Functional analysis of zinc hyperaccumulation related genes of *Noccaea* (*Thlaspi*) *caerulescens* for phytoremediation purposes

Zeshan Hassan

Thesis committee

Promotor
Prof. Dr *ir*. M. Koornneef
Personal chair at the Laboratory of Genetics

Co-promotor
Dr *ir*. M.G.M. Aarts
Associate Professor, Laboratory of Genetics

Other memebers
Prof. Dr R.G.F. Visser, Wageningen University
Prof. Dr F.J. Zhao, Rothamsted Research, Harpenden, United Kingdom
Prof. Dr J.C.H.M. Vangronsveld, Hasselt University, Hasselt, Belgium
Prof. Dr T. Bisseling, Wageningen University

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences

Functional analysis of zinc hyperaccumulation related genes of *Noccaea* (*Thlaspi*) *caerulescens* for phytoremediation purposes

Zeshan Hassan

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 28 January 2013
at 4 p.m. in the Aula.

Zeshan Hassan
Functional analysis of zinc hyperaccumulation related genes of *Noccaea* (*Thlaspi*) *caerulescens* for phytoremediation purposes, 223 pages.

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summary in English

ISBN 978-94-6173-430-3

Dedicated to my beloved parents…!!!

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

This chapter has been published in Journal of Environmental and Experimental Botany (2011) 72: 53-63.

1: Heavy metal contamination and remediation

Increasing environmental contamination of heavy metals is a growing risk for ecological and health associated hazards for the global human community (Sharma and Agrawal, 2005). The threats of these toxicants are higher than those of organic toxic compounds, especially in the long run, as they cannot be degraded and hence are difficult to remove from the environment. They are being spread in the environment by disposal of municipal wastes, use of irrigation water containing industrial effluents, agricultural use of sewage sludge and residues from metalliferous mining and smelting industries. Conventional methods of metal remediation like leaching, solidification, vitrification, electrokinetical treatment, chemical oxidation or reduction, excavation and off-side treatment are expensive, disturb soil structure and fertility and are limited to relatively small areas (Luo et al., 2000; Wu et al., 2010). The complications associated with conventional methods of remediation of metals have forced the researchers to find some alternate technologies.

The attractive concept of phytoremediation has been proposed, exemplifying it as a low cost and environment friendly technique, by which the dangers of hazardous heavy metals can be contained and controlled, or soils can even be cleaned from such metals, which can be recovered for reuse (Brooks et al., 1998; Lahner et al., 2003; McGrath and Zhao, 2003; Desbrosses-Fonrouge et al., 2005). Among different types of phytoremediation, the most interesting ones are phytostabilization and phytoextraction. "Phytostabilization" denotes the use of plants to stabilize harmful compounds in soil, by either converting the pollutants into a less bio-available form or by preventing the pollutants to distribute into nearby areas by run off, leaching, erosion, etc. Normally, phytostabilization strategies exemplify the use of local plant species to stabilize a certain metal contaminated site. An extensive root system and high biomass, so they keep most of the metals in their root zone, are desired properties of plants useful for phytostabilization (Wong, 2003). For applications dedicated to containment and control of toxic metals, plants that tolerate high toxic metal concentrations are needed. Adding additional value to contaminated land, by planting (engineered) metal tolerant high biomass plants (e.g. trees like poplar

and willow) for biofuel production, would be an advantageous application of phytostabilization and a requirement to make it commercially attractive. One of the important advantages of this strategy is that monitoring of a certain site is easier when biomass is harvested, as the condition of plants is easily visible and the plant metal concentration can be examined over time.

"Phytoextraction" refers to the use of plants to remove metals from contaminated soil by translocating them to their aboveground matter. This method is potentially very efficient, provided plants are able to concentrate metals to higher concentrations than in the contaminated soil, meaning that the total plant dry matter containing the metals will be less and easier to handle than the tons of soil to be treated by conventional ways of remediation. For commercially interesting phytoremediation crops yielding more than 20 tonnes/ha, such bioconcentrations factors should be over 10 to require <10 croppings to reduce the metal concentration in the soil by 50% (McGrath and Zhao, 2003). Another advantage of this method is that the fertility of soils, which otherwise is depleted by removal of top soil through engineering methods, can be maintained (Robinson et al., 2000). Valuable metals can be recovered from the plant material by incineration, along with the non-conventional advantage of biofuel production. Metal hyperaccumulator plants, accumulating metals to concentrations over 100-fold higher than conventional plants, will be suitable for phytoremediation purposes (Chaney et al., 1997; McGrath and Zhao, 2003). Natural metal hyperaccumulator plant species have been found, which evolved the ability to take up, tolerate and accumulate metals to exceptionally high concentrations in their aboveground biomass (Reeves and Baker, 2000). Low biomass and slow growth are the major limiting factors in the use of these natural hyperaccumulators. Often these species are (close to) wild accessions and require substantial adaptation to agricultural practices to produce sufficient metal containing biomass to avoid the need to culture many generations of a hyperaccumulator species and in fact many years to remediate a particular piece of soil (Chaney et al., 2005).

Two alternatives can be envisioned involving genetic engineering of

plants. One alternative focuses on improving growth and biomass production of known metal hyperaccumulators. In this review we focus on the other, and more attractive (Liang et al., 2009), alternative, which is to understand the molecular mechanisms involved in metal hyperaccumulation. This is largely based on the metal hyperaccumulator *Noccaea caerulescens* (previously known as *Thlaspi caerulescens*), and aims to identify the genes responsible for this property, which, if well characterized and elegantly utilized, could transform the high biomass producing plants species into metal tolerant and accumulating ones when properly expressed (Desbrosses-Fonrouge et al., 2005).

2. Heavy metals are essential micronutrients or resemble them

Many transition metals, such as Zn, Fe, Ni, Mo, Mn and Cu, that are toxic at high concentrations, are also essential micronutrients required for proper physiological and metabolic functioning of plants (Epstein and Bloom, 2005), and as such they cannot be avoided by plants. One of these important micronutrients is zinc (Zn). It is required by all organisms in small amounts. Despite their essentiality, Zn^{2+} ions can cause toxicity when accumulated in excess. In plants, an excess of Zn leads to the inhibition of root growth, decreased photosynthetic rates and chlorosis. When leaf Zn concentrations approach or exceed a critical toxicity level of 300 mg kg^{-1} dry tissue, then leaf necrosis and ultimately plant death occurs (Chaney, 1993; Marschner, 1995). Zn contaminated soils cause injury to soil microorganisms, reduce crop yield and hence are dangerous for food chains. In certain areas near industrial sites and agricultural fields, soil Zn concentrations have been found of more than 1000 mg Zn kg^{-1} dry soil (Audet and Charest, 2006), while normally around 100 mg Zn kg^{-1} dry soil is found in various agricultural soils (Marschner, 1995). Most of the Zn was concentrated as the residual fraction in those soils, which means most of this Zn was not readily bio-available. The non-residual fraction of Zn was present as Fe–Mn oxide, carbonate bound, organic, exchangeable and water soluble fractions.

Cadmium (Cd) is an important environmental pollutant and a potent toxicant to plants. It is also a carcinogenic metal (Clemens et al., 2012). Since it is widely distributed in the environment and used in industry, it currently ranks 7th on

US-Agency for Toxic Substances and Disease Registry (ATSDR) priority list of hazardous substances (http://www.atsdr.cdc.gov/cercla/07list.html). Sewage sludge is often contaminated with Cd and where Cd containing sewage sludge has been used as soil fertilizer, it readily accumulates in plants grown on such contaminated soils. Cd does not have a known biological function in plants, but as it resembles Zn and Fe, it may enter plants following the Zn or Fe uptake machinery (Pence et al., 2000; Vert, 2002. Although it may not be avoided by plants, plants are able to detect Cd or its effect and respond to exposure by alterations in gene and protein expression (Herbette et al., 2006; van de Mortel et al., 2008; Semane et al., 2010). Cd accumulation causes DNA repair inhibition (Banerjee and Flores-Rozas, 2005), reduction in photosynthesis, water and nutrient uptake (Sanita di Toppi and Gabbrielli, 1999), and visible symptoms of injury such as chlorosis, growth inhibition, browning of root tips, and finally death (Kahle, 1993).

Nickel (Ni) is also an essential micronutrient, although required in very small amounts. Wood et al. (2004) reported Ni deficiency in *Carya illinoinensis* trees in southeast USA. Plants need Ni as a co-factor for the enzyme urease. Deficiency results in leaf necrosis upon higher acclimation of the plants to urea (Gerendas et al., 1999). Ni is also well known for its injurious nature to plants when present in excess. Upon high Ni exposure, *Arabidopsis thaliana* showed significant loss of chlorophyll content and there was a large reduction in growth of shoot and root tissues (Freeman et al., 2004).

Copper (Cu) is important for many structural and functional roles in plants. Many oxidative enzymes such as Cu/Zn superoxide dismutase (Cu/Zn SOD), cytochrome oxidase, ascorbate oxidase, polyphenol oxidase and diamine oxidase and electron- transfer proteins (such as plastocyanin in chloroplasts) are Cu dependent (Bell and Dell, 2008). It also plays a distinct role in lignification of cell walls and is more required during the reproductive growth of plants compared to the vegetative growth (Bell and Dell, 2008). Cu deficiency can inhibit photosynthesis and reduce availability of leaf carbohydrates. Sterile pollen production, pendulousness of lateral branches, distortion of young

leaves, twisting and stem bending are a few Cu deficiency symptoms in plants (Marschner, 1995). Cu toxicity is also dangerous for plant growth as it causes reduced plant biomass production (Ghasemi et al., 2009).

Iron (Fe) plays a key role in the synthesis of chlorophyll, ethylene and in construction of the cell wall and being an electron donor, alterations in Fe concentrations compromise the electron transport chain (Bell and Dell, 2008). It is a component of heme and nonheme proteins and constitutes the heme Fe–porphyrin complex in cytochromes (Marschner, 1995). Fe deficiency can be distinguished by intervenal chlorosis and upon acute deficiency, necrotic lesions appear (Bell and Dell, 2008). At elevated concentrations, Fe becomes toxic and causes oxidative stress in plants (Guerinot and Yi, 1994). In rice plants, 50% reduction in total grain weight has been reported due to Fe toxicity (Genon et al., 1994).

Manganese (Mn) is essential for plants, as it is part of a Mn/Ca cluster crucial for photosynthesis (Rutherford and Boussac, 2004). It is a catalytically active metal, involved in cell protection against the detrimental effects of free radicals. Mn is also important as it activates the enzyme phosphoenylpyruvate carboxylase, which is vital for CO2 assimilation (Hansch and Mendel, 2009). Mn deficiency can be visualized as intervenal chlorosis in leaves. Upon Mn toxicity, brown speckles appear on leaves (Marschner, 1995). There is a competition of Mn with other metals like Cu, Fe and Zn, when they are in excess, probably due to shared specificities of metal uptake transporters.
In this review we will focus on Zn, Cd and Ni.

3: Metal hyperaccumulating plant species

Some plant species are extremely tolerant to exposure to excess of metals. A subclass of some 450 species even accumulate metals to very high levels. The term "metal hyperaccumulator" was first used to define plants that can concentrate 1000 mg kg^{-1} dry weight (DW) of Ni in shoot tissue (Brooks et al., 1977). After that, a range of Zn, Ni, Cd, Pb, Cu, Co and Mn hyperaccumulators has been described (recently summarized by Verbruggen et al. (2009)). The hyperaccumulator species that have been identified belong to 34 different families, with Brassicaceae being the larger family. The majority of

these species are Ni hyperaccumulators. Zn hyperaccumulators comprise the second largest group and only four species have been described as Cd hyperaccumulators: *N. caerulescens*, *Noccaea praecox*, *Arabidopsis halleri* and *Sedum alfredii* (Brown et al., 1995; Kupper et al., 2000; Yang et al., 2004; Vogel-Mikus et al., 2005). *Noccaea caerulescens* F.K. Meyer (J.&C. Presl) is an exceptional species, as it is known to hyperaccumulate Zn (30,000 mg kg^{-1} DW), Ni (4000 mg kg^{-1} DW) and Cd (2700 mg kg^{-1} DW) in shoots (Reeves and Brooks, 1983; McGrath et al., 1993; Brown et al., 1995; Lombi et al., 2000), but there is substantial natural variation regarding metal specificity and metal tolerance among different accessions. Accessions originating from serpentine soils are generally good at accumulating Ni. Accessions from calamine or non-metallicolous soils are good Zn accumulators. Cd hyperaccumulation so far has only been observed among populations found in the south of France, in the region around Ganges (Meerts and Isacker, 1997; Lombi et al., 2000; Assunção et al., 2003a; Roosens et al., 2003). *N. caerulescens* and shares about 88.5% coding region sequence similarity with the plant reference species *A. thaliana* (Rigola et al., 2006). Self-fertility, sufficient seed setting, adaptive traits of metal hyperaccumulation and tolerance and close relatedness to *A. thaliana*, are some of the many favourable characteristics of *N. caerulescens,* which make it an excellent model species for the study of metal hyperaccumulation (Assunção et al., 2003b; Peer et al., 2003; Milner and Kochian, 2008). Recently another *Noccaea* species, *N. praecox*, has been described to accumulate Cd to comparably high levels (Pongrac et al., 2009). Another good model species is *A. halleri*. It can accumulate high shoot Zn concentrations, ranging from 3000 to 22,000 mg kg^{-1} DW (Bert et al., 2000), while accumulation of more than 100 mg Cd kg^{-1} DW in leaf tissues has been documented for some individuals within populations of this hyperaccumulator (Dahmani-Muller et al., 2001; Talke et al., 2006). It is found on contaminated and uncontaminated sites in Eastern Europe, while in Western Europe its habitat consists only of metal contaminated soils (Bert et al., 2000). It is even closer related to *A. thaliana* than *N. caerulescens* and shares about

94% nucleotide sequence similarity with it in coding regions (Becher et al., 2004).

