Functional characterisation of three zinc transporters in *Thlaspi caerulescens*

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dedicated to the memories of my father

Contents

Chapter 1

General Introduction and scope of the thesis

The importance of metal homeostasis

Plants require a range of heavy metals as essential micronutrient for normal growth and development. Different metals have important roles in various pathways in plants. For instance, iron (Fe) is a key component of haem proteins (e.g. cytochromes, catalase) and Fe-S proteins such as ferredoxin and a range of other enzymes. Copper (Cu) is an integral component of certain electron transfer proteins in photosynthesis (e.g. plastocyanin) and respiration (e.g. cytochrome c oxidase) and is also involved in lignification (laccase). Manganese (Mn), which is less redox active than Cu, plays also a role in photosynthesis (e.g. O_2 evolution) (for details see Hall and Williams, 2003). Nickel (Ni) is a cofactor of urease and other enzymes such as hydrogenases and Ni-superoxide dismutase (Dalton et al., 1988; Watt et al., 1999). Zinc (Zn) ions are key structural or catalytic components in hydrolytic enzymes (e.g. Cu-Zn superoxide dismutase, alcohol dehydrogenase) and DNA-binding proteins like RNA polymerase (Marschner, 1995; Guerinot and Eide, 1999; Broadley et al. 2007). However, uncontrolled binding of metals to proteins can result in the inactivation of proteins. Few other heavy metals like cadmium (Cd), arsenic (As) and lead (Pb) are potentially toxic to plants, due to their similarity (especially in ionic radii) to some essential heavy metals, such as, Zn and Fe. This implies that plants need to balance metal uptake, intracellular compartmentalisation and partitioning to the various tissues and storage, to meet the specific requirements of metals in different tissues. This tight control and regulation of metal accumulation is called metal homeostasis.

Metal homeostasis in plants is affected by the mobilization and uptake from the soil, compartmentalisation and sequestration within the root, the efficiency of

xylem loading and transport, distribution between metal sinks in the aerial parts of the plants and sequestration and storage in leaf cells (Clemens et al 2002).

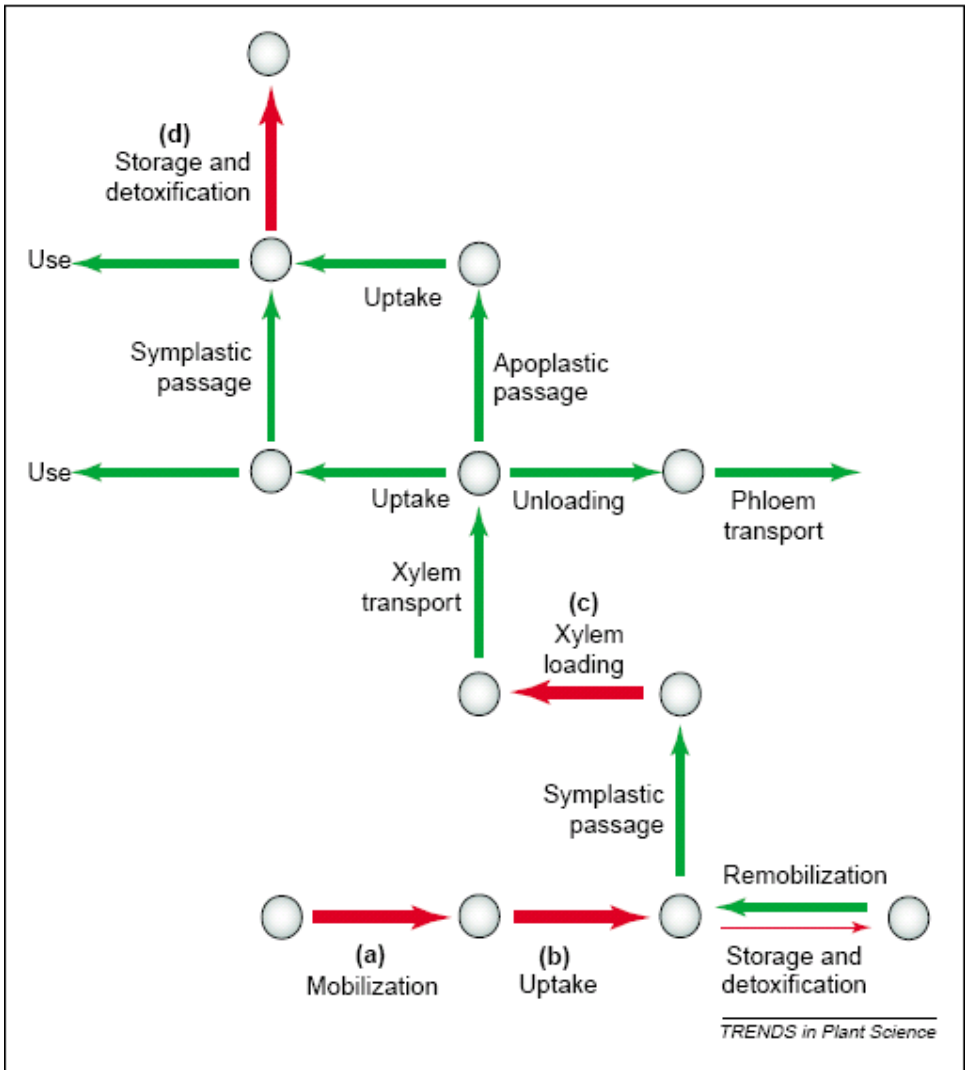

Figure 1: The path of a transition metal from the soil to the sites of use and storage in the leaf. The different processes governing metal accumulation are depicted in arrows. Red arrows highlight current engineering targets. These include attempts (a) to enhance mobilization by secretion of organic acids, (b) to increase uptake by overexpression or deregulation of transporters, (c) to stimulate uptake into the root and translocation via the xylem by overproduction of intracellular chelators, or (d) to increase the strength of metal sinks in the leaves by overexpression of storage and detoxification mechanisms. Processes that, according to our present knowledge, show distinct molecular characteristics in hyperaccumulator plants are represented by arrows of varying size. A thicker arrow indicates a process displaying higher activity in metallophytes as compared to non-metallophytes, for example, rates of uptake into the roots via plasma membrane-localised Zn transporters. A thinner arrow indicates a process displaying reduced activity in metallophytes. This applies to sequestration of metals in root tissues (Clemens et al., 2002)

Micronutrient levels in plants are also important for human health, as they are used as primary sources of food for humans (Grusak and Dellapenna, 1999). The most widespread nutritional problem in the world is Fe deficiency (Mäser, 2001). Similarly, Zn deficiency is a significant problem in agriculture, particularly in cereals, limiting crop production and quality (Guerinot and Eide, 1999; Hacisalihoglu et al, 2003). Increase of the ability of plants to provide higher levels of minerals will therefore have a dramatic impact on human health (Clemens et al., 2002; Mäser, 2001), especially when most of the diet depends on plant sources. Therefore, metal uptake/availability is of relevance, both because of its impact on plant growth where both deficiency and excess reduce growth and thereby crop yield, as well as because of its relevance for the nutritional quality of food and feed.

Metal hyperaccumulating species

The quantitative requirements of the metals are specific and vary greatly among different tissues in a plant. Similarly, the requirement of the metals varies from species to species and shows large differences among the metal 'non-hyperaccumulator' and the 'hyperaccumulator' species. 'Hyperaccumulator' species are defined as plants having a Zn concentration above 10,000 µg g^{-1} dw, Ni concentration higher than 1000 µg g^{-1} dw or Cd concentration above100 µg g^{-1} dw. In comparison, a non-accumulator plant contains 30-100 µg g^{-1} dw of Zn and 1–10 µg g^{-1} dw of Ni. A Cd concentration of more than 10 µg g^{-1} dw is toxic. *Thlaspi caerulescens* (Tc), a Zn/Cd/Ni hyperaccumulator species, can accumulate up to 30,000 µg Zn g^{-1} dw foliar concentration (Brown et al., 1995), 4000 µg Ni g^{-1} dw (Reeves and Brooks, 1983; McGrath et al., 1993) and 2700 µg

Cd g^{-1} dw (Lombi et al., 2000). *T. caerulescens* is the focus of this thesis. Approximately, 400 species have been reported as Ni, Zn, Cd, Pb, Cu, Co and Mn hyperaccumulators, which belong to a wide range of unrelated families (Baker and Brooks, 1989; Brooks 1994; Baker et al., 2000). Among these 400, 317 species are Ni hyperaccumulators and 12 are identified as Zn hyperaccumulators (Baker et al., 2000). Many Zn hyperaccumulators belong to the Brassicaceae family; 11 of them are *Thlaspi* species and one is an *Arabidopsis* species (*Arabidopsis halleri).* They are mainly found on calamine soils enriched in Zn, Pb and Cd, either naturally or due to mining or metal smelting in Southern, Western and Central Europe. Furthermore the species is naturalised in parts of Scandinavia (Tutin et al., 1993). Like other hyperaccumulators, *T. caerulescens* exhibits enhanced metal uptake, as well as enhanced metal translocation to the shoots (Lasat et al., 1996; Shen et al., 1997; Schat et al., 2000). *T. caerulescens* has been proposed to be a very convenient model plant species to study many aspects of this exciting research field and could provide answers to unresolved issues for two major reasons (Assunção *et al*., 2003). The first is that the adaptive traits associated with plant heavy metal hyperaccumulation and tolerances are simply not present in any of the current model species. The second, and equally important, reason is its close relatedness to *Arabidopsis thaliana.* The genetic and technical resources that have already been developed for *A. thaliana* can be applied for better understanding of the regulation of traits in *T. caerulescens* (Peer *et al*., 2006). In addition to these reasons, self-fertility and sufficient seed setting (Peer et al., 2006) also justifies *T. caerulescens* as a suitable model plant for the study of metal accumulation.

A great interest has developed recently in the use of terrestrial plants as a green technology for the remediation of surface soils contaminated with toxic heavy metals (Pence et al., 2000). This developed the new field of environmental biotechnology, termed phytoremediation, which uses plants to extract heavy metals from the soil and to concentrate them in harvestable shoot tissue (Salt et al., 1995). The practical utility of many hyperaccumulators for phytoremediation may be limited, because many of these species, including *T. caerulescens*, are slow-growing and produce little shoot biomass, severely constraining their potential for large scale decontamination of polluted soils (Ebbs et al., 1997). Transferring the genes responsible for the hyperaccumulating phenotype to higher shoot-biomass-producing plants has been suggested as a potential avenue for enhancing phytoremediation as a viable commercial technology (Brown et al., 1995). Progress toward this goal has been hindered by a lack of understanding of the basic molecular, biochemical and physiological mechanisms involved in heavy metal hyperaccumulation.

Metal uptake proteins

A primary control point for metal homeostasis appears to be the regulation of metal uptake across the plasma membrane into the cell (Guerinot, 2000). Metal ions are hydrophilic and do not cross cell membranes or other organelle membranes by passive diffusion (Cousins and McMahon, 2000). Plants have evolved mechanisms that allow the transport of metal ions, through different categories of metal transporters such as the ZIP (ZRT, IRT-like proteins) family (Guerinot, 2000), cation diffusion facilitators (CDFs) (Williams et al., 2000), heavy metal (or CPx-type) ATPases, the natural resistance-associated macrophage

proteins (Nramps) and the cation antiporters (Gaxiola et al., 2002), found to be located in different organelles within the cell.

ZIP family transporters

The ZIPs are involved in the transport of Fe, Zn, Mn and Cd with family members differing in their substrate range and specificity (Guerinot, 2000; Mäser et al, 2001). About 85 ZIP family members have now been identified from bacteria, archea and all types of eukaryotes, including 15 genes in *A. thaliana* (Mäser, 2001).

The ZIP proteins are predicted to have eight transmembrane (TM) domains with the amino- and carboxyl- terminal ends situated on the outer surface of the plasma membrane (Guerinot, 2000). The overall length varies widely, mostly because of a variable region between TM-3 and TM-4. This region is predicted to be on the cytoplasmic side and contains a potential metal-binding domain, rich in histidine residues. The most conserved region of these proteins lies in TM-4 and is predicted to form an amphipathic helix containing a fully conserved histidine that may form part of an intra-membranous metal binding site involved in transport (Guerinot, 2000; Mäser et al., 2001). Its transport function after heterologous expression in yeast is eliminated when conserved histidines or certain adjacent residues are replaced by different amino acids (Rogers et al., 2000).

The first member ZIP family identified from plant (Eide et al., 1996), was *AtIRT1* (Iron-regulated transporter 1), cloned from *A. thaliana* and identified by functional complementation of the Fe-uptake-deficient yeast double mutant *fet3 fet4*. *AtIRT1* is now thought to be the major transporter for high affinity Fe uptake by roots (Connolly et al., 2002; Vert et al., 2002). Plants overexpressing *AtIRT1*

also accumulate higher concentrations of Cd and Zn than wild types under Fe deficient conditions, indicating an additional role in the transport of these metals (Connolly et al., 2002). This is also supported by transport studies in yeast (Eide et al., 1996; Korshunova et al., 1999). The *irt1-1 A. thaliana* knockout mutant is chlorotic and shows severe growth defects that can be rescued by exogenous application of Fe (Vert et al., 2002). *AtIRT1* is expressed predominantly in the external layers of the root under Fe-deficient conditions and the protein is localized to the plasma membrane. Mutants of *AtIRT1* also showed significant changes in photosynthetic efficiency and developmental defects that are consistent with a deficiency in Fe transport and homeostasis (Henriques et al., 2002; Varotto et al., 2002). Interestingly, *AtIRT2* is also expressed in root epidermal cells under Fe deficiency. However, *AtIRT2* cannot complement the loss of *AtIRT1* (Grotz and Guerinot, 2002) and appears to have a greater specificity to substrates. Although the gene can complement Fe and Zn uptake mutants, the protein does not transport Cd or Mn in yeast (Vert et al., 2001). This suggests that these transporters have different functions in *A. thaliana*.

In tomato *LeIRT1* and *LeIRT2* were studied and both genes were shown to be expressed in roots (Eckhardt et al., 2001). Expression of *LeIRT1* was found to be strongly enhanced by Fe limitation, whereas this was not the case for *LeIRT2*. *LeIRT1* was also up-regulated by P and K deficiencies in the root medium. This suggests a possible co-regulation of the transporter genes for certain essential minerals (Wang et al., 2002). Studies in yeast suggest that *LeIRT1* and *LeIRT2* also have a broad range of substrate transport (Eckhardt et al., 2001). *OsIRT1* from rice, which has high homology to the *A. thaliana AtIRT1* gene, is also

predominantly expressed in roots and is induced by Fe- and Cu-deficiency (Bughioet al., 2002).

Based largely on yeast complementation studies, further information is available on the functional properties of other plant ZIP transporters. The ZIP1-3 transporters from *A. thaliana* restore Zn uptake in the yeast Zn uptake double mutant *zrt1 zrt2* and were proposed to play a role in Zn transport (Grotz et al., 1998; Guerinot, 2000). *ZIP1, ZIP3* and *ZIP4* are expressed in the roots of Zn-deficient plants, while *ZIP4* is also expressed in the shoots (Grotz et al., 1998; Guerinot, 2000). Wintz et al. (2003) demonstrated that two ZIP genes; *AtZIP*2 and *AtZIP4* are involved in copper transport.

The proposed role of ZIP transporters in Zn nutrition is supported by the characterization of homologues from other species. A member of the ZIP family, GmZIP1, has now been identified in soybean (Moreau et al., 2002). By functional complementation of the *zrt1 zrt2* yeast cells, *GmZIP1* was found to be highly selective for Zn, while yeast Zn uptake was inhibited by Cd. *GmZIP1* was specifically expressed in the nodules and not in roots, stems or leaves, and the protein was localized to the peribacteroid membrane, indicating a possible role in the symbiosis (Moreau et al., 2002).

The yeast ZIPs, *ZRT1* and *ZRT2*, were shown to be high and low affinity Zn transporters, respectively (Eide, 1998; Guerinot, 2000); *ZRT1* is glycosylated and present at the plasma membrane. A third ZIP homologue in yeast, *ZRT3*, is proposed to function in the mobilization of stored Zn from the vacuole (MacDiarmid et al., 2000).

Why do plants need so many ZIPs? This diversity may be required for a variety of reasons: (1) to provide the high and low affinity systems needed to cope with

varying metal availability in the soil; (2) to provide the specific requirements for transport at the different cellular and organellar membranes within the plant; and (3) to respond to a variety of stress conditions. High-affinity transporters are selective for their target metals and are tightly regulated according to metal need. Low-affinity transporters are less responsive to metal need and are somewhat less selective for the metals they transport (Radisky and Kaplan, 1999).

Metal efflux proteins

Plasma membrane controlled Zn regulation is not sufficient to control the free metal concentration in the cells. The vacuole, which occupies most of the plant cell volume, plays a major role in the regulation of ion homeostasis in the cell and in detoxification of the cytosol (Marschner, 1995). Several CDF genes involved in sequestration of metals in the cell have been identified in different plant species.

CDF family or cation efflux family

Over-expression of CDF family members are described to confer Zn, Cd, Co or Ni tolerance to plants (Paulsen and Saier, 1997; Eide, 1998). Several evidences suggest that these transporters either sequester metal ions within vacuoles or export metal ions out of the cells (Paulsen and Saier, 1997), though the mechanisms underlying the transport are not well understood for most of the CDF proteins. Proteins of the CDF family from diverse sources have the following features in common: (1) they share an N-terminal signature sequence that is specific to the family; (2) the proteins possess six trans-membrane-spanning regions; (3) they share a cation efflux domain; and (4) most of the eukaryotic members possess an intracellular histidine-rich domain that is absent from the

prokaryotic members (Paulsen and Saier, 1997). Several CDF family members have been studied from *Saccharomyces cerevisiae, Escherichia coli, Bacillus subtilis* and *Schizosaccharomyces pombe* and were found to contribute to the storage of and efflux of Zn and other metals from the cell (Li and Kaplan, 1998; MacDiarmid et al 2002; Miyabe et al 2001; Guffanti et al, 2002; Chao et al, 2004; Li and Kaplan, 2001; Clemens et al, 2002).

The genome of *A. thaliana* encodes 12 putative CDF genes, which are highly divergent in sequence, but share some characteristics of CDF family membrane transport proteins (Blaudez et al., 2003). *AtZAT* (Zn transporter of *A. thaliana*) later renamed as *AtMTP1* (Metal Tolerance Protein 1) was the first plant CDF, identified as a cDNA (van der Zaal et al., 1999; Mäser et al, 2001). Under normal or excess Zn supply *AtMTP1* transcripts were shown to be present at low levels in seedlings (van der Zaal et al., 1999). *35S::AtMTP1* transformed *A. thaliana* plants resulted in enhanced Zn tolerance of transgenic seedlings and slightly increased Zn accumulation in roots. Studies by Kobae et al. (2004) suggested *AtMTP1* to be a potential Zn transporter in *A. thaliana.* A further study by Desbrosses-Fonrouge et al. (2005) showed that AtMTP1 acts as a Zn transporter, localised in the vacuolar membrane, mediating Zn detoxification and leaf Zn accumulation. Bloβ et al (2002) expressed *AtMTP1* in *E. coli* and studied the purified protein in reconstituted proteoliposomes. The protein transported Zn into proteoliposomes by a mechanism that relied on the Zn gradient across the membrane and not on a proton gradient. Two of the 12 genes encoding putative CDF proteins in *A. thaliana*, *AtMTP2* and *AtMTP3*, are closely related to *AtMTP1* (64.4% and 67.6% identity respectively). *AtMTP2* was shown by expression profiling to be induced in a Zn deficiency condition (van de Mortel et al., 2006). AtMTP3 was found to

contribute to basic cellular Zn tolerance and was involved in the control of Zn partitioning (Arrivault et al., 2006). Silencing of *AtMTP3* causes Zn hypersensitivity and enhances Zn accumulation in above ground organs of plants exposed to excess Zn or to Fe deficiency. Over-expression of *PtdMTP1* from hybrid poplar (*Populus trichocarpa* x *P. deltoides*) in *A. thaliana* also conferred Zn tolerance (Blaudez et al., 2003). Heterologous expression of *PtdMTP1* from poplar in various yeast mutants was shown to confer resistance specifically to Zn, possibly as a result of transport into the vacuole (Kohler et al., 2003).

Persans et al. (2001) isolated CDF member genes (*TgMTPs*) from the Ni-hyperaccumulating species *Thlaspi goesingense*. These genes conferred metal tolerance to *S. cerevisiae* mutants defective in vacuolar *COT1* and *ZRC1* Zn transporters. They suggested that TgMTP1 transports metals into the vacuole. *In vivo* and *in vitro* immunological staining of hemagglutinin (HA)-tagged *TgMTP1::HA* revealed that the protein is localized in both vacuolar and plasma membranes in *S. cerevisiae*. It was assumed that TgMTP1 functions by enhancing plasma membrane Zn efflux thereby conferring Zn resistance in *S. cerevisiae*. Transient expression in *A. thaliana* protoplasts also revealed that *TgMTP1::GFP* is localized at the plasma membrane, suggesting that *TgMTP1* may enhance the Zn efflux in plants, which is different from the other endogenous *A. thaliana* CDFs. Delhaize et al. (2003) showed that the *ShMTP1* cDNA from *Stylosnathes hamata,* a tropical legume tolerant to acid soils with high concentrations of Mn, conferred Mn tolerance to yeast and plants by a mechanism that is likely to involve the sequestration of Mn into internal organelles. Expression studies on *AhMTP1* from *A. halleri* showed substantially higher transcript levels in the leaves and up-regulation upon exposure to high Zn concentrations in the roots of

A. halleri and vacuolar localization leading to Zn accumulation in the plant (Dräger et al., 2004).

Metal transport in *T. caerulescens*

Lasat *et al.* (1996) described the physiological characterization of Zn uptake of *T. caerulescens* and *T. arvense* (a non-hyperaccumulator species). A concentration-dependent Zn^{2+} influx into the root was recorded, using radiotracer flux techniques. In both species, there was a saturable component following Michaelis-Menten kinetics. V_{max} was much higher in *T. caerulescens* than in *T. arvense,* whereas the K_m values appeared similar for both species. This suggests a higher expression of functionally similar Zn transporters in *T. caerulescens* compared to *T. arvense* roots (Lasat and Kochian, 2000). Time-course analysis of Zn accumulation in roots and shoots supported this finding. The Zn content in the *T. caerulescens* roots was found to be two times higher than in the *T. arvense* roots at the start of the experiment, despite a much higher rate of Zn translocation to the shoot later on (Lasat *et al*., 1996). Further, Lasat *et al.* (2000) and Pence *et al.* (2000) isolated *TcZNT1*, a *ZIP* family Zn transporter gene from *T. caerulescens*, by functional complementation of the yeast *zhy3* mutant, defective in Zn uptake (Zhao & Eide, 1996). Assunção *et al.* (2001) cloned the *TcZNT1* gene as well as an apparent paralogue, the *TcZNT2* gene, based on the homology to the *A. thaliana AtZIP4* gene from accession La Calamine. In non-accumulator species, like *A. thaliana* or *T. arvense,* the orthologues of *TcZNT1* are mainly expressed in roots, but only under Zn deficiency conditions. At normal or elevated Zn supply their transcription is strongly down-regulated (Grotz *et al.*, 1998; Pence *et al.*, 2000; Assunção *et al.*, 2001). In contrast, in *T. caerulescens* both *TcZNT1* and

TcZNT2 are highly expressed in roots and at a lower level in shoots, not only under conditions of Zn deficiency, but also at normal or elevated Zn supply (Pence *et al*., 2000; Assunção *et al*., 2001). TcZNT1-mediated Zn uptake in yeast showed a saturable component (Pence *et al*., 2000) with a K_m value very similar to the one found for *T. caerulescens* (Lasat *et al*., 1996). Furthermore, the V_{max} of Zn influx in roots of *T. caerulescens* grown under different Zn concentrations correlated very well with the root *TcZNT1* transcript levels and the K_m values were very similar at all the Zn exposure levels tested (Pence *et al*., 2000). Similar experiments were done to show evidence of Cd transport of TcZNT1, concluding that TcZNT1 mediates high-affinity Zn uptake as well as low-affinity Cd uptake (Pence *et al*., 2000).

Why these genes are apparently over-expressed in *T. caerulescens* is still unknown. An increased expression of *TcZNT1* and *TcZNT2* may be one of the evolutionary changes on the way from non-accumulator to hyperaccumulator (Assunção *et al*., 2001). One possibility could be that a Zn-responsive element in the *TcZNT1* promoter has been altered, altering the Zn-imposed transcriptional down regulation (Assunção *et al*., 2001). However, since two genes (*TcZNT1* and *TcZNT2*) are over-expressed, an alteration in the Zn receptor and signal transduction machinery appears more plausible (Lasat *et al*., 2000; Pence *et al*., 2000). Recently, a gene encoding a basic helix-loop-helix (bHLH) transcription factor involved in the regulation of Fe status was identified in tomato. Mutation of this gene leads to much lower expression of the main root Fe transporter (Ling *et al*., 2002). One can envision that a similar transcription factor in *T. caerulescens* is involved in the regulation of Zn status. Modification of one transcription factor often changes the expression of several genes, as could be the case for the

TcZNT1 and *TcZNT2* paralogues (Assunção *et al*., 2001). Alternatively, if the Zn sequestration machinery is much more efficient in *T. caerulescens* than in non-accumulator species, this can create a state of 'physiological Zn deficiency' (Assunção *et al*., 2001). In this situation, cellular sensing machinery does not sense Zn at appropriate levels, even though the Zn supply rates and total cellular Zn concentrations would be adequate or even toxic for non-hyperaccumulator plants (Assunção *et al*., 2001).

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

Two related ZIP genes have been cloned from Ni hyperaccumulator *T. japonicum*, *TjZNT1* and *TjZNT2*. Expressing in yeast either *TjZNT1* or *TjZNT2* shows increased resistance to Ni^{2+} (Mizuno et al., 2005), highlighting a potential role for these genes in Ni tolerance. Further studies are necessary to determine if or how these proteins function to hyperaccumulate metals in plants and to understand the

differential regulation of the similar genes in the hyperaccumulator plants compared with non-accumulator plants.

Assunção et al. (2001) showed constitutively high expression of *TcZTP1* (Zn Transporter 1), a member of the CDF family in *T. caerulescens. TcZTP1* is very similar to *AtMTP1* of *A. thaliana.* It has 90% identity at the DNA level and 75% aa identity. A further study on *TcZTP1* has been reported in this thesis (chapter 5). Furthermore, it is fascinating that *T. caerulescens* and other hyperaccumulator plants cope with such high Zn and other heavy metal concentrations, which are very toxic to other non-accumulator plants. This is why the hyperaccumulator plants are assumed to have specific or higher activity of mechanisms for root-to-shoot transport, xylem loading and unloading (Lasat et al, 1998) and vacuolar sequestration of heavy metals, particularly in the leaf epidermal cells (Vázquez et al, 1994; Küpper et al, 1999), trichomes (Kramer et al, 1997), or stomatal guard cells (Heath et al, 1997) compared to the non-accumulating plants. Only few studies have been carried out to understand the mechanisms behind. Further researches are necessary to unravel the questions.

In this thesis, we tried to functionally characterise the genes *TcZNT1, TcZNT2* and *TcZTP1* from *T. caerulescens,* accession La Calamine. We examined the function of these three genes in metal homeostasis by studying the over-expression lines of *TcZTP1, TcZNT1* and *TcZNT2* in *A. thaliana. A. thaliana* was chosen as a reference non-accumulator species. An average of 88.5% sequence identity in transcript sequences between *Thlaspi* and *Arabidopsis* was found in the study of Rigola et al. (2006). This is in agreement with the study of Peer et al. (2006), which found 87–88% sequence identity for the intergenic transcribed spacer regions when several *Thlaspi* species were compared with *Arabidopsis.*

Such evidences and available genetic resources, makes *A. thaliana* an obvious choice as a comparative non-accumulator (Freeman et al., 2004). This allowed the comparisons between similar genes from hyperaccumulators and non-accumulators overexpressing these genes (Freeman et al., 2004).

Aim and Outline of the thesis

This thesis explores the function of the three Zn transporters of the Zn/Cd/Ni hyperaccumulator species, *Thlaspi caerulescens* and their regulation, in comparison with the non-accumulator model species *Arabidopsis thaliana.* The close relatedness of *T. caerulescens* to *A. thaliana* provides us the opportunity to study the genes from a hyperaccumulator in a non-hyperaccumulator, making use of the genetic and technical resources that have already been developed for the *A. thaliana* genome. Previous studies on the *TcZTP1*, *TcZNT1* and *TcZNT2* genes of *T. caerulescens* reported higher expression levels of these genes independent of the Zn status of the plant, whereas the homologous genes from related non-accumulator species, *T. arvense* and *A. thaliana*, show down-regulation under high Zn conditions. The research presented in this thesis is focused towards understanding the function of *TcZTP1*, *TcZNT1* and *TcZNT2* genes, by overexpressing the genes in *A. thaliana*. This also leads towards the study of the regulation of the gene, *TcZNT1* in comparison to *AtZIP4*, through the study of the *cis*-elements in these gene promoters.

Chapter 1 summarises the current state of knowledge on the metal homeostasis in plants with a focus on *T. caerulescens.*

In **chapter 2** a physiological study of the response to Zn in *A. thaliana* and *T. caerulescens* is described.

In **chapter 3** a functional characterisation of the ZIP (ZRT, IRT-like proteins) family member genes *TcZNT1* and *TcZNT2* is presented using the lines which over-expressed these genes in *A. thaliana*.

In **chapter 4** the regulation of *TcZNT1* was studied in comparison to *AtZIP4.* The *cis* elements of these two gene promoters were searched for, by comparative deletion analysis of promoter induced GUS expression in *A. thaliana*.

In **chapter 5** functional characterisation of the cation diffusion facilitator (CDF) family member gene *TcZTP1* was performed using over-expression lines of this gene in *A. thaliana.*

In **chapter 6** the combined findings of chapter 2 through 5 are discussed and suggestions for further research are proposed.

Chapter 2

Physiological study of *Arabidopsis thaliana* and *Thlaspi caerulescens* in response to zinc

Sangita Talukdar, Mark GM Aarts

Abstract

- The main objective of this research was to study the response of *Arabidopsis thaliana* and *Thlaspi caerulescens* to Zn deficiency and high Zn.
- The *A. thaliana* plants were exposed to Zn-deficiency (0 and 0.05 µM Zn) and compared to the plants grown on media containing standard Zn (2 µM). *T. caerulescens* plants were exposed to Zn-deficiency (0.05 µM Zn) and high Zn (1000 µM Zn) and compared to plants grown on standard Zn media (100 µM Zn).
- Both *A. thaliana* and *T. caerulescens* were found to be heavily affected by Zn deficiency, showing retarded growth and reduced reproduction. *T. caerulescens* plants were similarly affected when grown on high Zn concentrations, as by Zn deficiency, with comparable effects on reproductive tissues.

Introduction

Contamination of soils with heavy metals, either by natural causes or due to pollution, often has pronounced effects on the vegetation, often characterised by the appearance of metallophytes, heavy metal-tolerant and -hyperaccumulator plants. The zinc (Zn) hyperaccumulator species are defined to accumulate higher than 10,000 μg Zn g^{-1} of dry weight (dw) (1%, w/w) (Baker and Brooks, 1989), whereas most plants contain between 30 and 100 μg Zn g^{-1} dw and concentrations above 300 μg Zn g^{-1} dw are generally toxic (Marschner, 1995). *Thlaspi caerulescens* (Tc), is a model plant species to study metal hyperaccumulation and tolerance (Assunção et al. (2003). This species belongs to the Brassicaceae family and is known as a Zn hyperaccumulator, showing up to 30,000 μg Zn g^{-1} dw foliar concentration (Brown et al., 1995). In addition, hyperaccumulation of Cd or Ni has been reported for a number of natural *T. caerulescens* populations from calamine and serpentine soils (Reeves and Brooks, 1983; McGrath et al., 1993). Cd is a non-essential and toxic element for plants. However, in *T. caerulescens* foliar Cd concentrations up to 2700 μg g^{-1} dw (Lombi et al., 2000) have been found.

The model plant species *Arabidopsis thaliana*, a metal non-accumulator, also belonging to the Brassicacae family, is the reference species in this research. An average of 88.5% sequence identity in transcript sequences between *T. caerulescens* and *A. thaliana* was found in the study of Rigola et al. (2006). This is in agreement with the study of Peer et al. (2006), who found 87–88% sequence identity for the intergenic transcribed spacer regions when several *Thlaspi* species were compared with *Arabidopsis*. *A. thaliana* is a suitable species as a

comparative non-accumulator, based on this molecular similarity and because of its available genetic resources (Freeman et al., 2004).

All plants, either being an accumulator or non-accumulator, need micronutrients for healthy growth and development. Zn is the second most abundant transition metal (after Fe) in biological systems, including plants (Worlock and Smith, 2002). Zn is both stable and inert to oxidoreduction, in contrast to the neighbouring transition elements in the periodic table (for review see Vallee and Auld, 1990; Vallee and Falchuk, 1993). This makes Zn stable in a biological medium in which the redox potential is in constant flux. Zn is an essential element that is employed in a wide range of biochemical and biophysical roles. It is required to maintain the structural stability of macromolecules and to serve as a cofactor for more than 300 metabolic enzymes represented in all enzyme classes (Broadley et al., 2007), including Cu-Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase, etc. and DNA-binding proteins (Marschner, 1995; Guerinot and Eide, 1999). It also plays a prominent role in gene expression as a structural component in a large number of Zn–dependent transcription factors (Worlock and Smith, 2002). Among these, the largest group of Zn-binding proteins in *A. thaliana* are the Zn finger proteins (Broadley et al., 2007).

Zn available to plants is present in the soil solution or is adsorbed in a labile (easily desorbed) form. The soil factors affecting the availability of Zn to plants are those which control the amount of Zn in the soil solution and its sorption/desorption from/into the soil solution. These factors include: the total Zn content, pH, organic matter content, calcium carbonate content, redox conditions, microbial activity in the rhizosphere, soil moisture status, concentrations of other trace elements, concentrations of macro-nutrients (especially phosphorus) and

climate (Broadley et al., 2007). The availability of Zn can be limited in certain soils, especially in the calcareous ones (Grusak, 1999). Severe Zn deficiency generally leads to reduction in internodal growth with a consequent rosette-like development, small and discoloured leaves, poor root formation and reduced seed and fruit production (Bergmann 1992; Marschner 1995). Zn deficiency also leads to an impaired response to oxidative stress, likely due to a reduction in superoxide dismutase levels (Hacisalihoglu et al., 2003). Zn is the most common crop micronutrient deficiency (Broadley et al., 2007), and therefore it is a significant agricultural problem, particularly in cereals, limiting crop production and quality (Guerinot and Eide, 1999; Hascisalihoglu et al, 2003). On the other hand, excess of Zn leads to Zn toxicity, which induces chlorosis in young leaves, probably via competition with Fe and Mg (Marschner, 1995), and arrest root and eventually shoot growth. Additionally, plants have the ability to accumulate non-essential, but toxic metals resembling Zn, such as Cd and this ability could be used to remove pollutant metals from the environment (Guerinot, 2000).

In this study, the visible response on the plant growth and development to variations in Zn supply in the non-accumulator *A. thaliana* and the hyperaccumulator *T. caerulescens* is described.

Materials and methods

Plant growth conditions

To determine the response of *A. thaliana* (accession Columbia) and *T. caerulescens* (accession La Calamine) to Zn, the plants were grown on modified half Hoagland's solution supplied with different $ZnSO_4$ concentrations. *A. thaliana* was supplied with 0, 0.05 and 2 μM Zn and for *T. caerulescens* was supplied with 0.05, 100 and 1000 μM Zn. The nutrient medium was refreshed every week. *A. thaliana*, like any other non-accumulator, needs very low supply of Zn, hence 0 μM Zn (where Zn was left out of the media) was used to create a true Zn-deficient condition. In addition, plants were also grown on half Hoagland's solution supplied with 0.05 μM Zn to allow plants to be able to take up low amount of Zn from the medium. In case of plants grown on 0 μM Zn, the Zn content in the tissues are below the range of detection by Atomic Absorption Spectroscopy (AAS), which was used for the measurements of metal concentrations (Assunção et al., 2003). In case of *T. caerulescens* (accession La Calamine), 100 μM Zn was the concentration on which the plants grow healthy. 0.05 μM Zn was used as a low Zn concentration. In addition to low and normal supply, *T. caerulescens* was also exposed to high Zn (1000 μM Zn) supply, to which it is supposed to be tolerant. Twelve plants for each concentration of Zn were grown and the data were collected based on these plants. All the plants were first grown for two weeks on plates with half MS (Murashige and Skoog, 1962) media, containing 1% agar for normal growth and were then transferred to hydroponics using solutions of half Hoagland's and with different Zn concentrations.

Germination experiments were performed with around one month old seeds (from Zn-deficient and Zn-sufficient plants) in Petri dishes with water-imbibed filter paper. The seeds were incubated in a climate-controlled growth cabinet (25°C day; 16 h day with illumination at a light intensity of 120 µmol m^{-2} s^{-1}), either after cold-treatment of four days or no cold-treatment.

