plants have developed homeostasis mechanisms to regulate the uptake and distribution of these nutrients throughout the plant. These homeostasis mechanisms are supposed to be universal within plants. Some plants however, contain high concentrations of certain micronutrients on a dry mass basis in their foliage. These plants are so-called metal hyperaccumulators (Brooks et al., 1977). The term hyperaccumulator was first defined for plants, which contain nickel at concentrations higher than 1000 μg g^{-1} dry weight in their leaves (Brooks et al., 1977). With the discovery of plants accumulating large amounts of other metals, Baker and Brooks (1989) defined additional threshold concentrations for these metals, including 100 μg g^{-1} dry weight for cadmium, 1000 μg g^{-1} dry weight for copper, cobalt, lead and 10,000 μg g^{-1} dry weight for zinc and manganese. Soil pollution with metals is a global problem occurring through natural, industrial and agricultural processes. Hyperaccumulators have attracted considerable scientific interest. Researchers have been focusing on identifying new hyperaccumulating species and on exploring the physiological mechanisms of this trait because of the potential use of these hyperaccumulators for phytoremediation, cleaning up metal-contaminated soil and water (McGrath and Zhao, 2003), or for phytomining, using them to mine metals from high metal soils (Anderson et al., 1999). Plants ideal for phytoremediation should fulfill certain criteria. They should be (1) fast growing plants, (2) have high biomass, (3) deep roots, (4) easy to harvest and (5) tolerate and accumulate a range of heavy metals in their aerial and harvestable parts (Clemens et al., 2002). Until today no plant species has been identified that fulfils all these criteria. An approach that can be taken is to use a rapidly growing non-accumulator species producing high biomass, which can be genetically engineered to achieve some of the properties of hyperaccumulators (Clemens et al., 2002). Progress has been made in understanding the physiology behind the hyperaccumulation trait but the underlying molecular mechanisms for metal accumulation and tolerance in hyperaccumulators are still largely unknown.

The hyperaccumulating species *Thlaspi caerulescens* J. & C. Presl (Brassicaceae) (further referred to as *T. caerulescens*) has been chosen as one of the model species to study metal tolerance and hyperaccumulation (Assunção et al., 2003a). This species can hyperaccumulate

zinc, cadmium and nickel. It is a self-compatible species, which shows variable rates of outcrossing in nature. *T. caerulescens* is closely related to *Arabidopsis thaliana* (L.) Heynh., with on average 88.5 % DNA identity in coding regions (Rigola et al., 2006) and 87 % DNA identity in the intergenic transcribed spacer regions (Peer et al., 2003).

In this thesis, the underlying molecular genetics of metal tolerance and hyperaccumulation were studied by performing transcript profiling, which involved comparative heterologous microarray hybridization experiments of the hyperaccumulator *T. caerulescens* and the non-accumulator *Arabidopsis thaliana* (further referred to as Arabidopsis). The model species Arabidopsis was used as the reference species for heterologous transcript profiling because of the relative close phylogenetic similarity at molecular level to *T. caerulescens*, the availability of molecular and genetic resources and the availability of the complete genome sequence. The latter allows the construction of full transcriptome microarrays that are used for expression analysis. It is expected that gene expression studies of hyperaccumulator species will identify genes that are involved in the hyperaccumulation process, assuming that the main adaptive difference between hyperaccumulators and non-accumulator species lies in differential regulation of otherwise similar genes. Such analysis can only be performed by heterologous transcript profiling, as there are no full-transcriptome arrays available for *T. caerulescens* and this will allow direct comparison of two closely related species with contrasting traits. It will be inevitable that some genes of *T. caerulescens* may not be detected because of reduced homology to the Arabidopsis probes. The extent of such loss of information can be estimated beforehand by hybridizing the Arabidopsis micro-arrays with labeled genomic DNA of *T. caerulescens*.

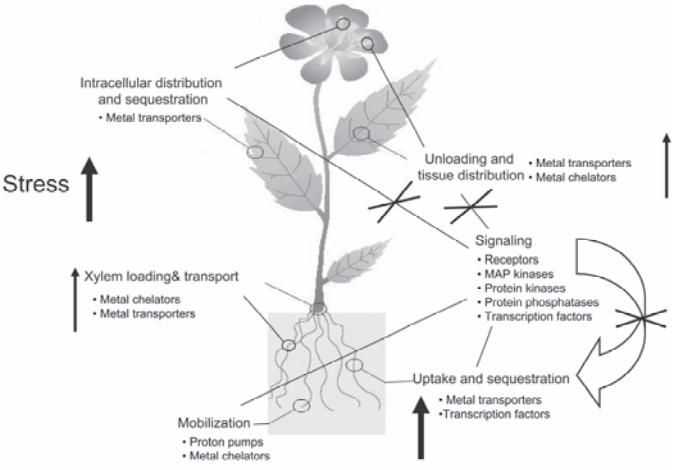

Figure 1. Molecular mechanism proposed to be involved in metal hyperaccumulation by *T. caerulescens*.

Plants use two methods to take up metals from the soil: (1) acidification of the rhizosphere through plasma membrane pumps and (2) by secretion of chelators. When metals are taken up in the root they are bound by the cell wall. The soluble metals can enter the root symplast by crossing the plasma membrane (PM) of the root epidermal, cortical or endodermal cells. Entering the root symplast and crossing these PMs is mediated by secondary transporters. The metals enter the xylem from where it is translocated to aerial tissues. Chelation with certain ligands such as histidine, nicotianamine and citrate facilitates the routing of these metals through the xylem. The metals reach the apoplast of the leaves in the xylem sap from where they are scavenged by the leaf cells. Transporters mediate uptake into the symplast, and distribution within the leaf occurs via the apoplast or the symplast. Once in the leaf cells, the metals can be sequestered into different subcellular compartments (cytosol, vacuole). Metal uptake, transport, sequestration, xylem loading and unloading are thought to be continuous processes within the hyperaccumulator species *T. caerulescens* and thus it is expected to require a constitutive expression (indicated with an arrow) of metal hyperaccumulation genes and stress-induced genes as an adaptation of the hyperaccumulator species to extreme environments. The signaling pathway necessary for regulation of metal uptake and distribution seems not to be regulated as effective as in non-accumulating species (indicated with a cross).