4. Metal physiology of hyperaccumulators

4.1. Metal uptake and root sequestration

"Metal homeostasis" is a property of an organism that regulates its internal metal environment (cellular and organellar metal concentration) so as to maintain a stable and constant condition. Metal homeostasis in plants helps them to survive both at metal deficiency and at higher metal concentrations. Metal mobilization and uptake from the soil, compartmentalization and sequestration within the root, xylem loading and transport, delivery between metal sinks in the aerial parts of the plants and sequestration and storage in leaf cells are the factors influencing metal homeostasis (Clemens et al., 2002). This whole series of processes can be summarized under three major components: (1) transport; (2) chelation; and (3) sequestration.

Metal hyperaccumulators have evolved special mechanisms to ensure enhanced metal accumulation in their aerial parts and maintain metal homeostasis. The enhanced metal influx into the root cells is the first step in the metal accumulation process. Metals enter the plants either by crossing the plasma membrane of the root endodermal cells through the symplast, or by crossing the root apoplast through the intercellular spaces. In the case of Zn, it has been reported that the maximum initial velocity of Zn influx in roots cells of the hyperaccumulator *N. caerulescens* is about 4.5- fold higher compared to the related non-hyperaccumulator *Thlaspi arvense*, suggesting that increased root absorption of Zn is one of the major factors in the mechanism of Zn hyperaccumulation (Lasat et al., 1996). Roots of *N. caerulescens* and *T. arvense* showed similar Km values in Zn kinetic studies performed by (Lasat and Kochian, 2000), while V_{max} was much higher in *N. caerulescens* compared to *T. arvense*. This suggests that transporters with very similar function are active in the roots of both species, while a higher expression of these transporters drives Zn in larger amounts into the hyperaccumulator *N. caerulescens* compared to *T.*

arvense. Candidates for such Zn transporters are members of the ZIP family. Upon comparison of *N. caerulescens* and *A. thaliana*, *ZIP4, ZIP10* and especially *IRT3* were found to be much higher expressed in *N. caerulescens* roots than in *A. thaliana* roots, even at different Zn exposures (van de Mortel et al., 2006). The *N. caerulescens* orthologues of the *AtZIP4* and *AtIRT3* genes have been cloned and are called *NcZNT1* and *NcZNT2* (Assunção et al., 2001). Contrary to expectations, *AtZIP4* is not expressed in the root epidermis, but in the endodermis and pericycle (Milner and Kochian, 2008), which is also the site of expression for *NcZNT1* (Chapter 2; Milner et al., 2012). This expression is in line with the idea that apoplastic Zn is actively transported into the stele and thus is available for translocation to the shoots. In *A. thaliana* these ZIP transporters are strongly induced by Zn deficiency. Similar induction is seen in *N. caerulescens*, but also overall expression levels are much higher than in *A. thaliana*, reflecting the constitutive nature of Zn uptake in this hyperaccumulator. Other *ZIP* genes that are higher expressed in *N. caerulescens* roots than in *A. thaliana* are *ZIP1, ZIP2 and ZIP5*. All three of these are induced by Zn deficiency in *A. thaliana* (van de Mortel et al., 2006). Only the *ZIP5*-orthologue, *NcZNT5*, has been cloned from *N. caerulescens* (Plaza et al., 2007; Kupper and Kochian, 2009; Wu et al., 2009). This gene is more or less constitutively expressed in *N. caerulescens* roots. Unfortunately the tissue of expression is not yet known and thus it is not clear if it acts redundantly or complementary to *NcZNT1* for Zn uptake in roots.

"Ganges" is a *N. caerulescens* accession from one of the southern France populations that accumulate high amounts of Cd in its leaves. 'Prayon' is an accession with much lower Cd accumulating potential. Lombi et al. (2001) reported a 5-fold higher V_{max} of Cd influx into the root cells of Ganges compared to Prayon, with a saturable component at low Cd concentrations. This suggests that there is a different, high-affinity Cd uptake mechanism active in the roots of Ganges which is absent in Prayon. Although Cd can be transported by *NcZNT1* (Pence et al., 2000), also the Fe uptake ZIP transporter IRT1 is able to transport Cd (Rogers et al., 2000; Vert et al., 2002). Since Fe deficiency enhances Cd uptake in Ganges but not in Prayon (Lombi et al., 2002),

Cd uptake in Ganges could very well be accommodated by expression of *NcIRT1*. Work by Plaza et al. (2007) showed that Ganges expresses a full-length copy of *NcIRT1*, whereas Prayon only expresses a truncated copy. Although heterologous expression of either copy in yeast indicated both can mediate Fe transport, this appears very unlikely to happen in plants and hence NcIRT1 remains an important candidate to confer high Cd uptake into the Ganges Cd hyperaccumulating accession.

Some accessions of *N. caerulescens*, such as 'Monte Prinzera', are good Ni hyperaccumulators (Assunção et al., 2003a), but also other *Noccaea* species like *N. goesingense* and *N. japonicum* can hyperaccumulate Ni (Krämer et al., 1997; Mizuno et al., 2002). In some serpentine *N. caerulescens* accessions, Ni uptake is inhibited in the presence of Zn, suggesting that Ni is taken up through Zn uptake transporters (AssunçãoAssunção et al., 2008). This may very well involve the NcZNT1 transporter, although Ni transport has not yet been demonstrated for this transporter. *NcZNT1* is highly expressed in roots of the serpentine accession Monte Prinzera (AssunçãoAssunção et al., 2001). In *A. thaliana*, the Zn deficiency induced expression of *AtZIP4*, the orthologue of *NcZNT1*, can be repressed by supplying additional Ni, but not by additional Cd or Fe (Zientara, AssunçãoAssunção and Aarts, unpublished), suggesting competition between Zn and Ni for uptake through AtZIP4/NcZNT1.

The second physiological step in metal hyperaccumulation is the mild metal sequestration in the root cells and enhanced transport through the root for increased xylem loading. Most hyperaccumulators reduce storage of metals in the root cells to ensure a continuous metal influx available for xylem loading. Lasat et al. (1998) reported that 2.5-fold more Zn is stored in the root cells of the non-accumulator *T. arvense* compared to *N. caerulescens* and that Zn is also exported quickly out of the vacuoles of root cells of *N. caerulescens* compared to *T. arvense*. Thus most of the Zn is available for further transport to the aerial parts of these plants. The enhanced efflux of Zn from root vacuoles may be due to higher expression of *NRAMP* genes, as is the case for *NcNRAMP3* and *NcNRAMP4* when compared to their *A. thaliana* orthologues *AtNRAMP3* and *AtNRAMP4* (Oomen et al., 2009). *A. thaliana nramp3nramp4* mutants are more sensitive to zinc and cadmium than wild type, which means that mobilization

of essential metals from the vacuole is also essential for tolerance to zinc and cadmium (Oomen et al., 2009). Enhanced Zn efflux from root vacuoles is not the only mechanism to enhance the translocation of metals towards the central stele. Recently, Richau et al. (2009) showed that *N. caerulescens* contains much more free histidine in roots compared to the Ni non-hyperaccumulator *T. arvense* and that histidine-complexed Ni was much less taken up by *N. caerulescens* vacuoles compared to *T. arvense* vacuoles. Therefore, the authors concluded that an increased root histidine concentration inhibits vacuolar His–Ni sequestration in *N. caerulescens* compared to *T. arvense* and contributes to enhanced histidine-mediated Ni xylem loading. Since an elevated free histidine concentration appears to be a constitutive trait in *N. caerulescens*, even in Ni non-hyperaccumulating accessions, it may well be that it also promotes xylem loading and reduced vacuolar sequestration of Zn.

4.2. Root-to-shoot transport

Enhanced metal xylem loading and translocation to the shoots is a next key physiological step in the metal hyperaccumulation trait that accounts for the increased metal flow towards the shoot, where metals are detoxified and stored. Both Zn root-to-shoot translocation and xylem sap Zn concentrations are enhanced by 10-respectively 5-fold, in *N. caerulescens* compared to *T. arvense* (Lasat et al., 1996, 1998). The main candidate gene responsible for enhanced xylem loading is *HMA4*. This encodes a P1B-type ATPase expressed in the stele of the root and located in the plasmamembrane, directing Zn outside the cell. Mutation of *AtHMA4* and another P1B-type ATPase gene, *AtHMA2*, renders *A. thaliana* unable to translocate Zn from roots to shoots (Hussain et al., 2004). Wong and Cobbett (2009) recently showed that upon silencing of *HMA2* and *HMA4* in *A. thaliana*, an almost complete inhibition of root-to-shoot Cd translocation was observed, suggesting both *HMAs* are the major controllers of Zn/Cd root-to-shoot translocation. The most compelling evidence that HMA4 is indispensable for metal hyperaccumulation was provided by Hanikenne et al. (2008), who showed that upon RNAi-mediated silencing of *AhHMA4* in the

Zn/Cd hyperaccumulator *A. halleri,* plants are impaired in both Zn/Cd root-to-shoot translocation and tolerance. *AhHMA4*, and also *NcHMA4* (Hammond et al., 2006; van de Mortel et al., 2006), are much higher expressed in *A. halleri* and *N. caerulescens* compared to *HMA4* expression in non-accumulators (O'Lochlainn et al., 2011; Craciun et al., 2012), which would argue for the possibility that increased expression of *HMA4* would be solely responsible for Zn/Cd hyperaccumulation. Whether related genes, such as *HMA2* and *HMA3*, are involved is not clear. In *A. thaliana*, *AtHMA2* and *AtHMA3* are neighbouring genes, expressed in similar tissues. Both proteins are very similar at the amino acid level, but with the strong difference that AtHMA3 lacks the C-terminal tail found in AtHMA2 and AtHMA4. Although a pseudogene in some accessions, a functional AtHMA3 protein contributes to vacuolar storage of Zn and Cd in *A. thaliana* (Morel et al., 2009). Based on heterologous microarrays analysis, *HMA3* is expressed at much higher levels in *N. caerulescens* than in *A. thaliana* (van de Mortel et al., 2006), and it was recently found to be a key factor in conferring tolerance to Cd (Ueno et al., 2011). So far there is no evidence for an HMA2 gene in *N. caerulescens*. Also the mechanism by which Ni is loaded into the xylem is still largely unknown.

Once in the xylem, metals are generally chelated to organic acids such as histidine (Krämer et al., 1996), nicotianamine, citrate, malate or oxalate (Senden et al., 1995), although also free Zn^{2+} has been found in xylem sap of *N. caerulescens* (Salt et al., 1999). Histidine has generally been reported as the ligand involved in the long distance root-to-shoot transport of Ni through xylem, such as in the Ni hyperaccumulator *Alyssum lesbiacum* (Krämer et al., 1996), but probably also in *N. caerulescens* (Morel et al., 2009). Krämer et al. (1996) showed that exogenously applied Ni to enhance the Ni content of *A. lesbiacum* plants, increased free histidine levels, while external application of histidine to non-accumulator *A. montanum* plants greatly enhanced root elongation and plant biomass (Ni tolerance), and Ni influx through the xylem. Citrate has been shown to be transported into the xylem by FRD3, a member of the MATE family of transporters. This protein is essential for efficient iron translocation via vascular tissues (Durrett et al., 2007). Citrate is probably also involved in Zn translocation as FRD3 is much higher expressed in roots of *N.*

caerulescens than those of *A. thaliana* (van de Mortel et al., 2006). This could be a side-effect though, of a high Zn uptake compromising Fe uptake.

Fe and few divalent metal ions like Zn, Ni and Cu are also chelated and transported in plants by nicotianamine (NA) (Ling et al., 1999; Pich et al., 2001; Takahashi et al., 2003). NA is synthesized by trimerization of S-adenosylmethionine by the enzyme nicotianamine synthase (NAS) (Shojima et al., 1990). *NAS2* has been shown to play a crucial role in metal hyperaccumulation in *A. halleri* (Deinlein et al., 2012). All four *NAS* genes are highly expressed in *N. caerulescens* compared to *A. thaliana* (van de Mortel et al., 2006), and often show a different pattern of expression, indicating their involvement in the hyperaccumulation of Zn, Cd and/or Ni. This could be direct or indirect. In the *chloronerva* mutant of tomato, which is impaired in NA biosynthesis (Ling et al., 1999), Fe, Zn and Mn xylem transport are not, or hardly, affected compared to wild type. Cu xylem transport, however, was strongly reduced and Cu accumulated in the roots (Pich and Scholz, 1996). NA is also important for Ni transport in *N. caerulescens*. Ouerdane et al. (2006) identified Ni–NA complexes in Ni-exposed *N. caerulescens* plants. They did not quantify Ni–His complexes, so it is not clear what the relative contributions of Ni–His and Ni–NA are to Ni root to-shoot translocation, but both appear to be important. Recently the *ZIF1* gene was found to play a crucial role in controlling vacuolar NA levels (Haydon et al., 2012).