Results

Response of A. thaliana plants to low Zn

The *A. thaliana* plants grown for four weeks under Zn-deficiency (0 µM Zn) are chlorotic and shorter compared with plants grown for the same time under Zn-sufficiency (2 µM Zn) (Figure 1A, 1B). Average shoot weight was reduced by ~6-fold and average root weight was reduced by ~4-fold (Figure 2A). Measuring the length of the main axis (stem) and root length over time shows a seven-to-ten fold difference at the end of an eight-week exposure (Figure 2C, 2D). The middle leaves were smaller in size, cripple and chlorotic (Figure 1C, 1D) and the mean leaf weight was reduced 2.7 fold (Figure 2B). These symptoms have been described in other species as the 'little leaf' syndrome (Broadley et al., 2007). The Zn deficient flower buds were yellow (Figure 1F) compared to those of the control plants (Figure 1E) and their flowers were poorly developed (Figure 1G, 1H). The Zn-deficient pistils were smaller in size than the Zn-sufficient ones (Figure 1I, 1J). The siliques were shorter in length than the Zn-sufficient siliques (Figure 1K) and the number of seeds per silique was less (Figure 1L, 1M). Silique length was reduced by two fold (Figure 2A) and silique weight by three fold (Figure 3B) compared to Zn-sufficient siliques. The Zn-deficient seeds were smaller in size and weight (Figure 1N, 1O) and the germination percentage of Zn-

deficient seeds was strongly reduced, probably because these seeds were not viable. The Zn contents of the Zn-deficient plants were significantly lower than those of the Zn-sufficient plants in all tissues that were analysed (Figure 4).

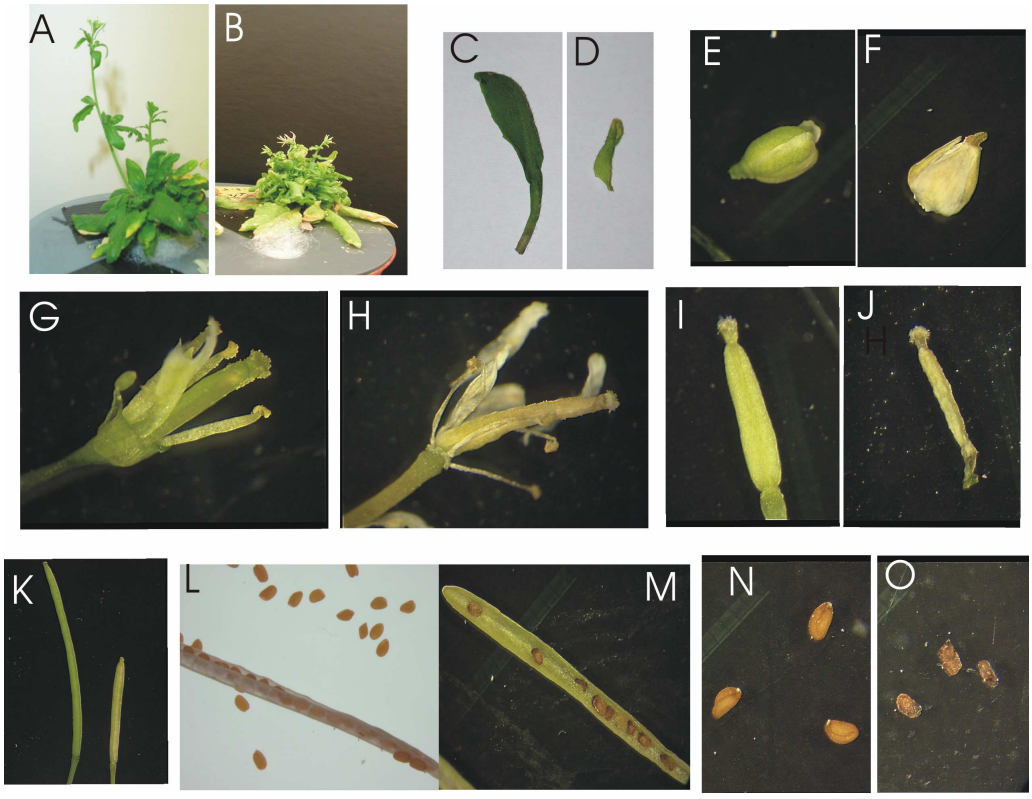

Figure 1: Phenotypic differences of *A. thaliana* grown under Zn-sufficient and Zn-deficient conditions.

A, C, E, G, I, L and M show parts of Zn sufficient plants; B, D, F, H, J, M and O show parts of Zn defiecient plants. *A. thaliana* plant grown under Zn-sufficiency (2 µM Zn) and Zn-deficiency (0 µM Zn) after four weeks **(A, B)**. The Zn-sufficient and Zn-deficient leaf **(C, D),** flower bud **(E, F),** flowers **(G, H)** pistil **(I, J),** silique **(K),** silique with seeds **(L, M)** and seeds **(N, O).**

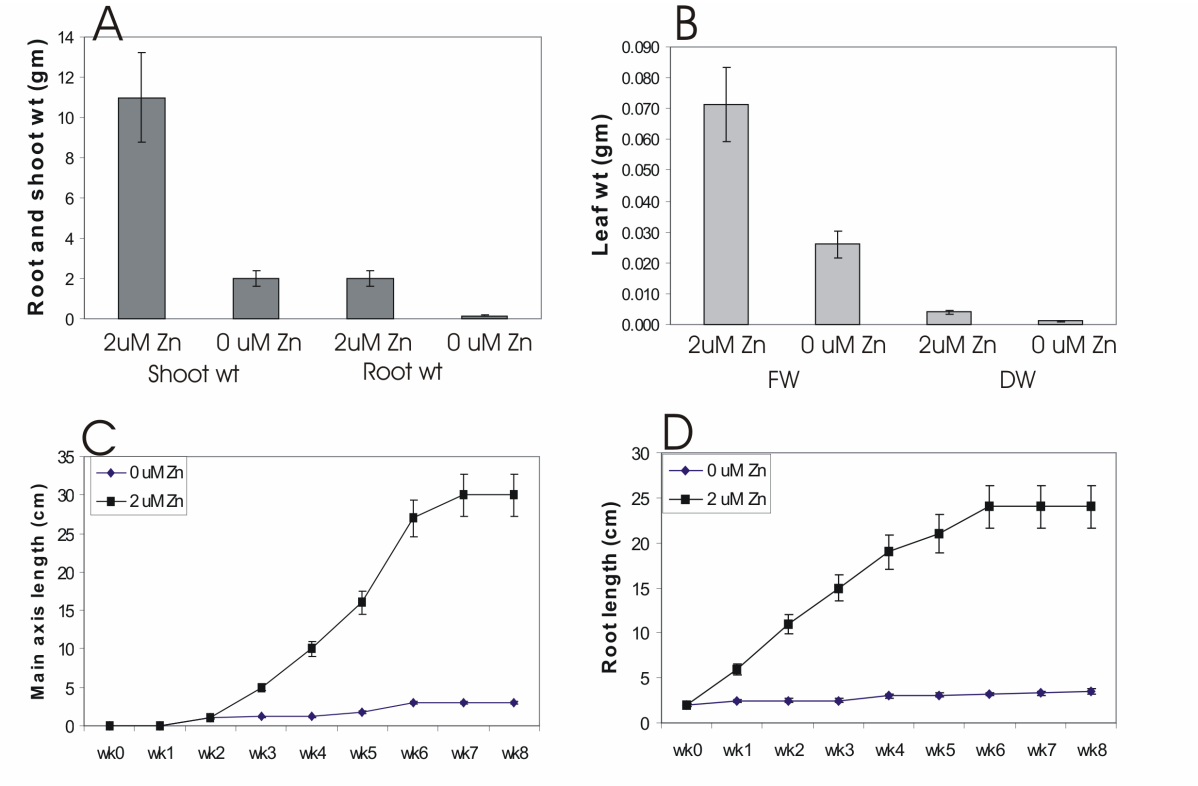

Figure 2: Shoot and root growth characteristics of *A. thaliana* grown under Zn sufficient (2 µM Zn) and Zn deficient (0 µM Zn) conditions. Shoot and root weight after eight weeks **(A)** ; Leaf weight after eight weeks (wk) **(B)** ; Main axis (stem) length **(C)** ; and root length during eight weeks of growth **(D).**

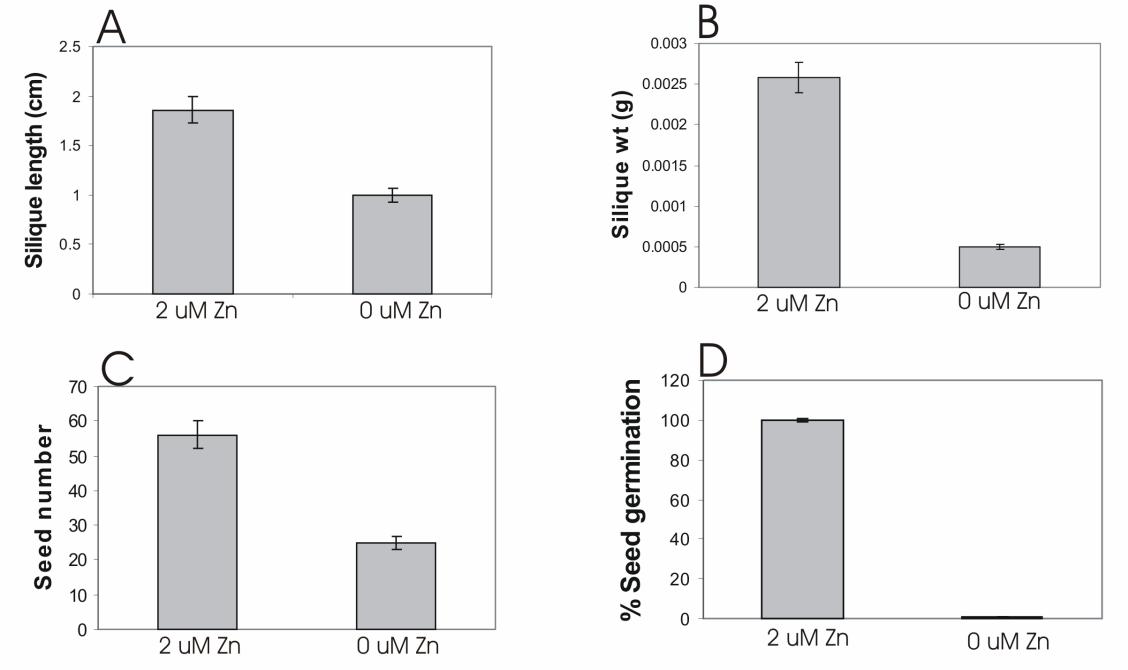

Figure 3: The effect of Zn supply on *A. thaliana* silique characteristics. Final silique length **(A)** and silique weight **(B)**; seed number per silique **(C)**; and percentage of seed germination **(D)**. umoles represents micromoles.

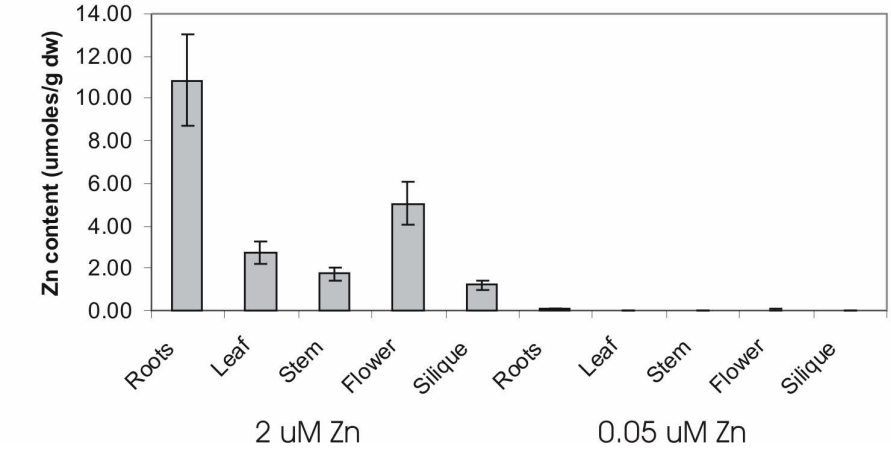

Figure 4 : Comparison of the Zn content in roots, leaves, stems, flowers, siliques of *A. thaliana* plants grown on hydroponic media with 2 µM Zn and 0.05 µM Zn for eight weeks. umoles represents micromoles.

Response of T. caerulescens plants to Zn

The response of *T. caerulescens* to different Zn supplies was studied in a similar way as in *A. thaliana.* However, as *T. caerulescens* is a Zn hyperaccumulator plant, Zn concentrations to which the plants were exposed were increased except for Zn deficiency. For *T. caerulescens*, accession La Calamine, the optimal hydroponic Zn concentration (in half Hoagland's solution) for growth of healthy plants on hydroponics is 100 μM Zn (van de Mortel et al, 2006). Zn defficiency was created by growing plants on half Hoagland's solution, supplied with 0.05 μM Zn. In addition to deficient and sufficient Zn, we also grew plants on a medium with high Zn supply (1000 μM), to see if plants showed visible phenotype. Overall plant size was smaller when the plants were grown under Zn-deficiency and high-Zn compared to sufficient Zn media (Figure 5A). Shoot and root weight was reduced by three-to-four fold under Zn-deficiency and high Zn conditions compared to 100 μM Zn compared to the (Figure 6A). The leaves are also smaller in plants grown on low and high Zn (Figure 5B). The leaves of high Zn plants were darker green than those from 100 μM Zn plants and the leaves of Zn-deficient plants were more chlorotic than the ones from Zn-sufficient plants. Reduction in leaf weight was 2.5-fold in Zn-deficiency and high Zn conditions, compared to Zn-sufficient plants (Figure 6B). The Zn-deficient and high Zn inflorescences are not as well developed and generally chlorotic compared to the Zn-sufficient inflorescences (Figure 5C, D, E). In total 12 plants grown on standard 100 μM Zn produced 30 inflorescences after 16 weeks of exposure, whereas plants grown on Zn-deficiency made 10 inflorescences and in case of high Zn only four. The flower buds in the Zn-deficient and high Zn plants were

chlorotic (Figure 5F, 5H). Most of the flower-buds in Zn-deficient plants failed to open. The siliques formed on Zn-deficient and high Zn plants were chlorotic (Figure 5I, 5K), whereas in the Zn-sufficient plants they were green (Figure 5J). The numbers of seeds in these siliques were lower than for the control plants. The Zn contents of different plant tissues from Zn-deficient, Zn-sufficient and high Zn plants were compared (Figure 6D). In each case the Zn content in the shoots were higher than in the roots. In the Zn-deficiency condition the Zn content was in a very low range. The Zn content in the roots of the plant grown on high Zn was 2.3-fold higher after 16 weeks of exposure than at 100 µM Zn and in shoots it was around three-fold higher. The shoot-to-root ratio after 16 weeks in 100 µM Zn was 2.7 and in 1000 µM Zn it was 3.1.

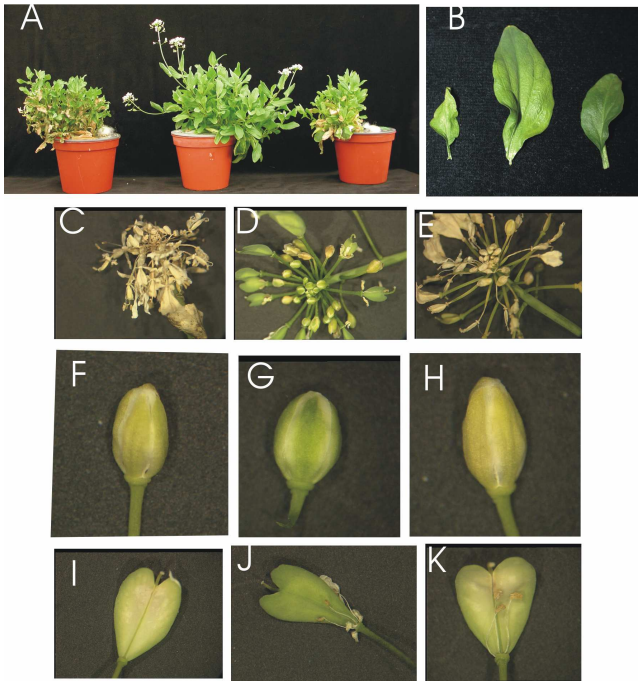

Figure 5: Phenotypic differences of *T. caerulescens* plants in response to deficient, sufficient and high Zn. *T. caerulescens* plants grown hydroponically under Zn-deficiency (0.05 µM Zn) **(A)**, Zn-sufficiency (100 µM Zn) and high Zn (1000 µM Zn) after 16 weeks. Leaves **(B)**; Inflorescences **(C, D, E)** Flower buds **(F, G, H)**; and Siliques **(I, J, K)** of *T. caerulescens* plants grown in respectively Zn-deficient **(C, F, I)**, Zn-sufficient **(D,G, J)** and high Zn **(E,H,K)** conditions .

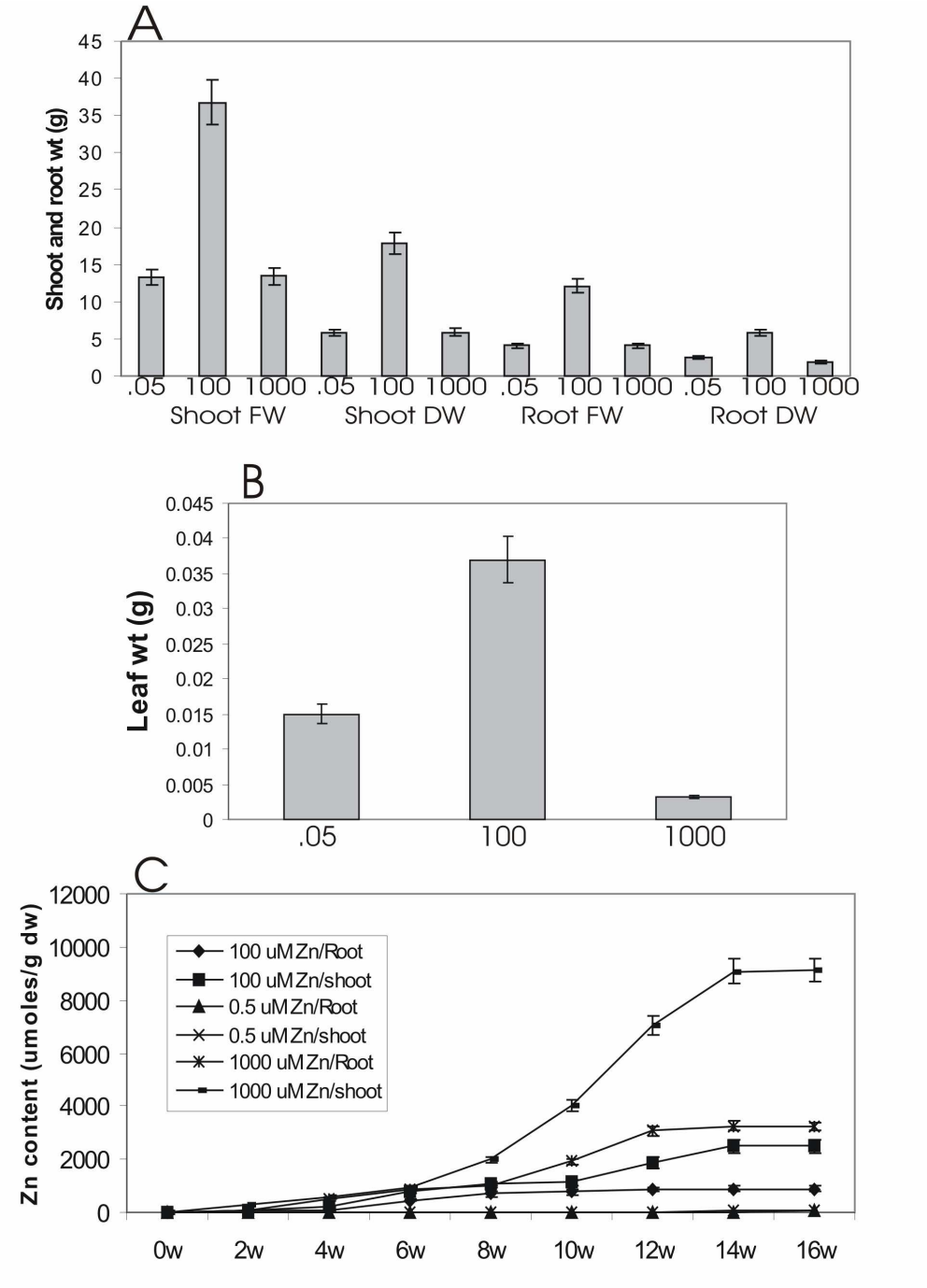

Figure 6: The effect of different Zn supplies on growth and Zn content of *T. caerulescens* **(A)** Root and shoot weight per plant (X-axis represents micromolar Zn concentration in the half Hoagland's media supplied to the plant) **(B)** Leaf fresh weight **(C)** Zn content comparison of the *T. caerulescens* plants grown on 0.05, 100 and 1000 μM Zn, measured every two weeks for 16 weeks. The X-axis represents the week number as 'w'. umoles represents micromoles.

Discussion

The Zn-deficiency phenotypes in *A. thaliana* plants were studied in this research. In general plants showed all the characteristics as described previously for Zn deficient plants in several other species: reduction of internodal growth with an enhanced rosette-like development, small and discoloured leaves, poor root formation and reduced seed and fruit production (Bergmann, 1992, Marschner 1995 and Broadley et al. 2007). The reduced height of Zn-deficient plants (0 μM Zn) compared with Zn-sufficient (2 μM Zn) plants could be due to a decrease of the growth hormone auxin correlating with decreased Zn levels in the plants. A previous study on the relationship of Zn and auxin has shown that a decrease in auxin precedes the appearance of visible symptoms of Zn deficiency in tomato and sunflower suggesting that Zn is required for auxin production. The effect of a reduction in auxin levels was only observed in Zn-deficient plants, whereas it was not observed in the Mn-deficient and Cu-deficient plants (Skoog, 1940). The 'little leaf' is typical for Zn-deficiency, as it is also found in other plants. The increasing chlorosis in the leaf is assumed to be due to the effect of decrease in Zn in the photosynthetic enzymes. In Zn-deficient plants it is also known to result in an impaired response to oxidative stress, likely due to a reduction in superoxide dismutase levels (Hacisalihoglu et al, 2003). Sharma et al., (1995) showed a requirement of Zn for stomatal opening in cauliflower. Zn-deficiency induced increased epicuticular wax deposits, lamina thickness, degree of succulence, water saturation deficit, diffusive resistance, and proline accumulation and decreases in carbonic anhydrase activity, water potential, stomatal aperture, and transpiration in the leaves of cauliflower plants (Sharma et al., 1995). These phenomena

together are assumed to lead to a severe imbalance of growth and development in plants including *A. thaliana.*

The reproductive tissues, the most important parts of the plant, were found to be heavily affected in the Zn-deficient *A. thaliana* plant. The Zn concentration in the flowers is the second highest (after the roots) in the Zn-sufficient plants, indicating the importance of Zn for proper development and function of the reproductive organs. In the Zn-deficient plants, the Zn content in flowers is much lower than in Zn-sufficient plants, like in all parts of the plants. Polar (1970) showed that anthers, and especially pollen contained the highest concentration of Zn when compared to other parts of *Vicia faba* plants. This Zn is used during the pollen tube growth and pollination. During the elongation of the pollen tubes RNA is synthesised (Mascarenhas and Goralnick, 1971), which needs RNA polymerase that requires Zn to be functional. All these reasons together show why the reproductive tissues are very affected by Zn-deficiency. In pea, the Zn-deficient plants were found to have smaller siliques and their seeds were generally not well developed compared to Zn-sufficient plants (Reed, 1944). Failure to produce good seeds was due to irregularities in the growth of the ovule and its generative structures (Reed, 1944). Necrosis of cells in the funiculus and adjacent portions of such ovules was frequently observed, surrounded by areas in which the cells were generally hypoplastic, vacuolated, often showing evidence of cytolysis which destroyed their protoplasmic integrity (Reed, 1944).

While a low Zn content affects plant development, a high Zn content in plants can be toxic. Zn hyperaccumulator species seem to have found a way to deal with a high Zn content in the plant. How do such plants respond to Zn deficiency and high Zn that is lethal to non-accumulator species? Therefore we also studied the

response of the hyperaccumulator *T. caerulescens* to Zn deficiency and high Zn compared it with *A. thaliana*. Both at high Zn (1000 µM Zn) and low Zn (0.05 µM Zn) the plants were found to be severely affected, with reduced root and shoot weight (Figure 6A). Similar to *A. thaliana*, the reproductive tissues in *T. caerulescens* plants were affected at high and low Zn. A clear difference between *A. thaliana* and *T. caerulescence* was the Zn content in the plants, when grown on medium with Zn. In *T. caerulescens*, the Zn concentration was higher in shoots compared to roots as also described in previous studies (Assunção, 2003), whereas in *A. thaliana* it was higher in roots than shoots. Hence, this study shows that high and low Zn both affect the *T. caerulescens* plants in a similar way, but probably by different mechanisms. The high Zn concentration in the cell damages the normal cellular functionality, which is caused via binding of the cation at inappropriate sites in proteins (Eide, 2003). Recent studies of the regulatory Zn sensors that control expression of transporters suggest that *E. coli* cells strive to maintain essentially no free Zn in their cytoplasm under steady state growth conditions (Outten and O'Halloran, 2001). Similarly, in the cytoplasm of eukaryotic cells, free Zn levels are estimated to be in the low nanomolar range under steady state conditions (Sensi et al., 1997). When the Zn concentration in the plant cell is too high, the cell cannot detoxify the high Zn concentration, the tissues are observed to be affected and chlorotic as observed in our study.

Acknowledgement

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Chapter 3

Over-expression of the *Thlaspi caerulescens* ZIP family transporter *ZNT1*, but not *ZNT2*, confers higher Zn and Cd accumulation to *Arabidopsis thaliana*

Sangita Talukdar, Ceasare Rietberg, Henk Schat, Mark GM Aarts

Abstract

- The functional characterization of ZIP transporter genes *TcZNT1* and *TcZNT2* from *Thlaspi caerulescens* (Tc) accession La Calamine is presented.
- The response of the over-expression lines of *TcZNT1* and *TcZNT2* in *Arabidopsis thaliana* was compared with non-transformed wild type plants with respect to metal accumulation after different Zn and Cd exposures. In addition, *TcZNT1*, *TcZNT2* and *TcZNT1/TcZNT2* silenced *T. caerulescens* plants were studied with respect to Zn accumulation.
- Over-expression of *TcZNT1* showed a higher Zn concentration in *A. thaliana* in shoots and roots and to a lower extent also for *TcZNT2*. *TcZNT1* over-expression lines also contained more Cd. The Zn content of *TcZNT1*- and *TcZNT1/TcZNT2*-silenced *T. caerulescens* roots was significantly reduced compared to empty vector transformed roots.

Introduction

Zinc (Zn) plays a critical role in plants as an essential component of key enzymes such as Cu-Zn superoxide dismutase, alcohol dehydrogenase, RNA polymerase, and DNA-binding proteins (Marschner, 1995; Guerinot and Eide, 1999). When Zn supply is limited to the plants, a range of deficiency symptoms can appear (Marschner, 1995, chapter 2). In addition, it results in an impaired response to oxidative stress, likely due to a reduction in superoxide dismutase levels (Hacisalihoglu et al., 2003). Zn deficiency is a significant agricultural problem, particularly in cereals, limiting crop production and quality (Guerinot and Eide, 1999; Hascisalihoglu et al, 2003). On the other hand, a high concentration of Zn in the cell can be toxic. So, the intracellular concentration of this essential but potentially toxic metal must be carefully controlled in a process called metal homeostasis. The primary control point for metal ion homeostasis appears to be regulation of metal uptake across the plasma membrane (Guerinot, 2000).

Zn ions are hydrophilic and do not cross cell or organelle membranes by passive diffusion (Cousins and McMahon, 2000). Plants have evolved mechanisms that allow transport of Zn ions across membranes, which involve transporter proteins such as heavy metal (or CPx-type) ATPases, natural resistance-associated macrophage proteins (Nramps), cation diffusion facilitators (CDFs) (Williams et al., 2000), the ZIP family proteins (Guerinot, 2000) and cation antiporters (Gaxiola et al., 2002). The ZIP (*ZRT, IRT*-like) proteins are involved in the transport of Fe, Zn, Mn and Cd with family members differing in their substrate range and specificity (Guerinot, 2000; Mäser et al, 2001). About 85 ZIP family members have now been identified from bacteria, archeae and eukaryotes, including 15 genes in *Arabidopsis thaliana* (Mäser, 2001). The ZIP proteins are

predicted to have eight transmembrane domains (TM) with the amino- and carboxy- terminal ends situated on the outer surface of the plasma membrane (Guerinot, 2000). They vary widely in overall length, due to a variable region between TM-3 and TM-4. This region is predicted to be on the cytoplasmic side and is a potential metal-binding domain, rich in histidine residues. The most conserved region of these proteins lies in TM-4 and is predicted to form an amphipathic helix containing a fully conserved histidine that may form part of an intra-membranous metal binding site involved in transport (Guerinot, 2000; Mäser et al., 2001). The transport function observed upon heterologous expression in yeast is eliminated when the conserved histidine or certain adjacent residues are replaced by mutation (Rogers et al., 2000).

AtIRT1 (Iron-regulated transporter 1), the first identified member of the ZIP family (Eide et al., 1996), was cloned from *A. thaliana* by functional complementation of a Fe-uptake-deficient yeast double mutant (*fet3 fet4*). *AtIRT1* is now thought to be the major transporter for high-affinity Fe uptake by roots (Connolly et al., 2002; Vert et al., 2002). Plants over-expressing *AtIRT1* also accumulate higher concentrations of Cd and Zn than wild types under Fe-deficient conditions, indicating an additional role in the transport of these metals (Connolly et al., 2002), which is supported by studies in yeast (Eide et al., 1996; Korshunova et al., 1999). An *A. thaliana* knockout *irt1-1* mutant was chlorotic and showed severe growth defects, which could be rescued by the exogenous application of Fe (Vert et al., 2002). The AtIRT1 protein is localized at the plasma membrane and, under Fe-deficient conditions, expressed predominantly in the external epidermal layers of the root. Mutants of *AtIRT1* also showed significant changes in photosynthetic efficiency and developmental defects that were consistent with a

deficiency in Fe transport and homeostasis (Henriques et al., 2002; Varotto et al., 2002). Interestingly, *AtIRT2* (another ZIP protein) is also expressed in root epidermal cells under Fe-deficiency. However, it cannot substitute for the loss of *AtIRT1* (Grotz and Guerinot, 2002) and it appears to have a greater substrate specificity. Although it can complement Fe and Zn uptake mutants in yeast, it does not seem to transport Cd or Mn (Vert et al., 2001), suggesting that AtIRT1 and AtIRT2 have different functions in *A. thaliana*.

Based largely on yeast complementation studies, further information is available on the functional properties of plant ZIP transporters. The *AtZIP1, 2, 3* transporters from *A. thaliana* restore Zn uptake in the yeast Zn uptake mutant *zrt1zrt2* and are proposed to play a role in Zn transport (Grotz et al., 1998; Guerinot, 2000). *AtZIP*s 1, 3 and 4 are expressed in the roots of Zn-deficient plants, while *AtZIP4* is also found in the shoots (Grotz et al., 1998; Guerinot, 2000). The proposed role of ZIP transporters in Zn nutrition is supported by the characterization of homologues from other species. Wintz et al. (2003) suggested that two ZIP genes (*AtZIP2* and *AtZIP4*) are also involved in copper transport.

Still, the knowledge about all the ZIP transporters is not yet complete. Further molecular studies of these transporters will provide the answers to many of the questions related to metal homeostasis in plants and might be used to improve the metal content of crops used for human and animal nutrition. In addition, this knowledge allows the further development of phytoremediation, a technology using plants to remove toxic elements from soil.

Recently, the regulation of the metal ions in Zn hyperaccumulator plants has drawn a lot of interest. The Zn hyperaccumulator species are defined to accumulate more than 10,000 μg Zn g^{-1} of dry weight (dw) (1%, w/w) (Baker and

Brooks, 1989), whereas most plants contain between 30 and 100 μg Zn g^{-1} dw and concentrations above 300 μg Zn g^{-1} dw are generally toxic (Marschner, 1995). *Thlaspi caerulescens* (Tc) is a model plant species to study metal hyperaccumulation and tolerance (Assunção et al., 2003). This species belongs to the Brassicaceae family and is known as a Zn hyperaccumulator, showing up to 30,000 μg Zn g^{-1} dw foliar concentration (Brown et al., 1995). However, hyperaccumulation of Cd or Ni also has been reported for a number of natural *T. caerulescens* populations from calamine or serpentine soils (Reeves and Brooks, 1983; McGrath et al., 1993). Cd is a non-essential but toxic element for plants. In *T. caerulescens* accession "Ganges", foliar Cd concentrations up to 2700 μg g^{-1} dw were found (Lombi et al., 2000).

TcZNT1, a *ZIP* homologue, from *T. caerulescens* was shown to mediate high-affinity Zn uptake and low-affinity Cd uptake following expression in yeast (Pence et al., 2000). Based on sequence identity, *TcZNT1* is most likely an orthologue of *AtZIP4*. *TcZNT1* shares 90% DNA identity and 76% aa identity with *AtZIP4* (Grotz et al., 1998). *TcZNT2* resembles *TcZNT1* but it is probably an orthologue of *AtIRT3* of *A. thaliana*. *TcZNT2* shares 86% DNA identity and 83% aa identity with *AtIRT3*. Assunção et al. (2001) reported that *TcZNT1* and *TcZNT2* were predominantly expressed in roots and less in shoots, and their expression was barely Zn responsive. By contrast, in the non-hyperaccumulator *T. arvense*, these genes were exclusively expressed under conditions of Zn-deficiency (Assunção et al., 2001). In *A. thaliana, AtZIP4* and *AtRT3* were found to be strongly induced under Zn-deficiency conditions compared to Zn-sufficient conditions (Grotz et al., 1998; van de Mortel et al., 2006)

To investigate the role of *TcZNT1* and *TcZNT2* in plants further, we functionally characterised the genes by studying over-expression lines of *TcZNT1* and *TcZNT2* in *A. thaliana* and by silencing the genes in *T. caerulescens.* An average of 88.5% sequence identity in transcript sequences between *T. caerulescens* and *A. thaliana* was found by Rigola et al. (2006). This is in agreement with the study of Peer et al. (2006), who found 87–88% sequence identity for the intergenic transcribed spacer regions when several *Thlaspi* species were compared with *Arabidopsis*. These evidences and available genetic resources makes *A. thaliana*
a suitable choice as a comparative non-accumulator (Freeman et al., 2004). This provides the opportunity to compare similar genes from hyperaccumulator and non-accumulator species by over-expressing these in the non-accumulator species *A. thaliana* (Freeman et al., 2004). In this study, we examine the function of *TcZNT1* and *TcZNT2* in metal homeostasis using lines over-expressing the genes in *A. thaliana.*

Materials and Methods

Construction of GFP-fusion and over-expression plasmids

DNA manipulation and cloning were performed using standard procedures (Sambrook et al., 1989). To make the over-expression construct of *TcZNT1 (35S:: TcZNT1),* a fragment of 1337 bp containing the *TcZNT1* cDNA (Genbank accession number AF275751) was cut from a cDNA library clone (Assunção et al., 2001), using BamHI and XbaI. The fragment was cloned into the BamHI-XbaI digested pEZR(H)-LN plasmid (kind gift of Dr. Gert-Jan de Boer, Ehrhardt laboratory, Dept. of Plant Biology, Carnegie Institution of Washington, USA), which was created by ligating the expression cassette from pEZS-LN (David Ehrhardt, Stanford University; http://deepgreen.stanford.edu/) into pCambia 1300

(http://www.cambia.org/daisy/bios/585.html). BamHI and XbaI digestion removed the GFP gene from the vector and ligation of the *TcZNT1* gene resulted in a CaMV 35S promoter-*TcZNT1* fusion. To make an over-expression construct of *TcZNT2 (35S::ZNT2)*, a fragment of 1623 bp of *TcZNT2* cDNA (Genbank accession number AF275752; Assunção et al., 2001) was cloned into the pEZR(H)-LN vector following the same method. To generate a construct encoding a chimeric fusion protein of N-terminal GFP and *TcZNT1 (35S::ZNT1-GFP)*, a *TcZNT1* cDNA fragment of 1289 bp was amplified from the cDNA library clone using primers P1 and P2 and Pfu DNA polymerase (MBI Fermentas, St. Leon-Rot, Germany). The PCR fragment was digested with HindIII and SalI and ligated into HindIII/SalI digested pEZR(H)-LN. Similarly, a *35S*::*TcZNT2-GFP* construct was made. A 1525-bp proof-reading PCR-amplified fragment of *TcZNT2* cDNA was obtained by using primers P1 and P3 (Table 1) and cloned into pEZR(H)-LN using HindIII and SalI sites. The inserted fragments were verified by DNA sequencing. The constructs were transformed to *A. thaliana* accession Columbia (Col), by *Agrobacterium tumefaciens*-mediated flower dipping transformation as described by Clough and Bent (1998).

Plant growth conditions

Seedlings of T_1 transformants containing the *35S::TcZNT1* and *35S::TcZNT2* constructs were selected on 12x12 cm square plates with half strength MS medium (Murashige and Skoog, 1962) (Duchefa Biochemie B.V., Haarlem, The Netherlands) containing 20 mg l^{-1} hygromycin B (Duchefa Biochem, Haarlem, The Netherlands) to select 10 independently transformed lines for each construct, which were propagated until homozygous T_3 lines. Plates were incubated in a

climate-controlled growth cabinet (25°C day; 16 h day with illumination at a light intensity of 120 µmol m^{-2} s^{-1}).