Plants have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001). A number of genes encoding potential metal transporters appear to be constitutively highly expressed in *T. caerulescens* compared to Arabidopsis. These belong to the heavy metal ATPases (*HMA3*, *HMA4*), the natural resistance associated macrophage protein family (*NRAMP3*, *NRAMP4*), the cation diffusion facilitator (CDF) (*AtMTP1*/*TcZTP1*; *MTP8*) family, the ZRT- and IRT-like

proteins (*AtZIP4*/*TcZNT1*; *AtIRT3*/*TcZNT2*) and the cation antiporters (Chapter 2; Pence et al., 2000; Assunção et al., 2001; Bernard et al., 2004). P_{1B}-ATPases are a subfamily of the P-type ATPases, which are known to transport heavy metals such as copper, zinc, cadmium and lead (Lutsenko and Kaplan, 1995; Axelsen and Palmgren, 2001; Argüello, 2003; Courbot et al., 2007). *ZIPs* and *NRAMPs* are suggested to mediate cellular metal uptake (Fox and Guerinot, 1998; Thomine et al., 2000). *CDFs* appear to mediate vacuolar sequestration of zinc (van der Zaal et al., 1999). Metal hyperaccumulation is a constitutive trait in *T. caerulescens* and thus we expected that it requires a constitutive expression of metal hyperaccumulation genes instead of specific induction at deficiency or excess (Figure 1), whereas in Arabidopsis most of these genes are induced under these conditions. It is complicated to identify such metal accumulation genes from the large set of more or less constitutively higher expressed *T. caerulescens* genes, as many genes will be involved in general species differences that might be unrelated to metal hyperaccumulation. Hammond et al. (2006) compared two Thlaspi species to each other but not to Arabidopsis. An interesting general trend that emerged from their analysis together with other expression analysis studies (Chapter 2 and 3; Becher et al., 2004; Weber et al., 2004, 2006) of the heavy metal adapted species was that many orthologous of genes that were induced by stress in general in Arabidopsis were higher expressed in the adapted species (Figure 1).

Other transporter genes in *T. caerulescens*, for example, the potassium transporters *HAK5* (Chapter 2 and 4), *KUP3* (Chapter 2) and *KAT* (Chapter 2) or the sulfate transporter *SULTR2;1*(*AST68*) (Chapter 3, Broadley et al., 2007) are also highly expressed in the hyperaccumulator *T. caerulescens.* Armengaud et al. (2004) found that *HAK5* was induced upon potassium starvation in Arabidopsis and we identified this gene as being also higher expressed in *T. caerulescens* roots compared to Arabidopsis roots (Chapter 2). *HAK5* was also higher expressed in the roots of *T. caerulescens* accession Ganges and La Calamine. The higher expression of *HAK5* (Chapter 4) in leaves of the cadmium tolerant La Calamine accession compared to Ganges suggests that *T. caerulescens* accession La Calamine becomes potassium deficient upon zinc and cadmium accumulation. The potassium level in the leaves of La Calamine should be measured to confirm this. Potassium is the most abundant cation in higher

plant cells and plays central roles in plant growth and development, including maintenance of turgor pressure, leaf and stomatal movement, and cell elongation. A recent study (Shin and Schachtman, 2004) identified reactive oxygen species (ROS) as a critical signaling molecule for plant response to potassium-deficiency conditions. Interestingly, ROS in root cells has been shown to elicit changes in cellular calcium and is required for root hair growth and mineral uptake (Foreman et al., 2003). Together, these studies suggest that the ROS signal is produced under low potassium conditions and ROS-induced calcium changes may be a crucial messenger for downstream responses. However, it is not known how the low-K^+-induced calcium signal is perceived by the cell. In addition to the low-K^+ response, calcium plays multiple roles in many other cellular pathways, including signaling processes in response to pathogen, abiotic stresses, and developmental signals (Shin and Schachtman, 2004). The low potassium response in *T. caerulescens* could trigger a calcium signal, which can protect the plant from heavy metal stress but this should be verified by analyzing the potassium and calcium fluxes in the hyperaccumulating species.

Phosphate is an essential element required for plant growth and development. Besides nitrogen, phosphate is the most limiting nutrient for plant growth, and it is a common limiting factor for crop production in arable soils. Plants have evolved general strategies for phosphate acquisition and use in limiting environments that include: mycorrhizal symbioses, decreased growth rate, remobilization of internal inorganic phosphate, modification of carbon metabolism bypassing phosphate-requiring steps, increased production and secretion of phosphatases, exudation of organic acids, modification of root architecture, expansion of root surface area, and enhanced expression of inorganic phosphate transporters (Hernández et al., 2007). Three phosphate transporters (*PHT3*, *PHT4*, *PHT5*) were much higher expressed in *T. caerulescens* roots compared to Arabidopsis (Chapter 2). Phosphate transporters are normally expressed in plant roots to accommodate inorganic phosphate uptake. Hammond et al. (2003) have shown that many genes that respond to phosphate stress in Arabidopsis shoots also respond to other environmental challenges, including salinity, wounding, pathogen attack, anoxia, and other nutrient stresses. For *A. halleri* a difference in expression of *PHT1;4* was found between

accumulator and non-accumulator F3 plants segregating in the progeny of an interspecific cross between *A. halleri* and *A. lyrata ssp. petrea* (Filatov et al., 2006). Another study on *A. halleri* grown in Zn-containing hydroponics showed a correlation between the concentration of Zn and the concentration of phosphate in the roots (Zhao et al., 2006). The zinc-phosphate correlation was attributed to zinc phosphate precipitates at the root surface. No zinc correlation with phosphate or organic acids was found in the leaves. Previous reports have also shown that zinc deficiency induces expression of high-affinity phosphate transporters in barley (Huang et al., 2000), although the plants are not phosphate deficient and actually contain more phosphate at low zinc conditions than at sufficient zinc conditions. These results together with the constitutive high expression of some ZIP family members suggest that *T. caerulescens* experiences constitutive zinc deficiency and thus is unable to down-regulate phosphate transporter expression. There also is a relationship between iron and phosphate homeostasis. This phenomenon is reinforced by the formation of insoluble complexes in the environment with many cations, affecting the solubility of both phosphate and associated ions (Hirch et al., 2006). This interaction is investigated for iron, a strong phosphate chelator. Depleting the medium in phosphate clearly resulted in an increase of iron content in Arabidopsis. These modifications triggered molecular responses linked with iron status (transport, homeostasis and accumulation). Interestingly, physiological modifications affecting iron storage were also observed (Hirch et al., 2006). The accumulation of phosphate/iron complexes in the vacuoles of plants grown in inorganic phosphate-rich medium disappeared in inorganic phosphate-depleted medium in favor of accumulation of iron inside the chloroplasts, likely associated with ferritin (Hirch et al., 2006). The constitutive high expression of these three phosphate transporters in *T. caerulescens* suggests that the hyperaccumulator suffers from constitutive phosphate deficiency or a probable other micronutrient stress. Zinc-phosphate precipitates could be formed at the root surface, which cause phosphate deficiency in the root and as a response to this starvation the expression of the phosphate transporters is induced. Another assumption may be that hyperaccumulation is associated with a state of zinc deficiency. Lowering the phosphate level in the apoplast through enhanced expression of the phosphate transporters could mobilize zinc and stimulates zinc uptake. Thus the higher expression of the phosphate transporters might also be a zinc deficiency response. These results

together with the constitutive high expression of three phosphate transporters *in T. caerulescens* show a clear correlation between phosphate and other nutrients and mineral content analysis should be performed to confirm this.