4.3. Metal storage

Metals distribution and detoxification in shoots, as well as their redistribution via phloem, starts with xylem unloading processes (Schmidke and Stephan, 1995). Subsequently, from the apoplast around the xylem, metals are either taken up into surrounding cells and further transported symplastically through the leaf tissues (Marschner, 1995), or they are distributed apoplastically over the leaf (Clemens et al., 2002). For symplastic transport, the chelation of metals to NA is important. For instance, in the *chloronerva* mutant of tomato (Ling et al., 1999), but also in the quadruple

nas1234 mutant of *A. thaliana* (Klatte et al., 2009), Fe accumulates in leaves, but is not distributed over the leaf and plants show strong indications of Fe deficiency. Most of the symplastic transport of NA-chelated metals, for instance to and from the vascular tissues after xylem transport (Waters et al., 2006), occurs through Yellow Stripe Like proteins (YSL) (DiDonato et al., 2004). Gendre et al. (2007) showed that three *N. caerulescens YSL* genes, namely *NcYSL3, 5,* and *7*, were highly expressed around vascular tissues, particularly in shoots. The pattern of expression of these genes was different and distinct, compared with their *A. thaliana* orthologues. The authors speculated that one of the aspirant functions of *NcYSL3*, among this group of genes, is to unload Ni–NA complex from the xylem tissues into the leaf cells and to further deliver it into its storage cells. Furthermore, the authors demonstrated NcYSL3 to be a Fe/Ni–NA influx transporter, illustrated by yeast complementation and uptake measurement studies. Since YSL proteins are particularly associated with Fe–NA transport (Curie et al., 2009), it seems likely that also in metal hyperaccumulators, they will mainly control Fe–NA transport, but considering the higher expression of NAS in metal hyperaccumulators and the occurrence of Zn–, Ni– and Cu–NA complexes, these are also likely to be transported by YSLs.

Leaf cell vacuoles are the sites of sequestration of excess essential and non-essential metals (Vogeli-Lange and Wagner, 1990). Kupper et al. (1999) found that the highest concentration of leaf Zn and Cd was present in leaf epidermal cells, which contained four times higher concentrations of these metals compared to mesophyll cells. The epidermis may be preferred, since most epidermis cells lack chloroplasts, which could be compromised by high metal concentrations. Milner and Kochian (2008) suggested the role of *NcZNT1* in facilitating uptake of Zn from leaf apoplast into bundle sheath and mesophyll cells in *N. caerulescens*. However the mechanism of Zn loading into epidermal cells is still unknown. *NcZNT5* and *NcZNT6* may be important in this regard. Both genes showed constitutively high expression in *N. caerulescens* compared to their *A. thaliana* orthologues *AtZIP5* and *AtZIP6*, but engineered high constitutive ectopic expression of the *NcZNT5* and *NcZNT6* genes in *A. thaliana* did not cause remarkable alterations in metal accumulation or tolerance

(Wu et al., 2009). This may be due to ectopic expression of these genes, since Kupper and Kochian (2009) showed that *NcZNT5* is mainly expressed in non-photosynthetic epidermal cells and bundle sheath cells, and constitutive expression in all leaf and root cells is unlikely to confer the same Zn sink in *A. thaliana* as the endogenous expression confers to *N. caerulescens*.

Once metals have reached their target destinations, they need to be stored in vacuoles. The main candidates for this function are members of the cation diffusion facilitator (CDF) protein family. These proteins have also been established as conferring tolerance to various metals like Zn, Mn, Cd, Co or Ni, by sequestering metals in the vacuoles (Montanini et al., 2007). In *N. caerulescens*, a CDF family member *NcMTP1* (similar to *AtMTP1/AtZAT*: previously called *ZTP1*) (van der Zaal et al., 1999; Desbrosses-Fonrouge et al., 2005) showed constitutively high expression and was suggested to play a role in Zn tolerance (Assunção et al., 2001). Over-expression of *NcMTP1* enhances tolerance and accumulation of Zn and Cd in *A. thaliana*, accumulation of Zn and tolerance to Zn, Cd and Ni in *N. tabacum* (Chapter 3).

Recently, Guimaraes et al. (2009) reported that shoots and roots have different roles in metal hyperaccumulation and hypertolerance in *N. caerulescens*. Reciprocal grafting experiments using *N. caerulescens* and the non-hyperaccumulator *Microthlaspi perfoliatum* showed that in *N. caerulescens,* Zn hyperaccumulation is mainly controlled by root processes, while shoot processes control the hypertolerance to Zn. The authors advocated that shoot-governed hypertolerance would be driven mainly by MTP1 (Assunção et al., 2001; Persans et al., 2001). This supports the idea that tissue specific expression of potential genes will be crucial in mimicking metal tolerance and hyperaccumulation traits in engineered high biomass plants useful for phytoremediation. Furthermore, one can hypothesize that since shoot tissues involved in tolerance do not seem to influence the hyperaccumulation trait, there appears to be no feedback mechanism from shoots to roots for metals that are loaded and stored in the shoot tissues. This suggests that hyperaccumulators are disturbed in the feedback signal transduction pathway

indicating to roots that shoots are becoming overloaded with metals and that metal uptake needs to cease. However, Hanikenne et al. (2008) postulated that, for *A. halleri*, strong activity of HMA4 in fact depletes root cells from Zn, even at high Zn concentrations, which somehow leads them to continue Zn uptake at a high rate.The various activities of genes involved in Zn and Cd uptake, translocation and sequestration are summarized in Fig. 1.

5. Genetic engineering, exploiting modern molecular tools for improved metal tolerance and accumulation in plants

For quite some time, it has been recognized that the metal hyperaccumulation and hypertolerance traits could be potentially transferred to other plant species by the use of genetic engineering (Chaney et al., 1997). Until now, the goal to transfer such proper ties to high biomass non-accumulator plants and to convert them into efficient crops for phytoremediation purposes has not been reached. However this goal may not be accomplished following a short-term scenario. A long-term strategy towards achieving this goal appears more realistic, when it is focusing on the functional analysis of individual genes, and hence first tries to unravel the underlying molecular mechanisms that control the metal tolerance and accumulation traits. Upon successful characterization of single and multiple genes in ameliorated combinations in model species like *A. thaliana* and tobacco, the actual focus could then shift towards utilizing these genes in plant species useful for field oriented remediation purposes.

As described above, most non-accumulator plants try to prevent toxic metals, like Cd, or excessive essential metals, like Zn and Ni, to enter the shoot. In contrast, most hyperaccumulators specifically mediate root xylem loading of Zn, Cd and Ni (Fig. 1). They accomplish this by: (1) enhancing metal uptake in root cells; (2) reducing metal sequestration capacities in the root cells close to the vasculature; (3) concomitantly enhancing the export of metals for loading into the xylem; (4) timely unloading of metals from xylem in shoots; (5) enhanced transport through neighbouring cells to metal storage cells (epidermis and mesophyll); and (6) enhanced sequestration of metals in these cells.

How to achieve this with the current knowledge on gene functions? While for several genes the understanding of their function, both in regular plants and hyperaccumulators is increasing, there are still many genes that are differentially expressed when comparing hyperaccumulators and non-hyperaccumulators, but which have not been examined at all (Weber et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006, 2008). Understanding the function of expression of these genes in *N. caerulescens* by cloning and analysis of promoter activities will be an important objective for further research, along with conferring functional expression to non-accumulators. Conferring the trait can first be studied in the model species *A. thaliana* or in *N. tabacum*, which is already producing high biomass, before attempting this in more efficient biomass producers such as poplar or willow. Primary candidate genes that deserve further attention are genes encoding for structural proteins, such as metal transporters, metal-chelator transporters and chelator biosynthesis genes, but also transcriptional regulators and signal transduction pathway regulators, controlling the endogenous metal homeostasis and shoot-to-root signal transduction.

Most metal or metal-chelate transporters show tissue specific, rather than the constitutive, expression (Curie et al., 2009), which allows for concentration gradients and supports sequential transport over tissues. This specific expression will make it difficult to functionally mimic high expression of transporters in hyperaccumulators by simply ectopically over-expresssing transporter genes under control of the strong and constitutive CaMV 35S promoter. This also explains why attempts so far to ectopically over-express metal (-chelate) transporters only resulted in few fold increases in metal accumulation, compared to the >100-fold higher metal accumulation of hyperaccumulators, when compared to non-accumulators (van der Zaal et al., 1999; Kim et al., 2005; Hanikenne et al., 2008; Oomen et al., 2009; Wu et al., 2009). Unfortunately, at the moment, the tissue specific expression of metal transporters has not been analysed in great detail and particularly not in metal hyperaccumulator species, which are much more difficult to transform with

promoter-marker gene constructs. Analysis of tissue specific expression of candidate hyperaccumulator genes, as has been performed by Kupper and Kochian (2009) for three *N. caerulescens* zinc transporters by in situ hybridisation, will be needed to properly mimic hyperaccumulator-like gene expression in non-hyperaccumulator plants. Since many transporter genes are differentially expressed, most likely a tissue specific multigene expression strategy needs to be followed.

5.1. Improving specific metal uptake

Proteins belonging to the ZRT-, IRT-like (ZIP) family play an important role in metal uptake within a plant (Guerinot, 2000). Mutation of the iron uptake ZIP transporter gene IRT1 in *A. thaliana*, renders these plants Fe deficient. Analysis of mutants of the *A. thaliana ZIP1, ZIP2, ZIP3* and *ZIP4* genes, which were the first putative plant zinc transporters cloned (Grotz et al., 1998), have not yet been reported, but considering the number of putative Zn transporting ZIPs (Guerinot, 2000), there is likely to be functional redundancy among them. *ZIP1, ZIP4, ZIP9* and *IRT3* are strongly induced by zinc deficiency in *A. thaliana* roots and the orthologues of these genes are constitutively highly expressed in *N. caerulescens* (Hammond et al., 2006; van de Mortel et al., 2006) or *A. halleri* (Becher et al., 2004; Weber et al., 2004). Expression of *AtZIP4* is not in the epidermis, which would be expected for an uptake transporter, but instead in the root endodermis and pericycle (Milner and Kochian, 2008). The same was seen for the *NcZNT1* orthologue (Chapter 2), suggesting that enhancing endodermis-specific metal uptake by tissue specific expression of metal uptake transporters would be the target for modification of Zn/Cd/Ni root uptake capacity.

5.2. Increased root-to-shoot metal translocation

Root-to-shoot translocation of metal ions is one of the key essential features of metal hyperaccumulators and is thus an obvious target of genetic engineering for efficient phytoremediation (Krämer and Chardonnens, 2001; Krämer, 2005; Hanikenne et al., 2008; Wong and Cobbett, 2009). A strong metal sink in the shoots, increased xylem loading and restricted metal sequestration in

root vacuoles, are possible ways to increase the root-to-shoot translocation. *HMA4*, encoding a P1B-type ATPase, has been shown to be essential for root-to-shoot transport of Zn and Cd in general (Papoyan and Kochian, 2004; Verret et al., 2004;Wongand Cobbett, 2009) and plays a crucial role in metal hyperaccumulation of *A. halleri* (Hanikenne et al., 2008). The high expression of *HMA4* in *A. halleri*, compared to *A. thaliana*, is caused by a combination of cis-regulatory sequences and copy number expansion of the *A. halleri* gene. Expression of *AhHMA4* in *A. thaliana* depleted the root pericycle cells of Zn, probably due to enhanced xylem loading, which corresponded with enhanced shoot Zn or Cd hypersensitivity when exposed to high Zn or Cd concentrations. However, enhanced accumulation of Zn or Cd could not be demonstrated in these overexpression plants, for reasons not yet understood (Hanikenne et al., 2008). Still, proper tissue specific expression of this gene, mimicking expression in *A. halleri*, is likely to be needed to ensure enhanced root-to-shoot transport, but this should be complemented with enhanced xylem unloading and storage of Zn and Cd in leaf tissues, to avoid toxicity problems.

5.3. Detoxification through metal chelation and sequestration

A strong sink to safely store toxic metals is crucial for tolerance when a plant accumulates higher amounts of damaging metals. In this way, the plants can avoid the undesired reaction of these metals to different cellular compounds. This consideration needs to be kept in mind for engineering plants useful for phytoremediation purposes. Storage and detoxification of metals in some metal accumulating species is due to metal storage in epidermal cells (Kupper et al., 1999, 2000; Clemens et al., 2002). Ma et al. (2005) reported that *N. caerulescens* stores metals intracellularly in the vacuoles of leaf epidermal or sub-epidermal cells.

Phytochelatins (PCs), metallothioneins (MTs), amino acids and organic acids are compounds that can chelate metals and thus play a role in detoxification of metals in plants (Ernst et al., 1992). PCs have been found in different organisms, ranging from yeast to plants (Kondo et al., 1984; Grill et al.,

1985). Glutathione is the precursor from which PCs are formed. PCs can form complexes with different metals ions including Cd, but affinity to Zn is much less (Maitani et al., 1996). Exposure to Cd normally induces the accumulation of PC to high levels, and although PC levels are increased upon overexpression of *PCS*, this did not lead to increased Cd accumulation in the shoot in *A. thaliana* (Li et al., 2004) or tobacco (Wojas et al., 2008) and even caused Cd sensitivity. On the other hand, Martinez et al. (2006) reported that expression of a PCS gene from *Triticum aestivum* enhanced accumulation of Cd, Pb and Cu in *Nicotiana glauca*. However, increasing PC concentration is not essential to obtain high Cd tolerance. Upon exposure to Cd, the natural Cd tolerant and accumulating *N. caerulescens* accession Prayon has lower PC levels in leaves than the related non-accumulator *T. arvense* (Ebbs et al., 2002). Increasing PC levels in roots might even reduce the root-to-shoot transport required for accumulation in shoots.

Improved NA-chelation of metals also contributes to improved tolerance. Over-expression of barley *NAS* in *A. thaliana* and tobacco increased the NA concentration in these plants and this conferred increased Mn, Zn, Fe, Cu and especially Ni tolerance to these plants (Kim et al., 2005). Over-expression of the *NAS3* gene from *N. caerulescens* also improved Ni tolerance of *A. thaliana* plants, which accumulated slightly more Ni in their aerial parts (Pianelli et al., 2005). Similarly, over-expression of the rice *NAS3* gene enhanced Fe, Zn and Cu accumulation in seeds of paddy-grown rice plants to ~2-fold higher levels compared to wild type plants (Kawachi et al., 2009).