Genomic DNA extraction and DNA blot

To confirm the presence of a T-DNA insert in the transgenic lines, DNA was extracted from flowers of *35S::TcZNT1* and *35S::TcZNT2* transformed *A. thaliana* T_1 plants, as described by Aarts et al. (2000). PCR was performed to amplify the fragments with the primers P2 and P4 (Table 1) for *TcZNT1* (fragment size: 1238 bp) and P3 and P5 (Table 1) for *TcZNT2* (fragment size: 1292 bp). To determine the insertion number of T-DNA, DNA-blot analysis was performed of 10 independent transformants for each of two constructs *35S::TcZNT1* and *35S::TcZNT2*. The fragments obtained for *TcZNT1* and *TcZNT2* by PCR amplification mentioned above, were used as probe for the DNA-blot hybridisations, according to Aarts et al. (2002). The transgenic plants with a single insertion were subsequently checked for expression levels of the transgene using semi-quantitative RT-PCR.

Isolation of total RNA and semi-quantitative Reverse Transcriptase-PCR (RT-PCR)

Total RNA of leaves and roots was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocol. One µg of total RNA was used to synthesize cDNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and an oligo(dT) primer (Invitrogen, Carlsbad, CA, USA). The RNA quantity was estimated based on the spectrophotometric measurements and its quality was checked by agarose gel electrophoresis. The RNA was treated with DNase (MBI Fermentas, St. Leon-Rot, Germany) before cDNA synthesis was

performed. PCR was performed on cDNA using *At-tubulin* (At1g04820) primers P6 and P7 (Table I) as a constitutively expressed control gene to check for any contamination with genomic DNA. To differentiate between amplifications from genomic DNA and cDNA, these primers were designed to span an intron of the genomic *At-tubulin* gene. The gene-specific PCR-amplification was performed with primers P2 and P4 for *TcZNT1* and P3 and P5 for *TcZNT2* (Table I). Three transgenic lines for each construct, with the highest expression level of *TcZNT1* or *TcZNT2* were chosen for further analysis.

Transient expression in Cowpea protoplast transformation

Cowpea protoplasts were prepared and transfected as described previously by Shah et al. (2002).

Metal exposure

To determine the effect of exposure to heavy metals on growth of seedlings, three independent transgenic lines containing either the *35S::TcZNT1* or *35S::TcZNT2* construct and the untransformed control *A. thaliana* (Col) line were grown vertically on 12x12 cm square Petri plates with half MS containing 1% agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). To determine the response of the transgenic and wild type plants to Zn or Cd, the plants were exposed to $ZnSO_4$ (0, 15, 50, 75, 150, 250, 400, 500 µM) or $CdSO_4$ (0, 25, 50, 75, 100, 150, 200, 250 µM) (Merck, Darmstadt, Germany). Plates were incubated in a climate-controlled growth cabinet (25°C; 16 h day with illumination at a light intensity of 120 µmol m^{-2} s^{-1}). Root lengths of individual seedlings were measured. Each experiment was repeated at least twice at different time points.

In case there was difference in the root growth of transgenic lines and wild type, further hydroponics experiments were done, to check the root growth and tolerance phenotype. Ten to fifteen plants for each of the three independent transformants of *35S::TcZNT1* and *35S::TcZNT2* were grown on modified half strength Hoagland's nutrient solution (Schat et al., 1996) containing 2 µM $ZnSO_4$, which is sufficient for *A. thaliana*, on Zn-deficiency medium (0 µM $ZnSO_4$, where Zn is not added to the modified half Hoagland's nutrient solution, and 0.05 µM $ZnSO_4$) or on high-Zn medium (50 µM $ZnSO_4$). Plants were grown in trays, each containing about nine litre of hydroponic medium. For each concentration the transgenic and control lines were grown in the same tray to avoid an effect of variation among the trays. Typically a maximum of 81 plants were grown per tray. The 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/day; 75% RH) for five weeks. The nutrient medium was refreshed every week. Root and shoot tissues were harvested for metal content analysis. Each hydroponics experiment was repeated twice at different time points keeping all the growth conditions the same.

To determine the response of *35S::TcZNT1* and *35S::TcZNT2* transformed *A. thaliana* plants to Cd, the same transgenic lines were grown hydroponically on modified half Hoagland's solution for two weeks and then transferred to the same media but containing 0 µM Cd-0 µM Zn, 0 µM Cd-2 µM Zn, 0.5 µM Cd-0 µM Zn, 0.5 µM Cd-2 µM Zn, 5 µM Cd-0 µM Zn or 5 µM Cd-2 µM Zn, keeping the rest of the minerals constant in the media. The nutrient solution was refreshed every week. For mineral content analysis the root and shoot tissues were harvested individually at the end of two weeks exposure.

Agrobacterium rhizogenes-mediated T. caerulescens transformation and RNAi

RNAi silencing constructs were made by PCR amplifying a 361-bp fragment of *TcZNT1* cDNA (bp 268-597 of the cDNA sequence (AF275751) and a 417-bp fragment of *TcZNT2* cDNA (bp 1160-1545 of the cDNA sequence (AF275752) with primers P10, P11 and P12, P13 respectively (Table 1) containing added restriction sites. Fragments were cloned into the vector pREDRoot according to Limpens et al. (2004) and transformed into *A. rhizogenes* strain MSU440 (Sonti et al., 1995). In order to silence both *TcZNT1* and *TcZNT2*, a construct was made in which the PCR amplified fragments from *TcZNT1* and *TcZNT2* were ligated into a 778 bp fragment using NsiI and cloned into pREDRoot following the steps mentioned above. For transformation, roots of five-day-old vertically grown *T. caerulescens* seedlings were removed from the hypocotyls and the wounded surface was inoculated with *A. rhizogenes* containing the binary plasmids. The seedlings were co-cultivated with *A. rhizogenes* for five days at 20°C (16h light-8 h darkness) and subsequently transferred to new plates with half-strength MS media containing 100 mg l^{-1} Ticarcillin (Duchefa Biochem, Haarlem, The Netherlands). The plants were grown vertically on 14 cm-square Petri plates for five weeks and during these period new roots formed that are potentially co-transformed with the T-DNA of the binary vector. Transformed roots were distinguished based on the expression of DsRED, co-expressed on the pREDRoot vector, and visualized by fluorescence using a Leica MZIII fluorescence stereomicroscope and a Nikon Optiphot-2 coupled to a mercury-lamp. Untransformed roots were cut off until no more untransformed roots appeared. Plants transformed with *A. rhizogenes* containing the empty pRedRoot vector were used as control for comparison to silenced plants.

After five weeks growing on plates these chimeric transformed Thlaspi plants were transferred to hydroponics pots (600 ml), one plant/pot. Each pot contained modified half Hoagland's nutrient solution containing 100 µM $ZnSO_4$, which is sufficient for *T. caerulescens*. Plants were grown for three weeks. The plants with silenced expression of *TcZNT1, TcZNT2* and *TcZNT1/TcZNT2* in roots were selected based on semi-quantitative RT-PCR (using primers P14, P15 for *TcZNT1* and P16, P17 for *TcZNT2*) performed on the root tissue from each of these plants. The root and shoot tissues of the silenced plants were harvested for Zn content measurement.

Root and shoot metal accumulation assay

Root and shoot tissues were analysed for their Zn, Fe, Mn and Cd content as described by Assunção et al. (2003).

Table 1: Primers used for the PCR amplification.

Primer name	**Sequence of oligonucleotides (5'-3')**	**Purpose**
P1	CCCAAGCTTACCCAAAAAAAGAGATCGAATT	Forward primer designed on cDNA clone pAD-GAL4-2.1 vector with HindIII site
P2	TAAAGTCGACGCCCAAATGGCGAGTGC	Reverse primer for the *TcZNT1* cDNA
P3	CAGTGTCGACACCCAAATGGCAAGAGAAGA	Reverse primer for *TcZNT2* cDNA
P4	TTCCCATGATCATCGCCGAT	Forward primer designed on *TcZNT1* cDNA
P5	GCAATCGTAAGACCCCAATGT	Forward primer designed on *TcZNT2* cDNA
P6	AAGCTTGCTGATAACTGTACTGGT	Forward primer for PCR amplification of *At-tubulin*
P7	GGTTTGGAACTCAGTGACATCA	Reverse primer for PCR amplification of *At-tubulin*
P8	CCTACGCACCAGTCATCTCT	Forward primer for PCR amplification of *Tc-tubulin*
P9	CGAGATCACCTCCTGGAACA	Reverse primer for PCR amplification of *Tc-tubulin*
P10	GCC**ACTAGT**GGCGCGCCAGGAAATCTCTTTGTAG CTGCTAAAGC	Forward primer for *TcZNT1* for silencing; containing SpeI and AscI sites
P11	GCC**GGATCC**ATTTAAATTCACCAAACGCTTTGCT ATCATTCC	Reverse primer for *TcZNT1* for silencing; containing BamHI and SwaI sites
P12	GCC**ACTAGT**GGCGCGCCGAACAAATCAGCAACCA TCATGGC	Forward primer for *TcZNT2* for silencing; containing SpeI and AscI sites
P13	GCCA**GGATCC**ATTTAAATGTGAAATTCGAGATTT GATTGATTAT	Reverse primer for *TcZNT2* for silencing; containing BamHI and SwaI sites
P14	CCTTCAAACCGAAGGAAATCTCTTT	Forward primer for semi quantitative RT-PCR of the *TcZNT1* silenced plant
P15	GATGATGTCTCCTCAACAACAGCTG	Reverse primer for semi quantitative RT-PCR of the *TcZNT1* silenced plant
P16	GAACAAATCAGCAACCATCATGGC	Forward primer for semi quantitative RT-PCR of the *TcZNT2* silenced plant
P17	GAAATTCGAGATTTGATTGATTATG	Reverse primer for semi quantitative RT-PCR of the *TcZNT2* silenced plant

Restriction sites incorporated in the primers are underlined and bold (if there are two sites on the same primer).

Results

Subcellular localization of 35S::TcZNT1-GFP and 35S::TcZNT2-GFP in cowpea protoplast

To check the predicted plasma membrane localization for both TcZNT1 and TcZNT2 (Assunção et al., 2001), the cDNAs of both genes were fused at their C-terminus in frame with a GFP gene. These fusion constructs were transiently expressed in cowpea protoplasts. The GFP fluorescence was localized to the plasma membrane of the cowpea protoplasts. This indicates that *TcZNT1* and *TcZNT2* localise to the plasma membrane (Figure 1).

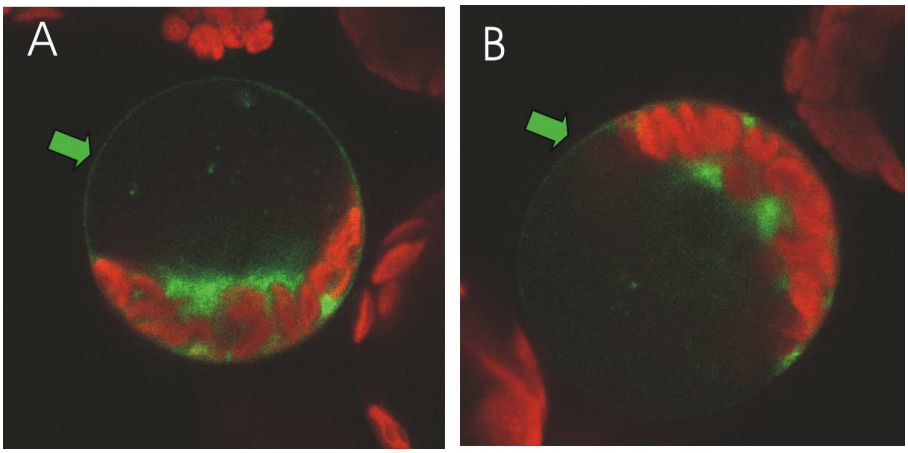

Figure1: A chimeric fusion protein transiently expressed from (A) *CaMV 35S::TcZNT1-GFP* or (B) *CaMV 35S::TcZNT2-GFP* constructs localizes to the plasma membrane (arrows) of cowpea protoplasts. The GFP signal was not seen in the vacuolar membrane or in the chloroplast membrane. The background staining of cytoplasm is probably due to the high expression of the construct due to the strong CaMV 35S promoter.

TcZNT1 and TcZNT2 over-expression lines confer early flowering and increased Zn content in roots and shoots

In order to understand the function of *TcZNT1* and *TcZNT2*, the *35S::TcZNT1* and *35S::TcZNT2* transformed *A. thaliana* homozygous lines were grown on modified half Hoagland's nutrient solution containing standard Zn (2 μM), no Zn (0 μM,

i.e. no Zn added in the media to induce deficiency) on excess Zn (50 μM, which is toxic to Arabidopsis) for five weeks. The plants grown with 2 μM Zn showed early flowering compared to the wild-type Col plants (Figure 2A, 2B). The *35S::TcZNT1* transgenic plants were also more sensitive to Zn deficiency (0 μM Zn) than the wild type plants (Figure 3A). The Zn content in the *35S::TcZNT1* plants grown with half Hoagland's solution containing 2 μM Zn and 0.05 μM Zn was significantly higher in shoots and roots than in wild type (Figure 2C, 2D and 3B, 3C respectively). The shoot Zn content in *35S::TcZNT2* plants was significantly higher but only by 1.2 fold compared to wild type when grown on half Hoagland's solution containing 2 μM Zn, but not on medium with 0.05 μM Zn (Figure 2C, 2D and 3B, 3C respectively).

The same *TcZNT1* overexpression lines grown on high Zn (50 μM Zn) had a markedly higher Zn content in roots and shoots compared to the wild type (Figure 3D, 3E). The effect of *TcZNT2* overexpression was less clear and not significant in roots.

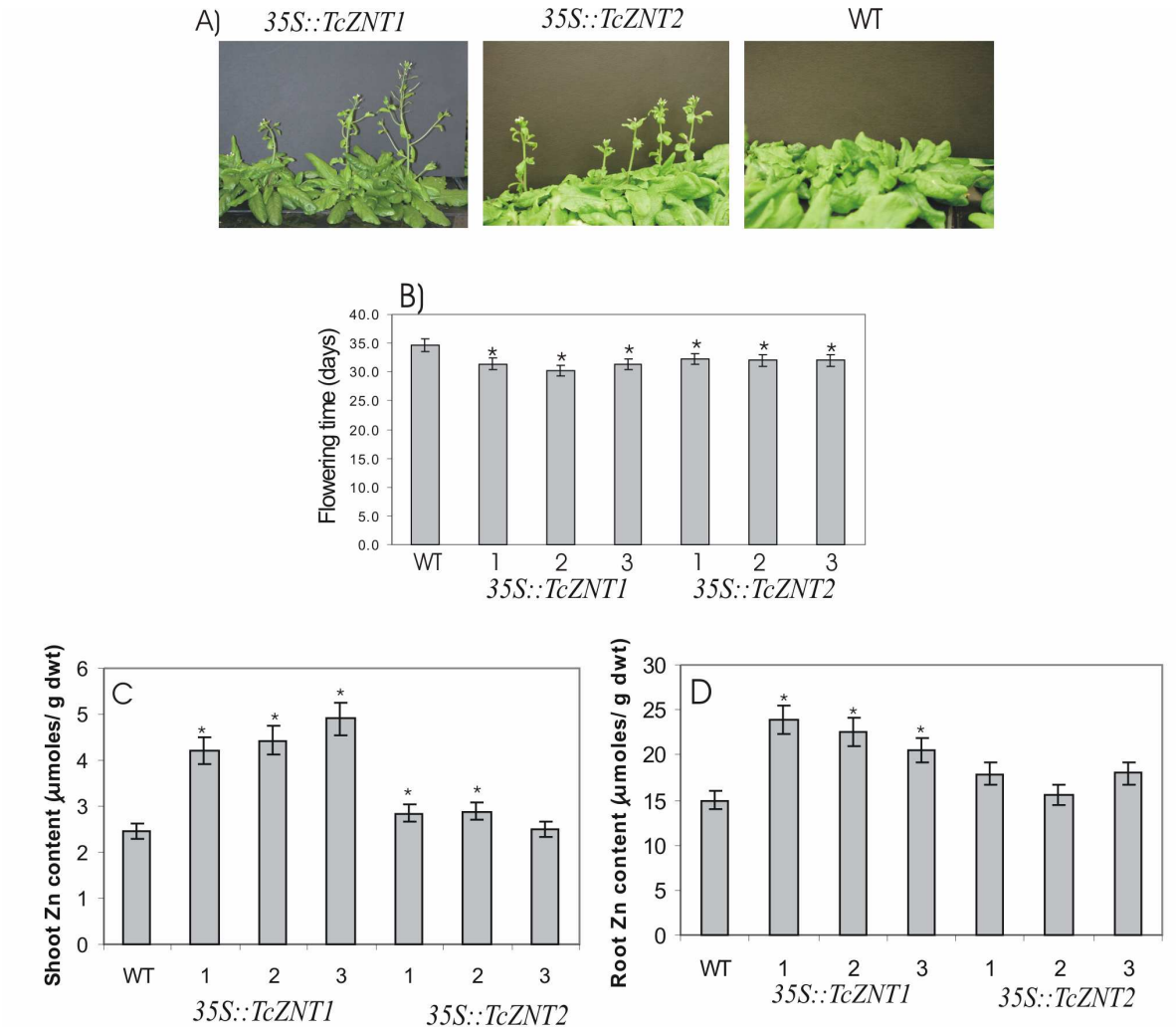

Figure 2: Flowering time and Zn content of transgenic *35S::TcZNT1* and *35S::TcZNT2 A. thaliana* lines compared to non-transformed wild type. **(A)** Three independently transformed *35S::TcZNT1* and *35S::TcZNT2 A. thaliana* lines grown in comparison to Col wild-type (WT) plants on half Hoagland's solution containing 2 µM Zn, show early flowering. Photograph was taken after five weeks since seed sowing. **(B)** Flowering time in days after sowing. **(C, D)** Zn content (µmoles g^{-1} dw; mean ± SE of 15 replica) in shoots (C) and roots (D) of the *35S::TcZNT1* and *35S::TcZNT2* transformed *A. thaliana* lines compared to wild-type plants after five weeks of growth in half Hoagland's solution (containing 2 µM Zn). The Zn content of three independent transformant lines is shown. Significant difference between the transgenic line and WT is indicated with * ($p<0.05$, Student's t test).

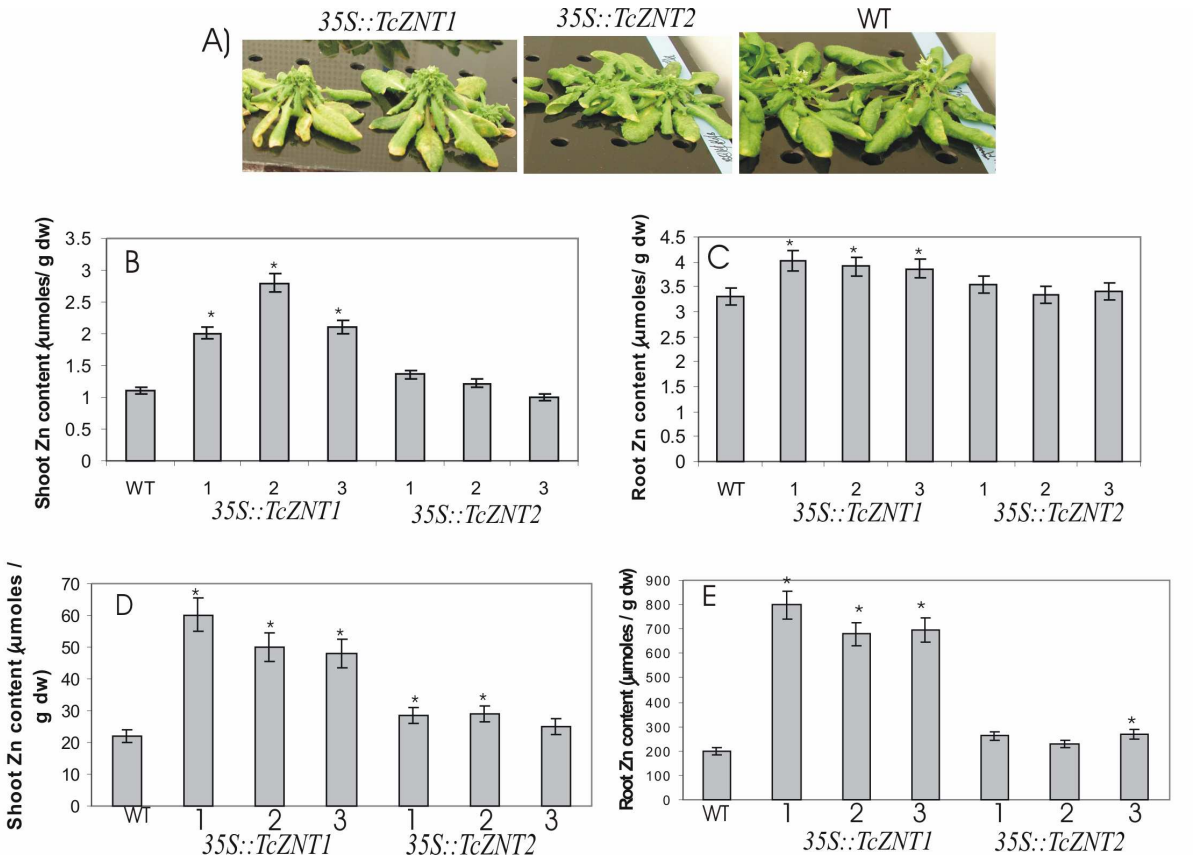

Figure 3: The response of transgenic *35S::TcZNT1* and *35S::TcZNT2 A. thaliana* lines to Zn deficiency and Zn excess. Plants were grown hydroponically on half Hoagland's media with modified Zn supplementation.

(A) Phenotypes of *35S::TcZNT1* and *35S::TcZNT2* plants grown on Zn deficient medium (0 µM $ZnSO_4$) *35S::TcZNT1* plants shows the highest sensitivity to Zn compared to *35S::TcZNT2* and WT plants, with typical Zn deficiency symptoms of 'little leaf' and chlorosis of leaves. Zn content (µmoles g^{-1} dw; mean ± SE of 15 replicates) after five weeks of exposure to half Hoagland's solution supplemented with 0.05 µM $ZnSO_4$ **(B, C)** and 50 µM $ZnSO_4$ **(D, E)**. The Zn contents of three independent transformant lines have been shown. Significant difference between the transgenic line and WT is indicated with * ($p<0.05$, Student's t test).

TcZNT1 over-expression lines show higher Cd content in shoots.

In order to determine the response of the *TcZNT1* and *TcZNT2* over-expression plants to Cd, the transgenic lines and wild type were grown hydroponically on modified half Hoagland's solutions containing 0 µM Cd-0 µM Zn, 0 µM Cd-2 µM Zn, 0.5 µM Cd-0 µM Zn, 0.5 µM Cd-2 µM Zn, 5 µM Cd-0 µM Zn and 5 µM Cd-2

μM Zn, keeping the other minerals constant in the media. *TcZNT1* overexpressing lines showed no visible phenotypic difference to the wild type, but all contained a significantly higher Cd content in shoots, while the root Cd content was lower in the overexpression lines than in the wild type (Figure 4A, B). Figure 4C and 4D show the Zn and Cd contents in the *TcZNT1* over-expression line (transformant number 1), grown on half Hoagland's containing 2 μM Zn, 5 μM Cd-2 μM Zn and 5 μM Cd-0 μM Zn. Similar results were observed also for two other transgenic lines (data not shown). The shoot Zn and Cd content in the transgenic plants under 2 μM Zn and 5 μM Cd-2 μM Zn is significantly higher than wild type plants (Figure 4C). But the Zn content in shoots of transgenic plants grown on 5 μM Cd-2 μM Zn is lower than in the plants grown on 2 μM Zn, whereas in wild type plants they show similar concentration. The Fe contents in the roots and Mn content in shoots of the transgenic plants in Cd exposed wild type and transgenic plants are higher than in the non-exposed plants (Figure 4E, 4F). The over-expression lines of *TcZNT2* neither showed any visible phenotype nor a Zn or Cd content difference compared to the wild type grown on the same media.

The plants were also grown on Cu, Mn, and Co deficient plates with half MS/agar media but no significant root growth difference compared to wild type was observed (data not shown).

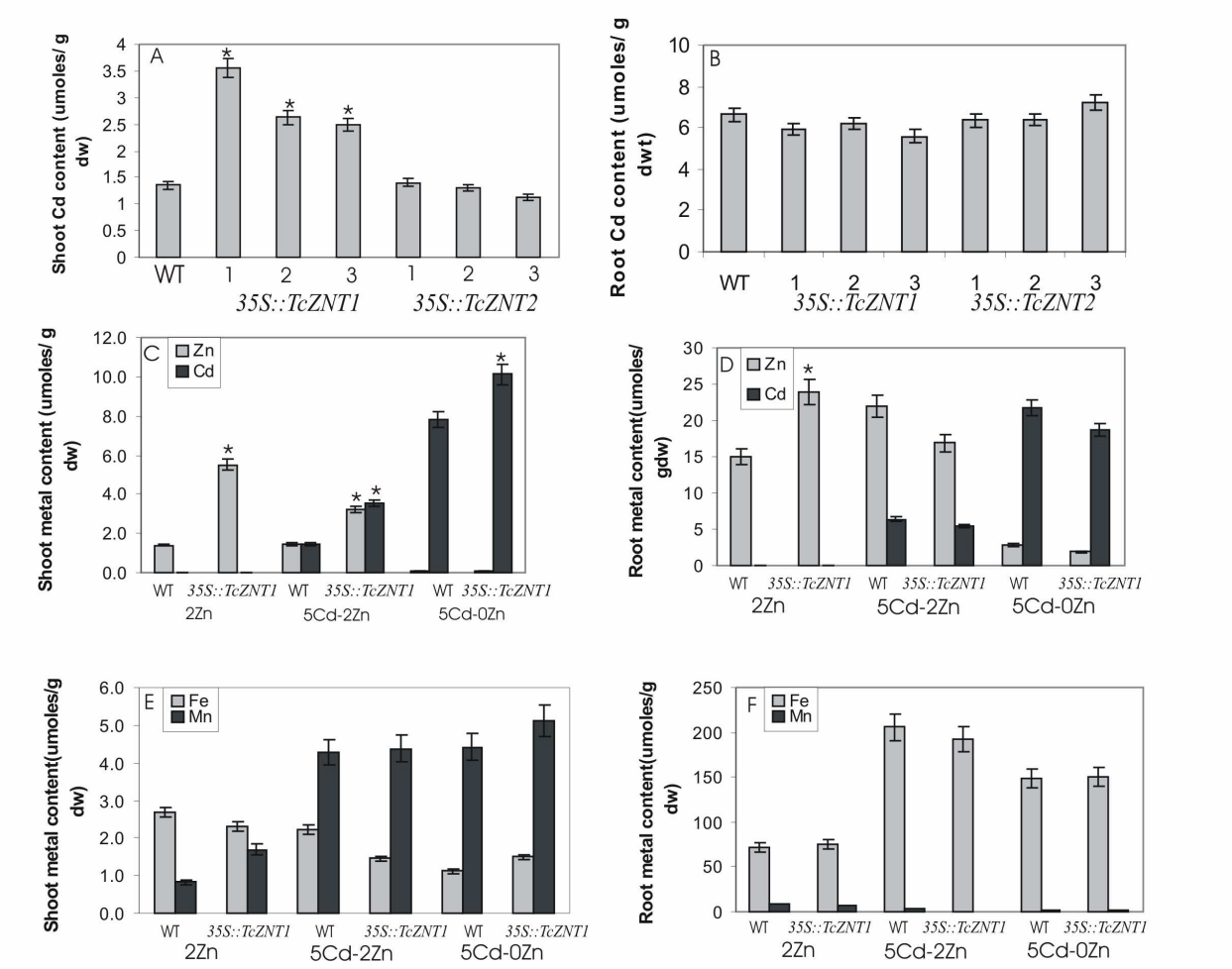

Figure 4: Phenotypic analysis of *TcZNT1* and *TcZNT2* overexpressing *A. thaliana* plants compared to wild type when exposed to excess Cd.

(A, B) Cd content (µM g^{-1}dw; mean ± SE) in (A) shoots and (B) roots of *35S::TcZNT1* and *35S::TcZNT2* transformed *A. thaliana* plants compared to wild type *A. thaliana* (Col) plants grown hydroponically on half Hoagland's media with 5 µM $CdSO_4$ and 0 µM $ZnSO_4$. The Cd contents of three independent transformants are shown with 15 replica per genotype. **(C, D)** Zn /Cd content (µM g^{-1}dw; mean ± SE of 15 replica) **(E, F)** Fe/Mn content (µM g^{-1}dw; mean ± SE of nine replica) content in roots and shoots of the *35S::TcZNT1* transformed *A. thaliana* plants in comparison to the wild type plants after two weeks exposure to half Hoagland's solution with 0 µM Cd-2 µM Zn, 5 µM Cd-2 µM Zn and 5 µM Cd-0 µM Zn. The asterisk shows the difference in metal content in wild type and transgenic is significant ($p<0.05$, Student's t test). umoles represents micromoles.

Suppression of TcZNT1 and TcZNT2 transcript levels in T. caerulescens by RNA interference results in reduction in Zn content in roots

In order to silence the *TcZNT1* and *TcZNT2* genes in *T. caerulescens*, *Agrobacterium rhizogenes*-mediated root transformation was performed on 450 *T. caerulescens* seedlings. 301 chimeric plantlets containing a transformed root system were selected based on *DsRED1* red fluorescence screening. These plants were grown with modified half Hoagland's solution containing 100 μM Zn for three weeks. Semi-quantitative RT-PCR was performed on two independently synthesised cDNAs of each seedling to check the expression level of *TcZNT1* and *TcZNT2* compared to control empty vector transformed plants. Nine plants for *TcZNT1*, six for *TcZNT2* and eleven for *TcZNT1/TcZNT2* were found to have silenced the target gene in roots (Table 2). The rest of the plants showed low to moderate expression of *TcZNT1* and *TcZNT2* transcripts. The Zn contents of the roots of *TcZNT1* and *TcZNT1/TcZNT2* silenced plants were significantly ($p<0.05$) lower than those of the empty vector transformed *T. caerulescens* plants (Figure 5). The *TcZNT2* silenced plants did not show a significant difference compared to the control plants.

Table 2: Overview of T. caerulescens plants with silenced TcZNT1 and/or TcZNT2 in roots

Genes silenced	**Total no. of plants used for transformation**	**No. of plants transformed**	**No. of silenced plants**	**Significance of Zn content ($p<0.05$)**
TcZNT1	120	94	9	Significantly lower than empty vector transformants
TcZNT2	120	79	6	Lower but not significant
TcZNT1 and *TcZNT2*	120	82	11	Significantly lower than empty vector transformants
pREDRoot (empty vector)	90	46	-	-

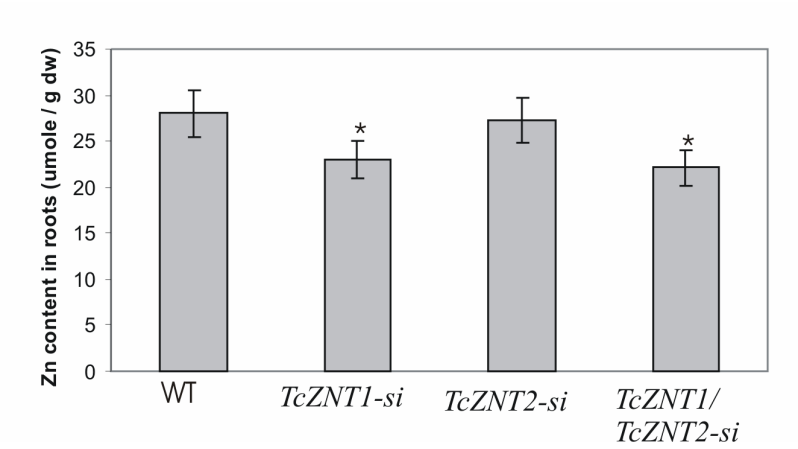

Figure 5: Silencing of *TcZNT1* and *TcZNT2* by RNA interference results in Zn sensitivity.

The Zn content (µmoles g^{-1} dw; mean ± SE) in roots of *TcZNT1, TcZNT2* and both *TcZNT1/TcZNT2* silenced *T. caerulescens* plants in comparison with empty vector (pREDRoot) transformed plants, grown on half Hoagland's solution supplied with 100 µM $ZnSO_4$. The asterisk shows the difference is significant ($p<0.05$, Student's t test). umoles represents micromoles.

Discussion

To investigate the mechanism of the hyperaccumulation phenomenon in *T. caerulescens,* functional characterization of *TcZNT1* and *TcZNT2* was performed in this study. The data presented here suggests that *TcZNT1* is involved in transport of Zn in *T. caerulescens. TcZNT1* is also involved in Cd transport in the plant. The studies were done by over-expressing the genes in a closely related non-accumulator species, *A. thaliana* and in silenced lines of *T. caerulescens*.

Based on the sequence similarity *TcZNT1* and *TcZNT2* both were predicted to be members of ZIP family and assumed to be plasma membrane localised (Assunção et al., 2001). This was confirmed in a cowpea protoplast transfection experiment with the *TcZNT1-GFP* and *TcZNT2*-GFP fusion proteins, which were found to be localized in the plasma membrane (Figure 1).

To check the function of these transporters in plant, the *TcZNT1* and *TcZNT2* over-expression lines were grown on half Hoagland's solution. The Zn contents of the over-expression lines were significantly higher than the wild type ones grown on the same media (Figure 3). This result, together with the plasma membrane localization suggests that the Zn cations are taken up by the *TcZNT1* transporter at the plasma membrane of the plant cells. The study by Pence et al. (2000) of *TcZNT1* showed that this gene complemented the *Saccharomyces cerevisiae* mutant *zhy3*, defective in the high- and low-affinity Zn transporters, ZRT1 and ZRT2 (Zhao and Eide, 1996) and provides evidence that *TcZNT1* can mediate a high-affinity Zn uptake, but only in yeast. Our analysis suggests that *TcZNT1* functions in the same way in plants. The effects of *TcZNT2* are hardly detectable, so that it either does not function in Zn transport or its effects in Zn content measurement is so weak that it is not detected in the present experiments.

The transgenic plants confer earlier flowering than the control (Figure 2A). Flowering in plants is a consequence of the transition of the shoot apex from vegetative to reproductive growth in response to environmental and internal signals (Colasanti et al., 1998). This transition is manifested as a change in properties of the shoot apical meristem, which stops producing leaves and instead starts producing the floral meristems that give rise to flowers. The observation that over-expression of *ZNT* genes leads to early flowering, might be due to the altered Zn status, which initiates the flowering signal through the zinc controlled proteins, earlier in the transgenic plants than in the wild type plants.

It is often assumed that Cd, and other toxic heavy metals are taken up by several transporters for essential elements, which lack substrate specificity (Lombi et al., 2002). Clemens (1998) showed that in non-accumulating plants a Ca transport pathway could be involved in the uptake of Cd, albeit with low affinity. In our study the *35S::TcZNT1* transformed *A. thaliana* lines showed significantly higher Cd content by around two fold in the shoots than the wild type, but the Cd contents in the roots were lower than the wild type (Figure 4). A few studies have been performed about Cd transport in *A. thaliana* (Howden and Cobbett, 1992; Gong et al., 2003). Gong et al. (2003) showed evidence of long distance root-to-shoot transport of phytochelatins (PCs) and Cd in *A. thaliana.* Our study suggests *TcZNT1* transports Cd into the plant cell and most probably PC-mediated translocation to the shoot of the plant for safer storage of the toxic elements. In case of *TcZNT2,* neither any visible phenotypic difference nor a higher Cd uptake was observed in the over-expression lines compared to the wild type. So Cd uptake is mediated specifically by TcZNT1. This data also correlates with the

previous study of Pence et al. (2000), which showed *TcZNT1* as low-affinity Cd uptake transporter in yeast.

Figures 4C and 4D show the effect of different combinations of Zn and Cd supply on the metal content in a *TcZNT1* over-expression line compared to the wild type. The shoot Zn and Cd content in the transgenic plants on 2 μM Zn and 5 μM Cd-2 μM Zn is significantly higher than in wild type plants (Figure 4C). But the Zn content in shoots of transgenic plants grown on 5 μM Cd-2 μM Zn is lower than that of the plants grown on 2 μM Zn, whereas in wild type plants they show similar concentrations. This data strongly suggest competition of Zn and Cd by the same uptake system. This observation again supports the study of Pence et al. (2000), which reported *TcZNT1* to be a high-affinity Zn transporter and low-affinity Cd transporter. Interestingly, the Cd content both in the shoots and roots were higher in transgenic and wild type plants grown on 5 μM Cd-0 μM Zn than those on 5 μM Cd-2 μM Zn. This suggests that in general the Zn transporters can accumulate higher Cd concentrations in the absence of Zn. Strikingly, the Mn content in shoots and Fe content in roots was higher in the transgenic and the wild type plants which were exposed to Cd, in contrast to the ones which were not exposed (2 μM Zn). These observations are not clearly understood, but as similar effects were observed in both wild type and transgenic plants they are considered to be a general effect of Cd in plants. Fe and Mn both are redox-active elements and are important for many biological reactions in plant. This is why probably Mn is higher in the shoots to protect the plants from the unwanted substitution of the essential photosynthetic enzymes. Fe is a key component in a range of enzymes in the plant, for which probably the plant tries to protect itself from the Cd exposure.