The tripeptide glutathione is a product of primary sulfur metabolism. A variety of enzymes or proteins are involved in sulfur assimilation including sulfate transporters (STs), ATP sulfurylase (ATPS), APS reductase (APSR), sulfite reductase (SiR), γ-glutamylcysteine synthetase, glutathione synthetase (GS) and phytochelatin synthase (PCS). These enzymes or proteins are upstream-regulated by cadmium at either the metabolic or the genetic level under metal stress. Increasing evidence shows that enhancement of sulfate uptake and reduction occurs with the production of phytochelatins (PC) in plants under heavy metal stress. A relationship between sulfur metabolism and heavy metal tolerance was also suggested by the higher expression of several sulfur metabolism genes upon exposure to cadmium (Chapter 3). The sulfate transporter *SULTR2;1* was already shown to be over-expressed in response to cadmium treatment in Arabidopsis (Herbette et al., 2006). In *Saccharomyces cerevisiae*, exposure of cells to cadmium leads to a global drop in sulfur-containing protein synthesis and in a redirection of sulfur metabolite fluxes to the glutathione pathway (Lafaye et al., 2005). Thus, at least in yeast, heavy metal exposition and sulphur homeostasis are clearly linked. Several other genes involved in sulfate assimilation were differentially expressed in *T. caerulescens* in response to cadmium (Chapter 3). Sulfur is present in the oxidized state as a sulfo-group in plant metabolites, which play various roles in plant defense against biotic and abiotic stress, like for example cadmium stress but this will need further attention to confirm this.

One mechanism for heavy metal detoxification in plants is the chelation of metal ions to ligands like organic acids, amino acids, peptides and polypeptides. Nicotianamine (NA) is such a chelator and it has been suggested that NA (1) is able to form more stable complexes with Fe^{2+} than with Fe^{3+} but also with other metals, (2) serves as a sensor for the physiological iron status within the plant and (3) might be involved in the transport of iron (Scholz et al., 1992; Stephan and Scholz, 1993; Ling et al., 1999). NA is made through the activity of nicotianamine synthase

(NAS). The *NAS* gene family (*NAS1*, *NAS2*, *NAS3* and *NAS4*) is constitutively highly expressed in *T. caerulescens* exposed to zinc and cadmium (Chapter 2-5) (Figure 1). Expression analysis of the *NAS* gene family members showed that the expression of three of the four *NAS* genes is different between Arabidopsis and *T. caerulescens* (Chapter 2-5), which may suggest a function for these genes in translocation of metals to the shoots in *T. caerulescens*. Comparative transcriptomic studies of *A. thaliana* with the hyperaccumulator species *A. halleri* (Becher et al., 2004; Weber et al., 2004) also revealed that the *NAS* genes were differentially expressed in this hyperaccumulating plant species in response to zinc. In *A. halleri* four *NAS* genes were identified and sequence analysis showed 94-98% similarity to the four *A. thaliana* genes (Weber et al., 2004). The four *T. caerulescens NAS* genes showed 88-91% similarity to the four orthologous *A. thaliana* genes (Chapter 5). Overexpression of the *T. caerulescens NAS* genes in Arabidopsis resulted in a phenotype more tolerant to excess nickel and zinc (Chapter 5). Overexpression of these metal homeostasis related genes therefore seems to be a good mechanism for improving phytoremediation properties of non-accumulators.

The relatively high expression of genes with a suggested function in lignin/suberin biosynthesis (Ehlting et al., 2005; Costaglioli et al., 2005) in *T. caerulescens* was very surprising (Chapter 2 and 3) and coincides well with the remarkable feature of *T. caerulescens* roots developing an extra endodermal layer of cells forming a compact cylinder surrounding the endodermis from the outer side (Chapter 2), which might be linked to the hyperaccumulation trait. This layer is composed of secondary cell walls impregnated by suberin/lignin and is not found in the non-accumulator species *T. arvense* (Broadley et al., 2007) or Arabidopsis (Chapter 2). Additionally, Broadley et al. (2007) described a difference in the endodermis between *T. caerulescens* and *T. arvense*. In *T. caerulescens* roots, the Casparian bands and suberin lamellae were developed much closer to the root tip than in *T. arvense*. However, we did not find a difference in the composition and content of suberin and lignin in four-week-old roots between *T. caerulescens* and Arabidopsis (data not shown). The reason can be that for the analysis whole roots were taken, rather then only root tips, which could have diluted the contribution of extra suberinization/lignification in *T. caerulescens* too much to be detectable. Four genes called

Disease resistance-responsive or Dirigent (*DIR11*, *DIR13*, *DIR20*, *DIR23*) were constitutively expressed in *T. caerulescens*. These genes are actually involved in lignin biosynthesis (Gang et al., 1999) and were identified to have the highest expression in the root endodermis of Arabidopsis (http://www.arexdb.org/index.jsp). Also the comparative transcriptional analysis of the cadmium response of the zinc/cadmium-hyperaccumulator *T. caerulescens* and the non-accumulator Arabidopsis emphasizes the role of genes involved in lignin biosynthesis (Chapter 3). A hypothesis is that this layer prevents excess efflux of metals from the vascular cylinder rather than to prevent uncontrolled influx (Chapter 2). The precise function of this layer of cells and its impact on apoplastic and symplastic fluxes of metals into the root stele remains unclear and further anatomical and physiological studies are necessary to study the differences in ion influx/efflux between hyperaccumulating and non-accumulating species.