Most Zn and Cd are stored in vacuoles. Members of the CDF protein family are involved in the sequestration of metals into vacuoles. Some CDF family members, like *AtMTP1*, *PtdMTP1, AtMTP3 and NgMTP1*, were shown to cause increased Zn tolerance and accumulation when ectopically or heterologously expressed in *A. thaliana* (van der Zaal et al., 1999; Blaudez et al., 2003; Arrivault et al., 2006; Gustin et al., 2009), 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 or as buffer in case of Zn deficiency. Song et al. (2003) reported that strong artificial metal sinks are generated in roots and shoots by expressing a vacuolar metal transporter under the control of a 35S promoter, ultimately resulting in enhanced metal accumulation. The *AtMTP1*

(or ZAT) gene is involved in Zn storage and enhanced expression confers tolerance to Zn (van der Zaal et al., 1999; Desbrosses-Fonrouge et al., 2005). Mutant *mtp1* plants exposed to Zn are unable to store much Zn in their roots. They proportionately accumulate more in the shoots compared to wild-type plants (Kawachi et al., 2009). *MTP1*-like genes have previously been shown to be higher expressed in *N. caerulescens, N. goesingense* and *A. halleri*, compared with non-accumulators *T. arvense*, *A. thaliana and B. juncea* (Assunção et al., 2001; Persans et al., 2001; Dräger et al., 2004). Expression of the *MTP1* gene from *N. goesingense* in Arabidopsis enhances root Zn accumulation and decreases shoot Zn accumulation, while enhancing tolerance to excessive Zn. Exclusive expression of *NgMTP1* in shoots enhanced accumulation of Zn in shoots, while exclusive expression in roots reduced Zn accumulation in shoots compared to plants expressing the gene constitutively in the whole plant (Guimaraes et al., 2009). Experiments to express the *MTP1* gene of *N. caerulescens* in *A. thaliana* or tobacco indicate that this also enhances tolerance to Cd (Chapters 3, 4, 5). It means that expression of *MTP1* is a useful tool to modulate vacuolar Zn, and perhaps even Cd, sequestration. One possibility of using this gene for enhanced accumulation of Zn is to reduce its expression in roots while keeping its expression higher in shoot cells. This should increase the availability of Zn for long distance transport into the shoot tissues.

Some other genes with a putative role in intracellular compartmentalization have been identified. These include genes for CPx-type ATPases, ABC transporters and CAX transporters (Krämer et al., 2007), but only few genes have been functionally analysed in detail. Mutation of the *cax4* gene in *A. thaliana* renders these plants more Cd sensitive, most likely due to reduced vacuolar Cd storage capacity in roots. However, CaMV 35S-mediated over-expression did not affect Cd tolerance, although it made plants more sensitive to high Mg or low Ca (Mei et al., 2009). These compartmentalization genes do not have to come from plants. Song et al. (2003) reported that CaMV 35S-mediated expression of the vacuolar ABC transporter *YCF1*, generated a strong

artificial Pb and Cd sink in roots and shoots of *A. thaliana*, and the ultimate result was enhanced metal accumulation.

5.4. Transcriptional regulation

Another tool to transfer metal hyperaccumulator traits to non-hyperaccumulator species is by modifying expression of transcriptional regulators, normally controlling the expression of several genes needed for metal hyperaccumulation. This avoids the need to identify many or all genes in the response pathway, to determine their expression in the metal hyperaccumulator and to modify it accordingly in the non-hyperaccumulator. The approach has worked very well in case of engineering drought tolerance in plants, in which constitutive ectopic over-expression of the *DREB1* gene, one of the transcriptional regulator of plant drought response (Liu et al., 1998), enhanced drought tolerance. However, the constitutive expression of drought stress response also caused severe dwarfism. Such could be circumvented by expressing the *DREB1* gene under control of the drought responsive rd29A promoter, thus only activating enhanced drought tolerance under drought conditions (Kasuga et al., 1999). A similar approach could be used once transcription factors controlling Zn tolerance or hyperaccumulation traits are identified, which is not yet the case. In *A. thaliana*, basic-region leucinezipper (*bZIP*) transcription factors (TFs) *bZIP19* and *bZIP23* were reported to regulate adaptation to Zn deficiency (Assunção et al., 2010). The *bzip19 bzip23* double mutants were sensitive to Zn deficiency. The authors have shown that these transcription factors induce the expression of a set of Zn deficiency responsive genes. These TFs, their binding domain (Zn deficiency response element; RTGTCGACAY) and their target genes were found to be conserved in higher plants. One striking characteristic of Zn hyperaccumulators is the high expression of Zn homeostasis genes (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006, 2008). In non-hyperaccumulators these genes are mainly induced upon Zn deficiency. Therefore, the transcription factors, like *bZIP19* and *bZIP23,* controlling the Zn deficiency response in plants are likely to be important regulators of hyperaccumulation traits, and cloning of these genes will be relevant to explore

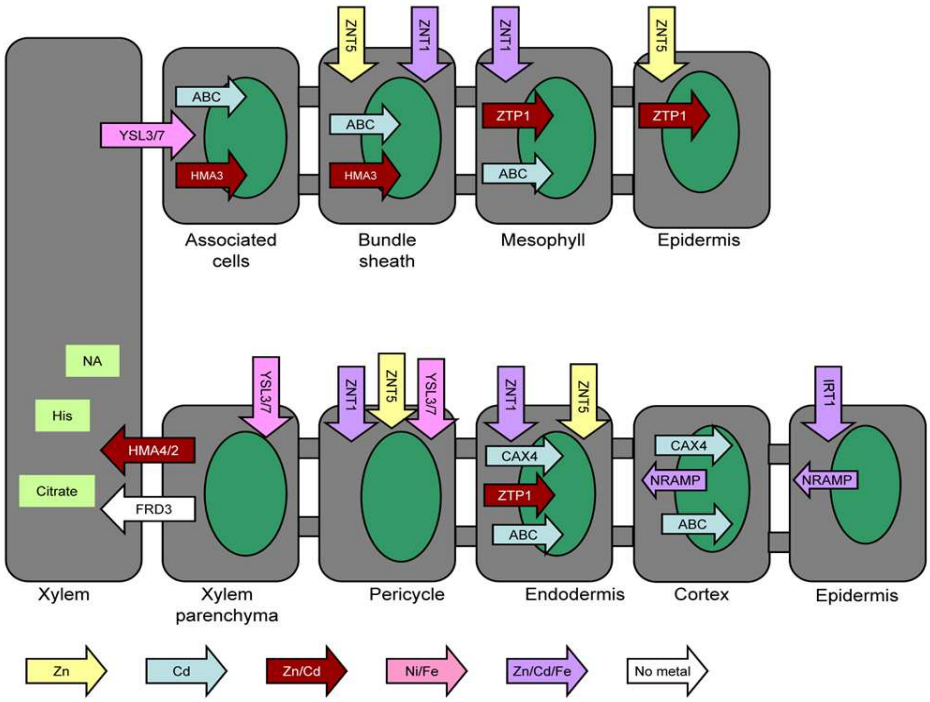

Fig. 1: Model for Zn, Cd and Ni transport and sequestration in root and shoot cells of the Zn/Cd/Ni hyperaccumulator *Noccaea caerulescens* Metal or metal-chelate transporters are depicted as arrows located at the plasma membrane or the vacuolar membrane of tissues that take part in metal translocation. The symplast is indicated in grey, the vacuole is indicated in green. The colour of the arrows indicates the metals these proteins are most likely to transport. IRT1 is an inward directed Fe/Zn/Cd plasmamembrane transporter. Vacuolar ABC transporters are thought to sequester phytochelatin-chelated Cd in vacuoles. NRAMPs are outward directed Zn/Fe/Cd vacuolar transporters. CAX4 is most likely a root vacuolar importer of Cd. ZNT1 is an inward directed Zn plasmamembrane transporter with affinity for Fe and Cd. ZNT5 is most likely an inward directed Zn plasmamembrane transporter. HMA2 and HMA4 are outward directed Zn/Cd plasmamembrane transporters. HMA3 is an inward directed vacuolar Zn transporter. FRD3 is a citrate transporter, essential for Fe/Zn translocation via the xylem. YSL3 and YSL7 are probably inward directed NA–Ni/Fe (and possibly NA–Cu/Zn) chelate plasmamembrane transporters. ZTP1 (now called MTP1) is an inward directed vacuolar Zn transporter. NA (nicotianamine), His(tidine) and citrate are ligands involved in metal translocation through the xylem.

this hypothesis.

In the absence of such Zn hyperaccumulation transcription factors, there is an option to use transcriptional regulators involved in Fe homeostasis, for modification of Cd accumulation, since Cd uptake in hyperaccumulators appears to go through the IRT1 Fe uptake transporter (Plaza et al., 2007). For Fe homeostasis, a number of transcriptional regulators have been identified in dicotyledonous plants, such as the *FER/FIT/bHLH29, bHLH38, bHLH39, bHLH100* and *bHLH101* genes (Bauer et al., 2007; Wang et al., 2007; Yuan et al., 2008). Constitutive over-expression of any of these to enhance Fe uptake may not be successful due to additional levels of regulation of protein expression, as was seen for IRT1 (Connolly et al., 2002; Kerkeb et al., 2008). For example, CaMV 35Smediated over-expression of *FIT* in A. thaliana increased expression of the down-stream genes, but only under Fe deficiency and not under Fe sufficient conditions (Colangelo and Guerinot, 2004). Thus, control of post-transcriptional regulation will be needed.

6. Conclusions and future perspectives

A wealth of knowledge has been generated about metal hyperaccumulation and tolerance using modern molecular tools. Different studies have unravelled distinct aspects of these fascinating biological processes and their underlying molecular mechanisms. Different genes have been cloned and their importance in metal accumulation has been established. *ZIP, MTP, NAS, YSL* and *HMA* genes are particularly important in this regard as they are found to play a significant role in these physiological processes. In general, genetic engineering has opened new horizons for understanding metal hyperaccumulation and hypertolerance properties. The use of modern molecular tools, helpful for developing of a practical phytoremediation technology, will have more milestones ahead. The better understanding of the molecular mechanisms controlling these traits needs attention. The promoters of different genes need to be isolated and characterized. This will help in understanding the regulation of different genes, which appears to be crucial in mimicking the hyperaccumulator phenotype in high biomass non-hyperaccumulator species useful for phytoremediation. Different studies have elaborated the role of single

genes in metal tolerance and accumulation, but combinations of two, or even more, genes have hardly been used to enhance these traits. It is thus rational to use a multigene strategy for improving metal accumulation in non-hyperaccumulators. As field oriented phytoremediation technology needs high biomass plants species with metal accumulation traits, a multigene strategy with tissue specific expression needs to focus on obtaining such high biomass plants. The large majority of metal-related research activities are being carried out in a lab, using optimal hydroponic growth conditions and single metal exposures. Although such conditions are excellent for dedicated phenotypic studies to understand physiological processes and new gene functions, it does not represent actual field conditions, where generally metals are mixed and less available than in hydroponics. It is therefore plausible to more reliably test the phytoremediation potential of transgenic plants in actual metal contaminated field soils.

7. Outline of the thesis

This thesis describes the functional analysis of two Zn transporter genes, *NcZNT1* and *NcMTP1*, from the metal hyperaccumulator plant species *N. caerulescens*. Furthermore, this study explores the applied implications of these genes in high biomass species *N. tabacum* useful for phytoremediation purposes. Functional characterization of the *NcZNT1* gene by expressing it in *A. thaliana* and the comparison of the promoter with its homologue *AtZIP4* in order to understand its regulation and tissue specific localization is described in chapter 2. Chapter 3 explores the functional analysis of the *NcMTP1* gene by expressing it in *A. thaliana* and by knocking down its expression in *N. caerulescens*. Chapters 4 and 5 describe the separate and combined expression of *NcZNT1* and *NcMTP1* genes in *N. tabacum* for enhanced metal tolerance and accumulation useful for phytoremediation, initially on hydroponic medium and finally on original metal-contaminated soil. The main findings of previous chapters and their future perspectives are discussed in chapter 6.

CHAPTER 2

The expression of the ZNT1 zinc transporter from the metal hyperaccumulator *Noccaea caerulescens* confers enhanced zinc and cadmium tolerance and accumulation to *Arabidopsis thaliana*

Zeshan Hassan[1]*, Ya-Fen Lin[1]*, Liu Hong[1], Sangita Talukdar[1], Alfred Arulandhu[1], Henk Schat[2] and Mark G.M. Aarts[1]

[1]Laboratory of Genetics, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB, Wageningen, the Netherlands

[2]Ecology and Physiology of Plants, Faculty of Biology, Free University, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

* authors have contributed equally

ABSTRACT

- The objective of this research was to perform a functional analysis of the *NcZNT1* gene from the metal hyperaccumulator *Noccaea caerulescens* and a comparative promoter study of *NcZNT1* from *N. caerulescens* and *AtZIP4* from *Arabidopsis thaliana*.

- Functional analysis of *NcZNT1* was carried out by analysing its expression in *N. caerulescens* shoots and roots in zinc (Zn) deficient, sufficient and excess conditions. Furthermore, expression of the full length *NcZNT1* cDNA in *A. thaliana* was performed and gene expression of known metal transporters was analysed in the transgenic lines. The expression pattern of *NcZNT1* and *AtZIP4* was studied in *A. thaliana* and *N. caerulescens* using promoter-GUS/GFP reporter constructs.