All these studies were done in *A. thaliana* expressing with the *T. caerulescens* genes, as stable transformation in *T. caerulescens* is not an easy process. Still the direct effect of the gene function can be shown when the targeted genes are silenced in *T. caerulescens.* This is why we introduced an *A. rhizogenes*-mediated silencing method in *T. caerulescens* according to the procedure described by Limpens et al. (2004) for *A. thaliana* and *M. truncatula.* Our study shows that *A. rhizogenes*-mediated RNAi silencing is a fast and efficient tool to knock down genes in the roots of *T. caerulescens. TcZNT1* and *TcZNT2* were silenced individually and also in combination in *T. caerulescens*. *TcZNT1* silenced *T. caerulescens* plants grown on 100 μM Zn showed a significantly lower Zn content than the empty vector transformed plants (Figure 5). This shows directly that *TcZNT1* plays a role in Zn uptake into *T. caerulescens* root cells. Still, Figure 5 shows there is uptake of Zn into the *T. caerulescens* cells, when the individual genes and even when both the genes are silenced. This suggests that *TcZNT1* is not the rate limiting step for Zn uptake in roots. *TcZNT2* silenced plants showed slightly lower Zn content, but not significant, than the empty vector transformed plants. This observation, together with the over-expression study suggests that *TcZNT2* probably has a very minor role or no role in Zn transport. The observation of reduced Zn content in the *TcZNT1* silenced *T. caerulescens* plants, together with the over-expression results establish *TcZNT1* as an impotant Zn transporter in *T. caerulescens.*

Acknowledgement

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Chapter 4

A comparative promoter study of *ZIP4*-like transporter genes of *Arabidopsis thaliana* and *Thlaspi caerulescens*

Sangita Talukdar, Ludmila Mlynárová, Mark GM Aarts

Abstract

- The main objective of this research was to do a comparative study of the Zn-deficiency-induced *ZIP4* transporter promoters from *Arabidopsis thaliana* and closely related species.
- The *ZIP4* promoters were isolated from *Arabidopsis thaliana, Arabidopsis lyrata, Arabidopsis halleri*, *Cochleria pyrenaica* and *Thlaspi caerulescens* and a sequence analysis was performed. The expression pattern of *AtZIP4* and *TcZNT1* was studied in *A. thaliana*, using promoter-GUS reporter constructs.
- Zn-deficient roots, shoots, flowers and seeds showed similar GUS staining in *AtZIP4pro-GUS* and *TcZNT1pro-GUS* transformed *A. thaliana* plants. Putative *cis* elements in both promoters were identified by 5' deletion analysis. For *AtZIP4* these were found between -263 bp and -115 bp from the start codon and for *TcZNT1* between -223 bp and -98 bp. The sequence analysis performed on the *ZIP4* promoters shows short conserved sequences in all five species, suggesting that these are essential promoter elements in both hyperaccumulator and non-accumulator species. The difference in expression between these species is apparently not due to differences in the *cis*-elements of the *ZIP4*-like gene promoters.

Introduction

Regulation of transcription is a major mechanism to control gene activity in complex biological systems. A key step in the regulation of gene expression is the sequence specific binding of transcription factors (TFs; the *trans* elements) to their DNA recognition sites (the *cis* elements or transcription factor binding sites; TFBS). The expression of genes can be tightly regulated, depending on the variation in *cis* element sequence, the number of *cis* elements and on the expression of transcription factors. Changes in spatial and temporal expression of the transcription factor genes will affect the expression patterns of down-stream target genes. On the other hand, differences in *cis* elements will alter the expression of orthologous genes in different species. It has been observed in several examples that the evolutionary differences in the *cis* or *trans* elements according to their needs lead to evolution of new species (de Folter and Angenent, 2006). This *cis-trans* interaction is an integral part of transcriptional regulatory networks and is fundamental for an understanding of all biological processes (reviewed in de Folter and Angenent, 2006; Takatsuji, 1998).

The *Arabidopsis thaliana* genome codes for at least 1533 transcriptional regulators, accounting for 5.9% of its estimated total number of genes, which is 1.3 times that of *Drosophila melanogaster* and 1.7 times that of *Caenorhabditis elegans* or *Saccharomyces cerevisiae* (Riechmann et al., 2000). Around 22% of the *A. thaliana* transcription factors are zinc (Zn)-coordinating proteins (Riechmann et al., 2000; Broadley et al., 2007). Therefore, Zn plays a central role in transcription. The first discovery of a Zn metalloprotein was made by Hanas et al. (1983), with the observation that transcription factor TFIIIA in *Xenopus* oocytes was a Zn protein. This protein is necessary for the transcription of the 5S

RNA gene by RNA polymerase III, was a Zn protein. Mutations in several genes encoding for Zn-finger proteins in plants, containing specific DNA binding domains, have been found to cause profound developmental aberrations or defective responses to environmental signals (Takatsuji, 1998).

For healthy development, plants need to supply Zn to all tissues. For this, Zn needs to be taken up from the soil and transported in the plant in a regulated way. The Zn transporters located in the plasma membrane and the organellar membranes are the central points for this regulation of Zn homeostasis. In yeast, Zn transporters are found to be regulated by Zn at both the transcriptional and translational level (Radisky and Kaplan, 1999). Transcriptional analysis showed that the genes encoding Zn transporters *ZRT1* and *ZRT2* in yeast were highly regulated by Zn with increased expression only under Zn-deficiency conditions. The *ZAP1* gene was identified as a Zn-regulated transcription factor (Zhao and Eide, 1997). When *ZAP1* was deleted, *ZRT1* and *ZRT2* were not expressed, resulting in severe Zn deficiency. Promoter analysis revealed that ZAP1 binds to specific zinc-responsive elements (ZREs) located within the promoter regions of each *ZRT1* and *ZRT2*. However, in general, limited or no information is available on the transcriptional regulation of Zn transporters in plants, nor of the *cis* regulatory elements that should be present in their promoters. In barley Fe-responsive *cis* elements IDE1 and IDE2 were identified in the promoter of a Fe-deficiency-inducible gene *IDS2,* by studying promoter deletions using promoter-GUS fusions (Kobayashi et al., 2003).

The *ZIP* (*ZRT* and *IRT*-like proteins) gene family in *A. thaliana* encodes an important group of metal transporters and shows high sequence similarity to *ZIP* genes in many other organisms, including yeast and humans. More than 100 *ZIP*

genes have been identified from bacteria, archeae and other eukaryotes, fifteen of them in *A. thaliana* (Mäser, 2001). In addition to Zn transport, ZIPs are also involved in the transport of Fe, Mn and Cd, thus differing in their substrate range and specificity (Guerinot, 2000; Mäser et al, 2001).

Although *ZIP* genes are now identified in many different plant species, only a few of them have been functionally characterized. They appear to play a crucial role in so-called 'heavy metal hyperaccumulator' plant species. Hyperaccumulator plants have developed the ability to tolerate and accumulate high metal contents in their shoots. *Thlaspi caerulescens* (Tc), as described by Assunção et al. (2003), is a model plant species to study metal hyperaccumulation and tolerance. This species belongs to the Brassicaceae family and is known as a Zn hyperaccumulator, showing up to 30,000 µg Zn g^{-1} foliar dw (Brown et al., 1995). A *ZIP* gene homologue, *TcZNT1* was identified from *T. caerulescens* (Pence et al., 2000; Assunção et al., 2001), which shares 90% DNA identity and 76% aa identity with the *AtZIP4* gene (Grotz et al., 1998). *TcZNT1* has been shown to mediate high-affinity Zn uptake and low-affinity Cd uptake following expression in yeast (Pence et al., 2000). Assunção et al. (2001) reported that *TcZNT1* was predominantly expressed in roots but also in shoots, and that its expression was barely Zn-deficiency responsive, in contrast to the *TaZNT1* gene in the non-hyperaccumulator *T. arvense* and the *AtZIP4* gene of *A. thaliana* (Grotz et al., 1998) which are strongly induced by Zn-deficiency.

An emerging picture is that many of the genes encoding ZIP proteins are induced by Zn deficiency in non-accumulators but highly and constitutively expressed in hyperaccumulators. This changed expressed might lead to increased metal transport which could be a component of the hyperaccumulation mechanism.

Since transcription levels are controlled both by *trans* and *cis* acting elements it is important to investigate what determines the difference in expression of metal transporters such as those of the ZIP family between related plant species.

Our objective was to study the regulation of *AtZIP4* and *TcZNT1*. We used the model plant *A. thaliana* as a system for studying the regulation of both promoters. The response of these two genes with respect to Zn exposure and the tissue specific expression were studied. The locations of essential *cis* elements of both the genes were identified, using promoter deletions combined with the GUS reporter gene.

Materials and Methods

Isolation of AtZIP4 and TcZNT1 promoters by polymerase chain reaction (PCR)

Genomic DNA was extracted from flowers of *A. thaliana* (accession Columbia) and *T. caerulescens* (accession La Calamine), as described by Aarts et al. (2000). To amplify the sequence containing the *AtZIP4* promoter, a PCR reaction was performed on genomic DNA of *A. thaliana* using the primers P5 and P15 (Table 1). To amplify the sequence containing the *TcZNT1* promoter, two forward primers (P1 and P2; Table 1) were designed on the gene upstream of *AtZIP4* (At1g10970) in *A. thaliana*. This gene, At1g10980, is predicted to encode a "membrane protein *PTM1* precursor isolog" by TAIR (www.arabidopsis.org). The reverse primer (P3; Table 1) was designed on the cDNA of *TcZNT1* (GenBank acc. No. AF275751) of *T. caerulescens* (Figure 1). Each fragment obtained by PCR amplifications was cloned into the pGEMT-easy vector (Promega, Leiden, The Netherlands) and plasmids from several colonies for each fragment were sequenced to identify the sequence containing the *TcZNT1* promoter.

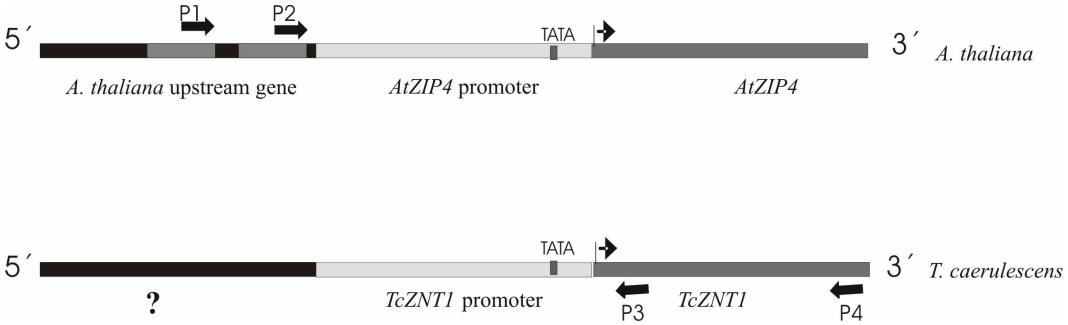

Figure 1: Scheme illustrating the isolation of the *TcZNT1* promoter by PCR.
The rectangular bars represent the *A. thaliana* and *T. caerulescens* genomic fragment, with the *AtZIP4* and *TcZNT1* genes on the right side of the bar. The upstream gene, At1g10980, is shown on the left side of the *A. thaliana* genome bar. The upstream gene of *TcZNT1* is not known, and thus indicated with '?'. In between *AtZIP4* or *TcZNT1* and the upstream gene is the predicted promoter of the corresponding genes, indicating the putative TATA box with a grey box and the translational start site with the short right arrow downstream of the TATA box. Primers are indicated with arrows: two arrows on the left side of the genome bar represent the forward primers (P1 and P2), designed on the transcribed region of At1g10980 and the arrow on the right side of the genome bar represent the reverse primer (P3), designed on the *TcZNT1* cDNA, used for isolation of the *TcZNT1* promoter. P4 is the primer designed at the 3' end of the *TcZNT1* cDNA.

Isolation of the promoters of AtZIP4-like genes from Cochleria pyrenaica, Arabidopsis halleri and *Arabidopsis lyrata*

In order to isolate the *ZIP4* promoters from three different species, *Cochleria pyrenaica* (accession La Calamine, Belgium), *Arabidopsis halleri* (accession Auby, France) and *Arabidopsis lyrata* (accession Unhost, Central Bohemia, Czech Republic; Macnair et al., 1999), DNA of which was kindly provided by Dr. Pierre Saumitou Laprade, the same primers as for amplification of the *TcZNT1* promoter were used for PCR amplification on genomic DNA of these plants. Each fragment obtained by the PCR amplifications was cloned into the pGEM-T-Easy vector (Promega, Leiden, The Netherlands) and plasmids from several colonies for each fragment were sequenced to determine the sequence containing the ZIP4-like promoter.

Construction of AtZIP4pro- and TcZNT1pro- GUS plasmids and plant transformation

To make the *AtZIP4pro-GUS* construct, a 1048 bp fragment obtained by PCR, using genomic DNA of *A. thaliana* and primers P5 and P15 (Table 1), was digested with HindIII and NcoI and cloned at the 5' end of the *uidA* (GUS) gene in the HindIII-NcoI digested pCAMBIA1301 vector (http://www.cambia.org /daisy/bios/585.html) (Ge et al., 2004). This vector contains the hygromycin B-resistance gene for selection in plants. The CaMV 35S promoter originally located upstream of the *uidA* gene was removed from the vector upon digestion with HindIII and NcoI. This *AtZIP4pro-GUS* construct was named F05 and the cloned fragment was verified with DNA sequencing.

For construction of *TcZNT1pro-GUS* the *TcZNT1* promoter cloned into pGEM-T-Easy (www.promega.com) was reamplified with primers P16 and P26 (Table 1), from the cloned ~900 bp fragment into pGEM-T-Easy, containing the *TcZNT1* promoter sequence. This fragment was used to make the *TcZNT1pro::GUS* reporter construct following the same steps as for F05 and named F16.

Ten forward primers based on the promoter sequences of *AtZIP4* and *TcZNT1* (P5 through P14 for *AtZIP4* and P16 through P25 for *TcZNT1*) containing the HindIII and NcoI sites, were designed in order to generate 5' deletions of the promoters (Table 1). In total, 22 constructs, 11 from *AtZIP4* and 11 from *TcZNT1,* were obtained following the same method, as described above for F05 and F16. Each construct was named after the forward primer. Two more constructs were made by digestion of the F05 and F16 vectors with SalI and subsequent self-ligation. These constructs were named F26 in case of *TcZNT1* and F15 for the construct

with the *AtZIP4* promoter. All these constructs were transformed into *A. thaliana*, accession Columbia, by the flower dipping method (Clough and Bent, 1998).

Plant growth conditions

T_1 transformants for all the constructs were selected on plates with half MS and 1% agar medium (Murashige and Skoog, 1962) containing 20 mg l^{-1} hygromycin. In general six independent transformants were selected for each construct, and all of them were propagated until homozygous T_3 lines. For qualitative GUS analysis, all homozygous lines were grown hydroponically on modified half strength Hoagland's nutrient solution (Schat et al., 1996), which supplied sufficient Zn to plant (2 µM $ZnSO_4$) and on Zn-deficiency medium (where Zn was not added). Three of these lines for each construct were selected for quantitative GUS assay, based on the GUS expression results obtained with the qualitative GUS assay. These three lines (six seedlings per line) were grown on vertical half MS and 1% agar plates for two weeks and transferred to normal and Zn deficient modified half Hoagland's nutrient solution (2 µM and 0 µM $ZnSO_4$). Roots were collected for quantitative GUS assay every week. The same was done for all other transformed *A. thaliana* plants with F05 through F26 constructs, except that roots were collected for quantitative GUS assay after three weeks only.

Table 1: Primers used for the PCR amplification

Restriction sites incorporated in the primers are underlined.

Primer	Sequence of oligonucleotides (5'-3')	Purpose
P1	5'-ATCGGCGATGATCATGGGAA-3'	Forward primer for *TcZNT1* promoter isolation; designed on At1g10980 gene
P2	5'-CCTCTTTTGGCCTCCATCGGAA-3'	Forward primer for *TcZNT1* promoter isolation; designed on At1g10980 gene
P3	5'-TTATAAGATCAATCAATAATAACA-3'	Reverse primer for *TcZNT1* promoter isolation; designed on cDNA of *TcZNT1*
P4	5'- TAAAGTCGACGCCCAAATGGCGAGTGC -3'	Reverse primer on *TcZNT1* cDNA
P5	5'-GTAAGCTTTTGGAAAGTGAAGTGGATTG-3'	Forward primer for *AtZIP4* promoter isolation
P6	5'-CCAAGCTTAGATCTTGTCTGTTTTGTACTAACATGT-3'	Forward primer on *AtZIP4*
P7	5'-ATAAGCTTTCCACTGCAGAAACCGGTA-3'	Forward primer on *AtZIP4*
P8	5'-TGAAGCTTCCCATCTTACAAAGTTACCG TCCT-3'	Forward primer on *AtZIP4* promoter
P9	5'-TTAAGCTTCTTAAGCTACTCCTAATCATCCTTTTA-3'	Forward primer on *AtZIP4* promoter
P10	5'-TTAAGCTTTAGACTTGACTTAATCGGATTTTCT-3'	Forward primer on *AtZIP4* promoter
P11	5'-TGAAGCTTTTTGGAACAAATTGATTTTCTGTTT-3'	Forward primer on *AtZIP4* promoter
P12	5'-GAAAGCTTAATAACGCGAAAATGTCGACAT-3'	Forward primer on *AtZIP4* promoter
P13	5'-TTAAGCTTAGTATAGACAAGATTGGGAAGCTCT-3'	Forward primer on *AtZIP4* promoter
P14	5'-AGAAGCTTTCACTCTTTCTCCAAGTTGCCTCCT-3'	Forward primer on *AtZIP4* promoter
P15	5'-ATCGACGAAGACCATGGGAACAAGAGT-3'	Reverse primer for *AtZIP4* promoter isolation
P16	5'-ATATCAAGCTTTCTGACTCTTTATCTGGCCTTTTA-3'	Forward primer on *TcZNT1* promoter
P17	5'-TTAGAAGCTTAATACCTGATCTTGTCTG-3'	Forward primer on *TcZNT1* promoter
P18	5'-GGAAGCTTTACGTAGCTGAAATGGAGGATGA-3'	Forward primer on *TcZNT1* promoter
P19	5'-GGGAAGCTTGAAACAATCCAATCCTTAACC-3'	Forward primer on *TcZNT1* promoter
P20	5'-TTAAGCTTCCGGTTTAGTGTGTTGAAGTTGTTAA-3'	Forward primer on *TcZNT1* promoter
P21	5'-TTAAGCTTTCGTTTTTTTGTATTTCATGAACAA-3'	Forward primer on *TcZNT1* promoter
P22	5'-AGAAGCTTCCATCATTACAATATTTTACTTGTCAAC-3'	Forward primer on *TcZNT1* promoter
P23	5'-TTAAGCTTAAAAGGTGAAAAGAGAGAATAACG-3'	Forward primer on *TcZNT1* promoter
P24	5'-AAATAAGCTTGTGTACAAGTGCCACGGAGC-3'	Forward primer on *TcZNT1* promoter
P25	5'-ACAAGCTTTCGCTCGTCGATTCCTTCTTTTT-3'	Forward primer on *TcZNT1* promoter
P26	5'-ATCGGCGATGACCATGGGAACAAAGA-3'	Reverse primer on *TcZNT1* promoter

GUS assay

(a) Qualitative GUS assay

For GUS staining the tissues were fixed with ice-cold acetone for an hour and rinsed three times with 0.1 M phosphate buffer to wash away the acetone. The staining was performed using 5-bromo-4-chloro-3-indolyl–β-D-glucuronide (Duchefa Biochemie B.V., Haarlem, The Netherlands) as a substrate (Jefferson et al., 1987) for maximum one hour at 37ºC in 0.1M phosphate buffer each time. A Nikon Diaphot DIC microscope was used to visualise the GUS staining and a Sony 3 CCD video camera attached to it was used to take images.

(b) Quantitative GUS assay

Kinetic GUS assays were performed as described by Nap et al. (1992)

Results

Isolation of the ZNT1 promoter from T. caerulescens

Two fragments of 600 bp and 900 bp respectively were obtained from the PCR amplifications using primers P1-P3 and P2-P3, on genomic DNA of *T. caerulescens.* The sequences from different colonies were found to be the same for each fragment. The sequences from both of the cloned fragments (~600 and ~900 bp) were overlapping at their 3' end. To verify the sequence as the *TcZNT1* promoter fragment, a PCR fragment was obtained with primers P2 and P4 (designed at the 3' end of the *TcZNT1* cDNA) and the fragment was sequenced. The sequence obtained for the fragment amplified with the primers P2 and P3, overlapped with the 5' end of the fragment amplified with the primers P2 and P4, suggesting the sequence obtained contains the promoter of *TcZNT1*.

Comparison of AtZIP4pro- and TcZNT1pro-GUS expression in root and shoot tissues

To study the response of *AtZIP4* to metal deficiency, the *AtZIP4pro-GUS* transformed *A. thaliana* plants were grown under deficiency of Zn, Fe, Cu, Cd or Mn. The qualitative GUS assay performed on these plants grown on hydroponics did not show any staining at Zn-sufficiency (2 μM Zn) whereas under Zn-deficiency (0 μM Zn) GUS staining was observed (Figure 3). A similar experiment performed with *TcZNT1pro-GUS* transformed *A. thaliana* plants showed expression only under Zn-deficiency (0 μM Zn).

The roots of Zn-deficient *AtZIP4pro-GUS* transgenic plants showed strong staining both in tap roots and lateral roots (Figure 3A), specifically in the endodermis (Figure 3B). A similar expression pattern was observed in *TcZNT1pro-GUS* transformed *A. thaliana* plants in Zn-deficient roots (Figure 4A, 4B, 4C). For both of the transgenic lines of *AtZIP4pro-GUS* and *TcZNT1pro-GUS*, intense staining was observed at the root tip, in the region of maturation of the root, in the rest of the root (Figure 3C and 4C). No staining was observed in the elongation zone of the root. All these observations were similar for all six independent transformants for both constructs. When examining later stages of development, GUS expression was also observed in Zn-deficient leaves, with most intense staining at the leaf edges (Figure 3D) and in the trichomes (Figure 3E). Similar expression patterns in the Zn-deficient leaves were also observed in *TcZNT1pro-GUS* transformed plants (Figure 4D).

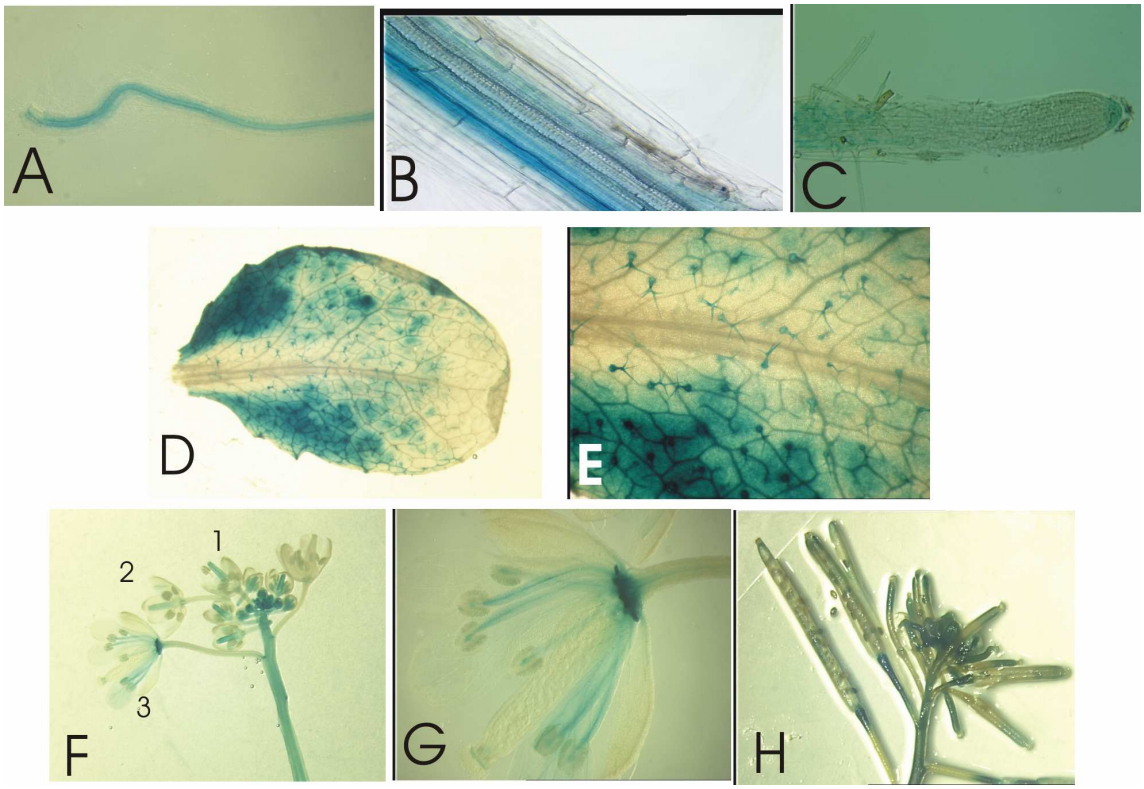

Figure 3: *AtZIP4pro-GUS* expression in Zn deficient *A. thaliana* plant tissues. *GUS* staining in (A) a detached lateral root; (B) close-up of (A) showing endodermis staining; (C) root tip and root hair zone; (D) leaf; (E) close up of (D) showing trichomes; (F) young inflorescence (G) close up of (F) showing flower organs ; (H) siliques.

AtZIP4pro-GUS and TcZNT1pro-GUS expression in flowers and siliques

To investigate the role of *AtZIP4* during the development of the inflorescence in *A. thaliana*, GUS staining was performed on flowers of *AtZIP4pro-GUS* transformed *A. thaliana* plants grown under Zn-deficient (-Zn) and Zn-sufficient conditions (+Zn). The expression pattern of *TcZNT1pro-GUS* in *A. thaliana* transformed plants was compared to *AtZIP4-GUS* staining. GUS staining was observed only in the Zn-deficient flowers, whereas the Zn-sufficient tissues did not give any staining, neither in *TcZNT1pro-GUS* nor in *AtZIP4pro-GUS* transformants. The highest staining intensity was observed in the young buds (Figure 3F). Strong staining was also observed in the pistils of older buds, in all

developmental stages, until just before flower opening. In the open flower (Figure 3G), the flower base is stained intensely in addition to the anther filaments. After fertilization, the siliques are typically stained at the distal ends; most prominently in young siliques (Figure 3H). Also the pedicles are stained. In case of *TcZNT1*, the same expression pattern was observed in the Zn-deficient *A. thaliana* transgenic plants in all six independent transformants (Figure 4E, F).

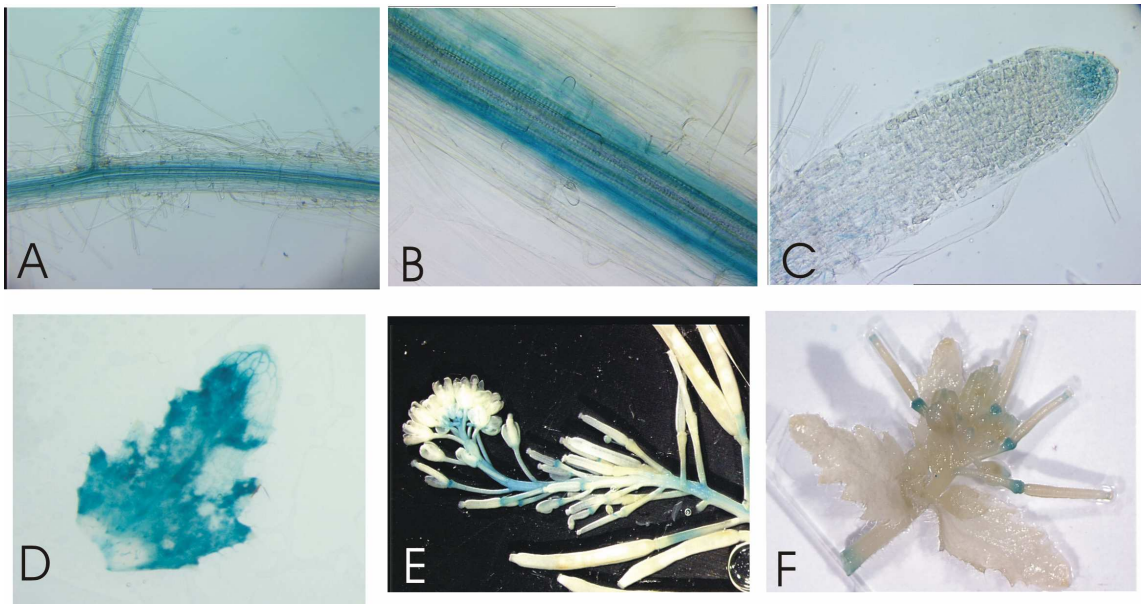

Figure 4: *TcZNT1pro::GUS* expression in Zn deficient *A. thaliana* plant tissues.
GUS staining in (A) a detached lateral root (B) close-up of (A) showing endodermis and inner cortex staining; (C) root tip and root hair zone; (D) leaf (E) flowers (F) siliques.

Quantitative comparison of AtZIP4pro::GUS and TcZNT1pro::GUS in A. thaliana shows highest expression in Zn-deficient roots

To investigate the response of the *AtZIP4* and *TcZNT1* promoters to Zn in *A. thaliana* in a quantitative way, the *AtZIP4pro-GUS* and *TcZNT1pro-GUS* transgenic plants were grown for eight weeks in half Hoagland's solution containing a concentration series of Zn (0, 0.1, 0.2, 0.5, 1, 2 µM) and the GUS activity was measured quantitatively every week (wk) starting from the first week

of exposure (Figure 5). The plants were transferred to the different Zn media after growing the plants on half MS/agar plates for two weeks. Both in *AtZIP4pro-GUS* and *TcZNT1pro-GUS* lines, the GUS activity in roots were induced by Zn deficiency. The GUS activity was found to increase gradually in wk1 and wk2 after transfer to 0 µM Zn (Figure 5A). The highest values were observed in the wk3, and from wk4 onwards the expression was gradually decreasing. The GUS activity at wk3 was increased by 31-fold compared to plants grown on 2 µM Zn. The plants grown on 0.1 µM Zn showed some GUS activity, although to a lesser extent than on 0 µM Zn. In plants grown on 0.2, 0.5, 1 and 2 µM Zn hardly any GUS activity could be detected. Comparable expression was observed for *TcZNT1pro-GUS* transgenic plants (Figure 5B).

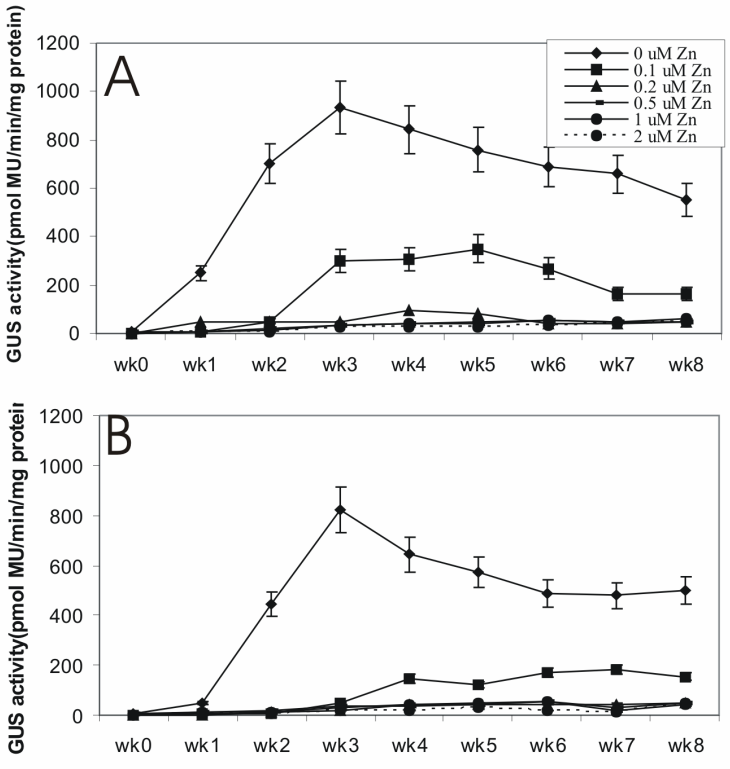

Figure 5: Quantitative comparison of GUS expression in **(A)** *AtZIP4pro-GUS* and **(B)** *TcZNT1pro-GUS* in *A. thaliana* roots. A time-course GUS assay was performed at weekly intervals on the transgenic plants grown on half Hoagland's solution containing 0, 0.1, 0.2, 0.5, 1, 2 µM Zn.

5' deletion analysis in A. thaliana roots under Zn-deficient and Zn-sufficient conditions

A 5' deletion analysis of the *AtZIP4* and *TcZNT1* promoters was performed to identify the *cis* elements in these two promoters. Truncated promoters were fused to GUS and compared to the complete *AtZIP4pro-GUS* construct results. The GUS activity was reduced significantly by ~200 fold in fragment F14 compared to the values obtained from F5 through F12 (Figure 6). This indicates that there are at least one or more *cis* elements in the region of -263 bp to -115 bp from the start codon of *AtZIP4*. GUS activity is reduced by around ~25-fold from F12 to F13 and ~9-fold from F13 to F14. This suggests that there is an essential *cis* element located between -263 bp and -232 bp. Sequence analysis of the two promoters *AtZIP4* and *TcZNT1* shows two conserved palindromic sequences. The first palindrome is 5'-ATGTCGACAT-3' which is a true palindromic sequence and the second sequence is 5'-ATGTCGACAC-3' which is an incomplete palindrome because of the last base (a C instead of T). Interestingly, the proposed first palindrome is located in the region between -263 bp and -232 bp. However, transcription is not completely inhibited upon removal of this palindrome as indicated by the residual GUS activity in the F13 transgenic plants. This suggests that there is another *cis* element between -232 bp and -115 bp. This region contains the second conserved palindromic sequence in the promoter. Fragment F15 gives a GUS activity similar to the control. This fragment contains only part of the second palindromic sequence as it lacks the first three bases of it. GUS activity for this fragment is diminished, like for F14, suggesting the complete palindrome is required for transcription. This strongly suggests that the conserved palindromic sequences in the *AtZIP4* promoter are essential *cis* elements for

transcription of *AtZIP4*. Similarly, the 5' deletions of the *TcZNT1* promoter show that the GUS activity is reduced ~16-fold when comparing the fragment F23 of 220 bp to fragment F24 of 166 bp. GUS activity is further reduced by two fold when comparing fragment F24 of 166 bp to fragment F25 of 98 bp. Fragments F26 of 103 bp (including only part of the second palindrome) and F25 of 98 bp (in which the second palindrome is present except for the first three bases) give the same GUS activity as the empty vector transformed control values. In summary, the 5' deletion analysis suggests that essential *cis* element(s) are localised in between -263 bp and -115 bp from the start codon for *AtZIP4*; between -223 bp and -105 bp for *TcZNT1*.

Isolation of the promoters of AtZIP4-like genes from Cochleria pyrenaica, Arabidopsis halleri* and *Arabidopsis lyrata

In order to isolate the *ZIP4* promoters from three different species, *Cochleria pyrenaica, A. halleri* and *A. lyrata,* the same primers used for amplification of the *TcZNT1* promoter were used for PCR amplification on the genomic DNA of these species. All these PCR amplifications gave single bands of different sizes from around 600 to 1200 bp, except for *A. halleri*. For *A. halleri,* two bands were obtained by PCR amplification for each primer pairs: between (a) between 750 and 1000 bp (primer pair P1 and P3) (b) 500 and 750 bp (primer pairs P2 and P3). The PCR amplification from *C. pyrenaica* resulted in a single band of around 600 bp only for the P1-P3 combination. The PCR amplification for *A. lyrata* resulted in single bands of around 1200 bp (primer pair P1 and P3) and 700 bp (primer pairs P2 and P3). All these fragments were cloned into the pGEM-T-Easy vector and sequenced. The sequences of these fragments from each species were

overlapping and the larger fragments (except for *C. pyrenaica* of which only one fragment was cloned) were used for further analysis. The lengths of these DNA sequences were 571 bp for *C. pyrenaica,* 905 bp and 746 bp for the two fragments of *A. halleri* and 1189 bp for *A. lyrata*.

Sequence comparison among the ZIP4 promoters from Arabidopsis thaliana, Arabidopsis lyrata, Arabidopsis halleri, Cochleria pyrenaica* and *Thlaspi caerulescens

The sequence comparison shows small segments of sequence similarity among the promoter fragments, of different species. The short *A. halleri* has a deletion of 154 bp compared to the long fragement, between -538 and -384 bp; the rest of the sequence is nearly identical, except few bases of mismatches and few single nucleotide polymorphisms, suggesting that these two fragments represent two different alleles in this self-incompatible and generally heterozygous species. Interestingly, the two palindromic sequences (5'-ATGTCGACAT-3' and 5'-ATGTCGACAC-3') are conserved in the *ZIP4* promoters from all five species (Figure 7). The location of the palindromes in *ZIP4* each species and the predicted TATA boxes are summarised in Table 2.