Two DOF-type zinc finger domain-containing proteins, *AtDOF24* and *AtDOF31*, were constitutively higher expressed (*AtDOF24*: 4-7 fold higher and *AtDOF31*: 42-65 fold higher) in *T. caerulescens* roots compared to Arabidopsis roots (Chapter 2). This DOF (DNA-binding with One Finger) family of transcription factors is specific to plants (Moreno-Risueno et al., 2007). DOF transcription factors have been suggested to participate in the regulation of vital processes exclusive to plants such as photosynthetic carbon assimilation, light-regulated gene expression, accumulation of seed-storage proteins, germination, dormancy, response to phytohormones, flowering time, and guard cell-specific gene expression (Lijavetzky et al., 2003; Moreno-Risueno et al., 2007). The 35S::*AtDOF24 t*ransgenic lines showed a strong glossiness of young stem and siliques. This is typical for alterations in epicuticular wax biosynthesis, mutants of which are generally described as *eceriferum* (*cer*) mutants in Arabidopsis (Kunst and Samuels, 2003). Glossiness in these mutants is often not caused by less wax deposition, but by alterations in the wax composition (Jenks et al., 1995). When comparing Arabidopsis and *T. caerulescens* roots, *CER3* and *CER6* were found to be much higher expressed in *T. caerulescens*. Especially the expression of *CER6* in *T. caerulescens* roots is remarkable, as the expression in Arabidopsis roots is low (Hooker et al., 2002) and wax deposition on roots has not been described. As wax biosynthesis and suberin formation are biochemically closely related and involve the elongation of fatty acids (Franke and Schreiber, 2007) it is not un-realistic to assume that *CER3* and *CER6*,

which are both involved in fatty acid elongation (Kunst and Samuels, 2003), also play a role in suberin formation. Arabidopsis *cer3* and *cer6* mutants have less wax on the stems compared to wild-type plants (Jenks et al., 1995), but have not been examined for suberin content. Overexpression of *CER6* in Arabidopsis epidermis causes a higher wax load, but no alteration in wax composition (Hooker et al., 2002) and a similar phenotype was observed upon overexpression of *CER3* (Hannoufa et al., 1996). The expression level of these genes therefore seems to be positively correlated with wax load. Although overexpression of combinations of *CER* genes has not been investigated yet, it seems unlikely that the glossy phenotype of the 35S::DOF plants is due to overexpression of *CER3* or *CER6*. Down-regulation of expression seems more likely, but this contrasts the high expression of all of these genes in *T. caerulescens* compared to Arabidopsis. There is of course the possibility that the DOF transcription factor needs to be co-expressed with another gene to accomplish overexpression and that overexpression of only one component acts as a dominant negative factor. It is clear that this deserves further attention.

In order to adapt to the highly contaminated soil as a new niche, most likely the signaling pathway has changed in the hyperaccumulator plants, which results in changing the activity of certain transcription factors (Chapter 2-4) (Figure 1). The change in activity of transcription factors might have resulted in a constitutive over-expression of certain genes like metal transporters, metal chelators, stress-inducible genes (Figure 1) and subsequent higher uptake or sequestration of the metals in the plant. To test the importance of transcription factors, we recently started overexpression studies of *bHLH100* (At2g41240) (Chapter 3) and the two DOF-type zinc finger domain-containing proteins (At1g64620, At1g21340) as discussed above (data not shown). An earlier study showed that the putative bHLH transcription factor, Fe-deficiency Induced Transcription Factor 1 (*FIT1*), regulates iron deficiency responses in Arabidopsis (Colangelo and Guerinot, 2004). *FIT1* is required for proper regulation of ferric chelate reductase activity and iron transport into the plant root. This is achieved by regulating the Fe^{3+} chelate reductase FRO2 at the level of steady state mRNA accumulation and by controlling protein accumulation of the Fe^{2+} transporter IRT1. *FIT1* also controls many genes implicated in iron homeostasis as well as many novel genes, as was demonstrated by microarray analysis of a *fit1*

mutant (Colangelo and Guerinot, 2004). The *bHLH100* transcription factor was higher expressed under zinc excess and high cadmium exposure in *T. caerulescens* and Arabidopsis roots and leaves (Chapter 2 and 3) and 35S::*AtbHLH100* transgenic lines outperformed the Arabidopsis wild-type Col-0 in terms of tolerance to excess zinc, excess nickel, excess cadmium and iron deficiency (Chapter 3). This gene therefore could be involved in the regulation of genes causing overall higher tolerance to the plant but could also imply involvement of this gene in the regulation of iron homeostasis genes as was suggested for *FIT1*. According to the semi-quantitative RT-PCR results (Chapter 2 and 3) *bHLH100* is more induced under excess zinc and cadmium in the roots and leaves of Arabidopsis but these conditions could as well induce iron deficiency in the plant. To confirm this, expression studies on the iron deficiency response genes *FRO2* and *IRT1* should be performed in a *bHLH100* knock-out mutant to identify if the expression level of these genes is influenced and also mineral content analysis could be performed in this mutant to identify if the iron content in the mutant has changed.

When considering the large number of genes that are constitutively overexpressed in *T. caerulescens* compared to Arabidopsis roots, it is very unlikely that all of these genes will have a functional role in accumulating heavy metals. Probably only mutant analysis in *T. caerulescens* will be a way to distinguish the genes that involved in metal accumulation. On the other hand, the large number of differentially expressed genes makes it also unlikely that altered regulation and overexpression of single genes will be sufficient to convert metal nonaccumulators into hyperaccumulators, although there still is the possibility that overexpression of only one or two key regulatory loci will have this effect (Guerinot and Salt, 2001).

In summary, the research in this thesis has revealed a large amount of information about the possible molecular mechanisms behind metal tolerance and hyperaccumulation in *T. caerulescens*. It remains extremely challenging to elucidate how the function of single proteins in heterologous systems relates to the behavior at the whole-plant level. Furthermore the use of homologous transformation and the increasing availability of *T. caerulescens*-specific sequence information will undoubtedly fasten this process.

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SUMMARY

Heavy metal tolerance and accumulation in *Thlaspi caerulescens*, a heavy metal hyperaccumulating plant species

Minerals are essential for humans, plants and animals and have an important micronutrient role in physiological and metabolic processes of plants. Next to this essential role of minerals, they can also be very toxic when available to the plant in elevated amounts. Plants therefore need to keep very tight control over the intracellular mineral concentrations in a process called metal homeostasis. Although the metal homeostasis mechanisms are supposed to be universal within plants, there are plant species that can tolerate and even accumulate large amounts of metals without any sign of toxicity. *Thlaspi caerulescens* J. & C. Presl (Brassicaceae), a close relative of the plant reference species *Arabidopsis thaliana* (Arabidopsis), is one of these natural metal hyperaccumulator species. The overall aim of this project is to unravel the molecular genetic mechanism of heavy metal tolerance and hyperaccumulation of the metal hyperaccumulating plant species *T. caerulescens*. To achieve this goal heterologous transcript profiling experiments were performed, which involved comparative microarray hybridization experiments of the hyperaccumulator *T. caerulescens* and Arabidopsis. Arabidopsis is used as the reference species for heterologous transcript profiling because of the availability of genetic resources and the complete genome sequence.