- We have found that *NcZNT1* was expressed in both shoot and root tissues while its expression was enhanced under Zn deficient conditions. The *pro35S::NcZNT1 A. thaliana* lines showed enhanced tolerance to Zn and cadmium (Cd) excess, increased accumulation of Zn and Cd and up-regulation of the Fe deficiency response compared to wild type plants. *N. caerulescens* plants with transgenic *proAtZIP4::*GUS roots showed GUS expression only under Zn deficiency while plants with *proNcZNT1::GUS* roots showed expression both in Zn deficient and sufficient conditions. Both *proAtZIP4::eGFP* and *proNcZNT1::eGFP* expressing roots showed GFP expression in cortex, endodermis, pericycle cells and vascular tissues when expressed in *A. thaliana* roots under Zn deficient conditions. In *N. caerulescens*, expression of *proNcZNT1::eGFP* is higher than of *proAtZIP4::eGFP*, especially under Zn sufficient conditions. Differences in *cis*- and *trans*-regulators are likely to account for the differences in expression between *A. thaliana* and *N. caerulescens*. *NcZNT1* is clearly an important factor in Zn and Cd tolerance and accumulation in *N. caerulescens*. Its expression in the stele of *N. caerulescens* roots proposes its involvement in long distance metals transport by maintaining the metal influx into cells responsible for xylem loading and ultimately shoot translocation of the metals.

INTRODUCTION

Zinc (Zn) is an essential component of several enzymes in plants like RNA polymerase, alcohol dehydrogenase, Cu/Zn superoxide dismutase and carbonic anhydrase (Marschner, 1995; Guerinot and Eide, 1999). Also, a large number of proteins contain Zn-binding structural domains such as the Zn finger domain. Poor growth and less biomass are among the major Zn deficiency symptoms which lead to reduced crop yields (Marschner, 1995). Although Zn is essential for plants, supra optimal concentrations of Zn can be toxic. The toxic effects are due to uncontrolled binding of Zn to proteins and cofactors and rendering them non-functional (Eide, 2003). It is known that cellular damaging Reactive Oxygen Species (ROS) are highly induced under excess Zn conditions in plants (Cuypers et al., 1999). Leaf chlorosis and growth reduction was reported due to Zn toxicity (Marschner, 1995). Cadmium (Cd) is toxic for plants having no known biological function but can be taken up by Zn transporters due to similarity with Zn (Pence et al., 2000). Cd is not able to initiate ROS production directly but enhanced ROS levels were found under Cd exposure (Cuypers et al., 2009). Cd toxicity has harmful effects in plants as it disturbs DNA repair mechanism, reduced water and nutrient uptake, lowered photosynthesis and ultimately leaf chlorosis and reduction in plant growth (Banerjee and Flores-Rozas, 2005; Sanita di Toppi and Gabbrielli, 1999). In order to deal with fluctuations in metal concentrations, plants have evolved "metal homeostasis" which is the ability to regulate their cellular and organellar metal concentration to maintain a stable and constant condition (Chapter 1).

There are few species that hyperaccumulate Zn from the soil and store it in their leaves. 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). Another characteristic of metal hyperaccumulators is that most of the hyperaccumulated metals are found in the shoots rather than in the roots, whereas generally plants try to reduce the shoot heavy metal concentration to avoid toxicity and negative interference with photosynthesis. Two of these Zn

hyperaccumulators, *Noccaea caerulescens* and *Arabidopsis halleri*, were examined at the transcriptional level, which showed that both species generally express genes constitutively that are normally induced by Zn deficiency and at higher levels than related non-hyperaccumulators (Becher et al., 2004; Hammond et al., 2006; van de Mortel et al., 2006).

Noccaea caerulescens is an exceptional metal hyperaccumulating species, as it is the only one to hyperaccumulate Zn (30,000 mg kg^{-1}), Ni (4000 mg kg^{-1} DW) and Cd (2700 mg kg^{-1} DW) in shoots (Brown et al., 1995; Assunção et al., 2003a). In addition, there is substantial natural variation regarding metal specificity and metal tolerance among different accessions. Accessions originating from serpentine soils are generally good at accumulating Ni. Accessions from calamine or non-metallicolous soils are good Zn accumulators. Cd hyperaccumulation so far has only been observed among populations found in the south of France, in the region around Ganges (Lombi et al., 2000; Assuncão et al., 2003a; Roosens et al., 2003). *N. caerulescens* is one of the few known Cd hyperaccumulator species together with *A. halleri* and *Sedum alfredii* (Yang et al., 2004). It belongs to the Brassicaceae family and shares about 88.5% coding region sequence similarity with the dicot plant reference species *A. thaliana* (Rigola et al., 2006).

Understanding the mode of action of plant metal hyperaccumulation is interesting for evolutionary and applied biology reasons. Most of the metal hyperaccumulation traits evolved relatively recently. For instance within the Brassicaceae family, metal hyperaccumulation is found in at least three genera: *Arabidopsis*, *Noccaea* (previously known as *Thlaspi*) and *Alyssum*. Although these genera are related (Koch et al., 2009), it is unlikely that their common ancestor was hyperaccumulating, considering there are only few hyperaccumulator species within the lineages derived from such common ancestor. It means that this trait has evolved several times independently of each other and thus this facilitates the study of the molecular origin of such a drastic adaptive evolution. This will provide interesting insight in the selective mechanisms that are prone to such evolutionary changes. The applied interest in metal hyperaccumulation resides in the use of metal hyperaccumulator plants for the remediation of metal polluted soils known as "phytoremediation" (Reeves and Baker, 2000). A

disadvantage of the plant species that are currently used for Zn/Cd phytoremediation is that either their biomass is insufficient to support economically viable phytoremediation projects, or their metal extraction capacity is too low (Chapter 1). Furthermore, "biofortification" is the fascinating concept of improving the human micronutrients deficiencies (e.g. Zn and Fe) by developing crops having improved bioavailable micronutrient content (Palmgren et al., 2008). With increased knowledge on the mode of action of Zn and Cd tolerance, uptake, translocation and accumulation in Zn/Cd hyperaccumulating species, it may be possible to engineer Zn/Cd hyperaccumulation and tolerance in a high-biomass species for Zn/Cd phytoremediation or in a crop species useful for Zn biofortification purposes. In order for this to be efficient, both the genes involved and their (post-) transcriptional regulation should be known and optimized.

So far, a number of metal transporters are identified and shown to be involved in the metal hyperaccumulation process (Chapter 1). Previously, we have cloned the *ZNT1* gene from *N. caerulescens*, encoding a ZIP-like transporter (Assunção et al., 2001). *NcZNT1* resembles most the *AtZIP4* gene from *A. thaliana* with 90 % cDNA and 87 % amino acid identity. Heterologous expression in yeast showed it to mediate high-affinity Zn uptake and low-affinity Cd uptake (Pence et al., 2000). The *AtZIP4* gene was not studied in great detail, but expression is known to be strongly induced in roots and shoots under Zn deficient conditions (Grotz et al., 1998; van de Mortel et al, 2006). Previously, it was reported that *NcZNT1* is predominantly expressed in roots and less in shoots in *N. caerulescens*, but expression of this gene is barely responsive to changes in Zn supply (Assunção et al., 2001). Only at very high Zn concentrations, the expression is somewhat reduced (Pence et al., 2000; van de Mortel et al., 2006). This deregulation of the *N. caerulescens* gene compared to its *A. thaliana* orthologue may be part of the metal adaptation phenomenon of the former species.

Recently, Milner and colleagues (2012) have reported that NcZNT1 is able to transport Zn but not Cd, contradicting its previously known Zn and Cd transport ability (Pence et al., 2000). *A. thaliana* lines expressing *NcNZT1* were found to be sensitive to excess Zn but not to Cd. However, they have used what

appears to be a 5′ truncated *NcZNT1* cDNA. In the current study we have performed a detailed analysis of transgenic *A. thaliana* lines expressing *NcZNT1* and of the *NcZNT1* promoter, to investigate the function of *NcZNT1* and its role in metal hyperaccumulation or tolerance. We have determined the response of *NcZNT1* gene expression to changes in Zn supply. We have also examined the phenotype of *A. thaliana* lines expressing full length *NcZNT1* cDNA under control of the strong CaMV 35S promoter and have analysed the gene expression of known metal transporters in these lines. To further determine the expression pattern of *NcZNT1* and *AtZIP4* genes, we have used full promoter-GUS fusion constructs of these genes and have studied GUS expression in *N. caerulescens* roots under different metal exposure conditions. The possible function of *NcZNT1* gene and the relevance for Zn/Cd hyperaccumulation of *N. caerulescens* is discussed. We conclude that *NcZNT1* plays an important role in Zn and Cd tolerance and accumulation and is involved in establishing a high metal influx into the root vasculature, important for xylem-mediated translocation of metals to the shoot.

MATERIALS AND METHODS

Construction of binary plasmids

The *pro35S::NcZNT1* construct was made by cloning a 1.48-kb *NcZNT1* (accession La Calamine, Belgium) cDNA fragment (Assuncao et al., 2001) upon restriction digestion with XbaI and HinDIII into the pGD121 (de Folter et al., 2006) vector harbouring the NPTII gene for selection on kanamycin resistance upon transformation. To develop the *proNcZNT1::GUS* construct, the *NcZNT1* promoter (896 bp) was amplified from *N. caerulescens* (accession La Calamine, Belgium) by using forward primer, 5′-CACCTCTGACTCTTTATCTGGCCT-3′ and reverse primer 5′-GGGAACAAAGAGTGTCTTCTTC-3′. To generate the *proAtZIP4::GUS* construct, the *AtZIP4* promoter (1046 bp) was amplified from *A. thaliana* (accession Colombia) by using forward primer, 5′-CACCTTTGGAAAGTGAAGTG-3′ and reverse primer 5′-GGGAACAAGAGTTTATTC-3′. The amplified fragments were cloned separately into pENTR™/D-TOPO® vectors (Invitrogen™, cat. K2400-20).

These entry vectors were recombined into the binary destination vector, pKGWFS7-RR, by using the Gateway® LR Clonase™ Enzyme Mix (Invitrogen™, cat. 11791-019). pKGWFS7-RR contains GUS as reporter protein and pAtUBQ10::DsRed as a selection marker, which was used to identify transformed roots based on red fluorescence under a stereo microscope using a DsRed filter (Op den Camp et al., 2011; Karimi et al., 2002). The destination constructs were sequenced to confirm correct cloning of the *NcZNT1* and *AtZIP4* promoters. The *proNcZNT1::eGFP* and *proAZIP4::eGFP* constructs were developed based on the pEZR(H)-LN vector (Narvaez-Vasquez, Pearce and Ryan, 2005), which harbours the eGFP gene for GFP expression and the hygromycin resistance HPT gene as a selection marker for transformation. The *proNcZNT1::eGFP* construct was made by replacing the CaMV35S promoter region of pEZR(H)-LN with the *NcZNT1* promoter upon restriction digestion with HindIII and NcoI. Similarly, the *proAtZIP4::eGFP* construct was developed by cloning *proAtZIP4* into pEZR(H)-LN upon restriction digestion with SacI and NcoI.

Plant transformation and growth conditions

The *pro35S::NcZNT1* construct was transformed to *A. thaliana*, accession Columbia (Col), by the *Agrobacterium tumefaciens*-mediated flower dipping transformation method as described by Clough and Bent (1998). T_1 transformed seedlings were selected on ½ MS agar plates (Murashige and Skoog, 1962) (no sugar, pH 5.8) supplemented with 50 mg L^{-1} kanamycin (Duchefa Biochemie B.V., Haarlem, The Netherlands) at 24 °C (16/8 hr, light/darkness). 50 independently transformed plants were tested for *NcZNT1* expression by semi-quantitative RT-PCR (data not shown) and 10 high expressing lines were propagated until homozygous T_3 lines. Three lines with the highest transgene expression in T_3 were used for experimentation. Plates were incubated in a climate-controlled growth cabinet (25°C 16/8 hr, light/darkness with illumination at a light intensity of 120 µmol m^{-2} s^{-1}). *proNcZNT1::GUS*, *proAtZIP4::GUS*, *proNcZNT1::eGFP* and *proAtZIP4::eGFP* constructs were transformed into *N. caerulescens* roots using modified *Agrobacterium rhizogenes*

mediated transformation method as described by Limpens et al. (2004). Seeds of *N. caerulescens* were sterilized and geminated on ½ MS agar plates (no sugar, pH 5.8) at 24 °C (16/8 hr, light/darkness). Seven-day-old seedlings were cut above the hypocotyl-root boundary and roots were removed. A dot of *Agrobacterium rhizogenes* (MSU440) containing *NcZNT1pro::GUS* or *AtZIP4pro::GUS* constructs was applied to the cut surface of each seedling and incubated for 5 day at 20°C /15 °C (day/night, 12 hours light). The *A. rhizogenes* inoculated seedlings were then transferred to ½ MS agar plates (no sugar, pH 5.8) containing 200 mg L^{-1} tricarcillin (Ticarcillin Disodium Mixture 15:1 & potassium Clarulanate; Duchefa, Netherlands) at 24 °C (16/8 hr, light/darkness). The non-transformed roots, which did not express DsRed or GFP, were cut off once every week under Leica MZ FLIII Fluorescence Stereo Microscope until only transgenic roots were growing.