Table 2: Summary of the ZIP4 promoters isolated

Species name	**Length of the promoter fragment isolated (bp)**	**First palindrome (bp) [5'-ATGTCGACAT-3']**	**Second palindrome (bp) [5'-ATGTCGACAC3']**	**Predicted TATA box (bp)**
A. thaliana	1048	-246 to -236	-118 to -108	-59 to -55
T. caerulescens	902	-189 to -179	-107 to -97	-70 to -66
C. pyrenaica	571	-235 to -225	-116 to -106	-59 to -55
A. halleri(long)	905	-221 to -211	-115 to -105	-58 to -54
A. halleri(short)	746	-221 to -211	-115 to -105	-58 to -54
A. lyrata	1189	-235 to -225	-116 to -106	-59 to -55

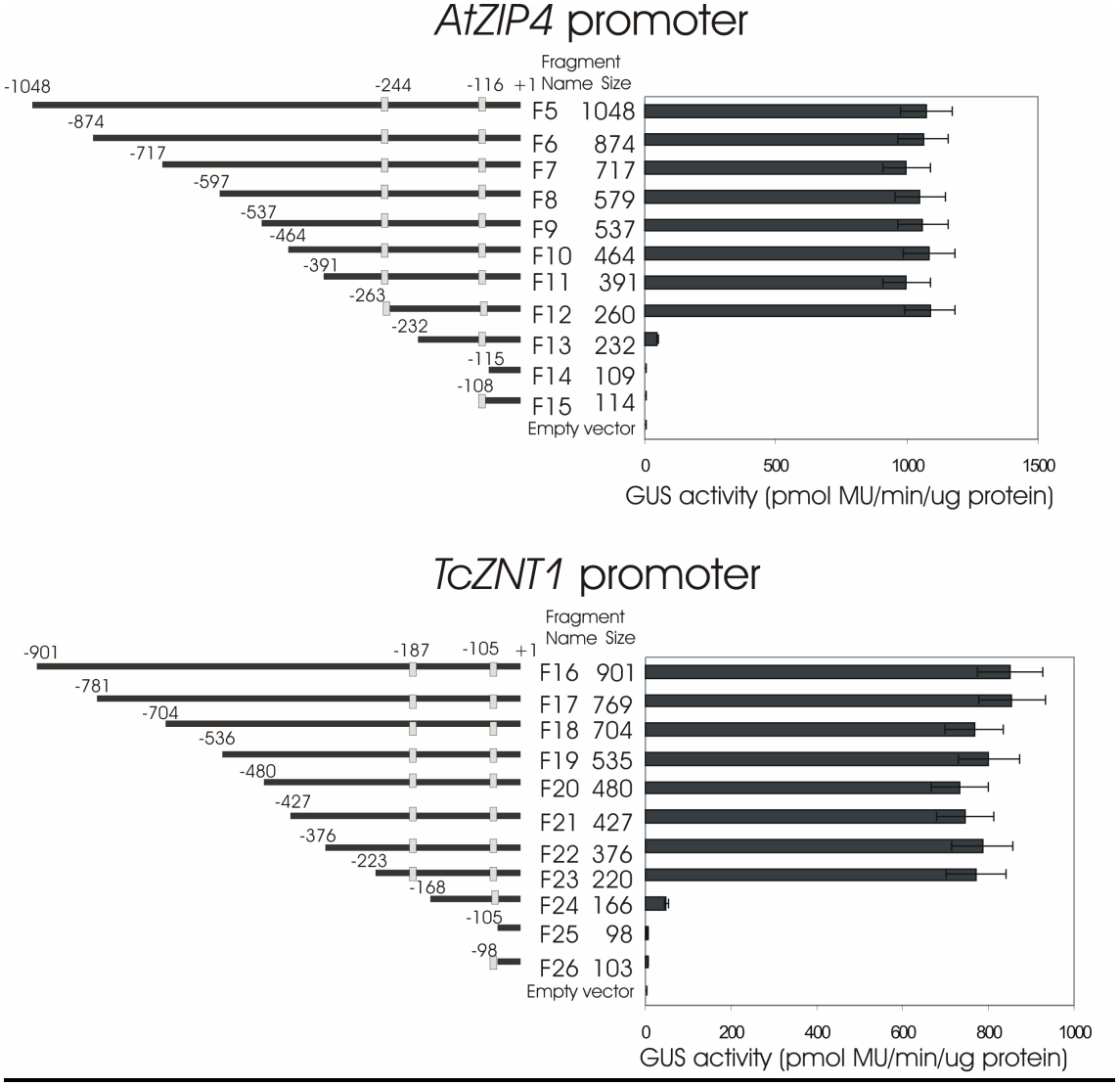

Figure 6: The effect of 5' deletions of the *AtZIP4* and *TcZNT1* promoters on GUS expression in transgenic *A. thaliana* plants. The GUS activity (pmole/min/µg protein) was tested in roots of transgenic plants exposed to 0 µM Zn.

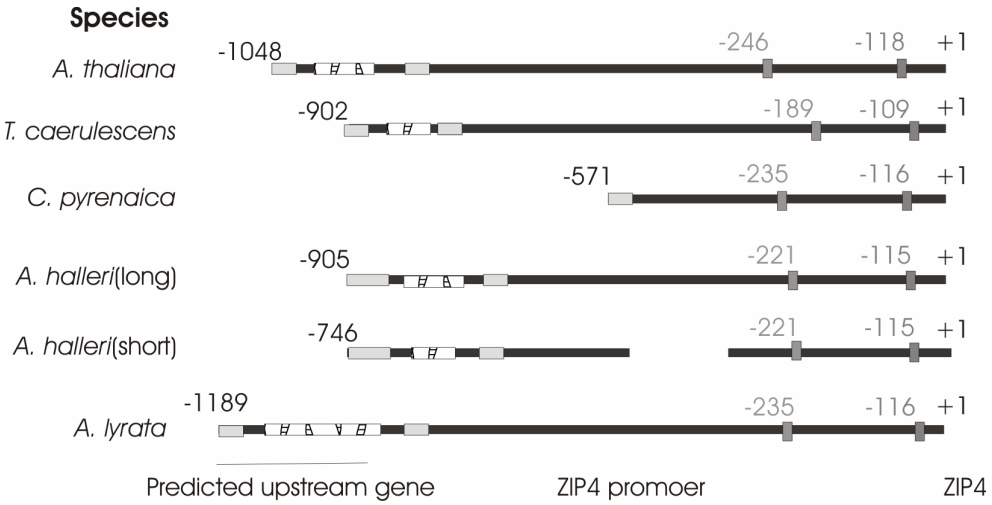

Figure 7: Schematic representation of the *ZIP4-like* promoters of *Arabidopsis thaliana, Arabidopsis lyrata, Arabidopsis halleri, Cochleria pyrenaica* and *Thlaspi caerulescens.* The black diagram represents the promoter sequence for each species. Two palindromic sequences (dark grey boxes) (5'-ATGTCGACAT-3' and 5'-ATGTCGACAC-3') at the 3' end of the sequences were found to be conserved in the promoters from all five species. On the 5' of the bar the conserved parts of the predicted upstream genes are shown with light grey and patterned boxes in five species.

Discussion

We cloned the promoters of the orthologous Zn transporters *AtZIP4* from *A. thaliana* and *TcZNT1* from *T. caerulescens.* Although both genes probably perform very similar functions in metal uptake on a cellular level (Pence et al., 2000; Assunção et al., 2001; Chapter 3 of this thesis), their expression is very different (van de Mortel et al., 2006). We analysed the expression of both genes in response to Zn-deficiency exposure, using *A. thaliana* plants stably transformed with promoter-GUS fusion constructs. Stable transformation was chosen over transient assay systems for characterising the promoter activities, including tissue-specific expression patterns and the response to the nutrient deficiencies. These analyses showed that the *AtZIP4* and *TcZNT1* promoters are the first plant

promoters to be fully responsive to Zn deficiency when tested in *A. thaliana*. The promoters are very specific to Zn deficiency and are not responsive to Fe, Mn or Cu-deficiencies. Previous microarray analysis already indicated that the *AtZIP4* promoter is strongly responsive to Zn-deficiency, with very little expression in roots under normal Zn supply conditions compared to Zn deficient conditions (van de Mortel et al. 2006), all in accordance with the first studies on this Zn transporter by Grotz et al. (1998).

Strong *AtZIP4pro-GUS* expression was found in the roots of the transgenic *A. thaliana* plants. However, with the exception of the region at the tip of the roots, where root hairs start to emerge, the GUS expression was restricted to the endodermis of the roots and clearly not in the epidermis of the root. Vert et al., 2002 showed that the *AtIRT1* gene is involved in Fe-uptake from the soil and found to be localised in the epidermis of *A. thaliana* roots. Our observations suggest that *AtZIP4* is probably not directly involved in Zn uptake from the soil, but more in transporting Zn from the root cortex to the stele, where it can be loaded into the xylem and transported to other parts of the plant. The expression is seen in the whole root system and not limited to the tip. Although *A. thaliana* roots are often not lignified, the expression in endodermis at older parts of the root where mineral uptake is less, it is beneficial to prevent Zn from leaking out of the stele and to ensure the full Zn supply for above ground tissues. The expression exclusively under Zn deficiency also indicates that *ZIP4* is not the major Zn transporter gene in *A. thaliana*. The previously identified *ZIP1, ZIP2* and *ZIP3* genes are more likely candidates for general Zn uptake by the plant. Although their expression is also enhanced by Zn deficiency, they all have a detectable basal level of expression under sufficient Zn supply (Grotz et al., 1998; Wintz et

al., 2003; van de Mortel et al., 2006). It is not known when the genes are expressed in the root.

AtZIP4pro-GUS staining was also observed in Zn-deficient leaves, mostly on the edges of the leaf, but only in parts that did not yet show the chlorosis that is typical for Zn deficiency. Remarkable was the intense GUS staining in the leaf trichomes. Several studies have shown trichomes to be a storage site for Zn in plants when in excess. In Zn hyperaccumulators trichomes contain higher amounts of Zn (Sarret et al., 2006; Sarret et al., 2002; Broadhurst et al., 2004; Küpper et al., 2001; Küpper et al., 2000). Therefore, we assume that trichomes are a strong sink for Zn, indicating higher Zn supply which results in stronger GUS expression.

GUS staining was observed also in flowers in Zn-deficient transgenic *A. thaliana* plants (Figure 3F). Interestingly, the GUS localisation in the flowers depends very much on the developmental stage, with the young buds having the highest staining, probably because they need more Zn than the older stages. Typical is also the expression of *AtZIP4* in the pistils of older flower buds, which is replaced by expression in the anther filaments upon opening of the buds, indicating non-overlapping requirements for Zn in subsequent developmental stages. Zn ions are the key structural or catalytic components in many important enzymes like Cu-Zn superoxide dismutase, alcohol dehydrogenase and many DNA-binding proteins like RNA polymerase and Zn-finger transcription factors, which are essential for the development of plants (Marschner, 1995; Guerinot and Eide, 1999), hence the higher expression, specially at the early stages. The tip of the pedicel just below the developing slilique is also intensely stained, whereas the rest of the pedicel is not stained. This suggests that the Zn supply to the flowers might be provided via

AtZIP4. Staining was also found in the seeds, suggesting a role for *AtZIP4* in Zn storage in the seeds, which is essential for seed germination. Therefore, the *AtZIP4* gene apperars to function in almost every tissue, from roots till seeds, when the plant is Zn-limited.

In addition to the localisation study, the quantitative GUS expression every week from wk0 through wk8 of exposure to Zn media shows, the induction and down-regulation of *AtZIP4* and *TcZNT1* in *A. thaliana* plants (Figure 5). The GUS activity was found to rapidly increase in wk1 and wk2 after exposure to 0 µM Zn (Figure 5A). The highest values were observed in wk3, and from wk4 onwards the expression was gradually decreasing on 0 µM Zn. Plant cells encounter a changing environment in which nutrient concentrations fluctuate. Cell survival requires physiological responses to such changes. The sensory systems detect changes in nutrient levels and the resulting signals are transduced into regulation of gene expression, hence this differentiation in the expression pattern in *AtZIP4pro-GUS.* As the highest requirements for the microelements are in the transition of developmental stages, the highest expression of *AtZIP4* and *TcZNT1* in the second and third week of the exposure might coincide with the phase that the plant actively develops its reproductive tissues.

A similar expression pattern of *TcZNT1* was observed in *TcZNT1pro-GUS* transformed *A. thaliana* plants (Figure 3 and 4). This means that the *trans* elements required for *AtZIP4* expression in *A. thaliana* do not distinguish between the *cis* elements in the *TcZNT1* promoter. This also implies that both the *TcZNT1* and *AtZIP4* promoter may have the same *cis* elements. To check this hypothesis further and in order to search for the specific *cis* element(s) in *AtZIP4* and *TcZNT1*, the 5' deletion analysis was performed for both of those promoters.

The sequence comparison of the promoters of *AtZIP4* and *TcZNT1* shows there are only two short stretches of similar DNA sequences, which contain two conserved palindromic sequences. Interestingly, these two palindromic sequences were found to be conserved in the *ZIP4* promoters isolated from the five related Brassicaceae species. This suggests that these two palindromic sequences might be essential *cis* elements for the ZIP4 gene in these five species. Deletion of the first palindrome in the fragment at -263 bp from the start site gives a 25 times reduced GUS value, suggesting essential *cis* elements to be present in this region. Still the transcription of *AtZIP4* was not completely diminished. Deletion of the second palindrome reduces the GUS activity nine times further. When both the palindromic sequences were deleted, in fragment F14 of 109 bp, the GUS activity was reduced to that of the empty vector transformed control plants, diminishing the complete transcription of *AtZIP4*. This suggests that the *cis* element(s) are localised in between -263 bp and -115 bp from the start codon. These data suggest that the major *cis* element is probably the first palindrome, which is a true palindromic sequence. In case transcription utilizing the first palindrome fails, the second palindrome is still utilized for rescuing the Zn-deficient plant for survival. The similar 5' deletion analysis of *TcZNT1* promoter shows that essential *cis* element(s) are located between -223 bp and -105 bp in agreement with the hypothesis that the palindromic sequences are important essential elements in the *ZIP4* promoters explaining why they are conserved in all five species tested. Such Zn-responsive elements (ZRE) have not been identified before in plants, whereas few other *cis* elements have been identified in few other micronutrient–responsive promoters (Kobyashi et al., 2003). Hence our data indicate that these ZREs are novel *cis*-acting elements that are needed for proper response to Zn-deficiency in

A. thaliana. The similar expression pattern of *TcZNT1* in *A. thaliana* suggests that the *trans* elements in this non-accumulator plant do recognise the *cis* elements in the *TcZNT1* promoter from the hyperaccumulator. This implies that both the *cis* elements in the *AtZIP4* and *TcZNT1* promoters are conserved, but either the mode of *cis*/*trans* interaction or the expression profile of the *trans* genes has changed in *T. caerulescens*. Most likely, the expression of the transcription factors in the hyperaccumulator plants have changed, as an adaptation to the highly contaminated soil as a new niche. This might have led to the constitutive over-expression of the gene *TcZNT1* and subsequent higher uptake or sequestration of the metals in the plant.

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Chapter 5

Over-expression of the Cation Diffusion Facilitator gene *ZTP1* of *Thlaspi caerulescens* confers Zn and Cd tolerance and accumulation to *Arabidopsis thaliana*

Sangita Talukdar and Mark GM Aarts

Abstract

- The functional characterization of the Cation Diffusion Facilitator family gene *ZTP1* from *T. caerulescens* (Tc) accession La Calamine is presented.
- The response of the over-expression lines of *TcZTP1* in *Arabidopsis thaliana* were investigated with respect to the accumulation of Zn, Fe and Cd in shoots and roots.
- Over-expression of the *TcZTP1* of *T. caerulescens* confers Zn and Cd tolerance and accumulation in *A. thaliana.* The over-expression lines also show tolerance to low Fe.

Introduction

Zinc (Zn) is an essential micronutrient element for plants, though excess of it has toxic effects. The optimal Zn concentration in cells is controlled in a process called Zn homeostasis, by which plants balance Zn uptake, intracellular compartmentalisation and partitioning to the various organs (Clemens et al., 2002). This is because free Zn in the cytoplasm is toxic and in eukaryotic cells labile Zn levels are estimated to be in the low nanomolar range under steady state conditions (Sensi et al. 1997). Toxicity due to high Zn in the cell can be caused via binding of the cations to inappropriate sites in proteins or co-factors (Eide, 2003). For example, excess Zn can interfere with mitochondrial aconitase activity and thereby impair respiration (Costello et al., 1997). Zn toxicity in plants is characterised by chlorosis in young leaves, probably via competition with Fe and Mg resulting in growth reduction (Marschner, 1995). Therefore, the essential but potentially toxic nature of Zn necessitates precise homeostatic control mechanisms.

Uptake and excretion of Zn into or out of the plant cell is controlled by transmembrane metal transporters, located at the plasma membrane. Still, plasma membrane controlled Zn regulation alone is not sufficient to control the Zn concentration in the cells. The vacuole, which occupies most of the plant cell volume, plays a major role in the regulation of ion homeostasis within the cell and in detoxification of metals in the cytosol (Marschner, 1995). In plant species, several genes have been identified, which are involved in the sequestration of metals in the cell, mostly by metal export into the vacuoles. The CDF family (also known as cation efflux family or metal transport/tolerance protein (MTP) family (Hall and Williams, 2003) is described to confer Zn, Mn, Cd, Co or Ni tolerance

to plants by sequestering the metals in the vacuole (for review see Colangelo and Guerinot, 2006). Several evidences suggest that these transporters sequester metal ions within the vacuoles (Paulsen and Saier, 1997). The mechanisms of transport and the driving force that allows the metal ions to be transported are not well understood for most of the CDF proteins. CDF proteins from diverse sources have the following features in common: (1) they share an N-terminal signature sequence that is specific to the family; (2) the proteins possess six trans-membrane-spanning regions; (3) they share a cation efflux domain; and (4) most of the eukaryotic members possess an intracellular histidine-rich domain that is absent in prokaryotic members (Paulsen and Saier, 1997). CDF family members have been studied from *Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus subtilis* and *Schizosaccharomyces pombe* and were found to contribute to the storage and efflux of Zn and other metals in the cell (Li and Kaplan, 1998; MacDiarmid et al 2002; Miyabe et al 2001; Guffanti et al, 2002; Chao et al, 2004; Li and Kaplan, 2001; Clemens et al, 2002).

The genome of the model plant *Arabidopsis thaliana* encodes 12 putative CDF genes, which are highly divergent in sequence, but share some characteristics of CDF family membrane transport proteins (Blaudez et al., 2003). The first reported plant CDF protein, identified as a cDNA, was *AtZAT* (Zinc transporter of *A. thaliana*), later renamed as *AtMTP1* (Metal Tolerance Protein 1) (van der Zaal et al., 1999; Mäser et al, 2001). *AtMTP1* transcripts were shown to be present at low levels in seedlings grown under normal or excess Zn supply (van der Zaal et al., 1999). Ectopic over-expression of the *AtMTP1* cDNA under the control of the cauliflower mosaic virus 35S promoter resulted in enhanced Zn tolerance of transgenic seedlings and slightly increased Zn accumulation in roots (van der Zaal

et al., 1999). Bloβ et al. (2002) expressed *AtMTP1* in *E. coli* and studied the purified protein in reconstituted proteoliposomes. The protein transported Zn into proteoliposomes by a mechanism that relied on the Zn gradient across the membrane and not on a proton gradient. Further studies by Desbrosses-Fonrouge et al. (2005) and Kobae et al. (2004) showed that AtMTP1 acts as a Zn transporter, localised in the vacuolar membrane and mediating Zn detoxification and leaf Zn accumulation. Two other putative CDF proteins of *A. thaliana*, *AtMTP2* and *AtMTP3*, are closely related to *AtMTP1* (64.4% and 67.6% aa identity respectively). Expression profiling showed *AtMTP2* to be induced under Zn-deficiency conditions (van de Mortel et al., 2006). *AtMTP3* was found to contribute to basic cellular Zn tolerance and involved in the control of Zn partitioning (Arrivault et al., 2006). Heterologous expression of *PtdMTP1* from hybrid poplar *Populus trichocarpa* x *Populus deltoides* in various yeast mutants conferred resistance specifically to Zn, which was assumed to be the result of transport into the vacuole (Kohler et al, 2003). Over-expression of *PtdMTP1* in *A. thaliana* also led to Zn tolerance (Blaudez et al, 2003). Delhaize et al. (2003) showed that over-expression of the *ShMTP1* cDNA from *Stylosnathes hamata*, a tropical legume tolerant to acid soils with high concentrations of Mn, conferred Mn tolerance to yeast and plants by a mechanism that is likely to involve the sequestration of Mn into internal organelles.

Recently, the regulation of the metal ions in Zn hyperaccumulator plants (Baker et al., 1992; Brooks, 1994) has drawn a lot of interest (Assunção et al., 2003a). The Zn hyperaccumulator species are found to accumulate more than 10,000 μg Zn g^{-1} of dry weight (dw) (1%, w/w) (Baker and Brooks, 1989), whereas most plants contain between 30 and 100 μg Zn g^{-1} dw and concentrations above 300 μg Zn g^{-1}

dw are generally toxic (Marschner, 1995). Few studies have been performed to unravel the mechanism of hyperaccumulation in plants and help understanding why these plant species are growing happily in contaminated soils compared to non-accumulator species. The hyperaccumulator species are in comparison to the non-accumulating species, assumed to have specific or higher activity of mechanisms for uptake, xylem loading and unloading (Lasat et al., 1998) and vacuolar sequestration of heavy metals, particularly in the leaf epidermal cells (Vázquez et al, 1994; Küpper et al, 1999) such as trichomes (Kramer et al, 1997) or stomatal guard cells (Heath et al, 1997). Transcriptional comparisons indicated that often similar genes are present in metal non-accumulating and accumulating plants, but the transcriptional control of especially metal homeostasis genes is very different (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006 and Talke et al., 2006, van de Mortel et al., 2006).

Persans et al. (2001) isolated CDF genes (*TgMTP1s*) from the Ni-hyperaccumulating species *Thlaspi goesingense*. These genes conferred metal tolerance to *S. cerevisiae* mutants defective in vacuolar *COT1* and *ZRC1* Zn transporters, suggesting that TgMTP1 transports metals into the vacuole. In vivo and in vitro immunological staining of hemagglutinin (HA)-tagged *TgMTP1::HA* revealed that the protein is localized in both the *S. cerevisiae* vacuolar and plasma membranes. It is assumed that TgMTP1 functions by enhancing plasma membrane Zn efflux, thereby conferring Zn resistance to *S. cerevisiae*. Expression studies of *AhMTP1* from the Zn/Cd hyperaccumulator *A. halleri* showed substantially higher transcript levels in the leaves compared to *A. thaliana* and *A. lyrata.* Vacuolar *AhMTP1 is* up-regulated in the roots of *A. halleri* upon exposure to high Zn concentrations (Dräger et al., 2004).

Thlaspi caerulescens is a suitable model plant for the study of Zn/Cd/Ni hyperaccumulation and tolerance (Assunção et al., 2003a). Like *T. goesingense* and *A. halleri*, this species belongs to the Brassicaceae family and is known to accumulate up to 30,000 µg Zn g^{-1} dw foliar concentration (Brown et al., 1995). Hyperaccumulation of Cd or Ni also has been reported for a number of natural *T. caerulescens* populations from calamine and serpentine soils (Reeves and Brooks, 1983; McGrath et al., 1993). Although Cd is a non-essential and toxic element for plants, in *T. caerulescens* a foliar Cd concentration up to 2700 µg Cd g^{-1} dw has been reported (Lombi et al., 2000). Assunção et al. (2001) showed constitutively high expression of the CDF family member *ZTP1* (Zinc Transporter 1) in *T. caerulescens* and suggested a role for this gene in Zn tolerance. *ZTP1* is most similar to *AtMTP1* of *A. thaliana* (90% DNA and 75% predicted protein identity). In this study, we examine the function of *TcZTP1* in metal homeostasis using lines over-expressing *TcZTP1* in *A. thaliana.* Here we demonstrate that over-expression of *TcZTP1* in *A. thaliana* confers Zn and Cd tolerance and accumulation.

Materials and Methods

Construction over-expression plasmids and plant transformation

DNA manipulation and cloning were performed using standard procedures (Sambrook et al., 1989). To make the over-expression construct of *TcZTP1*, a fragment of 1352 bp containing the *TcZTP1* cDNA (GenBank accession No. AF275750) was cut from a cDNA library clone, described in Assunção et al. (2001), using BamHI and XbaI. This fragment was cloned into pEZR(H)-LN, which was a kind gift of Dr. Gert de Boer, Ehrhardt laboratory, Dept. of Plant

Biology, Carnegie Institution of Washington, USA) and created by ligating the expression cassette from pEZS-LN (David Ehrhardt, Stanford University; (http://deepgreen.stanford.edu/) into pCambia 1300 (http://www.cambia.org /daisy/bios/585.html). BamHI and XbaI digestion removed the GFP gene from the vector and ligation of the *TcZTP1* gene resulted in a CaMV 35S promoter-*TcZNT1* fusion. The inserted fragments were verified by DNA sequencing. The construct was transformed to *A. thaliana,* accession Columbia (Col) by *Agrobacterium tumefaciens* mediated flower dipping transformation as described by Clough and Bent (1998).

Plant growth conditions

Seedlings of T_1 transformants containing the *35S::TcZTP1* construct were screened on plates with half strength MS medium (Murashige and Skoog, 1962) (Duchefa Biochemie B.V., Haarlem, The Netherlands) containing 20 mg l^{-1} Hygromycin (Duchefa Biochem, Haarlem, The Netherlands) to select 10 independently transformed lines which were propagated until homozygous T_3 lines were obtained. Three homozygous lines with high *TcZTP1* expression were selected by semi-quantitative RT-PCR analysis.

Genomic DNA extraction and DNA blot

To confirm the presence of a T-DNA insert in the transgenic plants in the T_1 generation, DNA was extracted from flowers of *35S::TcZTP1*-transformed *A. thaliana* plants, as described by Aarts et al. (2000) and PCR was performed to amplify a fragment of 1175 bp with the primers P2 and P3 (Table 1). In order to check the insertion number, DNA blot analysis was performed with 10

independent transformants of *35S::TcZTP1* transformed *A. thaliana* plants. A 1352 bp fragment obtained by PCR amplification with primers P2 and P3 (Table 1) was used as probe for the DNA blot (Aarts et al., 2000). The over-expression lines with a single insertion were chosen for further expression study.

RNA isolation and semi-quantitative Reverse Transcriptase-PCR (RT-PCR)

Total RNA of leaves and roots of *35S::TcZTP1* plants in T_1 and T_3 generation was extracted with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One µg of total RNA was used to synthesize cDNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and an oligo(dT) primer (Invitrogen, Carlsbad, CA, USA). The RNA quantity was determined spectrophotometrically and the quality was checked by agarose gel electrophoresis. The RNA was treated with DNase (MBI Fermentas, St. Leon-Rot, Germany) before cDNA synthesis was performed. The RNA was checked by PCR using the *At-tubulin* (At1g04820) primers P4 and P5 (Table I) for any residual genomic DNA. RT-PCR was performed with a cDNA aliquot of 2 µl and gene-specific primers P2 and P3 (Table I). To differentiate between amplifications from genomic DNA and cDNA, these primers were designed spanning an intron of the genomic *At-tubulin* gene.

Table 1: **Primers used for PCR amplifications.**

Primer	Sequence (5'-3')	Target Gene
P1	CCCAAGCTTACCCAAAAAAAGAGATC GAATT	Sense primer designed on *TcZTP1* pAD-GAL4-2.1vector with HindIII site
P2	TTGAGGTTAATGGAGGAAGATCTGA	Sense primer for *TcZTP1* cDNA
P3	CTTTGTCGACCGCTCGATTTGTACGGTT ACA	Antisense primer for *TcZTP1* cDNA with XbaI site
P4	AAGCTTGCTGATAACTGTACTGGT	Sense primer for *At-tubulin* (At1g04820)
P5	GGTTTGGAACTCAGTGACATCA	Antisense primer *At-tubulin*

Restriction sites incorporated in the primers are underlined.

Metal exposure

To check the response of the transgenic lines to metals, transgenic and wild type (Col) lines were grown vertically on 12x12 cm-square plates containing 0.5 x MS medium and 1% agar (Duchefa Biochemie B.V., Haarlem, The Netherlands). The response of transgenic lines exposed to $ZnSO_4$ (15, 50, 75, 100, 150, 250, 400 and 500 µM), $CdSO_4$ (50, 75, 100 and 200 µM) and Fe(Na)EDTA (0, 0.5, 50 µM) (Merck, Darmstadt, Germany) added to the media, was determined by measuring the root length of individual seedlings. Three independent transformants of *35S::TcZTP1* were used for each experiment. Plates were incubated in a climate-controlled growth cabinet (25°C day/night; 16 h day with illumination at a light intensity of 120 µmol m^{-2} s^{-1}). Each experiment was repeated at three different time points. The wild type and transgenic lines were grown together on the same MS/agar plate to minimize the effect of variation among plates.

In order to check the root growth and tolerance phenotype, ten plants for each three independent *35S::TcZTP1* transformants were grown on modified half Hoagland's nutrient solution (Schat et al., 1996), which supplies sufficient Zn to *A. thaliana* plants (2 µM $ZnSO_4$), and on the same medium to which no Zn was added, inducing Zn-deficiency to the plants. Plants were grown in trays, each containing about 9 L of hydroponic medium. A maximum of 81 plants were grown per tray. For each Zn concentration the transgenic and control lines were grown in the same tray to minimize the effect of variation among the trays. The 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) for five weeks. Root and shoot tissues were harvested for the metal accumulation assay. Each hydroponic experiment was performed three times.

To determine the response of *35S::TcZTP1* plants to Cd exposure, the same transgenic lines were grown hydroponically on modified half Hoagland's solution for two weeks and then transferred to the same media containing 0 μM Cd-0 μM Zn, 0 μM Cd-2 μM Zn, 0.5 μM Cd-0 μM Zn, 0.5 μM Cd-2 μM Zn, 5 μM Cd-0 μM Zn or 5 μM Cd-2 μM Zn, keeping the concentration of the other minerals constant. The root and shoot tissues were harvested at the end of two weeks exposure for mineral content analysis.

In order to determine the response of *35S::TcZTP1* plants to Fe, the same transgenic lines were grown hydroponically on modified half Hoagland's solution for two weeks and then transferred to the same media but containing 0.5 μM Fe for another two weeks, keeping the concentration of the other minerals the same. The transgenic and wild type plants were also grown on half Hoagland's as a control for four weeks in total. In all experiments the nutrient solution was refreshed every week.

Root and shoot mineral content assay

The root and shoot tissues were analyzed for Zn, Fe, Mn and Cd content as described by Assunção et al. (2003).

Sequence analysis of TcZTP1

The predicted coding region of the *TcZTP1* cDNA (GenBank accession no. AAK69428) was compared to other closely related MTP1 proteins obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/), using CLUSTALW (http://www.ebi.ac.uk/clustalw/) (Thompson et al., 1994) to identify sequence conservation. A BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/) using the TcZTP1 protein sequence was performed to identify MTP1-like proteins from *Thlaspi fendleri* (AAR83905), *Thlaspi goesingense* (AAS67026), *Arabidopsis*

halleri (CAD89013), *Thlaspi arvense* (AY483145), *Arabidopsis thaliana* (NP_182203), *Arabidopsis lyrata* (CAG28982), *Brassica juncea* (AAR83910), *Populus trichocarpa* x *Populus deltoides* (AAR23528), *Nicotiana glauca* (BAD89561), *Nicotiana tabacum* (BAD89563), *Medicago truncatula* (ABE83561), *Eucalyptus grandis* (AAL25646) and *Oryza sativa* (AAU10745). These proteins share between 63% and 91% amino acid identity with TcZTP1 (Table 2). Based on the amino acid sequence analysis a phylogenetic tree was constructed using the program MEGA 3.1 (Kumar et al., 2004) and Treeview (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). An unrooted phylogenetic tree was constructed using the BLOSUM matrix and the UPGMA tree-building algorithm (Saitou et al., 1987). Bootstrap values based upon 100 iterations provided estimations of statistical support for the tree.

Results

TcZTP1 over-expression enhances Zn tolerance and Zn accumulation in A. thaliana

To study the response of *35S*::*TcZTP1* transformed *A. thaliana* plants to high Zn, three homozygous lines overexpressing the *TcZTP1* gene were generated and grown in three replicates on vertical plates with half MS-agar media containing 15, 50, 75, 100, 150, 250, 400 or 500 μM Zn. A standard concentration of 15 μM of Zn is sufficient for normal growth of *A. thaliana*. The over-expression plants grown on 15, 50, 75 and 100 μM Zn did not show any difference compared to wild type Columbia (Col) plants, whereas the over-expression plants grown on 150 and 250 μM Zn formed longer roots than wild type plants (Figure 1A, B). The root growth difference between transgenic and wild type plants was statistically

significant ($p<0.05$) on 150 μM Zn, in all three replicates. The transgenic and wild type plants were both similarly affected by Zn concentrations higher than 250 μM. To study further the root growth phenotype of the *TcZTP1*over-expression lines, the plants were grown on modified half Hoagland's hydroponic media provided with standard (2 μM $ZnSO_4$) and high Zn (50 μM $ZnSO_4$). The transgenic plants grown on 50 μM Zn media showed better Zn excess tolerance than the wild type plants: the transgenic plants had a larger rosette size (Figure 1C) and longer roots after five weeks of exposure (Figure 1D). The fresh weight of the shoots and roots (Figure 1E, 1F) and the mean rosette radius (Figure 1G) of the transgenic plants were two-to-three fold larger than those of the wild type plants. The Zn concentration in the shoot and root samples of the transgenic lines grown on 50 μM Zn, were two-to-four fold higher than those of wild type ($p<0.001$) (Figure 1H and 1I) in two independent experiments. The Zn concentration in the transgenic plants grown with half Hoagland's solution (2 μM Zn) showed only a 1.4 fold higher Zn concentration in roots. However, no significant differences between wild type and overexpression plants were detected for Zn content in shoots in this condition (data not shown).

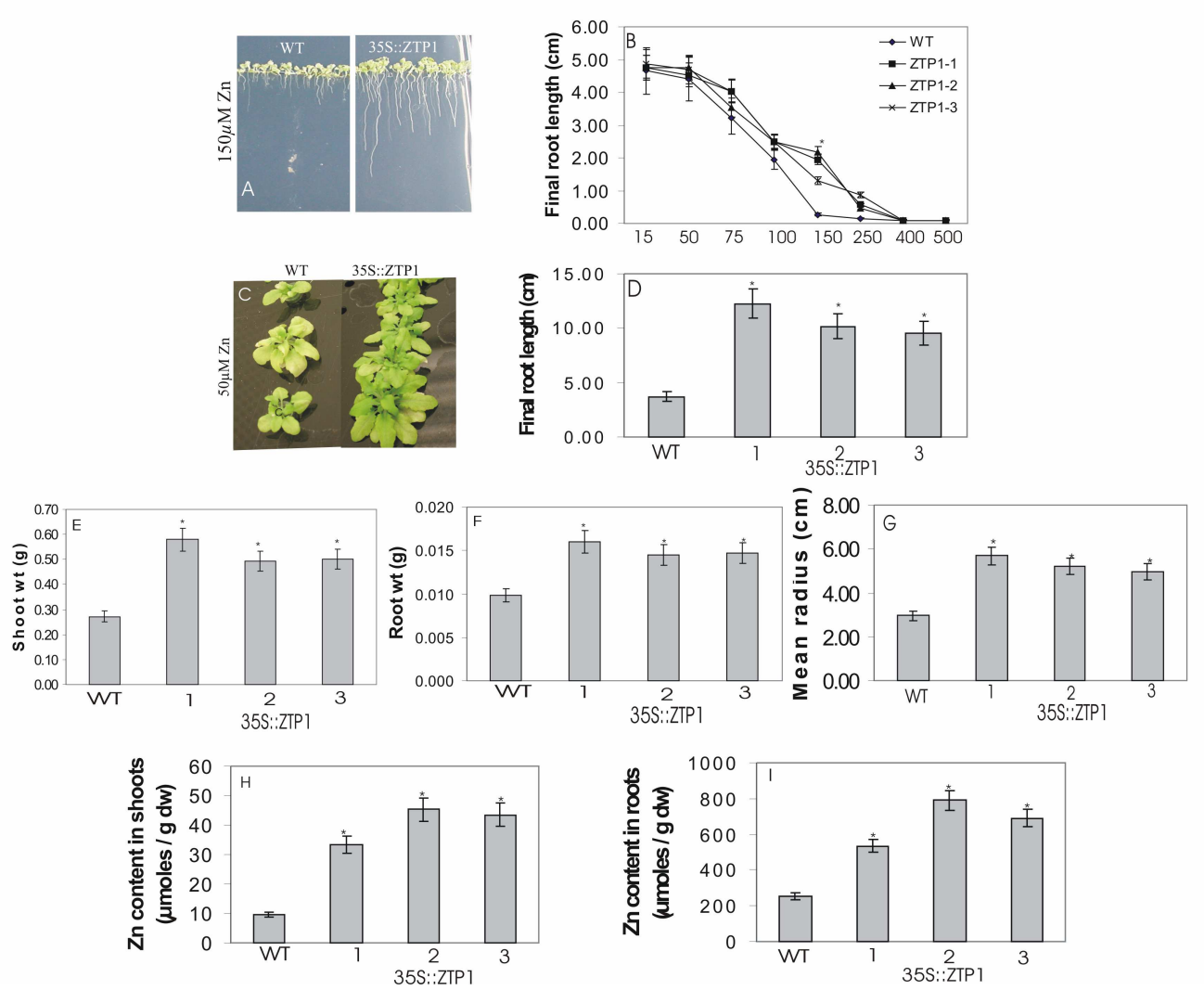


Figure 1: Phenotypic analysis of three independent *35S::TcZTP1* transformed *A. thaliana* lines (ZTP1-1, ZTP1-2, ZTP1-3) overexpressing *TcZTP1* in *A. thaliana* plants compared to wild type when exposed to excess Zn. **(A)** *35S::TcZTP1* transformed *A. thaliana* plants (right) compared to wild type *A. thaliana* Columbia (Col) (WT) plants (left) on vertical plates with half MS/agar media with additional supplied 150 µM $ZnSO_4$. Photograph was taken 14 days after germination of seeds. **(B)** Final root length in comparison to wild type Col grown on 15, 50, 75, 100, 150, 250, 400 and 500 µM Zn containing half MS/agar plates. (**C)** The *35S::TcZTP1* transformed *A. thaliana* plants (right) compared to wild type plants (left) on half Hoagland's hydroponic solution containing 50 µM $ZnSO_4$ at five weeks after seed germination. **(D)** Final root length **(E)** Shoot biomass, **(F)** Root biomass and **(G)** mean radius of the rosette, after five weeks since seed germination. **(H, I)** Zn concentration in shoots (H) and roots (I) after five weeks of growth in half Hoagland's solution with 50 µM Zn. The asterisk indicates that the difference between the value for transgenic line is significantly different from wild type ($p<0.05$, Student's t test).