The micronutrient zinc has an essential role in physiological and metabolic processes in plants as a cofactor or structural element. *Thlaspi caerulescens* can accumulate up to 3% of zinc on a dry weight basis without any sign of toxicity. The question postulated here is if this has drastic effects on the zinc homeostasis mechanism. We examined in detail the transcription profiles of roots of Arabidopsis and *T. caerulescens* plants grown under deficient, sufficient and excess supply of zinc (**Chapter 2**). A total of 608 genes were detected in Arabidopsis and 352 in *T. caerulescens* that responded transcriptionally to changes in zinc supply. Only 14% of these genes were also zinc-responsive in Arabidopsis. When comparing Arabidopsis and *T. caerulescens* at comparable zinc exposures, over 2200 genes were significantly differentially

expressed. While a large fraction of these genes are of yet unknown function, many genes with a different expression between Arabidopsis and *T. caerulescens* appear to function in metal homeostasis, in abiotic stress response and in lignin biosynthesis. The high expression of lignin biosynthesis genes corresponds to the deposition of lignin in the endodermis. Contrary to Arabidopsis roots, which have one endodermal cell layer, we found there are two endodermal layers in *T. caerulescens* roots. This extra physical barrier could enhance the control of metal fluxes in the plant, in addition to the higher expression of metal transporters in the root.

Cadmium is a widespread, naturally occurring non-essential element that is toxic for plants in higher concentrations. In **chapter 3** we compare between and within species transcript profiles of Arabidopsis and *T. caerulescens* roots exposed to cadmium, with the aim to establish which genes are most likely to be relevant for the tolerance to cadmium exposure of *T. caerulescens*. The comparative transcriptional analysis of the cadmium response of roots of the *T. caerulescens* and Arabidopsis emphasizes the role of genes involved in lignin-, glutathione- and sulfate metabolism. Furthermore two transcription factors, *MYB72* and *bHLH100*, with an altered expression after exposure to cadmium, are studied for their involvement in metal homeostasis. Analysis of a *myb72* knock-out mutant showed enhanced sensitivity to excess zinc or iron deficiency. Rather than controlling Cd tolerance, this gene appears to be involved in iron homeostasis, affecting the response to Cd indirectly. Arabidopsis transformants overexpressing the transcription factor *bHLH100* showed enhanced zinc and nickel tolerance, and although the exact role of this gene still needs to be resolved, the genes appears to have a role in metal homeostasis in Arabidopsis.

T. caerulescens accessions exhibit distinct metal accumulation, translocation and tolerance characteristics. *T. caerulescens* accession Ganges can accumulate high amounts of cadmium and is extremely tolerant to cadmium, whereas the La Calamine accession is also tolerant to cadmium but accumulates much less cadmium compared to Ganges. The transcription profiles of leaves and roots of *T. caerulescens* accessions Ganges and La Calamine plants grown with and without cadmium were examined using the Qiagen-Operon Arabidopsis Genome Array and results are described in **chapter 4**. A total of 161 genes were differentially expressed between the two *T. caerulescens* accessions in response to changes in cadmium supply and 38 genes were

differentially expressed in *T. caerulescens* accession Ganges leaves in response to cadmium. The comparative transcriptional analysis emphasizes that there are just minor differences between the two accessions but the genes which are differentially expressed could play an important role in the hyperaccumulation of cadmium in Ganges. The microarray data suggest that especially genes involved in cell wall modification and stress response relate to the major difference between the two accessions in cadmium hyperaccumulation.

Plants have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals. One potential mechanism for heavy metal detoxification in plants is the chelation of metal ions to ligands like organic acids, amino acids, peptides and polypeptides. This mechanism is important for the distribution of metal ions by keeping metal ions mobile within the plant. In plants metals are often found to be chelated to nicotianamine. Nicotianamine is formed by trimerization of S-adenosylmethionine, which is catalyzed by the enzyme nicotianamine synthase. Arabidopsis contains four nicotianamine synthase (*NAS*) genes. Also in *T. caerulescens* four full-length cDNAs encoding nicotianamine synthase members were identified (**Chapter 5**). The four genes were named *TcNAS1*-*TcNAS4*, analogous to the corresponding closest homologue in Arabidopsis. Arabidopsis plants over-expressing *TcNAS1*, *TcNAS2*, *TcNAS3* or *TcNAS4* that were tested for their response to growth on media with different zinc, iron, nickel or cadmium supply, provided evidence that the *Thlaspi* genes all have a genuine NAS function because they complement the NAS deficiency in specific triple knock-out Arabidopsis mutants. Evidence for a functional role in metal homeostasis was sought in studying the Arabidopsis single, double, triple and a quadruple nicotianamine synthase T-DNA insertion mutants. The combination of null mutations in three or four *AtNAS* genes, results in a severe phenotype that includes interveinal chlorosis and altered metal concentrations in leaves, roots and seeds. Arabidopsis transformants overexpressing *TcNAS3* or *TcNAS4* showed enhanced zinc and nickel tolerance compared to wild type plants.

The research described in this thesis does contribute to a better understanding of heavy metal hyperaccumulation in *T. caerulescens* and it can be concluded that it seems unlikely that altered regulation and overexpression of single genes will be sufficient to convert metal

nonaccumulators into hyperaccumulators. However, the possibility that overexpression of one or two key regulatory loci have this effect remains.

SAMENVATTING

Zware metalen tolerantie en accumulatie in *Thlaspi caerulescens* (Zinkboerenkers), een zware metalen hyperaccumulerende plantensoort

Voedingsstoffen zijn essentieel voor mens, plant en dier en spelen een belangrijke rol in fysiologische en metabolische processen in de plant. Naast deze essentiële functie kunnen deze voedingsstoffen ook erg toxisch zijn wanneer deze in te hoge concentraties beschikbaar zijn voor de plant. Planten hebben daarom een mechanisme ontwikkeld om de intracellulaire concentratie te controleren, dit wordt ook wel metaal homeostase genoemd. Hoewel metaal homeostase mechanismen universeel lijken te zijn in planten, zijn er plantensoorten die hoge concentraties metalen kunnen tolereren en zelfs accumuleren zonder toxiciteitsymptomen te vertonen. *Thlaspi caerulescens* J. & C. Presl (Zinkboerenkers) (Brassicaceae), familie van de model plant *Arabidopsis thaliana* (Arabidopsis), is één van deze metaal hyperaccumulerende soorten. Het doel van het onderzoek dat in dit proefschrift beschreven staat is het identificeren van het genetisch mechanisme wat betrokken is bij zware metalen tolerantie en hyperaccumulatie in de metaal hyperaccumulerende plantensoort *T. caerulescens*. Om dit doel te bereiken zijn heterologe microarray hybridisaties uitgevoerd, door vergelijkende microarray analyses te doen met de hyperaccumulator *T. caerulescens* en *Arabidopsis*. De model plant Arabidopsis wordt gebruikt als referentie vanwege de aanwezigheid van genetische hulpmiddelen en de beschikbaarheid van de complete genoom sequentie.