Metal exposure

To determine the *NcZNT1* expression in response to various Zn treatments, seeds of *N. caerulescens* (La Calamine) were grown in modified half strength Hoagland's nutrient solution (Schat et al., 1996) containing 10 µM $ZnSO_4$. After three weeks, the seedlings were supplied with different Zn concentrations, Zn deficiency (0.05 µM $ZnSO_4$), Zn supply (2 µM $ZnSO_4$), Zn sufficient (10 µM $ZnSO_4$), or excess Zn (1000 µM $ZnSO_4$). After another four weeks, shoots and roots were collected separately for gene expression analysis. To determine the metal tolerance and accumulation of transgenic *pro35S::NcZNT1 A. thaliana* lines, nine plants for each of three independent transgenic lines and one control *A. thaliana* wild type (WT) line were grown hydroponically in modified half strength Hoagland's nutrient solution containing sufficient Zn (2 µM $ZnSO_4$) and excess Zn (60 µM $ZnSO_4$). For each treatment, the transgenic and control lines were grown in the same tray to avoid any effect of variation among the trays. Each tray was containing about nine litres of hydroponic medium. 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). Plants were grown for five weeks for the flowering time and Zn deficiency analysis experiments, while for the excess Zn experiment, plants were grown for four weeks. For the

first two weeks, plants were grown in sufficient Zn and for the rest of the period in respective treatments. The nutrient medium was refreshed twice every week. Root and shoot tissues were harvested for metal concentration analysis. Each hydroponics experiment was repeated twice at different time points, while keeping all growth conditions the same. To determine the response of *pro35S::NcZNT1* transformed *A. thaliana* plants to Cd, the same transgenic lines were grown hydroponically on modified half strength Hoagland's solution with sufficient Zn (2 µM $ZnSO_4$) for two weeks and then transferred to the same media but containing sufficient Zn (0 µM $CdSO_4$-2 µM $ZnSO_4$) and/or excess Cd (2 µM $CdSO_4$-2 µM $ZnSO_4$) while keeping the rest of the minerals constant in the media. The nutrient solution was refreshed every week. Plants were grown for four weeks. For mineral concentration analysis the root and shoot tissues were harvested individually. To compare *NcZNT1* and *AtZIP4* promoter expression in response to Zn treatment in *N. caerulescens* roots, *proNcZNT1::GUS* and *proAtZIP4::GUS* expressing *N. caerulescens* were grown hydroponically on modified half strength Hoagland's solution with deficient Zn (0.05 µM $ZnSO_4$) and sufficient Zn (10 µM $ZnSO_4$). The nutrient solution was refreshed every week. Plants were grown for four weeks.

To observe the root tissues localization of *proNcZNT1* and *proAtZIP4* in *A. thaliana*, the *proNcZNT1::eGFP* and *proAtZIP4::eGFP* expressing seedlings were grown in modified half Hoagland's solution with Zn deficiency (0 µM $ZnSO_4$) or with Zn supply (25 µM $ZnSO_4$) for three weeks. Similarly, *N. caerulescens* plants expressing *proNcZNT1::eGFP* and *proAtZIP4::eGFP* in roots were transferred to half strength Hoagland's solution with Zn deficiency (0.05 µM $ZnSO_4$) or Zn supply (100 µM $ZnSO_4$) for one week.

RNA isolation and quantitative Reverse Transcriptase-PCR (qRT-PCR)

To determine *NcZNT1* expression in *N. caerulescens* under different Zn exposure conditions and the expression of known metal transporters in *pro35S::NcZNT1* expressing *A. thaliana* lines exposed to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM) and excess Cd (2 µM) treatment, qRT-PCR was carried out. Total RNA was extracted by RNeasy® Plant Mini kit (Qiagen). On-column DNase digestion was performed to eliminate genomic DNA contamination. RNA concentration

and quality were measured by a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific). All RNA samples had A260/230 ratios of over 2.1, and a A260/A280 ratio of 2.1, so that the RNA quality was good enough for further qRT-PCR analysis. The first strain cDNA was synthesized from 1 µg RNA by using the iScriptTM cDNA Synthesis Kit (Bio-Rad). Clathrine (Gendre et al., 2006) was used as reference gene for the normalization of *NcZNT1* expression in *N. caerulescens* and *AtUBP6* (At1g51710) for the normalization of *AtBHLH100, AtIRT1, AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtNRAMP3, AtYSL3, AFRD3, AtMTP1* and *NcZNT1* in *pro35S::NcZNT1* expressing *A. thaliana* lines. Primers used for this gene expression analysis are shown in Table 2. Samples to which no reverse-transcriptase enzyme was added (NRT) were used as control to ensure the absence of genomic DNA in every sample. qRT-PCR was performed by using the kit of iQTMSYBR® Green Supermix (Bio-Rad), including 12.5 µL of iQ SYBR Green Supermix, 5 pmol of forward and reverse primers, and 5 µL of 10 times diluted cDNAs (corresponding to 5 ng/µL RNA) in a total volume of 25 µL. The qRT-PCR conditions were 3 min at 95°C , followed by 40 cycles of 10 sec at 95 °C and 1 min at 62 °C. The fluorescence signal was detected by a CFX96TM Real-Time Detection System (Bio-Rad). Melting curves were analysed to confirm the absence of primer dimers and nonspecific products. Three biological repeats per genotype or treatment and two technical repeats per biological repeat were used for the qRT-PCR analysis. The difference between technical repeats was less than 0.2 cycles. Relative transcript levels (RLT) were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *NcZNT1* expression of leaves under excess Zn (1000 µM $ZnSO_4$) was used as the calibrator in *N. caerulescens* plants, which means its RLT value is 1. In case of *pro35S::NcZNT1* expressing *A. thaliana* and WT lines, expression of *NcZNT1, AtIRT1, AtIRT2, AtFRO2, AtBHLH100, AFRD3, AtMTP1, AtYSL3, AtNRAMP3 AtHMA4* and *AtHMA3* genes in transgenic and WT lines was normalized to the respective WT shoot under sufficient Zn condition (2 µM Zn). *NcZNT1* expression in the *pro35SNcZNT1* expressing *A. thaliana* line was normalized to its *AtHMA4* shoot expression grown in sufficient Zn. All other gene transcripts were normalized to their respective WT shoot transcripts under sufficient Zn exposure. Each point is the average of two technical repeats of three biological

samples (each biological sample was again a pool of 3 biological samples). The stability of housekeeping genes was calculated by geNorm in qBasePLUS software (Biogazelle) and the reference genes with a geNorm M value lower than 0.5 were taken as stable genes (Hellemans et al., 2007). Heat map representing q-PCR data was developed by using "BAR Heat Mapper Plus Tool" via website http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi.

Root and shoot metal accumulation assay

Shoot and root samples were collected at the end of the metal exposure experiments and minerals like Zn, Cd, Fe, and Mn were measured spectrophotometrically as described by Assunção et al. (2003b).

GUS staining assay

The transformed roots of *N. caerulescens* were cut off and incubated at 37 °C for 3 hours in GUS staining solutions (pH 7.4) having 50 mM sodium phosphate, and 1 mg/ml 5-bromo-4-chloro-3-indolyl b-D-glucuronide (X-Gluc). The stained roots were washed thrice with 70% ethanol. A Nikon Eclipse 80i microscope was used to visualize the GUS expression in root tissues and images were captured by NIS Elements D3.1 software.

GFP visualization

Transgenic roots identified by a Leica MZ FLIII Fluorescence Stereo Microscope were either or not immersed in 1 µg/mL propidium iodide for 1-5 minutes, and then washed with deionized water before imaging. Propidium iodide was used to stain red and identify the cell walls. Images were acquired with an inverted laser scanning confocal microscope (LSCM) system, Zeiss LSM 510 Meta (Carl Zeiss, Jena, Germany) or Zeiss LSM 5 PASCAL. The eGFP (green) signal was visualized with an excitation wave length set at 488 nm and assembling emission signals between 505 to 530 nm. The signal for plant cell wall was visualized with excitation wave length set at 543 nm and assembling emission signals at 560 nm. A ×63 Plan Apochromate/ 1.4 oil DIC objective was used for the observation of *A. thaliana* transgenic roots, and EC Plan-Neofluar 20x or LD

Plan-Neofluar 40x objectives were used for *N. caerulescens* transgenic roots. Digital images were processed using LSM 510 3.5 or LSM 5 Image Examiner software. Confirmation of the 5′ region of *NcZNT1* in three *N. caerulescens* accessions. To verify the presence of an ATG start codon in *NcZNT1* cDNA 5′ to the one indicated by Milner et al. (2012), in *N. caerulescens* accessions, a forward primer (5′- GCTTTCTGCTCCTTGATCC -3′) and reverse primer (5′-CGATGAGAGGTATGGCTACA-3′) were designed according to the *NcZNT1* cDNA sequence of La Calamine (Assunção et al., 2001) and q-PCR was performed for *NcZNT1* in accessions La Calamine (LC), Prayon (PY), and Ganges (GA). The forward primer was designed at the corresponding 5′-UTR region of the orthologous *A. thaliana ZIP4* gene. RNA of LC, PY, and GA were isolated using the RNeasy® Plant Mini kit (Qiagen), and cDNA was synthesized by using the M-MLV Reverse Transcriptase (Invitrogen). The amplification of *NcZNT1* cDNA fragments was performed by Pfu DNA polymerase (Fermentas). The amplified fragments were then cloned into the pGEMT-easy vector (Promega) for sequencing. The whole DNA sequence of *NcZNT1* was also amplified by using the same forward primer (5′-GCTTTCTGCTCCTTGATCC-3′), but different reverse primer, (5′-CTAAGCCCAAATGGCGA-3′) designed at the 3′ end of the *NcZNT1* coding sequence. DNA of LC, PY, and GA were extracted by a modified nuclear extraction protocol (Aarts et al., 2000). The amplified fragments were also cloned into the pGEMT-easy vector (Promega) for sequencing. cDNA, DNA and predicted protein sequence results were compared by using MultAlin software (Multiple sequence alignment by Florence Corpet http://multalin.toulouse.inra.fr/multalin).

Statistical analysis

Where needed, data were analysed for significance at $p<0.05$ by using Student's t-test, two-way ANOVA and ANOVA (Least Significance Difference) in the SPSS v. 12 software package for MS Windows.

RESULTS

NcZNT1 expression is up-regulated under Zn deficiency in *N. caerulescens*

Previously, van de Mortel et al. (2006) showed that the *NcZNT1* gene expressed constitutively in roots of *N. caerulescens*, almost irrespective of Zn supply status, in contrast to its *A. thaliana* orthologue, *AtZIP4*, whose expression is strongly up-regulated in roots under Zn deficiency. To quantify the *NcZNT1* expression in response to different Zn treatments, in roots and shoots of *N. caerulescens*, we performed a quantitative RT-PCR using material from plants exposed to four different Zn concentrations. Compared to Zn sufficient conditions (Zn2 and Zn10), *NcZNT1* expression was significantly induced under Zn deficient conditions (Zn0.05), especially in shoots (8.8 fold), but also in roots (up to 2 fold); while *NcZNT1* expression was repressed by excess Zn treatment (Zn1000) (Fig. 1 A). There was no significant difference in expression when comparing both Zn sufficient conditions.

Heterologous expression of *NcZNT1* confers increased Zn tolerance and accumulation in *A. thaliana*

NcZNT1 was found to be expressed at higher levels in *N. caerulescens* when compared to its orthologues from *A. thaliana* or *T. arvense* (Pence et al., 2000; Assunção et al., 2001; Hammond et al., 2006; van de Mortel et al., 2006), corresponding with high Zn uptake, suggesting the involvement of this gene in high Zn uptake from the growth medium. In order to investigate if high expression of the gene as such would be sufficient to increase Zn uptake in plants, we expressed the *NcZNT1* gene to high levels in *A. thaliana* under control of the CaMV 35S promoter (Fig. 1 B). Homozygous *pro35S::NcZNT1 A. thaliana* lines were grown on modified half Hoagland's nutrient solution containing sufficient Zn (2 µM $ZnSO_4$) or excess Zn (60 µM $ZnSO_4$). No abnormal visible phenotype was discerned at the sufficient Zn treatment in transgenic lines, however, the Zn concentration in both shoots and roots of the *pro35S::NcZNT1* plants was significantly higher than in the WT plants (data not shown).

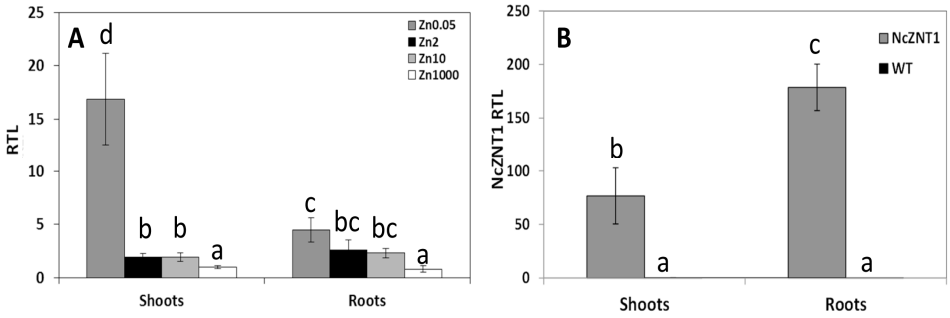

Fig. 1: Quantitative reverse transcriptase PCR analysis of *NcZNT1* expression in response to Zn in *N. caerulescens* and in *pro35S::NcZNT1* expressing *A. thaliana* (A) *N. caerulescens* plants were grown in ½ Hoagland's nutrient solution with different Zn conditions i.e. Zn deficiency (0.05 µM ZnSO4), Zn supply (2 µM ZnSO4), Zn sufficient (10 µM ZnSO4) and Zn excess (1000 µM ZnSO4) for four weeks. (B) *NcZNT1* expression in *pro35S::NcZNT1* expressing *A. thaliana* grown in sufficient Zn (2 µM ZnSO4) conditions. The gene expression was normalized to the AtUBP6 housekeeping gene in pro35S::NcZNT1 line and WT lines. Different letters indicate the significant differences between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of 4 replica).

At sufficient Zn supply, the transgenic lines showed reduced dry shoot and root weight compared to the WT line, although morphologically there was no difference (data not shown). The reverse was seen when plants were exposed to excess Zn. This high Zn supply affected growth of the WT line severely than that of the transgenic lines, also leading to significantly higher shoot and root dry weights in the transgenic lines (Fig. 2 A,B). The WT line also displayed purple anthocyanin pigmentation of the older leaves while transgenic lines did not show this phenotype and generally appeared to be greener. The *pro35S::NcZNT1* lines grown on excess Zn had a markedly higher Zn concentration in shoots and roots compared to the WT (Fig. 2 C, D). Transgenic lines also showed enhanced Mn, but not Fe, in shoots while there was no difference in Fe and Mn concentration in roots of transgenic and WT lines (Fig. 2 E, F).