Over-expression of TcZTP1 enhances Cd tolerance and Cd accumulation in A. thaliana

To determine the response of Cd on the *TcZTP1* over-expression lines, the same transgenic lines were grown on vertical half MS/agar plate containing 0, 50, 75, 100 and 200 μM $CdSO_4$. After 14 days the transgenic plants grown on 100 μM Cd showed hardly any reduction in root growth compared to plants grown on 75 μM Cd, whereas the roots of wild type had approximately 3.5-fold shorter roots compared to plants grown on 75 μM Cd. The difference in root length between over-expression lines and wild type was statistically significant ($p<0.05$) (Figure 2A). To investigate this root growth phenotype further, the same lines were grown hydroponically on modified half Hoagland's solution for two weeks and then transferred to the same medium but containing 0 μM Cd-0 μM Zn, 0 μM Cd-2 μM Zn, 0.5 μM Cd-0 μM Zn, 0.5 μM Cd-2 μM Zn, 5 μM Cd-0 μM Zn and 5 μM Cd-2 μM Zn, without changing the content of other minerals in the medium.

TcZTP1 over-expression plants grown at 5 μM Cd-2 μM Zn showed higher tolerance and were larger in size with larger leaves compared to wild type plants (Figure 2C). The wild type plants were severely affected by this high concentration of Cd and the leaves were very small. The shoot and root fresh weights (Figure 2D, 2E) of the over-expression plants were two-to-three fold higher (significant at $p<0.05$) than those of the wild type plants. Also the Cd content in shoots and roots of all three over-expression lines was two-to-three fold higher than that of wild type (Figure 2F, 2G). Exposing to 5 μM Cd-0 μM Zn reduced the Zn content and increased the Cd content compared to exposure to 5 μM Cd-2 μM Zn, but the difference between wild type and *35S::TcZTP1* plants were comparable (Figure 2H and 2I). Remarkably, over-expression of *AtMTP1* (*ZAT*) was not reported to show any effect on Cd accumulation or tolerance (van der Zaal et al., 1999). Therefore we compared the *35S::AtMTP1* (*35S::AtZAT*) over-expression lines and the corresponding wild type on the same trays with hydroponic medium with one *TcZTP1* overexpressing line and the corresponding wild type. No increased

Cd tolerance was observed for the *35S::ZAT* plants based on visible plant phenotype and rosette diameter (data not shown), confirming the initial observations by van der Zaal et al. (1999) and showing that only *TcZTP1, but not AtMTP1,* over-expression confers Cd tolerance and accumulation to *A. thaliana*.

The comparison of the Zn, Fe, Mn and Cd content in the *TcZTP1* over-expression line with the highest phenotypic effect (ZTP1-1) compared to wild type grown on 2 μM Zn, 5 μM Cd-2 μM Zn and 5 μM Cd-0 μM Zn, are shown in Figures 2H -K for shoots and roots respectively. The other two independent transformant lines showed similar results (data not shown). When *A. thaliana* was grown on medium with Cd, the root Fe concentration was found to be significantly higher ($p < 0.05$) than in plants grown without Cd. For this effect the transgenic lines do not differ significantly from wild type. The Mn concentration in shoots is significantly higher in Cd exposed plants than the non-exposed ones, which effects are also similar for wild types and overexpression lines.

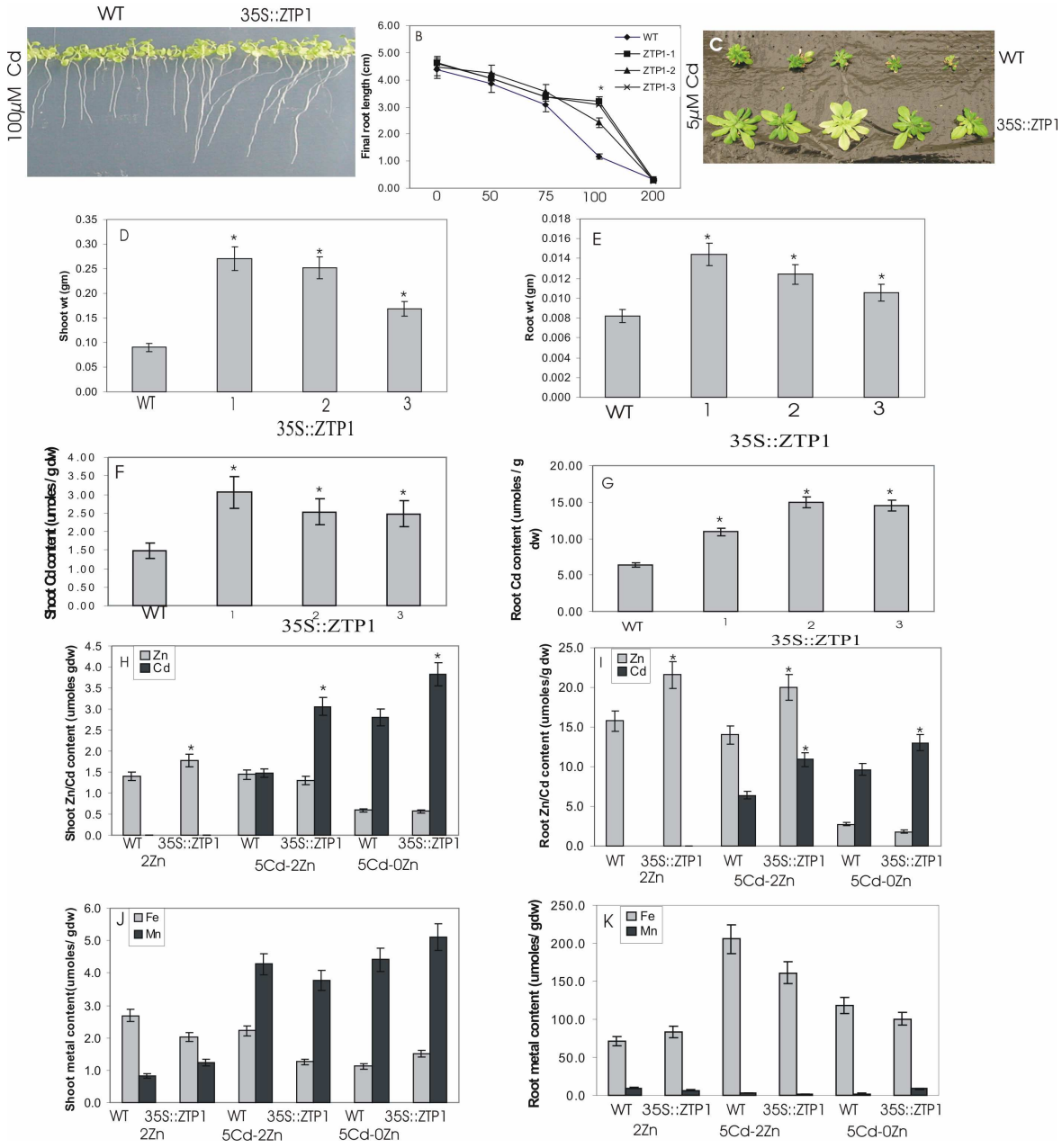


Figure 2: Phenotypic analysis of three independent transformants (ZTP1-1, ZTP1-2, ZTP1-3) overexpressing *TcZTP1* in *A. thaliana* plants compared to wild type when exposed to excess Cd. **(A)** The *35S::TcZTP1* transformed *A. thaliana* plants (right) grown in comparison to wild type *A. thaliana* Columbia (Col) (WT) plants (left) on vertical plates with half MS/agar media with additional supplied 100 µM $CdSO_4$ after 14 days since germination of seeds. **(B)** Final root length after 14 days since seed germination in comparison to the wild type plants grown on 0, 50, 75, 100 and 200 µM Cd containing half MS/agar plates. **(C)** The *35S::TcZTP1* transformed *A. thaliana* plants (below) exposed to Cd (5 µM $CdSO_4$) for two weeks after growing them on half Hoagland's solution for two weeks in comparison to wild type plants (above). Root **(D)** and Shoot biomass (E) after two weeks Cd exposure Cd concentration in shoots **(F)** and roots **(G)** of treatment shown in Figure 2C. **(H, I, J, K)** Zn, Cd, Fe, and Mn concentration in shoots and roots of the *35S::TcZTP1* transformed *A. thaliana* plants in comparison to the wild type plants (Figure 2C). The asterisk indicates that the difference between the value for transgenic line is significantly different from wild type ($p<0.05$, Student's t test). umoles represents micromoles.

TcZTP1 overexpressing A. thaliana plants reduces sensitivity to Fe deficiency

Zn homeostasis is often correlated with Fe homeostasis. Over-expression of *TcZTP1* could therefore have an affect on the ability to withstand low Fe supply. To check the response to low Fe, all three *TcZTP1* over-expression lines were grown on vertical half MS/agar plates containing 0 or 0.5 μM Fe and compared to growth on plates with 50 μM Fe, which is standard in half MS medium. The over-expression lines all showed longer roots, by around 1.5 fold compared to the wild type plants on 0 and 0.5 μM Fe, suggesting increased tolerance to Fe deficiency (Figure 3A). The transgenic lines grown with half MS (containing 50 μM Fe) did not show a significant difference in root length. To investigate the root growth phenotype further, the same over-expression lines were grown hydroponically on modified half Hoagland's solution with low Fe supply (0.5 μM Fe) and compared to the wild type plants grown at the standard 20 μM Fe. The over-expression plants grown on low Fe media were larger in size (Figure 3C and 3D), also when grown on hydroponics and after two weeks of exposure compared to wild type plants. When grown on normal half Hoagland's solution containing 0.5 μM Fe, the Zn contents in shoots and roots were significantly higher (Figure 3E, 3F), whereas no difference was found in the Fe content of these plants (data not shown). The Zn concentration in the transgenic plants grown with half Hoagland's solution (containing 20 μM Fe) showed a 1.4 fold higher Zn concentration in roots (Figure 3H), but in shoots the difference was not significant (Figure 3G).

The plants were also grown on Cu, Mn, and Co deficient plates with half MS/agar media but no significant root growth differences compared to wild type were observed (data not shown).

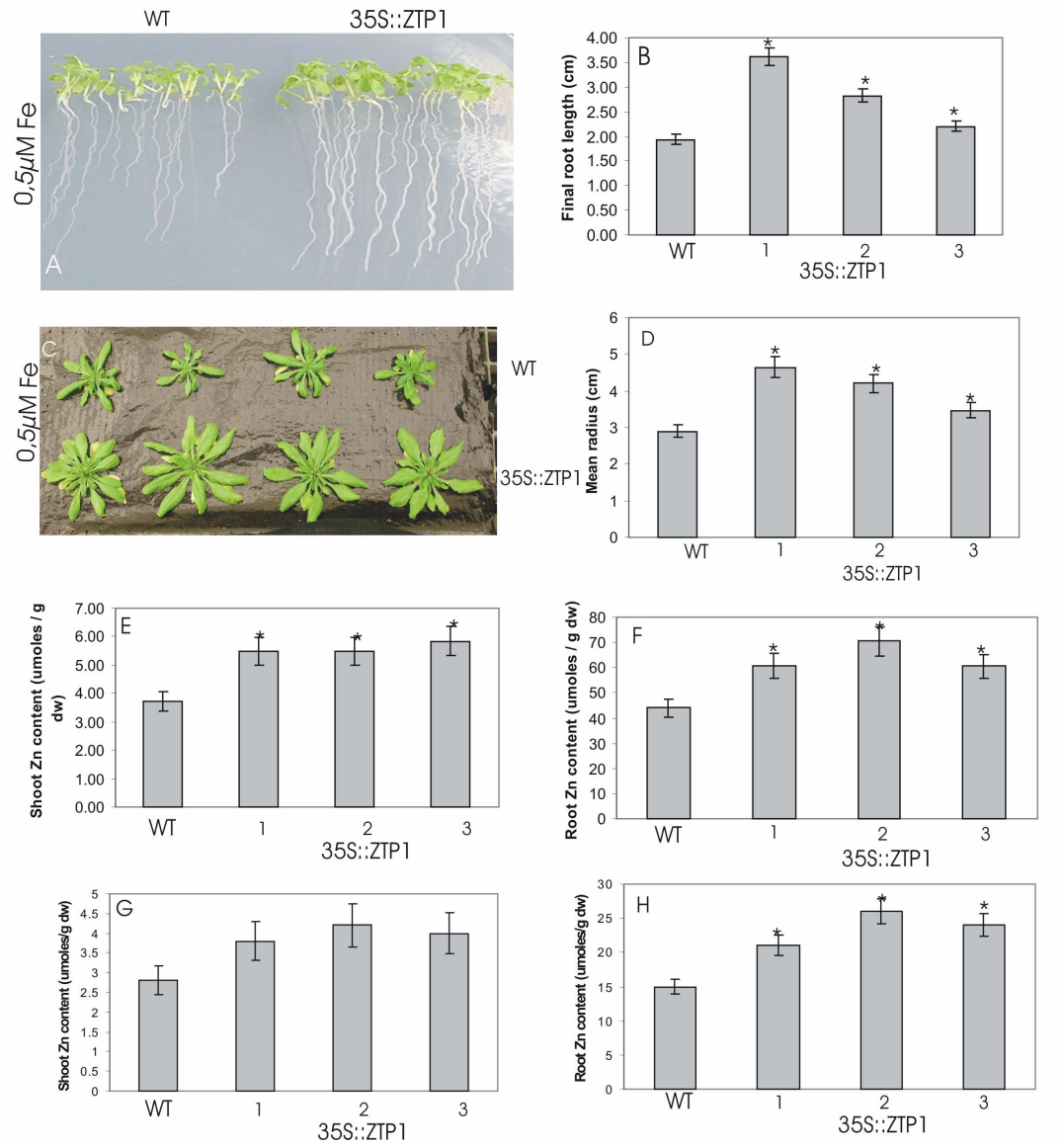


Figure 3: Phenotypic analysis of three independently transformed *35S::ZTP1* lines, overexpressing *TcZTP1* in A. *thaliana* plants, compared to wild type when exposed to low Fe. **(A)** The *35S::TcZTP1* transformed *A. thaliana* plants (right) grown in comparison to wild type *A. thaliana* (Columbia) (WT) plants (left) on vertical plates with half MS/agar media with low (0.5 µM Fe) after 14 days since germination of the seeds. **(B)** Final root length after 14 days since seed germination. **(C)** *35S::TcZTP1* transformed *A. thaliana* plants (top) grown in comparison to wild type plants (bottom) on half Hoagland's solution with low (0.5 µM Fe) for two weeks after growth for two weeks on half Hoagland's solution with normal Fe (20 µM Fe). Mean radius of the rosette **(D),** Zn content (µmoles g^{-1}; mean ± SE of 10 replica) in shoots **(E)** and roots **(F)**, Zn content in shoots **(G)** and roots **(H)** after four weeks of growth on half Hoagland's solution (which contains standard 20 µM Fe). The asterisks indicate that the transgenic line is significantly different from wild type ($p<0.05$, Student's t test). umoles represents micromoles.

Multiple alignment of TcZTP1 with related proteins from other species

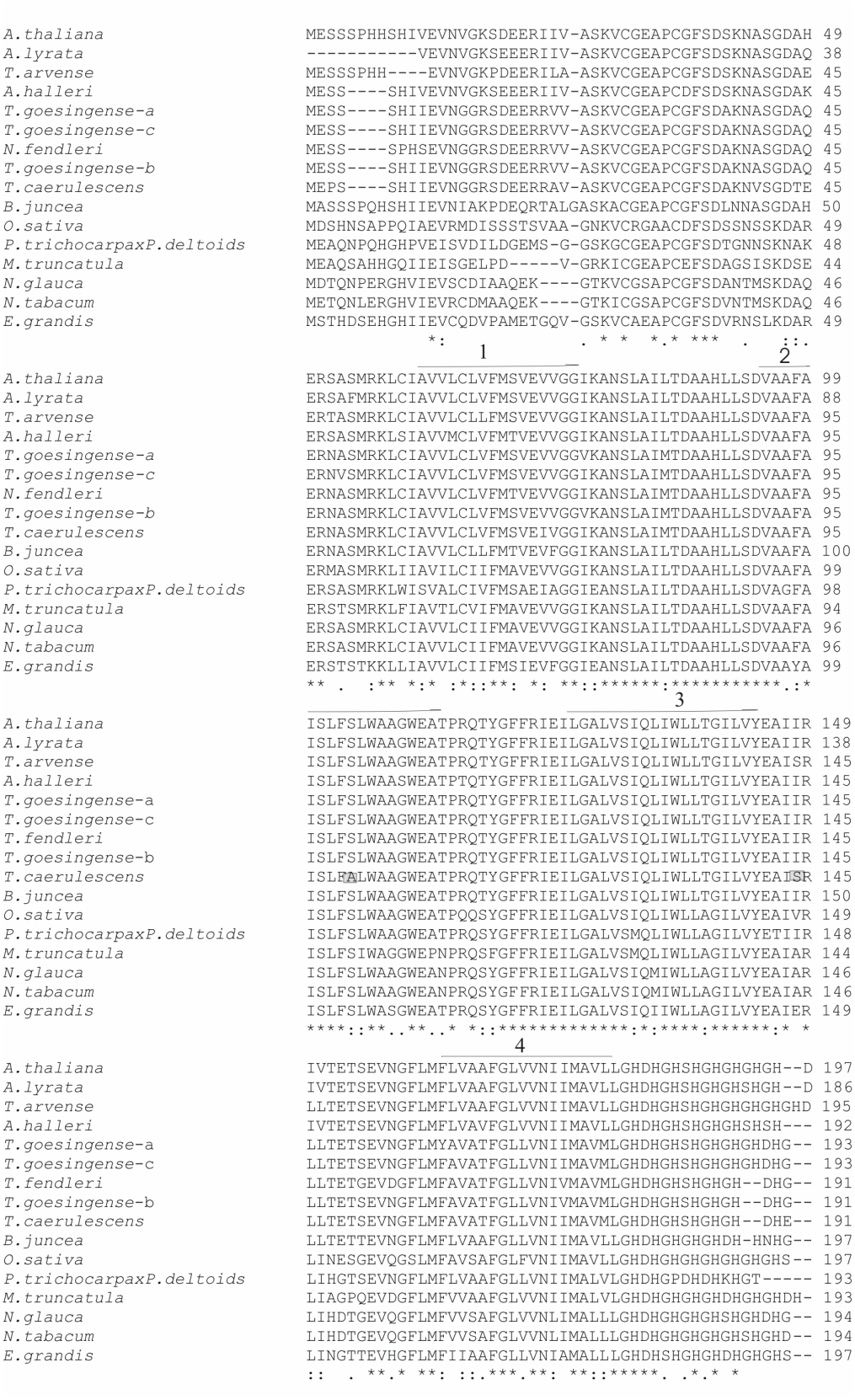
Considering the effect of *TcZTP1* over-expression on Cd tolerance and accumulation on *A. thaliana*, compared to *AtMTP1* over-expression, we decided to compare the predicted coding region of the *TcZTP1* cDNA of *T. caerulescens* to other MTP1 proteins obtained from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) that may explain the apparent difference in specificity. Multiple alignment of the TcZTP1 amino acid sequence and related proteins from *Thlaspi fendleri* (AAR83905), *Thlaspi goesingense* (AAS67026), *Thlaspi arvense* (AY483145), *Arabidopsis thaliana* (NP_182203), *Arabidopsis halleri* (CAD89013), *Arabidopsis lyrata* (CAG28982), *Brassica juncea* (AAR83910), *Populus trichocarpa* x *Populus deltoides* (AAR23528), *Nicotiana glauca* (BAD89561), *Nicotiana tabacum* (BAD89563), *Medicago truncatula* (ABE83561), *Eucalyptus grandis* (AAL25646) and *Oryza sativa* (AAU10745) shows considerable of sequence conservation in the MTP1 proteins of these 14 species (Figure 4). The percentages of amino acid identity of all these MTP1 proteins compared to TcZTP1 are summarized in Table 2. A phylogenetic tree has been made based on this protein sequence comparison (Figure 5), which shows that TcZTP1 is in the same group with other *Thlaspi* species *T. fendleri* and *T. goesingense* (a, b, c). *T. goesingense* is a Ni-hyperaccumulator species and *T. fendleri* is a non-accumulator species (Kim et al., 2004; Küpper et al., 2002; Koch et al., 2001). Few other closely related species, e.g., *A. thaliana, A. lyrata and A. halleri* are found to be clusterd together in this tree. The significance of branches in the tree is supported by the high bootstrap values shown at the branch origin. But the bootstrap values below 60 are not significant and considered not reliable.

Interestingly, the TcZTP1 sequence differs at five conserved amino acids from the MTP1-like sequences of all other species, with the exception of S144, which is also found in *T. arvense* and T365, which is also found in *O. sativa* (Figure 5). The alterations are not towards similar

amino acids: A100 in the second transmembrane domain is S in all other proteins; S144, just after the 3rd transmembrane domain is A, I, V in all other proteins except for E in *E. grandis* and except for *T. arvense*; T365 is K, R, M or I in all other proteins except for *O. sativa*; Q367 is D or E in the other proteins; and H375 is D or N in the other proteins.

Table 2: Summary of the amino acid identity of MTP1-like sequences found in GenBank, compared to TcZTP1

Species name	**NCBI protein ID**	**Protein length (aa)**	**% identity with TcZTP1 amino acid sequence**
Thlaspi caerulescens	AAK69428	396	-
Thlaspi goesingense-a	AAS67024	390	87%
Thlaspi goesingense-b	AAS67025	390	88%
Thlaspi goesingense-c	AAS67026	396	91%
Thlaspi fendleri	AAR83905	392	90%
Arabidopsis halleri	CAD89013	389	78%
Arabidopsis thaliana	NP_182203	398	81%
Arabidopsis lyrata	CAG28982	385	86%
Thlaspi arvense	AY483145	396	78%
Brassica juncea	AAR83910	385	76%
Populus trichocarpa x Populus deltoides (Ptd)	AAR23528	393	69%
Nicotiana glauca	BAD89561	418	66%
Nicotiana tabacum	BAD89563	418	64%
Medicago truncatula	ABE83561	407	67%
Eucalyptus grandis	AAL25646	421	63%
Oryza sativa	AAU10745	418	65%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A.lyrata ----------VEVNVGKSEEERIIV-ASKVCGEAPCGFSDSKNASGDAQ 38
T.arvense MESSSPHH----EVNVGKPDEERILA-ASKVCGEAPCGFSDSKNASGDAE 45
A.halleri MESS----SHIVEVNVGKSEEERIIV-ASKVCGEAPCDFSDSKNASGDAK 45
T.goesingense-a MESS----SHIIEVNGGRSDEERRVV-ASKVCGEAPCGFSDAKNASGDAQ 45
T.goesingense-c MESS----SHIIEVNGGRSDEERRVV-ASKVCGEAPCGFSDAKNASGDAQ 45
N.fendleri MESS----SPHSEVNGGRSDEERRVV-ASKVCGEAPCGFSDAKNASGDAQ 45
T.goesingense-b MESS----SHIIEVNGGRSDEERRVV-ASKVCGEAPCGFSDAKNASGDAQ 45
T.caerulescens MEPS----SHIIEVNGGRSDEERRAV-ASKVCGEAPCGFSDAKNVSGDTE 45
B.juncea MASSSPQHSHIIEVNIAKPDEQRTALGASKACGEAPCGFSDLNNASGDAH 50
O.sativa MDSHNSAPPQIAEVRMDISSSTSVAA-GNKVCRGAACDFSDSSNSSKDAR 49
P.trichocarpaxP.deltoids MEAQNPQHGHPVEISVDILDGEMS-G-GSKGCGEAPCGFSDTGNNSKNAK 48
M.truncatula MEAQSAHHGQIIEISGELPD-----V-GRKICGEAPCEFSDAGSISKDSE 44
N.glauca MDTQNPERGHVIEVSCDIAAQEK----GTKVCGSAPCGFSDANTMSKDAQ 46
N.tabacum METQNLERGHVIEVRCDMAAQEK----GTKICGSAPCGFSDVNTMSKDAQ 46
E.grandis MSTHDSEHGHIIEVCQDVPAMETGQV-GSKVCAEAPCGFSDVRNSLKDAR 49
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A.lyrata ERSAFMRKLCIAVVLCLVFMSVEVVGGIKANSLAILTDAAHLLSDVAAFA 88
T.arvense ERTASMRKLCIAVVLCLLFMSVEVVGGIKANSLAILTDAAHLLSDVAAFA 95
A.halleri ERSASMRKLSIAVVMCLVFMTVEVVGGIKANSLAILTDAAHLLSDVAAFA 95
T.goesingense-a ERNASMRKLCIAVVLCLVFMSVEVVGGVKANSLAIMTDAAHLLSDVAAFA 95
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T.goesingense-b ERNASMRKLCIAVVLCLVFMSVEVVGGVKANSLAIMTDAAHLLSDVAAFA 95
T.caerulescens ERNASMRKLCIAVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFA 95
B.juncea ERNASMRKLCIAVVLCLLFMTVEVFGGIKANSLAILTDAAHLLSDVAAFA 100
O.sativa ERMASMRKLIIAVILCIIFMAVEVVGGIKANSLAILTDAAHLLSDVAAFA 99
P.trichocarpaxP.deltoids ERSASMRKLWISVALCIVFMSAEIAGGIEANSLAILTDAAHLLSDVAGFA 98
M.truncatula ERSTSMRKLFIAVTLCVIFMAVEVVGGIKANSLAILTDAAHLLSDVAAFA 94
N.glauca ERSASMRKLCIAVVLCIIFMAVEVVGGIKANSLAILTDAAHLLSDVAAFA 96
N.tabacum ERSASMRKLCIAVVLCIIFMAVEVVGGIKANSLAILTDAAHLLSDVAAFA 96
E.grandis ERSTSTKKLLIAVVLCIIFMSIEVFGGIEANSLAILTDAAHLLSDVAAYA 99
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T.arvense ISLFSLWAAGWEATPRQTYGFFRIEILGALVSIQLIWLLTGILVYEAISR 145
A.halleri ISLFSLWAASWEATPTQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIR 145
T.goesingense-a ISLFSLWAAGWEATPRQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIR 145
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T.fendleri ISLFSLWAAGWEATPRQTYGFFRIEILGALVSIQLIWLLTGILVYEAIIR 145
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P.trichocarpaxP.deltoids ISLFSLWAAGWEATPRQSYGFFRIEILGALVSMQLIWLLAGILVYETIIR 148
M.truncatula ISLFSIWAGGWEPNPRQSFGFFRIEILGALVSMQLIWLLAGILVYEAIAR 144
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N.tabacum ISLFSLWAAGWEANPRQSYGFFRIEILGALVSIQMIWLLAGILVYEAIAR 146
E.grandis ISLFSLWASGWEATPRQSYGFFRIEILGALVSIQIIWLLAGILVYEAIER 149
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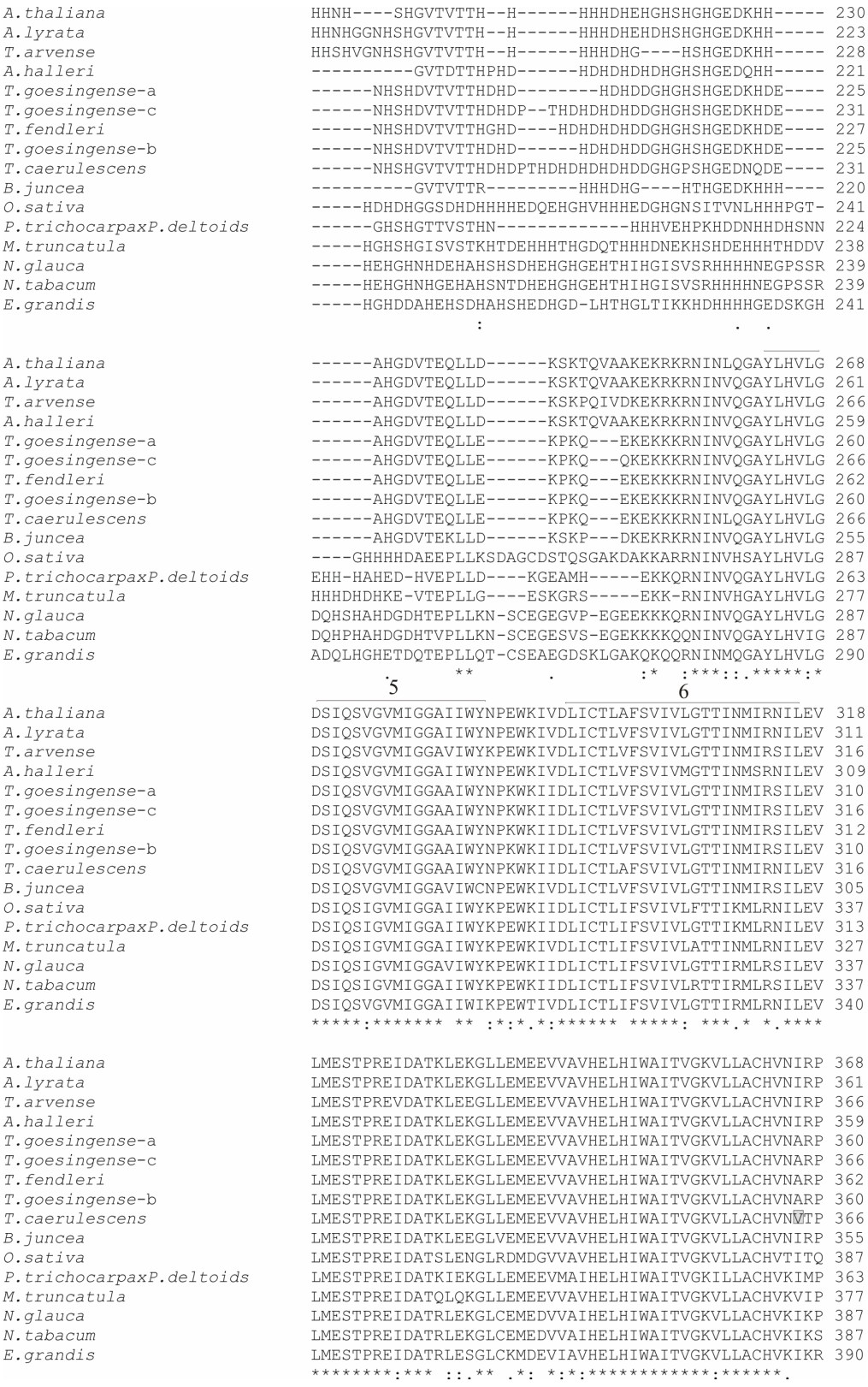
A.lyrata IVTETSEVNGFLMFLVAAFGLVVNIIMAVLLGHDHGHSHGHGHSHGH--D 186
T.arvense LLTETSEVNGFLMFLVAAFGLVVNIIMAVLLGHDHGHSHGHGHGHGHD 195
A.halleri IVTETSEVNGFLMFLVAVFGLVVNIIMAVLLGHDHGHSHGHGHSHSH--- 192
T.goesingense-a LLTETSEVNGFLMYAVATFGLLVNIIMAVMLGHDHGHSHGHGHGHDHG-- 193
T.goesingense-c LLTETSEVNGFLMFAVATFGLLVNIIMAVMLGHDHGHSHGHGHGHDHG-- 193
T.fendleri LLTETGEVDGFLMFAVATFGLLVNIVMAVMLGHDHGHSHGHGH--DHG-- 191
T.goesingense-b LLTETSEVNGFLMFAVATFGLLVNIVMAVMLGHDHGHSHGHGH--DHG-- 191
T.caerulescens LLTETSEVNGFLMFAVATFGLLVNIIMAVMLGHDHGHSHGHGH--DHE-- 191
B.juncea LLTETTEVNGFLMFLVAAFGLLVNIIMAVLLGHDHGHGHGHDH-HNHG-- 197
O.sativa LINESGEVQGSLMFAVSAFGLFVNIIMAVLLGHDHGHGHGHGHGHGHS-- 197
P.trichocarpaxP.deltoids LIHGTSEVNGFLMFLVAAFGLLVNIIMALVLGHDHGPDHDHKHGT----- 193
M.truncatula LIAGPQEVDGFLMFVVAAFGLVVNIIMALVLGHDHGHGHGHDHGHGHDH- 193
N.glauca LIHDTGEVQGFLMFVVSAFGLVVNLIMALLLGHDHGHGHGHSHGHDHG-- 194
N.tabacum LIHDTGEVQGFLMFVVSAFGLVVNLIMALLLGHDHGHGHGHGHSHGHD-- 194
E.grandis LINGTTEVHGFLMFIIAAFGLLVNIAMALLLGHDHSHGHGHDHGHGHS-- 197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T.arvense HHSHVGNHSHGVTVTTH--H------HHHDHG----HSHGEDKHH----- 228
A.halleri ----------GVTDTTHPHD------HDHDHDHDHGHSHGEDQHH----- 221
T.goesingense-a ------NHSHDVTVTTHDHD--------HDHDDGHGHSHGEDKHDE---- 225
T.goesingense-c ------NHSHDVTVTTHDHDP--THDHDHDHDDGHGHSHGEDKHDE---- 231
T.fendleri ------NHSHDVTVTTHGHD----HDHDHDHDDGHGHSHGEDKHDE---- 227
T.goesingense-b ------NHSHDVTVTTHDHD------HDHDHDDGHGHSHGEDKHDE---- 225
T.caerulescens ------NHSHGVTVTTHDHDPTHDHDHDHDHDDGHGPSHGEDNQDE---- 231
B.juncea ----------GVTVTTR---------HHHDHG----HTHGEDKHHH---- 220
O.sativa -----HDHDHGGSDHDHHHHEDQEHGHVHHHEDGHGNSITVNLHHHPGT- 241
P.trichocarpaxP.deltoids ------GHSHGTTVSTHN-------------HHHVEHPKHDDNHHDHSNN 224
M.truncatula -----HGHSHGISVSTKHTDEHHHTHGDQTHHHDNEKHSHDEHHHTHDDV 238
N.glauca -----HEHGHNHDEHAHSHSDHEHGHGEHTHIHGISVSRHHHHNEGPSSR 239
N.tabacum -----HEHGHNHGEHAHSNTDHEHGHGEHTHIHGISVSRHHHHNEGPSSR 239
E.grandis -----HGHDDAHEHSDHAHSHEDHGD-LHTHGLTIKKHDHHHHGEDSKGH 241
A.thaliana ------AHGDVTEQLLD------KSKTQVAAKEKRKRNINLQGAYLHVLG 268
A.lyrata ------AHGDVTEQLLD------KSKTQVAAKEKRKRNINVQGAYLHVLG 261
T.arvense ------AHGDVTEQLLD------KSKPQIVDKEKRKRNINVQGAYLHVLG 266
A.halleri ------AHGDVTEQLLD------KSKTQVAAKEKRKRNINVQGAYLHVLG 259
T.goesingense-a ------AHGDVTEQLLE------KPKQ---EKEKKKRNINVQGAYLHVLG 260
T.goesingense-c ------AHGDVTEQLLE------KPKQ---QKEKKKRNINVQGAYLHVLG 266
T.fendleri ------AHGDVTEQLLE------KPKQ---EKEKKKRNINVQGAYLHVLG 262
T.goesingense-b ------AHGDVTEQLLE------KPKQ---EKEKKKRNINVQGAYLHVLG 260
T.caerulescens ------AHGDVTEQLLE------KPKQ---EKEKKKRNINLQGAYLHVLG 266
B.juncea ------AHGDVTEKLLD------KSKP---DKEKRKRNINVQGAYLHVLG 255
O.sativa ----GHHHHDAEEPLLKSDAGCDSTQSGAKDAKKARRNINVHSAYLHVLG 287
P.trichocarpaxP.deltoids EHH-HAHED-HVEPLLD----KGEAMH-----EKKQRNINVQGAYLHVLG 263
M.truncatula HHHDHDHKE-VTEPLLG----ESKGRS-----EKK-RNINVHGAYLHVLG 277
N.glauca DQHSHAHDGDHTEPLLKN-SCEGEGVP-EGEEKKKQRNINVQGAYLHVLG 287
N.tabacum DQHPHAHDGDHTVPLLKN-SCEGESVS-EGEKKKKQQNINVQGAYLHVIG 287
E.grandis ADQLHGHETDQTEPLLQT-CSEAEGDSKLGAKQKQQRNINMQGAYLHVLG 290
5
6
A.thaliana DSIQSVGVMIGGAIIWYNPEWKIVDLICTLAFSVIVLGTTINMIRNILEV 318
A.lyrata DSIQSVGVMIGGAIIWYNPEWKIVDLICTLVFSVIVLGTTINMIRNILEV 311
T.arvense DSIQSVGVMIGGAVIWYKPEWKIVDLICTLVFSVIVLGTTINMIRSILEV 316
A.halleri DSIQSVGVMIGGAIIWYNPEWKIVDLICTLVFSVIVMGTTINMSRNILEV 309
T.goesingense-a DSIQSVGVMIGGAAIWYNPKWKIIDLICTLVFSVIVLGTTINMIRSILEV 310
T.goesingense-c DSIQSVGVMIGGAAIWYNPKWKIIDLICTLVFSVIVLGTTINMIRSILEV 316
T.fendleri DSIQSVGVMIGGAAIWYNPKWKIIDLICTLVFSVIVLGTTINMIRSILEV 312
T.goesingense-b DSIQSVGVMIGGAAIWYNPKWKIIDLICTLVFSVIVLGTTINMIRSILEV 310
T.caerulescens DSIQSVGVMIGGAAIWYNPKWKIIDLICTLAFSVIVLGTTINMIRNILEV 316
B.juncea DSIQSVGVMIGGAVIWCNPEWKIVDLICTLVFSVIVLGTTINMIRSILEV 305
O.sativa DSIQSIGVMIGGAIIWYKPEWKIIDLICTLIFSVIVLFTTIKMLRNILEV 337
P.trichocarpaxP.deltoids DSIQSIGVMIGGAIIWYKPEWKIIDLICTLIFSVIVLGTTIKMLRNILEV 313
M.truncatula DSIQSIGVMIGGAIIWYKPEWKIVDLICTLIFSVIVLATTINMLRNILEV 327
N.glauca DSIQSIGVMIGGAVIWYKPEWKIIDLICTLIFSVIVLGTTIRMLRSILEV 337
N.tabacum DSIQSIGVMIGGAIIWYKPEWKIIDLICTLIFSVIVLRTTIRMLRSILEV 337
E.grandis DSIQSVGVMIGGAIIWIKPEWTIVDLICTLIFSVIVLGTTIRMLRNILEV 340
A.thaliana LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNIRP 368
A.lyrata LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNIRP 361
T.arvense LMESTPREVDATKLEEGLLEMEEVVAVHELHIWAITVGKVLLACHVNIRP 366
A.halleri LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNIRP 359
T.goesingense-a LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNARP 360
T.goesingense-c LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNARP 366
T.fendleri LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNARP 362
T.goesingense-b LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNARP 360
T.caerulescens LMESTPREIDATKLEKGLLEMEEVVAVHELHIWAITVGKVLLACHVNVTP 366
B.juncea LMESTPREIDATKLEEGLVEMEEVVAVHELHIWAITVGKVLLACHVNIRP 355
O.sativa LMESTPREIDATSLENGLRDMDGVVAVHELHIWAITVGKVLLACHVTITQ 387
P.trichocarpaxP.deltoids LMESTPREIDATKIEKGLLEMEEVMAIHELHIWAITVGKILLACHVKIMP 363
M.truncatula LMESTPREIDATQLQKGLLEMEEVVAVHELHIWAITVGKVLLACHVKVIP 377
N.glauca LMESTPREIDATRLEKGLCEMEDVVAIHELHIWAITVGKVLLACHVKIKP 387
N.tabacum LMESTPREIDATRLEKGLCEMEDVVAIHELHIWAITVGKVLLACHVKIKS 387
E.grandis LMESTPREIDATRLESGLCKMDEVIAVHELHIWAITVGKVLLACHVKIKR 390