Het micronutriënt zink speelt als cofactor of structureel element een belangrijke rol in fysiologische en metabolische processen in planten. *T. caerulescens* kan tot 3% zink accumuleren zonder toxiciteit symptomen te krijgen. De vraag hier is of dit een drastisch effect heeft op het zink homeostase mechanisme in de plant en daarom werd het transcriptie niveau in Arabidopsis en *T. caerulescens* wortels gegroeid op zink deficiënt, sufficiënt en overmaat geanalyseerd (**Hoofdstuk 2**). In totaal komen er 608 genen differentieel tot expressie in Arabidopsis en 352 genen differentieel tot expressie in *T. caerulescens* in respons op zink. Maar 14% van de 352 zinkgereguleerde genen in *T. caerulescens*, zijn zinkgereguleerd in Arabidopsis. In de

vergelijking tussen *T. caerulescens* en Arabidopsis komen meer dan 2200 genen differentieel tot expressie. Een groot percentage van deze genen zijn genen waarvan de functie niet bekend is; de genen waarvan de functie wel bekend is, zijn betrokken bij metaal homeostase, abiotische stress-respons en lignine biosynthese. Het hoge expressieniveau in *T. caerulescens* van genen betrokken bij lignine biosynthese houdt verband met een lignine laag in de endodermis. In vergelijking tot de wortels van Arabidopsis, die één endodermale cellaag hebben, heeft *T. caerulescens* twee endodermale cellagen. Deze extra fysische barrière zou de metaalfluxen in de plant kunnen controleren, wat tezamen met de hogere expressie van metaal transporteurs in de wortel kan leiden tot een veranderde metaal homeostase in vergelijking met Arabidopsis.

Cadmium is een wijd verspreid, natuurlijk voorkomend element in de bodem, water, planten en dieren. Cadmium is een niet essentieel element in planten en is erg toxisch. In **hoofdstuk 3** vergelijken we Arabidopsis en *T. caerulescens* wortels blootgesteld aan cadmium, zodat achterhaald kan worden welke genen relevant zijn voor de adaptatie van cadmium tolerantie in *T. caerulescens*. De vergelijkende transcriptieanalyse van de cadmium respons in wortels van *T. caerulescens* en Arabidopsis benadrukt de rol van genen betrokken bij lignine-, glutathion- en sulfaat metabolisme. Twee transcriptie factoren, *MYB72* en *bHLH100*, die een veranderd expressieniveau hebben na blootstelling aan cadmium, werden bestudeerd met betrekking tot hun functie in metaal homeostase. Het analyseren van een *myb72* knock-out mutant heeft aangetoond dat MYB72 gevoeliger is voor blootstelling aan een hoge concentratie zink en ijzerdeficiëntie. Deze resultaten suggereren dat dit gen niet betrokken is bij cadmium tolerantie maar bij de regulatie van de ijzer homeostase dat een indirect effect heeft op de cadmium respons. Arabidopsis *bHLH100* overexpressietransformanten zijn toleranter voor blootstelling aan een hoge concentratie zink en nikkel, en hoewel de exacte functie nog niet bekend is lijkt dit gen een functie te hebben in metaal homeostase.

T. caerulescens accessies vertonen vaak grote onderlinge verschillen in de mate van metaalaccumulatie, -translocatie en -tolerantie. De *T. caerulescens* accessie Ganges heeft het vermogen om grote hoeveelheden cadmium te accumuleren en is ook extreem tolerant voor cadmium, terwijl de La Calamine accessie tolerant is maar veel minder het vermogen heeft om cadmium te accumuleren in vergelijking met Ganges. Het transcriptie niveau van *T. caerulescens*

accessie Ganges en La Calamine wortel en blad gegroeid met en zonder cadmium is getoetst (**Hoofdstuk 4**) door gebruik te maken van de Qiagen-Operon Arabidopsis genoom array. In totaal komen 161 genen differentieel tot expressie tussen de twee *T. caerulescens* accessies en 38 genen komen differentieel tot expressie in het blad van Ganges gegroeid met en zonder cadmium. De vergelijkende transcriptie analyse benadrukt dat er slechts geringe verschillen zijn tussen de twee accessies en dat de genen die differentieel tot expressie komen een rol kunnen spelen in de hyperaccumulatie van cadmium in *T. caerulescens* accessie Ganges. De microarray data suggereren dat vooral genen welke betrokken zijn bij celwand modificatie en stress respons het verschil uitmaken tussen de twee accessies.

Planten hebben een complex netwerk ontwikkeld om de opname, accumulatie, transport en detoxificatie van metalen te reguleren. Een potentieel mechanisme voor zware metalen detoxificatie in planten is de binding van metaalionen met verbindingen zoals organische zuren, aminozuren, peptiden en polypeptiden. Dit mechanisme is belangrijk voor de distributie van metaalionen door metaalionen mobiel te houden in de plant. In planten worden metalen vaak gebonden met nicotianamine. Nicotianamine wordt gevormd door de trimerisatie van S-adenosylmethionine, en deze reactie wordt gekatalyseerd door het enzym nicotianamine synthase. Zowel Arabidopsis als *T. caerulescens* hebben vier nicotianamine synthase (*NAS*) genen (**Hoofdstuk 5**). De vier *NAS* genen in *T. caerulescens* zijn *TcNAS1-TcNAS4* gebaseerd op de homologe genen in Arabidopsis. Arabidopsis *TcNAS* overexpressie planten zijn gefenotypeerd door deze te laten groeien op medium met verschillende zink-, ijzer-, nikkel- en cadmium-concentraties. De resultaten bewijzen dat de Thlaspi genen een NAS functie hebben omdat ze het NAS deficiëntie fenotype van de specifieke Arabidopsis *NAS* tripel knock-out mutant kunnen complementeren. Bewijs voor een functionele rol in metaal homeostase werd bestudeerd in de Arabidopsis enkel, dubbel, tripel en quadrupel nicotianamine synthase T-DNA insertie mutanten. De combinatie van nul mutanten in drie van de vier *AtNAS* genen resulteerde in een vergeling van het weefsel tussen de nerven en een verandering in metaal concentratie in blad, wortel en zaad. Arabidopsis *TcNAS3* en *TcNAS4* overexpressie planten hebben een zink en nikkel tolerant fenotype in vergelijking tot het Arabidopsis wilde type.