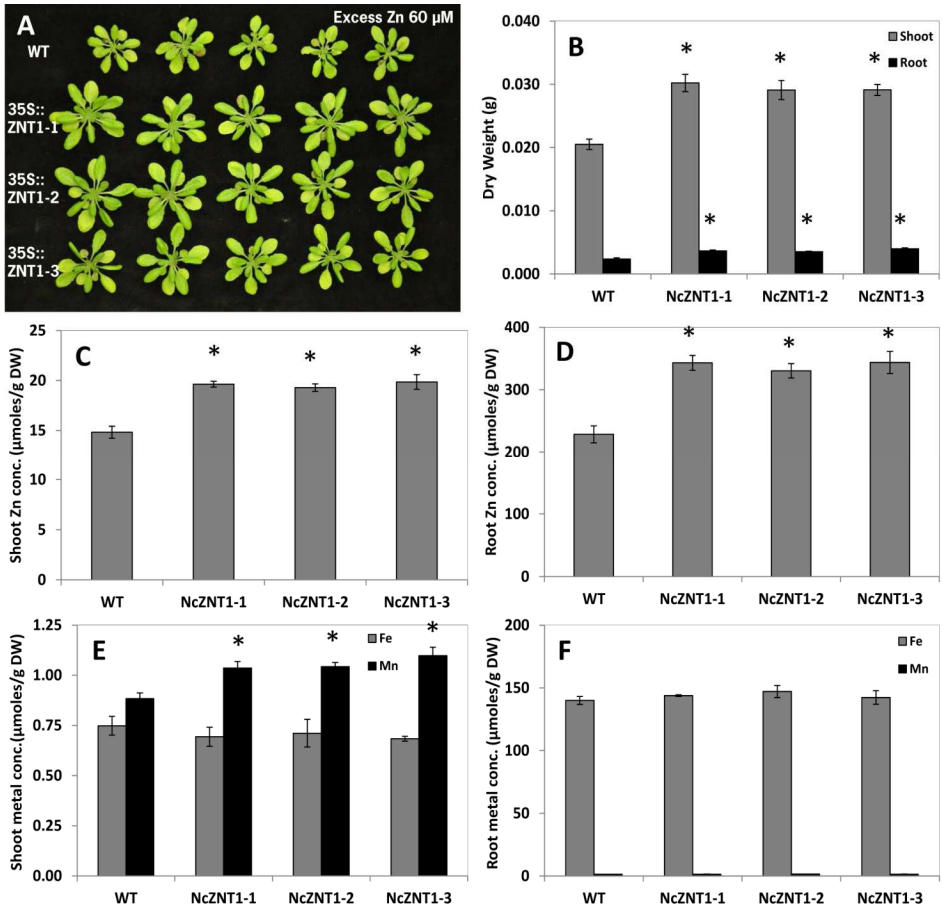

Fig. 2: The phenotypic response of transgenic *pro35S::NcZNT1* and Col wild-type (WT) *A. thaliana* lines to excess Zn. Three independently transformed lines (NcZNT1-1, NcZNT1-2, NcZNT1-3) and WT line were grown hydroponically on half Hoagland's media with excess Zn (60 µM $ZnSO_4$) for four weeks. (A) Visible phenotype of *pro35S::NcZNT1* and WT plants grown on Zn excess medium (B) Dry shoot weight (C) Zn concentration (µmoles/g DW) in shoots (D) and in roots (E) Fe and Mn concentrations (µmoles/g DW) in shoots (F) and in roots. Photographs were taken at the end of the 4th week since seed sowing. * indicates a significant difference (at $p < 0.05$ in two-way ANOVA) between transgenic and WT type line (mean ± SE of four replicates.

NcZNT1 expressing _A. thaliana_ lines showed enhanced Cd tolerance and accumulation

Since NcZNT1 was reported to be able to transport Cd, in addition to Zn (Pence et al., 2000), we also determined the response of the *pro35S::NcZNT1* plants to excess Cd exposure. The same three trangenic lines and the WT line as used in the previous experiment were grown hydroponically on modified half Hoagland's solutions containing sufficient Zn (2 µM $ZnSO_4$) or excess Cd (2 µM $CdSO_4$ + 2 µM $ZnSO_4$). Like for excess Zn exposure, the transgenic lines were more tolerant to Cd exposure than the WT line, with larger rosette size and leaves with less chlorosis (Fig. 3 A). Also the shoot and root dry weights of the transgenic lines were significantly higher than those of the WT line (Fig. 3 B). Cd exposure also increased the shoot Zn and Cd but reduced Zn concentrations in roots of the transgenic lines, although the shoot Cd concentrations remained much lower than the root Cd concentrations (Fig. 3 C, D). Shoot Fe concentrations decreased in the transgenic lines upon Cd exposure while shoot Mn concentrations of the transgenic line were same as that of WT line (Fig. 3 E) There was no difference in Fe and Mn concentration in roots of transgenic and WT lines (Fig. 3F).

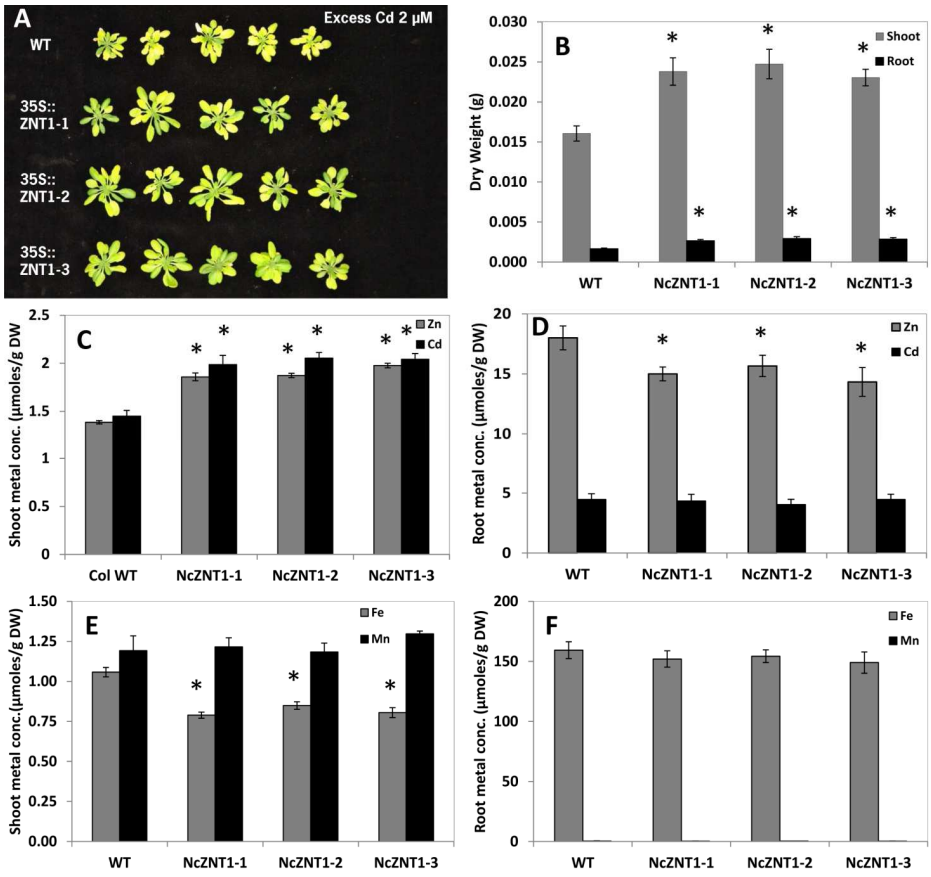

Fig. 3: The phenotypic response of transgenic *pro35S::NcZNT1* and Col wild-type (WT) *A. thaliana* lines to excess Cd. Three independently transformed lines (NcZNT1-1, NcZNT1-2, NcZNT1-3) and WT line were grown hydroponically on half Hoagland's media with excess Zn (2 µM $CdSO_4$) for four weeks. (A) Visible phenotype of *pro35S::NcZNT1* and WT plants grown on Cd excess medium (B) Dry shoot weight (C) Zn and Cd concentration (µmoles/g DW) in shoots (D) and in roots (E) Fe and Mn concentrations (µmoles/g DW) in shoots (F) and in roots. Photographs were taken at the end of 4th week since seed sowing. * indicates a significant difference (at $p < 0.05$ in two-way ANOVA) between transgenic and WT line (mean ± SE of four replicates).

Expression of *NcZNT1* alters the expression of other metal homeostasis genes in *A. thaliana*

Considering that the expression of *NcZNT1* alters Zn and Cd accumulation and tolerance in *A. thaliana*, we determined the expression of Zn and Fe homeostasis genes *AtBHLH100*, *AtIRT1*, *AtIRT2*, *AtFRO2* (involved in Fe uptake), *AtNRAMP3* (involved in Fe remobilization), *AtHMA4* (involved in Zn/Cd translocation), *AtYSL3*, *AFRD3* (involved in Zn/Fe translocation) and *AtMTP1* (involved in Zn excess tolerance) upon exposure of transgenic and WT lines to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$ + 2 µM $ZnSO_4$). Of the genes involved in Fe uptake, *AtIRT1, AtIRT2* and *AtFRO2* are mainly expressed in roots, whereas *AtbHLH100* is expressed both in roots and shoots. Transcription levels of all of these genes go up upon excess Zn and excess Cd exposure in roots of *A. thaliana* and often also in shoots, even if expression levels are low (Fig. 4: Table 1). This supports the idea that *A. thaliana* will induce a Fe deficiency response upon excess Zn and Cd exposure (van de Mortel et al., 2006). The *pro35S::NcZNT1* line exhibited reduced *AtbHLH100* expression in shoot and root tissues under excess Zn treatment while showing significantly higher expression upon excess Cd treatment. Expression of *AtIRT1*, *AtIRT2* and *AtFRO2* in roots of transgenic line was significantly reduced upon excess Zn treatment. Excess Cd had this effect only on *AtIRT1* expression, while it increased the expression of *AtIRT2*. Similar effects were seen for gene expression in shoot, though the biological relevance of this appears little considering the low expression of these genes in shoots. Also the expression of *AtFRD3* was significantly decreased in roots of transgenic line compared to WT under sufficient and excess Zn but not excess Cd (Fig. 4: Table 1). *AtMTP1* is expressed in both roots and shoots, with higher expression in both tissues of the transgenic line upon excess Zn and Cd, while at sufficient Zn, only the expression in shoots is reduced compared to the WT line (Fig. 4:

Table 1). Both *AtYSL3* and *AtNRAMP3* show only increased expression in roots of transgenic plants upon excess Zn, when compared to WT plants, while expression upon other treatments and tissues was similar in both genotypes (Fig. 4: Table 1). *AtHMA4* expression levels were found to be higher in root tissues of both lines compared to shoot (Fig. 4: Table 1), with transgenic line showing increased expression in roots upon sufficient Zn supply and decreased expression in roots upon excess Zn supply compared to the WT line. In general, *HMA3* was higher expressed in roots than in shoot. Expression of *AtHMA3* was higher in shoot and root tisuues of WT line in excess Zn condition (Fig. 4: Table 1). Transgenic line had higher *HMA3* expression in roots under sufficient Zn supply.

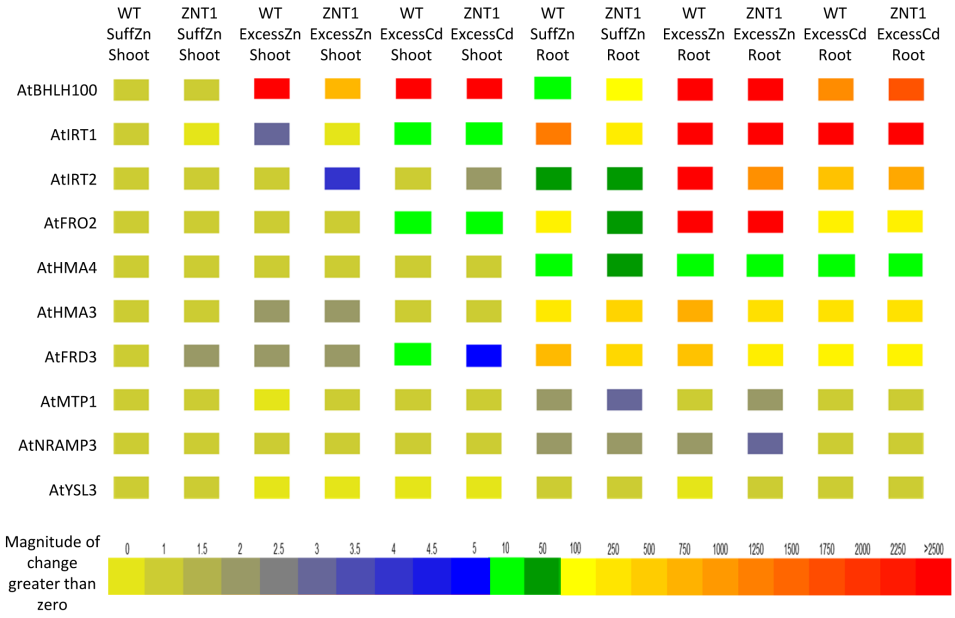

Fig. 4: Heat map representing relative gene expression analysis of known metal transporter genes *AtBHLH100, AtIRT1, AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtFRD3, AtMTP1, AtNRAMP3 and AtYSL3* in shoot and root of *pro35S::NcZNT1* and Col wild-type (WT) in response to sufficient Zn (SuffZn) (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in respective Col-WT in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data.