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A.thaliana                  EADADMVLNKVIDYIRREYNISHVTIQIER- 398
A.lyrata                    EADADMVLNKVIDYIRREYNISHV------- 385
T.arvense                   EADADMVLNKVIDYIRREYNISHVTIQIER- 396
A.halleri                   EADADMVLNKVMDYIRREYNISHVTIQIER- 389
T.goesingense-a             DADADMVLNKVVDYIRREYNISHVTIQIER- 390
T.goesingense-c             DADADMVLNKVVDYIRREYNISHVTIQIER- 396
T.fendleri                  EADADMVLNKVVDYIRREYNISHVTIQIER- 392
T.goesingense-b             DADADMVLNKVVDYIRREYNISHVTIQIER- 390
T.caerulescens              QADADMVLHKVVDYIRREYNISHVTIQIER- 396
B.juncea                    EADADMVLNKVIDYIRREYNISHVTIQIER- 385
O.sativa                    DADADQMLDKVIGYIKSEYNISHVTIQIERE 418
P.trichocarpaxP.deltoids    EANADMVLDNVISYLRREYNISHVTIQIER- 393
M.truncatula                EADADVMLDKVVDYIRRVHNISHVTIQIER- 407
N.glauca                    DADADTVLDKVIDYIKREYNISHVTIQIERE 418
N.tabacum                   DADADTVLDKVIDYIKREYNISHVTIQIERE 418
E.grandis                   DANADMVLDKVVDYIRREYKISHVTIQVERE 421
                            :*:** :*.:*:.*::  ::****
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Figure 4: Multiple ClustalW alignment of TcZTP1 from *Thlaspi caerulescens* compared to related proteins from other species. MTP1-like amino acid sequences were found for *Thlaspi fendleri* (AAR83905), *Thlaspi goesingense* (AAS67026), *Arabidopsis halleri* (CAD89013), *Thalspi arvense* (AY483145), *Arabidopsis thaliana* (NP_182203), *Arabidopsis lyrata* (CAG28982), *Brassica juncea* (AAO83659), *Populus trichocarpa* x *Populus deltoides (Ptd)* (AAR23528), *Nicotiana glauca* (BAD89561), *Nicotiana tabacum* (BAD89563), *Medicago truncatula* (ABE83561), *Eucalyptus grandis* (AAL25646) and *Oryza sativa* (AAU10745). Identical amino acids in all sequences are indicated with '*' below the aligned columns, ':' indicates conserved substitutions, '.' indicates semi-conserved substitutions. The membrane spanning domains for *TcZTP1* (Assunção et al., 2001) are shown as lines above the sequences. The distinct aa residues in *T. caerulescens* identified are marked in grey highlight.

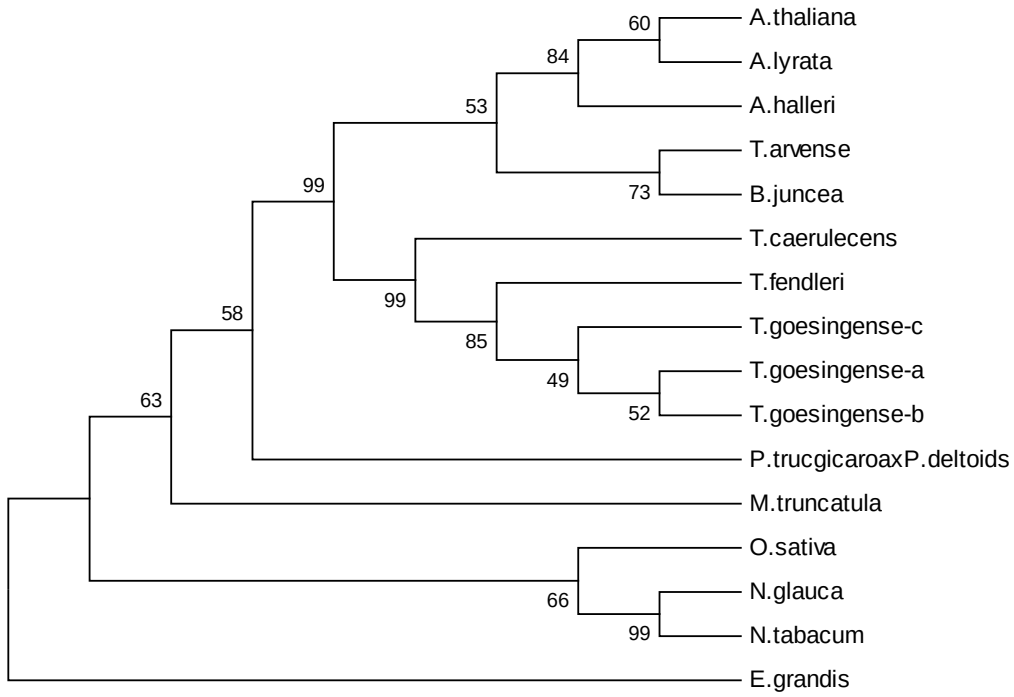

Figure 5: Phylogenetic tree based on the amino acid sequence of MTP1 of the 14 species.

Discussion

A major objective in studying metal hyperaccumulators is to identify the mechanisms that confer metal tolerance and accumulation to these species. The *TcZTP1* over-expressing plants were found to have enhanced Zn tolerance and accumulation in *A. thaliana* plants while grown on 50 μM Zn, which is comparable to the Zn level also found in Zn contaminated soil solution (Pence et al., 2000). Similar results were also found in previous studies ectopically over-expressing MTP1 proteins like AtMTP1 and PtdMTP1 in *A. thaliana,* suggesting that their normal function is most likely to create a sink for Zn in the vacuoles of plant cells in case of intracellular Zn excess (Blaudez et al., 2003; van der Zaal et al., 1999, Arrivault et al., 2006; Desbrosses-Fonrouge et al., 2005; Blaudez, et al., 2003; Kobae et al., 2004). Over-expression of *AtMTP3*, another CDF family member, also increased Zn excess tolerance in *A. thaliana* and accumulation of Zn in roots as well as in rosette leaves (Arrivault et al., 2006), similarly as we found for over-expression of *TcZTP1*. Constitutively elevated expression of *MTP1*-like genes has previously been shown to occur in the metal hyper-accumulators species *T. caerulescens, T. goesingense* and *A. halleri,* compared to the non-accumulators *T. arvense, A. thaliana* and *B. juncea* (Assunção et al., 2001; Dräger et al., 2004; Persans et al., 2001). Based on these studies, it was suggested that high expression of *MTP1* is important for especially Zn hypertolerance and accumulation (Assunção et al., 2001; Dräger et al., 2004; Persans et al., 2001). In line with this, the observation for *TcZTP1* suggests Zn hypertolerance and accumulation probably occurs through detoxification of Zn in the vacuolar compartments.

A novel observation for the function of *TcZTP1* was that the over-expression also has an effect on Cd tolerance and accumulation and on resistance to low Fe nutrition in *A. thaliana*. *T. caerulescens* is one of the few known Cd hyperaccumulating species (Baker et al., 2000), but this characteristic is not found in all accessions of the species. For instance the accession La Calamine (LC), from which we isolated the *TcZTP1* cDNA, is very tolerant to Cd, but

does not hyperaccumulate this element. This in contrast to several accessions from the south of France, which are both Cd tolerant and Cd accumulating (Assunção et al., 2003b; Reeves et al., 2001). Marqués et al. (2004) found very high and constitutive Zn tolerance and inducible Cd tolerance at the cellular level in the accession Ganges, one of the southern France *T. caerulescens* accessions, accompanied with enhanced Zn sequestration in the vacuoles. Cd sequestration was not tested. Several studies have been performed to understand the mechanism behind Cd hyperaccumulation, and the response of non-accumulators to Cd. Phytochelatin synthase (PCS) is induced in response to Cd exposure in *A. thaliana* and the accumulated phytochelatins (PCs) detoxify Cd in this non-accumulator species (Gong et al., 2003). However, PC accumulation is found not to be responsible for Cd tolerance in the tolerant accession of *T. caerulescens* (Cobbett and Goldsbrough, 2002; Ebbs et al., 2002; Schat et al., 2001). Therefore, this hyperaccumulator has developed other mechanisms for internal detoxification of Cd to shield it from metabolically active cellular sites (Clemens et al. 2002; Hall 2002). Zn was found to be sequestered predominantly in the epidermal vacuoles in the leaves of *T. caerulescens* (Küpper et al. 1999), while in another Zn-hyperaccumulator, *A. halleri*, Zn was localized in the base of trichomes as well as in mesophyll cells (Küpper et al. 2000). Thus, *TcZTP1* is likely to play a similar role in *T. caerulescens* in which it is constitutively overexpressed compared to the non-accumulators *T. arvense* and *A. thaliana* (Assunção et al., 2001; van de Mortel et al., 2006). In view of the localization of AtMTP1, AtMTP3 and PtdMTP1 to the plant vacuolar membrane, and the similar effect of *TcZTP1* over-expression on Zn/Cd tolerance and accumulation in *A. thaliana* compare to the other three constructs, we assume that also TcZTP1 is localized in the vacuolar membrane, effluxing Zn into the vacuole. The correlation between Cd tolerance and Cd accumulation in *A. thaliana* upon over-expression of this gene suggests that TcZTP1 itself is able to transport Cd into plant vacuoles. Enhanced Cd tolerance is a consequence of the ability to detoxify

more Cd than wild type plants, by storing it in vacuoles. This characteristic is not known for any other plant MTP protein, also not for AhMTP1 from *A. halleri*, which is also Cd tolerant. However in nature *A. halleri* is not Cd hyperaccumulating, which may explain the evolution of the Cd transport function of TcZTP1 in this species.

We tried to identify possible amino acid changes that can contribute to the novel characteristics with other MTPs. Surprisingly, there are several amino acid residues that are generally conserved among MTP1-like proteins, but which are different in TcZTP1 (Figure 4). Studies by Rogers et al. (2000) identified a number of conserved residues near or in the transmembrane domains that appear to be essential for all transport function and important for substrate selection, in AtIRT1, by heterologous expression in yeast. Amino acid substitution experiments are necessary to confirm a crucial role of these altered "conserved" amino acids in Cd transport.

The mechanisms that control Fe, Mn content in wild type and over-expression plants exposed to Cd, are not completely understood. Cd toxicity can be caused by improper binding of Cd in the essential enzymes in the plant. The higher Mn content in shoots and Fe content in the roots in the wild type and over-expression lines may reflect the response of the plants to the toxic concentration of Cd. Fe and Mn are known as important redox elements for photosynthesis. This is why probably Mn is higher in the shoots to protect the plants from the unwanted substitution of the essential photosynthetic enzymes. Fe is a key component in a range of enzymes in the plant, for which probably the plant tries to protect itself from the Cd exposure.

The same *TcZTP1* over-expression lines were found to be more tolerant to grow on media with low (0.5 µM) Fe nutrition compared to wild type (Figure 3). Several studies have been done to understand the effects on *A. thaliana* and other species when they are supplied with low Fe (Connolly et al., 2003; Vert et al., 2002). Fe is an essential element for plant and when plants are grown under low Fe supply, plants suffer from chlorosis of leaves and retardation in

growth (Varotto et al., 2002; Connolly et al., 2002). Fe limitation, either due to actual soil deficiency or due to reduced bioavailability at neutral or basic pH, causing many agricultural problems. This problem can be reduced when plants are more tolerant to low Fe (Guerinot and Yi, 1994). In this study, the transgenic plants had higher Zn content compared to wild-type plants. Previously we found that Zn and Fe content are often correlated in (wild-type) A. *thaliana* (van de Mortel et al., 2006). Under Fe deficiency, the increased demand for Fe induces expression of high affinity Fe transporters like *AtIRT1* and *AtIRT2* in *A. thaliana*, which are low affinity Zn transporters (Korshunova et al., 1999; Vert et al., 2001). This causes inadvertent increased uptake of Zn without any increase in capacity to store for maintenance of Zn (and Fe) homeostasis. *TcZTP1* overexpression provides extra Zn storage capacity in vacuoles, thus improving the ability to withstand Fe deficiency conditions much better than wild-type plants. This additional effect of *TcZTP1* overexpression, and probably that of other *MTP1*-like genes as well, can be interesting for phytoremediation and Fe efficiency application improvements. When there is severe soil Fe deficiency, over-expression of *MTP1* genes will probably not be so effective, as the plants will not be able to compensate the Fe deficiency by enhancing uptake capacity and thus lack of Fe will be limiting growth.

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Chapter 6

General discussion

Pollution due to metals is a global environmental problem that has resulted from mining, industrial, agricultural and military practices (Nriagu and Pacyna, 1988). Soil and water contamination by heavy metals is widespread in Eastern Europe, and is increasingly recognized as dramatic in large parts of the developing world, especially in India (Meharg, 2004) and China (Cheng, 2003). Many pollutants accumulate in the food chain and threaten human health (Krämer, 2005).

A great interest has developed recently in the use of terrestrial plants as a green technology for the remediation of contaminated soils with toxic heavy metals, which may help to reduce or even reverse these pollution problems (Pence et al., 2000). This developed a new field of environmental biotechnology, termed phytoremediation, which uses plants to extract heavy metals from the soil and to concentrate them in the harvestable shoot tissue (Salt et al., 1995).

Several criteria must be met before a plant is considered to be well-suited for phytoremediation (Song et al., 2003). An 'ideal' phytoremediator should be fast growing, develop a large biomass, be tolerant to and accumulate high concentrations of toxic metals in the shoot, and be easily cultivated and harvested. Although natural hyperaccumulators can tolerate and accumulate high concentration of toxic metals, they usually produce little biomass, they grow slowly and cannot be easily cultivated. Now with the advent of molecular biology, scientists can use genetic engineering to improve the metal accumulation capacity of fast-growing and high-biomass plants.

Several researchers have focused their attention on various metal hyperaccumulator species, including *Thlaspi caerulescens. T. caerulescens* has been proposed as a very convenient model plant species, to study the molecular physiology of this research field (Assunção *et al*., 2003). With the *A. thaliana* genome sequence now available, focus on closely related metal hyperaccumulator species opened up the powerful approaches of both genetics and genomics, to study the mechanism of metal hyperaccumulation and tolerance in plants. In addition, self-

fertility, good seed setting and competence for transformation at least with *Agrobacterium rhizogenes* in *T. caerulescens* made it the model of choice.

In this thesis, the reasons for metal hyperaccumulation of *T. caerulescens* were investigated, focusing mainly on functional characterization of three Zn transporters. The genes *TcZTP1*, *TcZNT1* and *TcZNT2* were reported to be highly and constitutively expressed in *T. caerulescens*, regardless of the Zn status of the plant (Assunção et al., 2003). The structural and functional analyses of the members of these Zn transporters could provide us clues about the molecular bases underlying the transport mechanism of CDF and ZIP family members. In addition, this research is likely to catalyze a rapid progress towards engineering of an 'ideal' hyperaccumulator.

In this study, the non-accumulator model plant *A. thaliana* was chosen as the reference plant. The study by Rigola et al. (2006) showed an average of 88.5% sequence identity between *T. caerulescens* and *A. thaliana* in transcribed sequences. This is in agreement with the analysis of Peer et al. (2006), who found 87–88% sequence identity for the intergenic transcribed spacer regions when comparing several *Thlaspi* species with *Arabidopsis*. Based on this relative strong homology and available genetic resources, *A. thaliana* is an obvious choice as comparative non-accumulator species (Freeman et al., 2004). This allows comparisons to be made between hyperaccumulator and non-accumulator genes by over-expressing them in a non-accumulator (Freeman et al., 2004).

At least three different engineering approaches to enhance metal uptake can be envisioned (Clemens 2002). (i) The number of uptake sites could be increased, (ii) the specificity of uptake systems could be altered, so that the competition among the unwanted cations is reduced, and (iii) the sequestration capacity could be enhanced by increasing the number of intracellular high-affinity binding sites or the rates of transport into organelles (Clemens 2002).

The study described in this thesis dealing with the over-expression of *TcZTP1*, *TcZNT1* and *TcZNT2* in *A. thaliana* brought molecular insights into the methods to be applied for improving phytoremediation technologies. *TcZNT1* is an uptake transporter, localized in the plasma membrane and its over-expression in *A. thaliana* results in a higher uptake of Zn and Cd into the plant, compared to its wild type (chapter 3). Because no significant effects could be detected in *TcZNT2* overexpressors, this gene could have only a minor or no role in Zn transport.

Because free Zn ions in the cytosol are toxic, plants are stressed when they are exposed to high Zn and when they accumulate high Zn concentrations. This can be improved when the non-accumulator is engineered to cope better with the accumulated metal ions better. The over-expression of *TcZTP1* in *A. thaliana* shows three-to-five fold higher Zn content and the plants were much more tolerant compared to wild type, suggesting improvement in the sequestration of the metals into plant storage organelles like vacuoles (chapter 5). Hence, if the plants are modified to be equipped with mechanisms for proper storage of the metal ions, preceded by increased uptake system; it can make the plant more suitable as phytoremediator. For this reason combining overexpression of the sequestration gene *TcZTP1* with overexpression of uptake genes such as *ZNT1* in the same plant, might increase both the uptake and sequestration in the plant.

Improving the efficiency of metal uptake of a plant is another goal in relation to improvement of plant nutrition. This can help a plant to survive in Zn/Fe deficient soil, which is an important agricultural problem in this world. Both *A. thaliana* and *T. caerulescens* are sensitive for Zn deficiency (chapter 2) and therefore can act as a suitable model to study this phenomenon and to find solutions. *TcZTP1* over-expression in *A. thaliana* plants resulted in a higher amount of Zn when plants were grown on Fe-deficient media, whereas the Fe content was not different from wild type (chapter 5). This finding could help in the engineering of Fe-

efficient plants using this and other Zn transport genes. Thus, the same plant variety can be used for different purposes: cleaning up the soil in Zn polluted soils and an efficient metal uptake from Fe deficient soils.

Furthermore, substrate specificity of the transporters could be increased by engineering the genes, as proposed by Clemens et al. (2002). It would be also useful to increase the specificity of the transporters, in such a way that it would uptake or sequester more than one metal. For example, Zn can be taken up together with other toxic elements, like Cd, collectively depending on the human need. In our study, we have shown that over-expressors of both *TcZNT1* and *TcZTP1* in *A. thaliana* mediate highly efficient uptake and sequestration of both Zn and Cd. This would be useful for practical purposes in phytoextraction of both Zn and Cd using the same plant. To our knowledge this is the first study to show a CDF family member to transport both Zn and Cd. The over-expression lines of *TcZTP1*, *TcZNT1* or *TcZNT2* in *A. thaliana* did not show any effect for other metals like Ni, Co, Cu, suggesting these transporters are specific for Zn and Cd.

The sequences of MTP1 (Metal Transporter Proteins) genes belonging to CDF family, including *TcZTP1*, from 13 related species of *T. caerulescens* has been analysed, in order to study the conservation of the amino acids in the proteins (chapter 5). Compared to 13 other species, several amino acids were found to be unique in the *TcZTP1* protein sequence, suggesting *TcZTP1* as a unique transporter among the group. These amino acids can be studied further, to see the specific roles of these residues in the functions of *TcZTP1*. *AtIRT1* was studied by substituting various conserved residues with alanine. This produces mutant versions of *AtIRT1*, which apparently no longer could transport Fe^{2+} and Mn^{2+}, yet retained Zn^{2+} and Cd^{2+} transport activity (Rogers et al., 2000). Similarly, the conserved and the distinct amino acids in *T. caerulescens* and other related species can be studied using site directed mutagenesis to find the essential amino acids for the transporters.

In addition, the regulation of the Zn transporters from hyperaccumulator *T. caerulescens* and non-accumulator *A. thaliana* was studied in this thesis (chapter 4). *AtZIP4pro-GUS* transformed *A. thaliana* plants were tested when grown on Zn-deficiency and Zn-sufficiency media. The Zn-sufficient plants did not show GUS staining, but the Zn-deficient plants showed staining in different tissues indicating that these genes are Zn induced. Strong *AtZIP4pro-GUS* expression was found in the endodermis of the roots, of the transgenic *A. thaliana* plants, suggesting that *AtZIP4* has a role in Zn transport in roots. *AtZIP4-GUS* staining observed in the Zn-deficient leaves and trichomes, suggests supply and storage possibility of Zn in these tissues. Interestingly, the GUS localisation in the flower is developmental stage specific. Strong staining within flowers was observed in the pistil in all developmental stages, except in the fully developed flower. In the completely developed flowers, the anther filaments were typically stained. Siliques were stained distinctly at the bottom and at the top. This study shows *AtZIP4* gene could function in almost every tissue, especially the reproductive tissues, including the seeds of *A. thaliana*, suggesting an important role for Zn translocation and storage in the end product of a plant. In nature, the role of every species is to pass on its genes to the next generation. Apparently, even when the plant is Zn-deficient, it will try to supply the crucial microelement Zn, required for DNA replication, at least to the reproductive tissues, to make reproduction possible. This information can be used further for improvement of Zn storage in the seeds or other tissues requiring high Zn content.

The expression pattern of the *AtZIP4* from non-accumulator *A. thaliana* and *TcZNT1* from *T. caerulescens*, was studied using reporter GUS constructs and the *cis* elements of these genes were searched for. Transforming the *TcZNT1 promoter-GUS* from the hyperaccumulator and *AtZIP4promoter-GUS* of *A. thaliana* into the non-accumulator *A. thaliana* showed a similar expression pattern. This reveals that *cis* elements in both the related species are similar and can be recognized in a similar way by the *A. thaliana* transcription factors. The *cis* element(s)

of *AtZIP4* are located between -263 bp and -115 bp from the start codon and for *TcZNT1* between -223 bp and -98 bp. Further characterization of the promoters to find the specific bases which are essential for the *cis-trans* interaction can be done by point mutations in the *cis* elements found in this study. The knowledge of the *cis* elements of the *AtZIP4* and *TcZNT1* promoters can also be used for searching of the corresponding trans-acting elements that should interact with these promoters. Change in spatial and temporal expression of the transcription factor genes may control the expression patterns of down-stream target genes. In this case, it looks that the expression or action of the transcriptional elements makes the difference between the hyperaccumulator and the non-accumulator. The expression profile of the transcription factors in the hyperaccumulator plants may have changed, in order to adapt to the highly contaminated soil as a new niche. This might have led to the constitutive over-expression of the genes like *TcZTP1* and *TcZNT1* or *TcZNT2*, and subsequent higher uptake or sequestration of the metals in the plant. However, not all over-expressed genes might have a functional role (or limiting factor) in accumulating heavy metals.

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Summary

Heavy metal hyperaccumulation in plants is a poorly understood phenomenon. Transmembrane metal transporters are assumed to play a key role in this process. In the research described in this thesis, genes encoding Zn transporters of *Thlaspi caerulescens*, a heavy metal hyperaccumulator plant, are studied and compared to their orthologues in *Arabidopsis thaliana*, a non-hyperaccumulator plant.

The *TcZNT1* and *TcZNT2* genes of *T. caerulescens* are members of the Zrt, Irt (*ZIP)*-like gene family and show 89% and 87% similarity with the *AtZIP4* and *AtIRT3* genes of *A. thaliana*, respectively. *AtZIP4* and *AtIRT3* genes are induced exclusively by Zn-deficiency in Arabidopsis. In limiting Zn conditions both *Arabidopsis* and *Thlaspi* show characteristic symptoms of Zn deficiency manifested both in vegetative and reproductive tissues. However, in *T. caerulescens*, *TcZNT1* and *TcZNT2* are constitutively and highly expressed in roots, irrespective of the Zn concentration in the medium. This observation suggested a role for these genes in Zn uptake and possibly in explaining the Zn hyperaccumulation trait in *Thlaspi*. The proteins are localized in the plasma membrane, conferring zinc uptake into the cytoplasm. *TcZTP1,* another Zn transporter gene shows 85% sequence similarity in its coding region with the *AtZAT*/*MTP1* of *A. thaliana* and is assumed to be localized in the vacuolar membrane. This gene belongs to the Cation Diffusion Facilitator (CDF) family.

The responses of the over-expression lines of *TcZNT1*, *TcZNT2* and *TcZTP1* in *A. thaliana* were investigated with respect to deficiency and toxicity of Zn, Fe and Cd. Over-expression of *TcZNT1* showed higher Zn accumulation in *A. thaliana*, whereas this effect was small and not significant for *TcZNT2*. *TcZNT1* over-expression lines also showed increased Cd accumulation. In addition, the *TcZNT1*, *TcZNT2 and TcZNT1/TcZNT2* silenced *T. caerulescens* showed reduced Zn content in roots. These results, together with the plasma membrane localisation of *TcZNT1* and *TcZNT2* in cowpea protoplast, suggest a role in metal uptake of these two proteins in the plant. Over-expression of the *TcZTP1* confers Zn and Cd tolerance and accumulation in *A. thaliana.* The regulation of expression of these genes was studied in comparison to the orthologous genes in *A. thaliana* by the study of the *TcZNT1* and *AtZIP4* promoter. The *TcZNT1* promoter was isolated by PCR using forward primers designed on the *A. thaliana* gene upstream of *AtZIP4* and a reverse primer on the *T. caerulescens TcZNT1* cDNA). The promoters of *ZIP4* orthologues were isolated by a similar method from *Arabidopsis halleri, Arabidopsis lyrata and Cochleria pyrenaica.* The sequence analysis performed on the *ZIP4* promoters shows the presence of two conserved palindromic

sequences in all five species, suggesting that these are essential promoter elements for both the hyperaccumulators and non-accumulators. Using (deleted) promoter: GUS fusions transformed to *A. thaliana*, the cis elements of *AtZIP4* were identified between -263 bp and -115 bp from the start codon and for *TcZNT1* between -223 bp and -98 bp by 5' deletion study of the promoters. These regions also contain the two palindromic sequences in both promoters, indicating the importance of these conserved palindromes in the hyperaccumulators and non-accumulators. The *AtZIP4pro-GUS* transformed *A. thaliana* plants showed high expression in Zn-deficient roots, shoots, flowers and seeds. Similar expression pattern was observed in *TcZNT1pro-GUS* transformed *A. thaliana* plants. This suggests both the cis elements in the *AtZIP4* and *TcZNT1* promoters are conserved. To explain the high expression of *TcZNT1* in *T. caerulescens*, the mode of cis/trans interaction and/or the expression profiles of the trans genes in both species need to be studied. Most likely, the expression profile of the transcription factors and consequently many other genes functioning in metal uptake, transport and sequestration have been changed, in the hyperaccumulator plants as an adaptation strategy to survive in the highly contaminated soil. The knowledge obtained can be used to design strategies to generate genetically engineered plants that can be used for phytoremediation and/or that have an improved nutritional quality.

Samenvatting

Accumulatie van zware metalen is een fenomeen waarover nog weinig bekend is. Transmembraan metaaltransporteiwitten lijken een belangrijke rol te spelen in dit proces. In het onderzoek beschreven in dit proefschrift zijn Zn transporteiwitten onderzocht van *Thlaspi caerulescens*, een plantensoort die zware metalen hyperaccumuleert, en vergeleken met overeenkomstige genen uit *Arabidopsis thaliana*, een niet-hyperaccumulerende plantensoort.

De *TcZNT1* en *TcZNT2* genen van *T. caerulescens* maken onderdeel uit van de Zrt, Irt (*ZIP*) familie van Zn transporteiwitten. Ze zijn 89% en 87% identiek met respectievelijk de *AtZIP4* en *AtIRT3* genen van *A. thaliana*. De expressie van *AtZIP4* en *AtIRT3* genen wordt uitsluitend geïnduceerd door Zn-deficiëntie. De bijbehorende eiwitten bevinden zich in de plasmamembraan van de cel, waarbij ze zorgen voor zinkopname in het cytoplasma. Onder Zn-beperkte omstandigheden laten zowel *Arabidopsis* als *Thlaspi* verschijnselen van Zn-deficiëntie zien, zichtbaar aan zowel vegetatieve als generatieve weefsels. In *T. caerulescens* komen de *TcZNT1* en *TcZNT2* genen constitutief en hoog tot expressie in wortels onafhankelijk van de Zn concentratie in het medium. Deze observatie suggereert een rol voor deze genen in de opname van Zn, en is een mogelijke verklaring voor de Zn-hyperaccumulatie eigenschap van *Thlaspi*.

Het *TcZTP1* gen, coderend voor een ander Zn transporteiwit van *T. caerulescens*, is 85% identiek aan het *AtZAT/MTP1* gen van *A. thaliana* in het eiwitcoderende gebied van het gen. Dit transporteiwit bevindt zich hoogstwaarschijnlijk in het vacuolemembraan van de cel. Het *TcZTP1* gen behoort tot de familie van “Cation Diffusion Facilitator” (CDF) genen.

De respons van *A. thaliana*-lijnen die de *TcZNT1*, *TcZNT2* of *TcZTP1* genen tot overexpressie brengen zijn onderzocht voor wat betreft gevoeligheid voor Zn-, Fe- en Cd-deficiëntie en Zn- en Cd-overmaat. Overexpressie van *TcZNT1* zorgde voor een hogere Zn-accumulatie in *A. thaliana* vergeleken met wildtype planten. Het effect van overexpressie van *TcZNT2* was daarentegen gering en niet significant. Lijnen met overexpressie van *TcZNT1* lieten ook een verhoogde Cd-accumulatie zien. Daarnaast lieten *T. caerulescens* planten waarin de expressie van *TcZNT1*, *TcZNT2* of *TcZNT1/TcZNT2* in de wortels sterk gereduceerd werd, een verlaging van het Zn-gehalte in de wortels zien. Deze resultaten, gevoegd bij de plasmamembraanlokalisatie van TcZNT1 en TcZNT2 zoals aangetoond in ogenboonprotoplasten, suggereren een rol van deze twee eiwitten bij metaalopname in de plant. Overexpressie van *TcZTP1* zorgt voor verhoogde Zn- en Cd-tolerantie en -accumulatie in *A. thaliana* vergeleken met wildtype planten.

De regulatie van expressie van deze genen is bestudeerd aan de hand van promotorvergelijkingen van de *TcZNT1* en *AtZIP4* genen. De *TcZNT1* promotor werd geïsoleerd door middel van PCR, waarbij primers gebruikt zijn deels ontworpen voor het *A. thaliana* gen dat stroomopwaarts van het *AtZIP4* gen ligt, en deels ontworpen op basis van de cDNA-volgorde van het *TcZNT1* gen van *T. caerulescens*. Op een soortgelijke manier zijn ook de promotors van de *ZIP4* genen van *Arabidopsis halleri, Arabidopsis lyrata* en *Cochlearia pyrenaica* geïsoleerd. De analyse van de DNA-volgorde van de verschillende *ZIP4* promotors toonde de aanwezigheid aan van twee geconserveerde palindrome DNA motieven in alle vijf de promotors. Dit suggereert dat dit essentiële promotorelementen zijn voor zowel hyperaccumulator- als niet-accumulatorsoorten. Door middel van 5' promotordeletie studies in promotor-GUS fusieconstructen zijn de *cis* elementen van de *AtZIP4* (tussen de posities -263 en -115 baseparen (bp) vóór het translatie startcodon), en *TcZNT1* (tussen de posities -223 en -98 bp) bepaald. Deze regio's bevatten ook beide geconserveerde palindrome motieven, hetgeen het belang van deze motieven voor de expressie van *ZIP4/ZNT1* genen in hyperaccumulator en niet-accumulator soorten verder onderschrijft. *A. thaliana* planten die getransformeerd waren met het *AtZIP4pro-GUS* construct lieten hoge expressie zien in Zn-deficiënte wortels, scheuten, bloemen en zaden. Vergelijkbare expressie werd gezien in *A. thaliana* planten getransformeerd met het *TcZNT1pro-GUS* construct. Dit suggereert dat de *cis* elementen in beide promoters geconserveerd zijn. Om de hoge expressie van *TcZNT1* in *T. caerulescens* afdoende te verklaren is het nodig om de interactie tussen *cis* en *trans* elementen en/of het expressie profiel van *trans*-werkende transcriptiefactorgenen te bestuderen. Meest waarschijnlijk is dat het expressieprofiel van de transcriptiefactoren in de hyperaccumulatorplanten veranderd is, en als gevolg daarvan de expressie van veel andere genen betrokken bij metaalopname, -transport en –verdeling over de plant, als een aanpassing voor overleving in sterk vervuilde grond.

Naar verwachting kan de kennis die in het beschreven onderzoek verkregen is gebruikt worden om genetische gemodificeerde planten te ontwikkelen die gebruikt kunnen worden voor fytoremediatie van met Zn en Cd vervuilde grond. Daarnaast kan deze kennis ook gebruikt worden om de Zn-mineraalgehaltes van planten te verhogen waardoor de voedingswaarde stijgt.