De resultaten in dit proefschrift dragen bij aan een beter begrip wat betreft metaal hyperaccumulatie in *T. caerulescens* . Het lijkt onwaarschijnlijk dat een verandering in regulatie en overexpressie van één enkel gen voldoende is om een metaal non-accumulator te veranderen in een hyperaccumulator. Echter, de mogelijkheid dat overexpressie van één of twee belangrijke regulerende loci dit effect wel heeft blijft bestaan.

LIST OF PUBLICATIONS

Published articles from this thesis:

van de Mortel JE, Almar Villanueva L, Schat H, Kwekkeboom J, Coughlan S, Moerland PD, Ver Loren van Themaat E, Koornneef M, Aarts MGM (2006) 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(3):1127-47.

van de Mortel JE, Aarts MGM (2006) Comparative transcriptomics -- model species lead the way. New Phytologist 170(2):199-201.

Additional articles:

Fedorova M, **van de Mortel J**, Matsumoto PA, Cho J, Town CD, VandenBosch KA, Gantt JS, Vance CP (2002) Genome-wide identification of nodule-specific transcripts in the model legume *Medicago truncatula*. Plant Physiology 130(2):519-37.

CURRICULUM VITAE

Judith van de Mortel werd op 21 april 1977 in Deurne geboren. Na het behalen van het MAVO diploma in 1993 en het behalen van certificaten op de HAVO in 1995 begon zij haar studie MLO- Medische Microbiologie. Tijdens deze studie liep zij stage bij de Gezondheidsdienst voor Dieren (GD) te Boxtel. In 1998 begon zij haar studie HLO-Technische Microbiologie en de afstudeeropdracht werd uitgevoerd bij de University of Minnesota (VS), Department of Agronomy and Plant Genetics in de groep van Carroll Vance. Na het afstuderen in 2001 werkte zij nogmaals 10 maanden in de groep van Carroll Vance om vervolgens in augustus 2002 als onderzoeker in opleiding te gaan werken aan het identificeren van het genetisch mechanisme wat betrokken is bij zware metalen tolerantie en hyperaccumulatie in de metaal hyperaccumulerende plantensoort *Thlaspi caerulescens* bij de leerstoelgroep Erfelijkheidsleer van Wageningen Universiteit in de groep van Mark Aarts. De resultaten van dit onderzoek staan beschreven in dit proefschrift. Met ingang van 21 mei 2007 werkt zij als postdoc bij de leerstoelgroep fytopathologie van Wageningen Universiteit als onderdeel van het STW project Biosurfactant production in plant-beneficial *Pseudomonas*.

NAWOORD

Na bijna vijf jaar is het dan zover. Het proefschrift is klaar en mijn promotietijd zit erop. Bij dezen wil ik in het laatste deel van mijn proefschrift alle mensen bedanken die mij op welke wijze dan ook geholpen en gesteund hebben tijdens deze periode.

Allereerst wil ik Mark Aarts bedanken voor de kans om AIO te worden in zijn groep en ik wil je natuurlijk ook bedanken voor je begeleiding tijdens deze periode. Ook wil ik mijn promotor Maarten Koornneef bedanken voor al je hulp bij het tot stand komen van dit proefschrift. Verder wil ik Hetty Blankestijn in het bijzonder bedanken voor het groeien en oogsten van vele planten, zonder haar had ik nooit zoveel verschillende aspecten in dit onderzoek kunnen bekijken. Ik doe ook een speciaal woordje voor Corrie Hanhart, je was een heel leuke collega en ik wil je bedanken voor al je goede zorgen en natuurlijk voor de gezellige koffiepauzes. Daarnaast wil ik mijn kamergenoot Joost bedanken voor de goede en gezellige samenwerking. Natuurlijk wil ik ook al mijn andere collega's die deel uitmaken of uitgemaakt hebben van de botanische genetica groep bedanken voor de gezellige en goede sfeer; Ana, Artak, Diana, Du Jian, Emile, Gerda, Hedayat, Huajie, Lèonie, Mohamed, Sangita, Wessel, Wu Jian. Ik wil ook de rest van de vakgroep bedanken en in het bijzonder Anita voor het vullen van de puntjes, het doen van de afwas en het gezellige kletsen tijdens de pauze. Jan van Kreel voor al zijn technische hulp. Aafke en Corrie Eekelder voor het draaiende houden van de vakgroep. Zonder de ondersteuning van Aart, Taede en Casper voor het groeien van de planten had ik ook al dit werk niet kunnen doen. Ook ontbreken in dit dankwoord niet alle studenten die ik heb mogen begeleiden in volgorde van verschijnen, Barbara, Björn, Laia, Ewa, Zizy, Marcel en Liana; I enjoyed it very much working with you all and although not all the work you did is included in this thesis most of it was very useful.

Mijn onderzoek is niet alleen binnen de leerstoelgroep erfelijkheidsleer uitgevoerd. Het werk dat in hoofdstuk 2, 3, 4 en 5 beschreven staat was in samenwerking met Henk Schat van de Vrije Universiteit in Amsterdam, Henk bedankt! Furthermore I would like to thank the other people in the group of Henk Schat; Antoinne Deniau, Veerle Grispen, Kersten Richau, Mattijs

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Het werk dat in hoofdstuk 2, 3 en 4 beschreven staat was ook in samenwerking met het Bioinformatica Laboratorium van het Academisch Medisch Centrum Amsterdam. Antoine van Kampen, Lisa Gilhuijs-Pederson, Perry Moerland, Emiel Ver Loren van Themaat en Diederik Wehkamp wil ik bedanken voor de goede samenwerking.

Ik wil ook Wilbert van Workum, André Wijfjes en Jeroen Kwekkeboom van Service XS BV Leiden bedanken voor hun goede samenwerking bij het tot stand komen van de microarray experimenten (hoofstuk 2 en 3). Furthermore, I also collaborated with Sean Coughlan from Agilent. Sean thanks for performing the genomic DNA microarray experiments (hoofdstuk 2 en 3). Also I would like to thank David Galbraith of the University of Arizona for giving me the opportunity to do a microarray experiment in his lab.

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Dan rest mij nog om de allerbelangrijkste mensen in mijn leven te bedanken. Jochem dank je voor al je liefde, begrip en hulp de afgelopen jaren, zonder jou was het me zeker niet gelukt om zo ver te komen, jij bent mijn steun en toeverlaat. Vele weekenden heb jij mij geholpen als ik weer eens naar het lab moest. Jij hebt mij geleerd dat niet alles in het leven draait om het leveren van goed werk maar dat er ook momenten zijn dat je voor jezelf moet kiezen. Ik weet dat ik de laatste jaren veel tijd heb doorgebracht om mijn promotieonderzoek zo goed mogelijk af te ronden, maar ik weet dan ook dat nu deze belangrijke fase in mijn leven voorbij is wij een ijzersterk team zijn. Ik houd van je! Pap, mam en Gijs, jullie ook heel erg bedankt voor al jullie steun en ondanks dat jullie toch ver weg zijn, zijn jullie altijd in mijn hart. Zonder jullie was ik nooit geworden wat ik nu ben en mam heel erg bedankt voor regelmatig een goed luisterend oor. Henny, Marian en Walter heel erg bedankt voor jullie interesse en steun in alles wat ik doe. En dan natuurlijk niet te vergeten al onze vrienden, vriendinnen en familie die altijd heel erg veel interesse hebben getoond voor het onderzoek wat ik deed.

Judith

Education Statement of the Graduate School
Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Judith E. van de Mortel**
Date: **12 September 2007**
Group: **Laboratory of Genetics, Wageningen University**

	date
1) Start-up phase	
► **First presentation of your project**	
Heavy metal tolerance and accumulation in *Thlaspi caerulescens* , a heavy metal hyperaccumulating plant species.	Sept 2, 2002
► **Writing or rewriting a project proposal**	
Writing a review or book chapter	
► **MSc courses**	
Laboratory use of isotopes	
► Safe handling with radioactive materials and sources, level 5B, Wageningen University	Nov, 2002
Subtotal Start-up Phase	*3.0 credits**

	date
2) Scientific Exposure	
► **EPS PhD student days**	
Vrije Universiteit Amsterdam, Netherlands	Jun 3, 2004
Radboud University Nijmegen, Netherland	Jun 2, 2005
► **EPS theme symposia**	
EPS theme 3, 'Metabolism and Adaptation' symposium	Oct 25, 2004
EPS theme 1, 'Developmental Biology of Plants' symposium	Apr 26, 2005
EPS theme 3, 'Metabolism and Adaptation' symposium	Nov 24, 2005
► **NWO Lunteren days and other National Platforms**	
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 7-8, 2003
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 5-6, 2004
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 4-5, 2005
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 3-4, 2006
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren)	Apr 2-3, 2007
► **Seminars (series), workshops and symposia**	
Seminar Tour: Analysis of Gene expression, Vught, Netherlands	Oct 7, 2003
Seminar series Plant Physiology	2003-2007
Seminar Philip Benfey	Oct 24, 2005
Symposium SMS: Salt, Metals and Stress, Amsterdam Vrije Universiteit, Amsterdam	Oct 1, 2004
Symposium SMS: Salt, Metals and Stress, Amsterdam Vrije Universiteit, Amsterdam	Jan 27, 2006
Seminar Series Frontiers in Plant Development	2003
Microarray Workshop, University of Arizona, USA	Nov 11-15, 2003
Workshop 'Patenting: the practice'	Jun 23, 2005
► **Seminar plus**	
P. Benfey	Oct 24, 2005
► **International symposia and congresses**	
ISPMB meeting Barcelona, Spain	Jun 23-28, 2003
Plant Responses to Abiotic Stress meeting, Santa Fe, USA	Feb 19-24, 2004
Phytotechnologies to promote sustainable land use management and improve food safety (EU-COST) Parma, Italy	Nov 4-6, 2004
16th Intern. Conference on Arabidopsis Research, University of Wisconsin-Madison, USA	Jun 15-19, 2005
PlantGEMs 2005, Amsterdam, Netherlands	Sep 20-23, 2005
Plant Biotechnology meeting Bonn, Germany	Oct 5, 2005
ASPB Plant Biology meeting Boston, USA	Aug 5-9, 2006
► **Presentations**	
ISPMB meeting Barcelona, Spain (Poster)	Jun 23-28, 2003
Plant Responses to Abiotic Stress meeting, Santa Fe, USA (Poster)	Feb 19-24, 2004
16th Intern. Conference on Arabidopsis Research, University of Wisconsin-Madison, USA (Poster)	Jun 15-19, 2005
PlantGEMs 2005, Amsterdam, Netherlands (Poster)	Sep 20-23, 2005
Plant Biotechnology meeting Bonn (Oral)	Oct 5, 2005
ASPB Plant Biology meeting Boston, USA (Poster)	Aug 5-9, 2006
Seminar series Plant Physiology (3 x Oral)	2003-2007
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren) (Oral)	Apr 7-8, 2003
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren) (Oral)	Apr 5-6, 2004
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren) (Oral + Poster)	Apr 4-5, 2005
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren) (Poster)	Apr 3-4, 2006
NWO-ALW Meeting on Experimental Plant Sciences (Lunteren) (Poster)	Apr 2-3, 2007
EPS theme 3 symposium (Oral)	Nov 24, 2005
► **IAB interview**	Jun 3, 2005
► **Excursions**	
La Calamine, Belgium	May, 2004
Subtotal Scientific Exposure	*29.8 credits**

	date
3) In-Depth Studies	
► **EPS courses or other PhD courses**	
EPS Summerschool Wageningen: The analysis of natural variation within crop and model plants	Apr 22-25, 2003
EPS Summerschool Utrecht: Functional genomics: theory and hands-on data analysis	Aug 25-28, 2003
VLAG Bioinformation Technology-1	May 12-21 , 2003
EPS Summerschool Utrecht: Environmental signalling: Arabidopsis as a model	Aug 22-24, 2005
► **Journal club**	
Genetics	2003-2007
► **Individual research training**	
Microarray Experiment, University of Arizona, USA	Nov 16 - Dec 4 2003
Atomic Absorption Spectrometry, Vrije Universiteit Amsterdam, Netherlands	Aug 28 - Sep 15 2006
Subtotal In-Depth Studies	*11.4 credits**

	date
4) Personal development	
► **Skill training courses**	
Career Orientation Course	May 27- Jun 24, 2005
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*1.5 credits**

TOTAL NUMBER OF CREDIT POINTS*	**45.7**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

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