Acknowledgement: Dr. Mark Aarts for translation

সার-সংক্ষেপ

ভারী ধাতু সঞ্চয়কারী উদ্ভিদ কি ভাবে ভারী ধাতু গ্রহণ ও সঞ্চয় করে, সে সম্পর্কে বিশেষ কিছুই জানা নেই| একথা মনে করা হয় যে কিছু কোষ-পর্দা পরিব্যাপী ধাতু পরিবাহী প্রোটীন এই প্রক্রিয়ায় মুখ্য ভূমিকা গ্রহণ করে| এই গবেষণা পত্রে বিবৃত কাজের জন্য *Thlaspi caerulescens*, একটি ভারী ধাতু সঞ্চয়কারী উদ্ভিদের সম্ভাব্য জিঙ্ক (Zn) পরিবহনকারী জিন গুলিকে পরিক্ষা করা হয়েছে এবং তাদের *Arabidopsis thaliana*-র, একই পরিবারভুক্ত জিনের সঙ্গে তুলনা করা হয়েছে| *Arabidopsis thaliana* ভারী ধাতু সঞ্চয় করতে পারে না|

Thlaspi caerulescens -এর একটি জিন, *TcZNT1*, কে যখন কৃত্রিম ভাবে অতিরিক্ত পরিমাণে *Arabidopsis thaliana*-র মধ্যে তৈরী করা হয়, তখন *Arabidopsis thaliana* স্বাভাবিকের তুলনায় বেশী জিঙ্ক ও ক্যাডমিয়াম গ্রহণ ও সঞ্চয় করে| এছাড়া, *Thlaspi caerulescens* এর মধ্যে যখন কৃত্রিম ভাবে *TcZNT1*, *TcZNT2* এবং *TcZNT1/TcZNT2* জিনগুলিকে নিষ্ক্রিয় করে দেওয়া হয়, তখন *Thlaspi caerulescens* এর মূলে স্বাভাবিকের তুলনায় কম জিঙ্ক পাওয়া যায়|

উপরোক্ত পর্যবেক্ষণ দুটি *TcZNT1* এবং *TcZNT2* এর ধাতু পরিবহনকারী ভূমিকাকে সমর্থন করে| এই গবেষণা থেকে পাওয়া আরও একটি পর্যবেক্ষণ হল, *TcZNT1* এবং *TcZNT2* এর কোষ-পর্দা পরিব্যাপী অবস্থান যা কোষের বাইরে থেকে ভিতরে ধাতু পরিবহন করার জন্য জরুরী|

উপরোক্ত জিন দুটিকে নিয়ে পরীক্ষা করার জন্য *TcZNT1* ও *AtZIP4* জিনের প্রোমোটার অঞ্চলের ভূমিকার তুলনা করা হয়েছে প্রোমোটার এর গুরুত্বপূর্ণ অঞ্চল (*cis* elements) আবিষ্কারের উদ্দেশ্যে| পরীক্ষায় দেখা গেছে যে *AtZIP4* এর প্রোমোটার এর পূর্বসুরী গুরুত্বপূর্ণ অঞ্চল প্রোটীন শুরু হওয়ার আগে ২৬৩ থেকে ১১৫ নিউক্লিওটাইডের মধ্যে অবস্থিত (between -263 & -115 bp from the start codon)| একইভাবে, *TcZNT1* এর প্রোমোটার এর পূর্বসুরী গুরুত্বপূর্ণ অঞ্চল প্রোটীন শুরু হওয়ার আগে ২২৩ থেকে ৯৮ নিউক্লিওটাইডের মধ্যে অবস্থিত (between -223 & -98 bp from the start codon)| এই পর্যবেক্ষণ দুটির থেকে ধারণা করা যায় যে, *AtZIP4* ও *TcZNT1* এর প্রোমোটারের গুরুত্বপূর্ণ অঞ্চল (*cis* elements)দুটি সংরক্ষিত|

এই গবেষণায় লব্ধ ফল থেকে ভবিষ্যতে কৃত্রিম জিন সম্বলিত উদ্ভিদ (genetically engineered plant) তৈরী করার আশা আছে যারা মাটি থেকে জিঙ্ক ও ক্যাডমিয়াম এর মত ক্ষতিকর মৌল সরাতে (Phytoremediation) সাহায্য করতে পারে| এছাড়াও, এরা উদ্ভিদের সঞ্চিত জিঙ্কের পরিমাণ বাড়াতে পারে যাতে উদ্ভিদের খাদ্যগুণ বৃদ্ধি পাবে|

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Coming to my project members I can keep on writing another thesis, but to be short I had so much fun with you all, that only reminds me my childhood….laughing without any reason!!! I am glad that I could mingle with some other crazy people like me, from different parts of Europe and was lucky to meet you all☺. I am grateful to be part of the EU METALHOME project for all the presentations and scientific discussions/courses which helped me grow in this project. I would specially thank Kerstin Richau from Germany and Aleksandra Trampczynka from Poland, who are now one of my best friends. I would like to thank specially Andrew Smith, Henk Schat, Sebastien Thomine, Pierre Saumitou-Laparde and Ronald Oomen for the scientific helps in my project.

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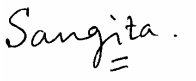
Love you all☺



Curriculum vitae

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1998: B.Sc. in Chemistry (Honours), Physics & Mathematics from University of Calcutta, India.

2000: M.Sc. in Bio-Chemistry from University of Calcutta, India.

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2001-2: Worked in Inforev technologies limited, India, as a biological programmer

2002: Worked as a project assistant in Human Genetics & Genomics of Indian Institute of Chemical Biology division, Kolkata, India.

2003: Started PhD in Genetics department, Wageningen University, as a part of EU METALHOME Research Training Network of 8 European countries. This resulted in this thesis "Functional characterisation of three zinc transporters in *Thlaspi caerulescens*" (Feb, 2003-Feb, 2007).

Publication:

Mukhopadhyay A, **Talukdar S**, Bhattacharjee A and Ray K (2004): Bioinformatic approaches for identification and characterization of olfactomedin related genes with a potential role in pathogenesis of ocular disorders, *Molecular Vision*, 10, 304-314. (http://www.molvis.org/molvis/v10/a39/).

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Talukdar S and Aarts MGM: Metal homeostasis in hyperaccumulator plants (*submitted*)

Talukdar S and Aarts MGM: Physiological study of *Arabidopsis thaliana* and *Thlaspi caerulescens* in response to zinc (*submitted*)

Talukdar S and Aarts MGM: Over-expression of the Cation Diffusion Facilitator gene *ZTP1* of *Thlaspi caerulescens* confers Zn and Cd tolerance and accumulation to *Arabidopsis thaliana* (*submitted*)

Talukdar S, Rietberg C, Schat H, Aarts MGM: Over-expression of the *Thlaspi caerulescens* ZIP family transporters *ZNT1*, but not *ZNT2*, confers higher Zn and Cd accumulation to *Arabidopsis thaliana* (*to be submitted*)

Talukdar S, Mlynárová L, Aarts MGM: A comparative promoter study of *ZIP4*-like transporter genes of *Arabidopsis thaliana* and *Thlaspi caerulescens* (*to be submitted*)

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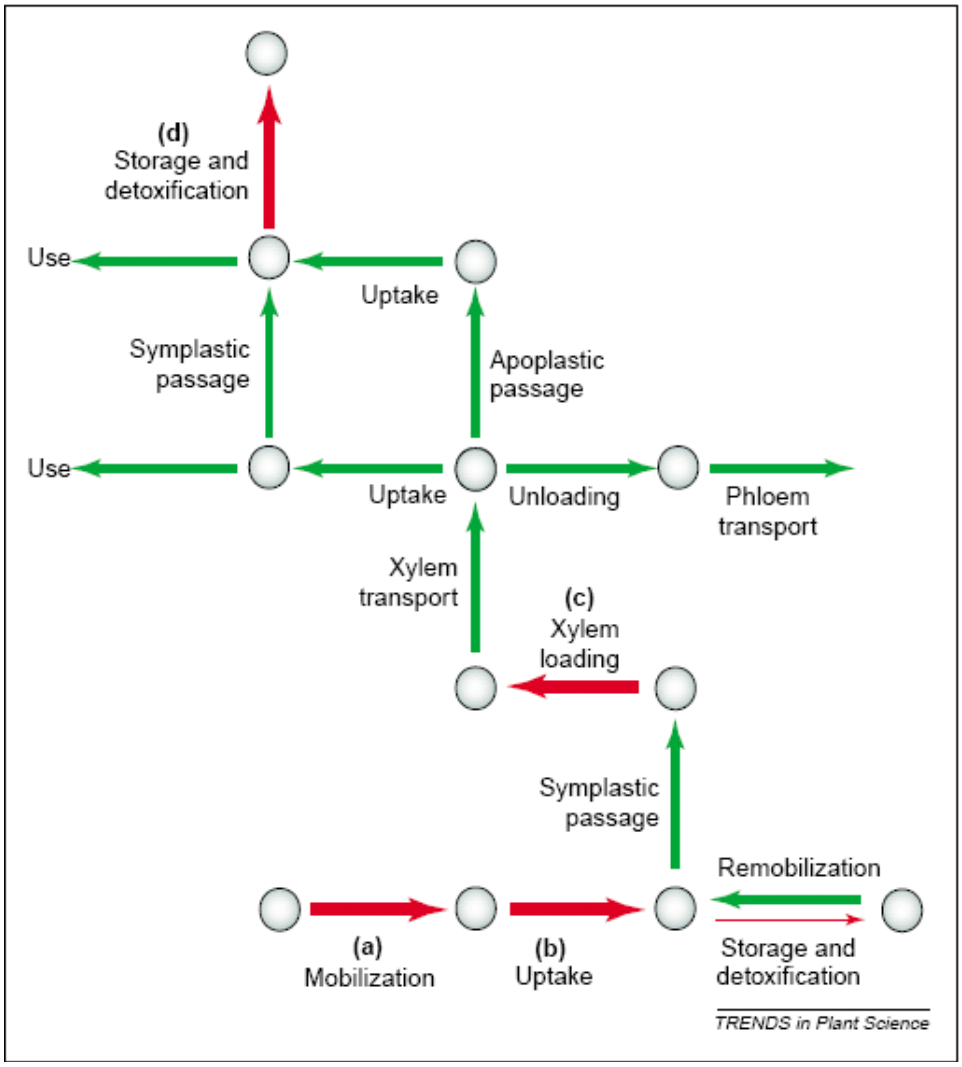
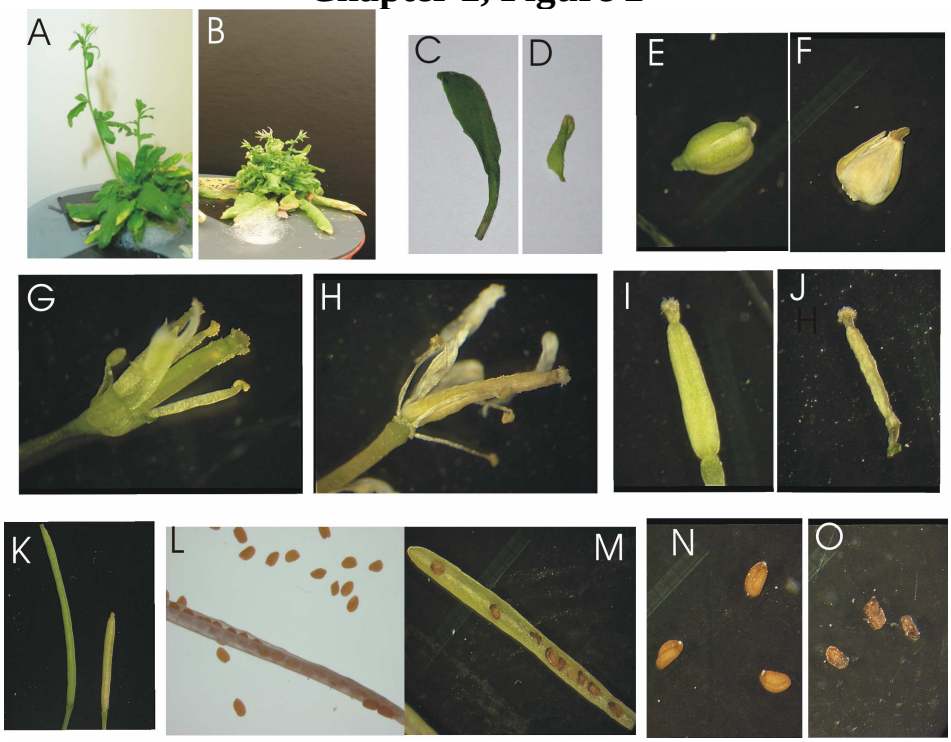
Chapter 1, Figure 1



Chapter 2, Figure 2



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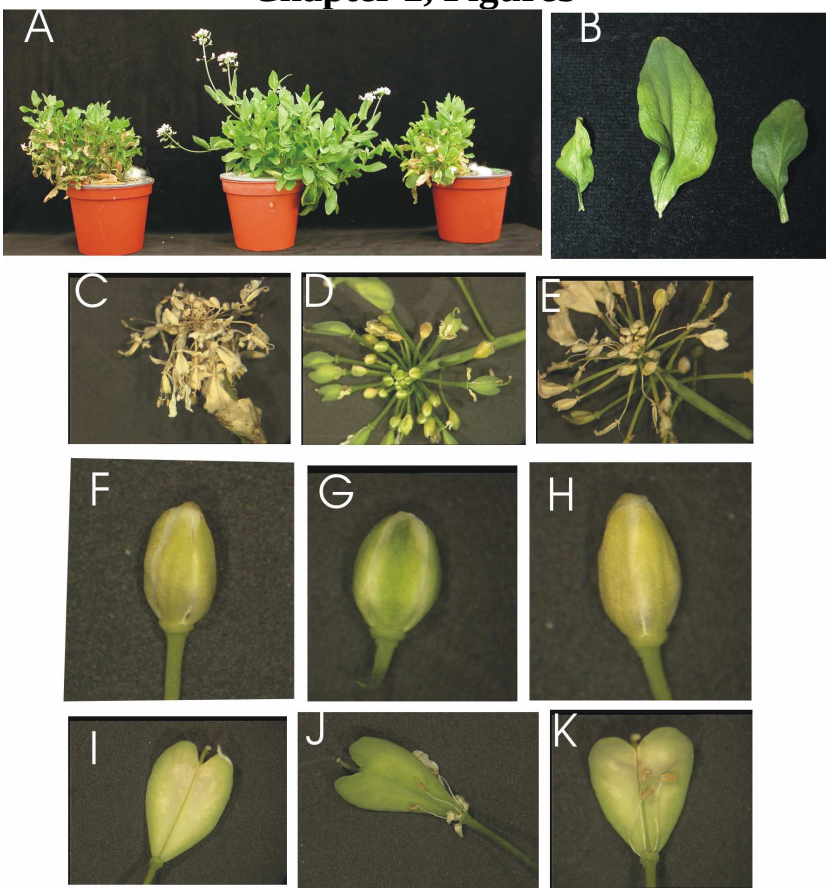
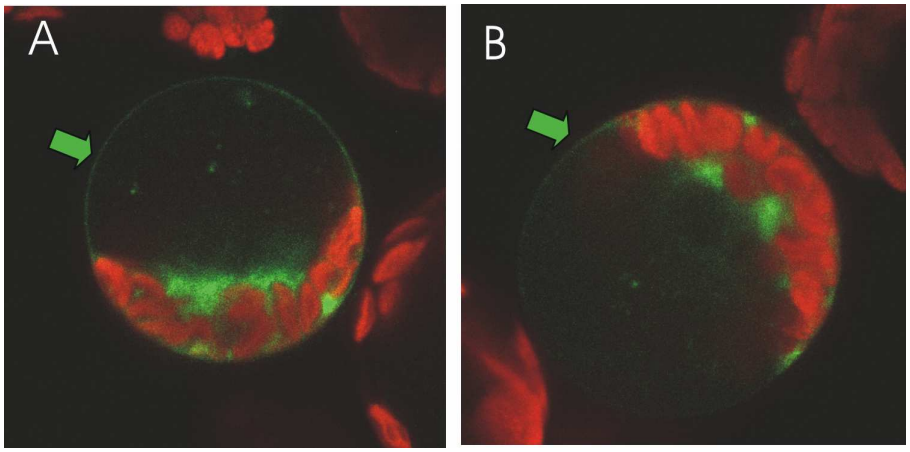
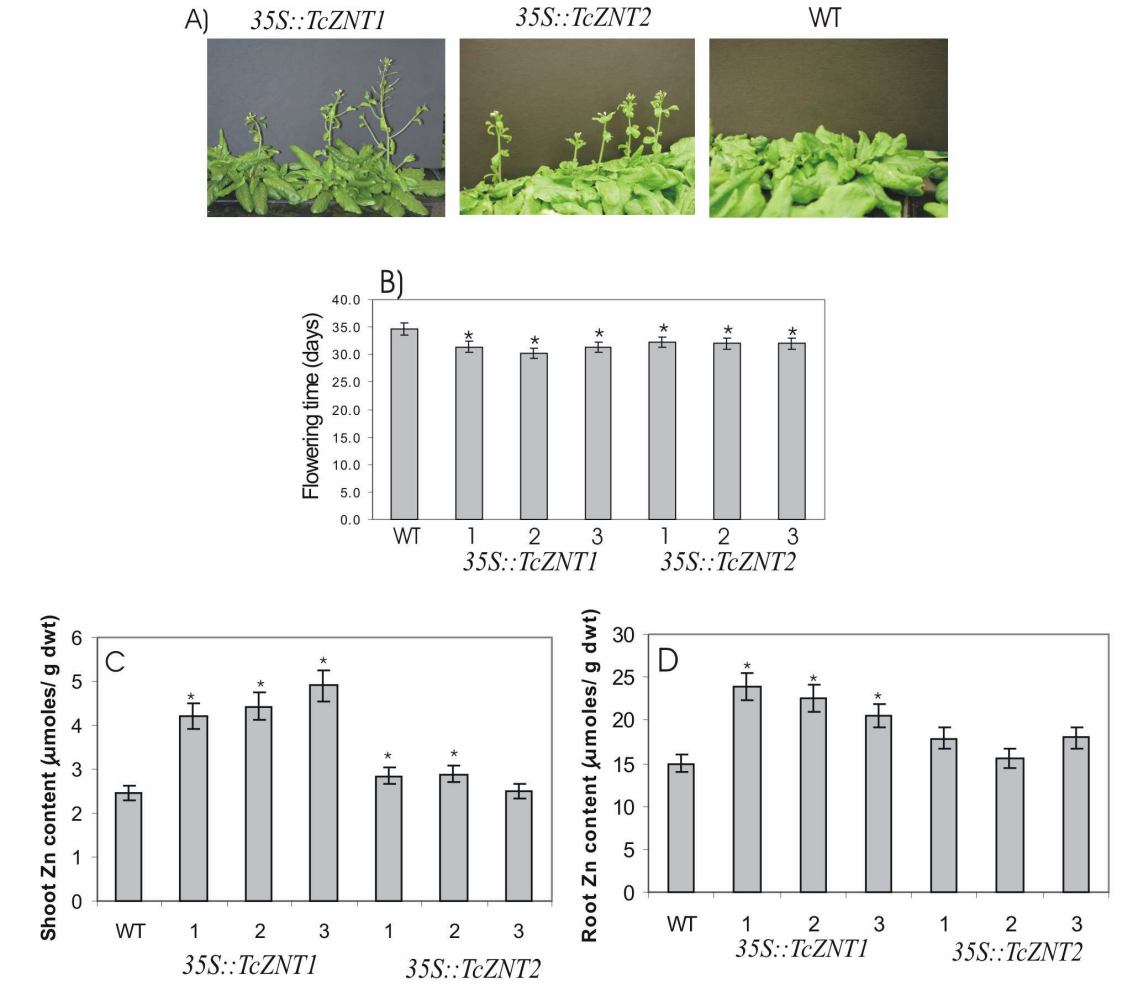


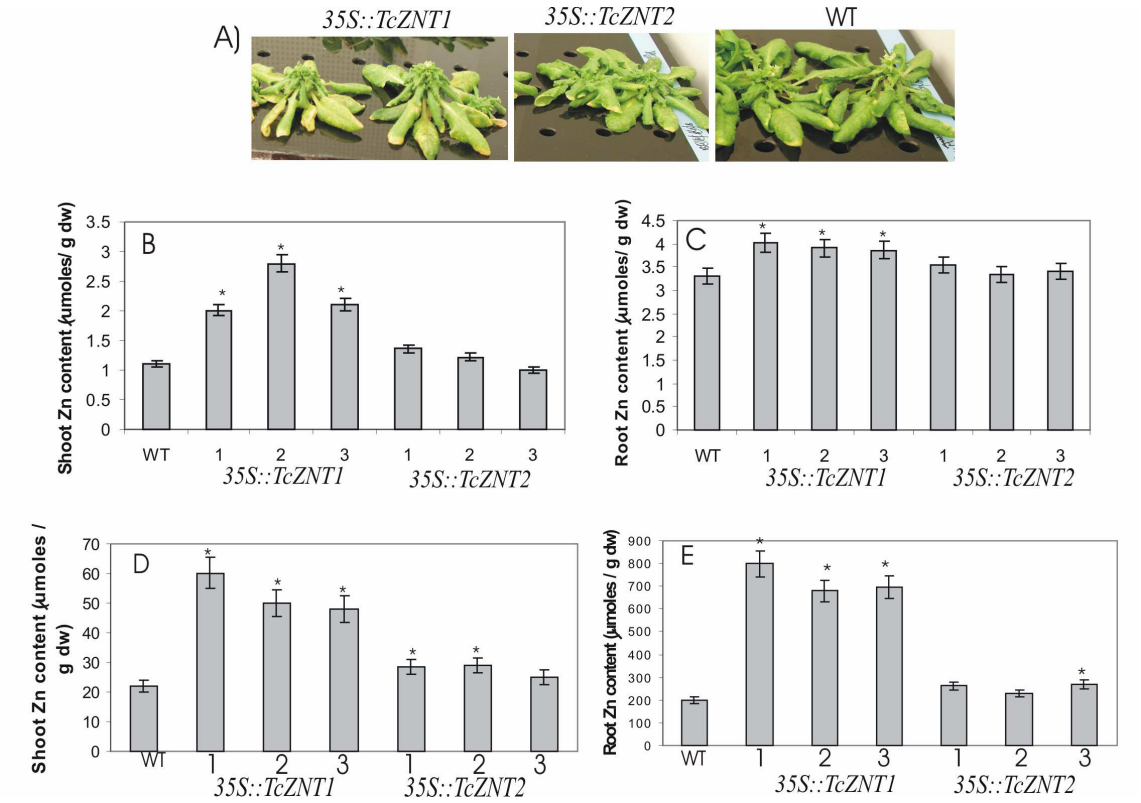
Chapter 3, Figure 1



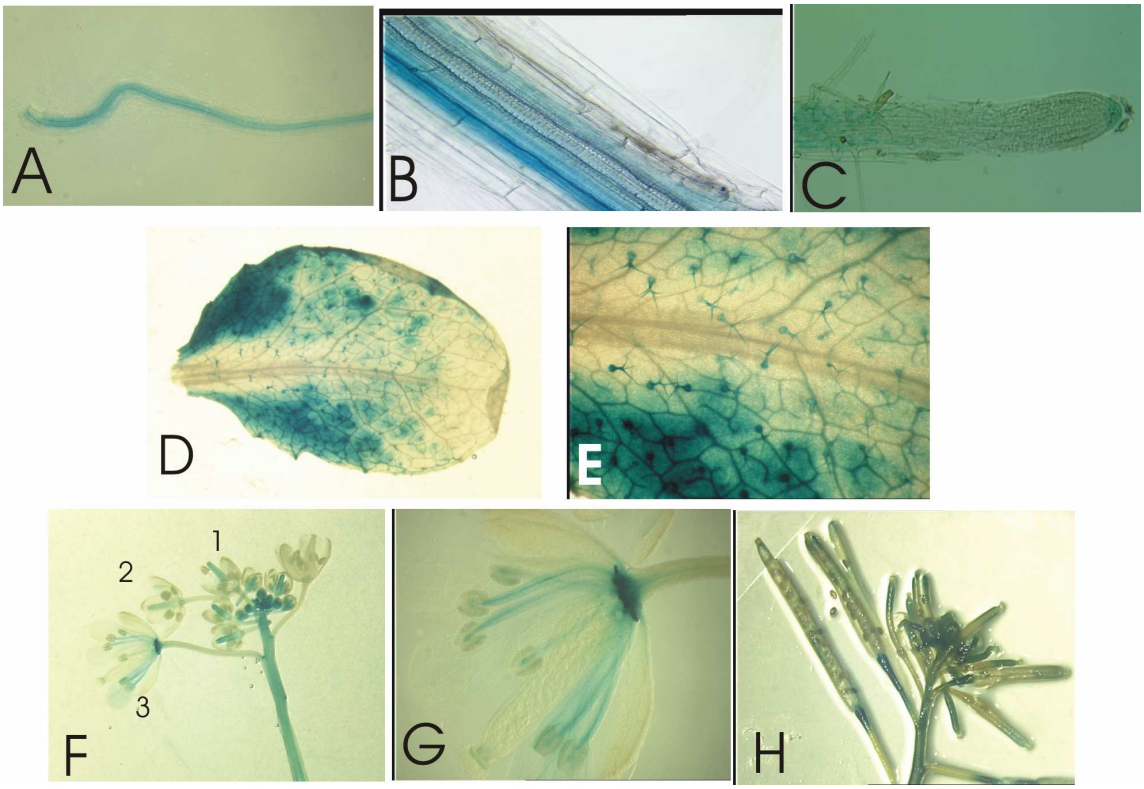
Chapter 3, Figure 2



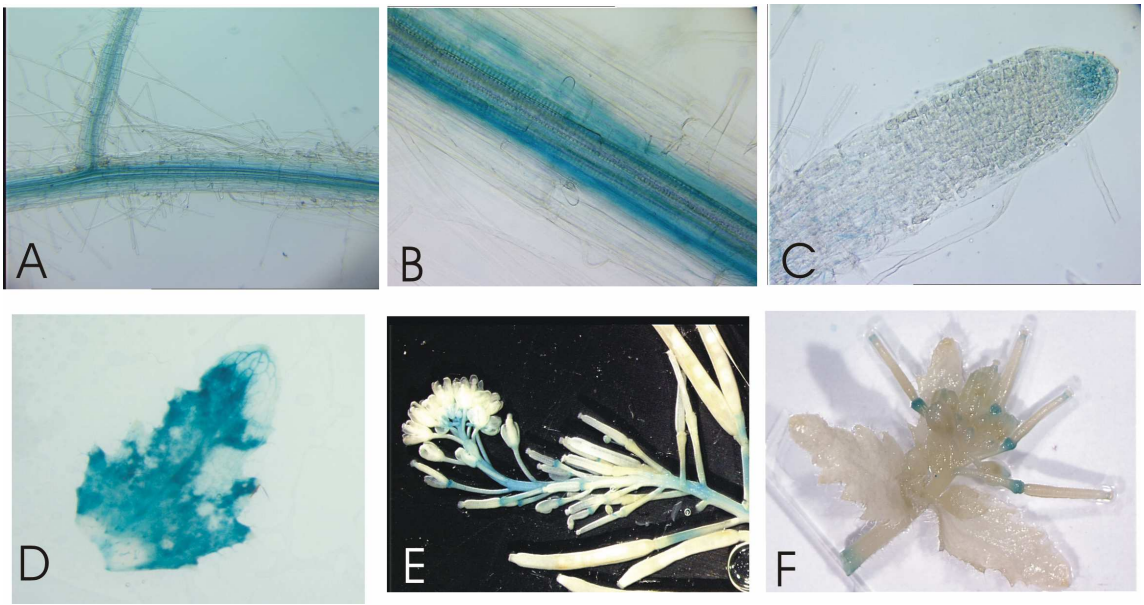
Chapter 3, Figure 3



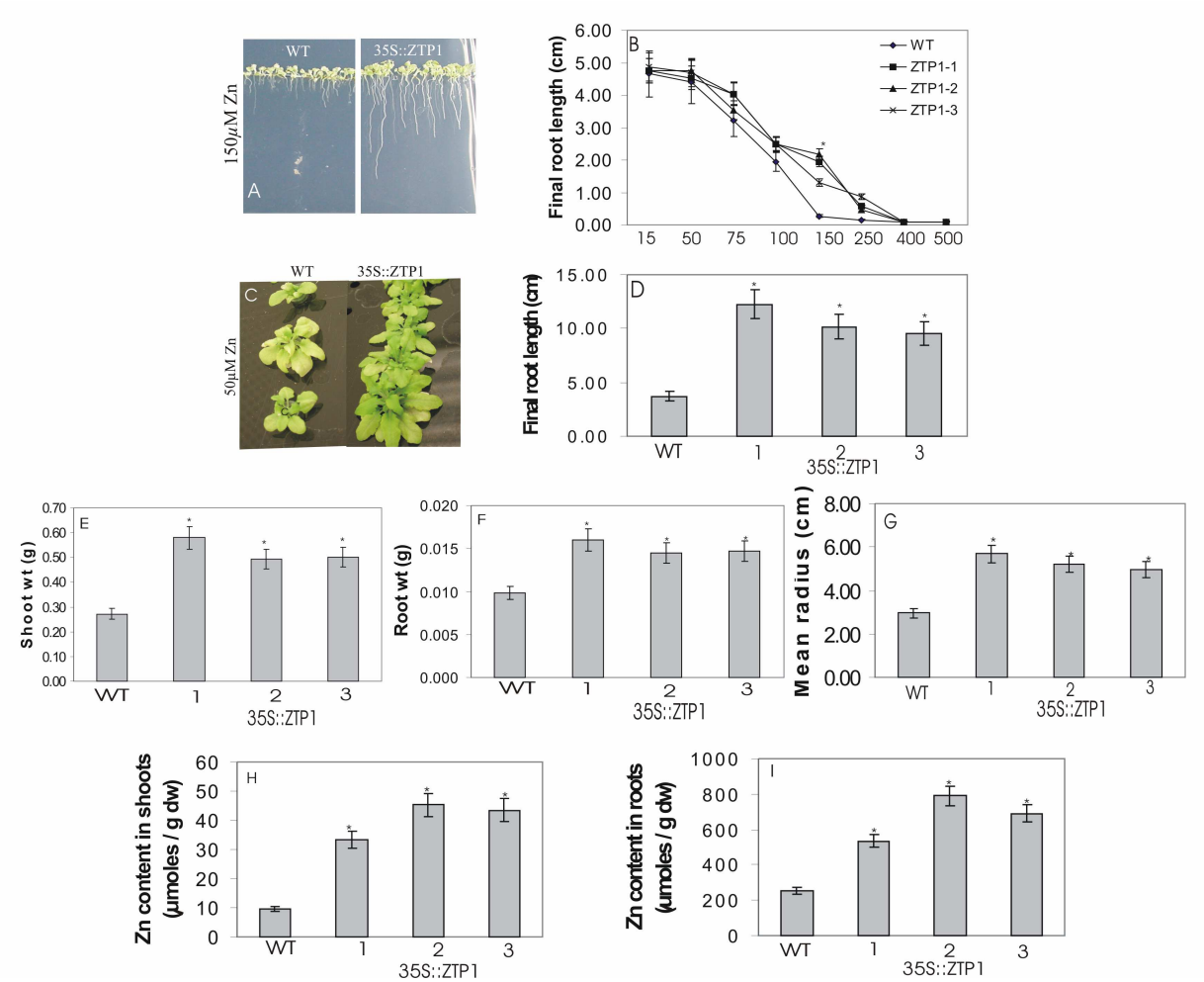
Chapter 4, Figure 3



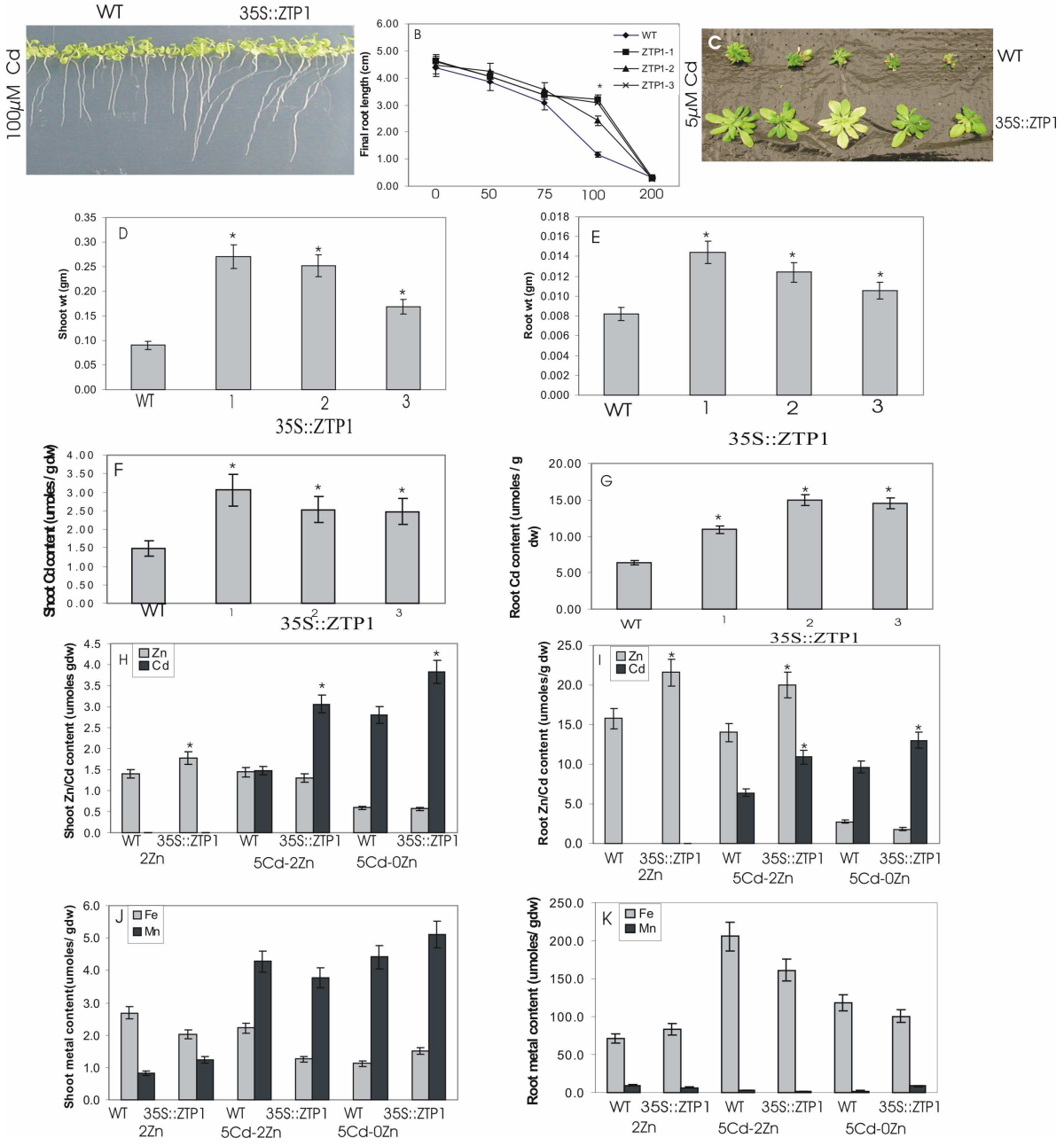
Chapter 4, Figure 4



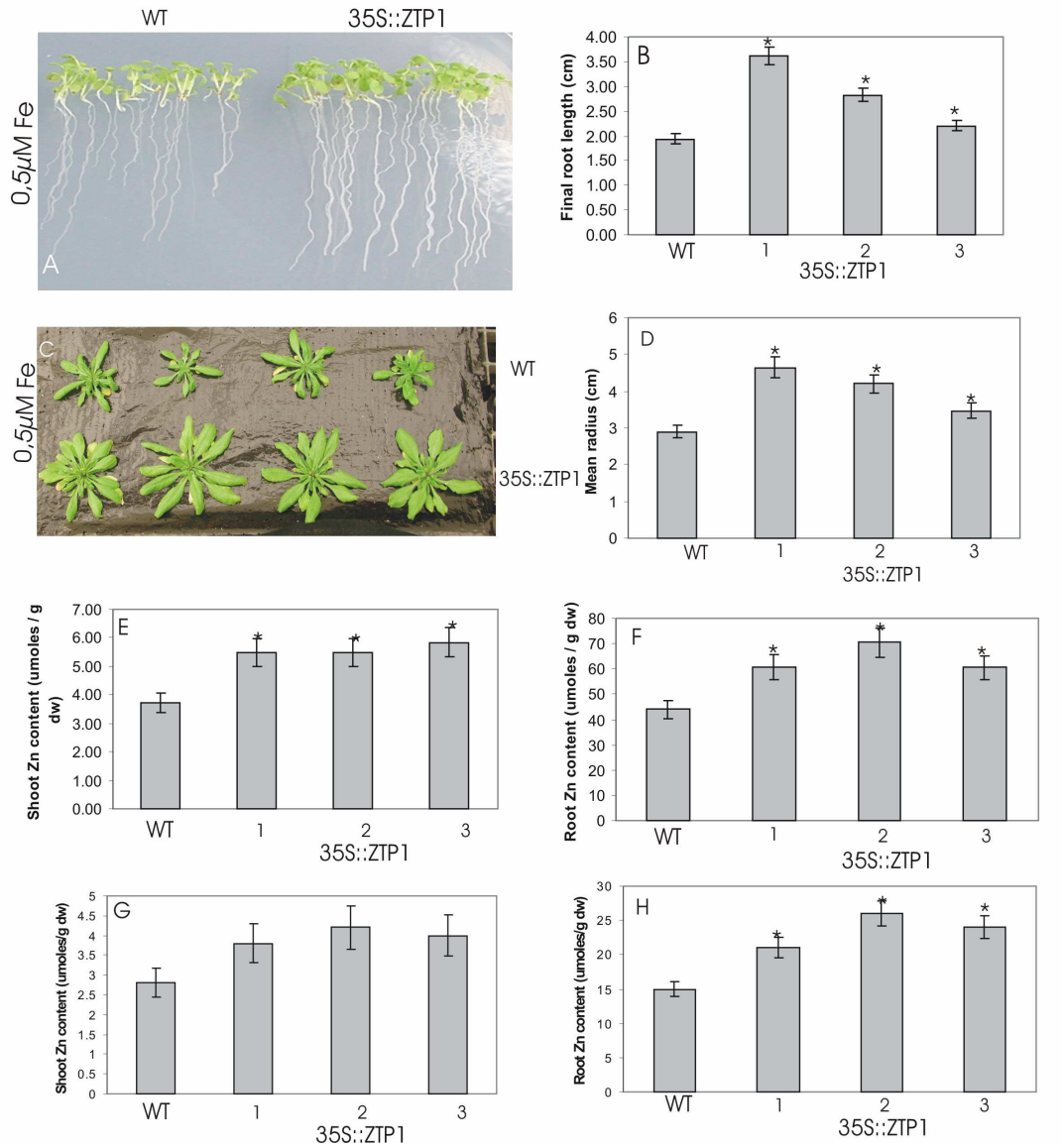
Chapter 5, Figure 1



Chapter 5, Figure 2



Chapter 5, Figure 3



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