	WT SuffZn Shoot	ZNT1 SuffZn Shoot	WT ExcessZn Shoot	ZNT1 ExcessZn Shoot	WT ExcessCd Shoot	ZNT1 ExcessCd Shoot	WT SuffZn Root	ZNT1 SuffZn Root	WT ExcessZn Root	ZNT1 ExcessZn Root	WT ExcessCd Root	ZNT1 ExcessCd Root
AtBHLH100	a 1.0 ±0.1	a 0.8 ±0.3	f 2760 ±362	c 698 ±78	h 13046 ±595	i 16240 ±1919	b 5.5 ±0.7	b 4.4 ±1.7	g 5377 ±760	g 4274 ±625	d 1127 ±66	e 1685 ±222
AtIRT1	ab 1.0 ±0.2	a 0.5 ±0.3	b 3.0 ±0.5	a 0.4 ±0.0	c 5.9 ±1.5	c 6.2 ±0.7	e 1286 ±91	d 175 ±44	h 81904 ±15916	g 11084 ±1771	g 11651 ±2095	f 2389 ±86
AtIRT2	a 1.0 ±0.2	a 1.0 ±0.3	a 1.3 ±0.1	c 4.2 ±0.1	a 1.3 ±0.0	b 2.3 ±0.1	d 18 ±2.2	d 23 ±6.4	g 3661 ±494	f 1082 ±131	e 597 ±121	ef 850 ±52
AtFRO2	a 1.0 ±0.1	a 0.8 ±0.3	a 1.5 ±0.2	a 1.0 ±0.3	b 5.7 ±0.1	c 8.2 ±0.9	e 123 ±8.2	d 47 ±7.5	g 6018 ±665	f 2650 ±605	e 129 ±5.7	e 141 ±4.9
AtHMA4	a 1.0 ±0.1	a 0.8 ±0.0	a 0.7 ±0.1	a 0.7 ±0.2	a 0.6 ±0.1	a 0.7 ±0.0	c 9.5 ±0.9	d 14 ±1.1	c 10 ±0.6	b 6.4 ±0.5	b 7.8 ±1.0	b 6.9 ±0.5
AtHMA3	a 1.0 ±0.1	a 1.1 ±0.6	b 2.0 ±0.1	ab 1.6 ±0.6	a 0.7 ±0.1	a 1.1 ±0.3	c 207 ±2.0	e 412 ±32	f 792 ±114	d 295 ±8.7	d 284 ±37	d 271± 2.3
AtFRD3	a 1.0 ±0.2	a 2.0 ±1.1	a 1.6 ±0.1	a 1.5 ±0.1	b 6.2 ±0.6	b 5.0 ±0.3	g 671 ±60	e 379 ±66	f 598 ±4.1	d 159 ±28	cd 114 ±23	c 86 ±7.6
AtMTP1	c 1.0 ±0.1	ab 0.5 ±0.1	a 0.4 ±0.0	bc 0.7 ±0.1	ab 0.5 ±0.0	c 1.1 ±0.1	e 2.2 ±0.3	e 2.6 ±0.4	c 1.2 ±0.1	d 1.7 ±0.1	bc 0.8 ±0.1	c 1.3 ±0.2
AtNRAMP3	a 1.0 ±0.1	a 0.9 ±0.0	a 1.0 ±0.1	a 0.9 ±0.2	a 0.9 ±0.1	a 0.8 ±0.1	b 2.0 ±0.1	b 2.1 ±0.2	b 2.0 ±0.3	c 2.9 ±0.3	a 1.1 ±0.1	a 1.0 ±0.0
AtYSL3	cd 1.0 ±0.1	cd 1.1 ±0.0	a 0.3 ±0.0	ab 0.4 ±0.1	ab 0.4 ±0.0	ab 0.5 ±0.1	cd 1.1 ±0.1	d 1.2 ±0.1	a 0.3 ±0.0	b 0.6 ±0.0	b 0.6 ±0.1	bc 0.7 ±0.0

Table 1: Relative gene expression analysis of known metal transporter genes *AtBHLH100, AtIRT1, AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtFRD3, AtMTP1, AtNRAMP3 and AtYSL3* in shoot and root of *pro35S::NcZNT1* and Col wild-type (WT) in response to sufficient Zn (2 μM $ZnSO_4$), excess Zn (60 μM $ZnSO_4$) and excess Cd (2 μM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in respective wild-type in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data. ZNT1 represents *pro35S::NcZNT1* line, WT is for wild-type and "suff" represents sufficient. Different letters indicate the significant difference in gene expression of the respective gene between lines grown in given treatments ($p<0.05$, ANOVA, Least Significant Difference) (mean ± SE of 4 replica).

Comparison of *AtZIP4* and *NcZNT1* promoter GUS activity in *N. caerulescens*

In order to analyse the differential regulation of *NcZNT1* and *AtZIP4*, we transformed promoter::GUS constructs for both genes into *N. caerulescens*. Since there is no efficient stable transformation system available for *N. caerulescens*, we used the *A. rhizogenes*-mediated root transformation method modified from Limpens et al. (2004). This results in chimeric plants, with a transgenic root system supporting a non-transgenic rosette. Both *AtZIP4* and *NcZNT1* promoters were up-regulated under Zn deficiency, but repressed by Zn supply. The expression of *proAtZIP4::GUS* in *N. caerulescens* roots under Zn deficiency was restricted to the root cap and stele (Fig. 5 A, C). After 3 hours of GUS staining, *proAtZIP4::GUS* transgenic roots grown in sufficient Zn did not show any GUS expression (Fig. 5 B,D); however, if the roots were stained overnight, they had very low expression in the stele (data not shown). When *proAtZIP4::GUS* expressing *N. caerulescens* roots were transferred from Zn deficient to sufficient treatment, reduced GUS activity was observed supporting the low expression of *proAtZIP4::GUS* under sufficient Zn (data not shown). Under Zn deficient condition, *proNcZNT1::GUS* was ubiquitously expressed in root tissues, including root tips, root hairs, epidermis, cortex, pericycle and phloem (Fig. 5 E,G). The strongest expression was found in pericycle. With a slight increase of Zn supply (to 10 µM ZnSO4), the expression of *NcZNT1* promoter at the root tip was not changed, but the GUS staining was weaker than under Zn deficiency (Fig. 5 H); in contrast to root tips, the expression in mature roots was limited to the stele, particularly in pericycle and phloem (Fig. 5 F). In general, the *NcZNT1* promoter exhibited stronger GUS staining activities in roots of *N. caerulescens* than the *AtZIP4* promoter whether grown either in Zn deficient or sufficient conditions. Thus, it seems that *N. caerulescens* has a transcription factor binding to the *NcZNT1* promoter which may not bind very well to the *AtZIP4* promoter or that *N. caerulescens* might have a transcription factor binding to the *NcZNT1* promoter which is not present in *A. thaliana.*

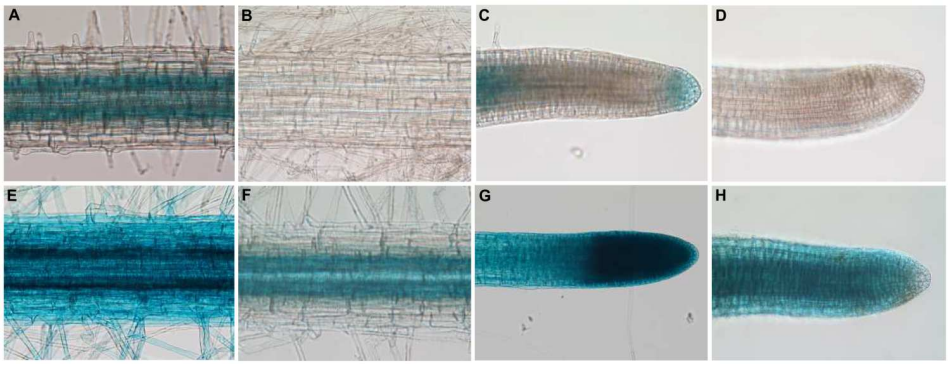

Fig. 5: *proAtZIP4::GUS* and *proNcZNT1::GUS* expression in *N. caerulescens* roots. *The d*ifferential expression of both *AtZIP4* and *NcZNT1* promoters in response to Zn is presented. Three-hour-GUS staining of transgenic *proAtZIP4::GUS* (A-D) and *proNcZNT1::GUS* (E-H) roots under Zn deficient (0.05µM $ZnSO_4$) conditions (A,E,C,G) and Zn sufficient (10µM $ZnSO_4$) conditions (B,F,D,H) condition is shown. Mature roots (A,B,E,F) and root tips (C,D,G,H) are displayed respectively.

GFP expression of *AtZIP4* and *NcZNT1* promoters in A. *thaliana* and *N. caerulescens*

Although the GUS assay is a quick and convenient method to detect the localization of promoters, the diffusion of the GUS protein under high GUS expression is still a limiting factor for precise localization analysis. Therefore, we used GFP visualization for the analysis of the *NcZNT1* and *AtZIP4* promoters, both in *A. thaliana* and in *N. caerulescens*. *proAtZIP4::eGFP* was expressed in the pericycle and cortex in *A. thaliana* roots (Fig. 6 A-C); whereas, it was expressed throughout the *N. caerulescens* roots (Fig. 6 D-F). *proNcZNT1::eGFP* expression was not changed in response to Zn in *A. thaliana* and it was localized in pericycle (Fig. 6 G-N); however, its localization in *N. caerulescens* was in xylem and pericycle cells (Fig. 6 O-W).

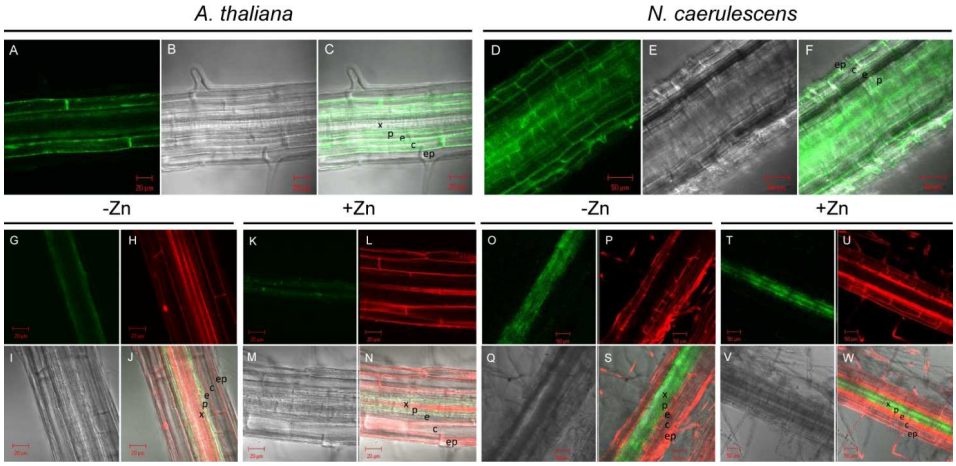

Fig. 6: *proAtZIP4::eGFP* and *proNcZNT1::eGFP* expression in *A. thaliana* and *N. caerulescens* roots under Zn deficient (-Zn) or sufficient (+Zn) conditions. *Agrobacterium tumefaciens* mediated transgenic *proAtZIP4::eGFP A. thaliana* (A-C) and *proNcZNT1::eGFP A. thaliana* (G-N) were grown on half Hoagland solution containing Zn deficiency (0 µM $ZnSO_4$) (A-C, G-J) or Zn supply (2 µM $ZnSO_4$) (K-N). *Agrobacterium rhizogenes* mediated transgenic *proAtZIP4::eGFP N. caerulescens* (D-F) and *proNcZNT1::eGFP N. caerulescens* (O-W) were grown on half Hoagland solution containing Zn deficiency (0.05 µM $ZnSO_4$) (O-S), or Zn supply (100 µM $ZnSO_4$) (T-W). GFP images of transgenic roots were acquired by an inverted laser scanning confocal microscope (LSCM) system, Zeiss LSM 5 PASCAL. (A), (D), (G), (K), (O), and (T) show the GFP florescence; (H), (L), (P), and (U) show the roots stained by Propidium iodide; (B), (E), (I), (M), (Q), and (V) are DIC microscopy; (C), (F), (J), (N), (S), and (W) are merging images. Root tissues are denoted by alphabets i.e. x (xylem), p (pericycle), e (endodermis), c (cortex) and ep (epidermis). Scale bar is 50µm.

ZNT1 sequence comparison

When comparing the coding sequence (CDS) of *AtZIP4* (At1g10970.1), *NcZNT1*-LC (Genbank: AF275751.1, from *N. caerulescens* accession La Calamine, LC), and *NcZNT1*-PY (Genbank: AF133267.1, *N. caerulescens* accession Prayon, PY), we noticed that the first ~90 bp were missing in the published *NcZNT1* CDS from PY (Milner et al., 2012), when comparing to LC. This results in N terminal deletion of 33 amino acids which may very well change the functional properties of the NcZNT1 protein. To clarify if this first ATG in LC and *A. thaliana* also exists in PY, we amplified the 5′ regions of *NcZNT1* from three *N.*

caerulescens accessions, LC, PY, and GA, using qRT-PCR. Our sequencing results showed that all three accessions contain the first 90 bp at theirs 5′ ends of CDS (Fig. 7). We thus conclude that the *NcZNT1* CDS sequence (Genbank AF133267.1) and published by Pence et al. (2000) and by Milner et al. (2012) is incomplete, which results in the production of N-terminally truncated protein. The expression of this N-terminally truncated protein is likely to affect the results of the functional studies described by Milner at al. (2012). To further clarify the gene structure, we also amplified the full length *NcZNT1* coding genomic DNA regions from these three accessions. The DNA sequence length (from start codon to stop codon) of *NcZNT1* from LC is 1589 bp; from GA is 1582 bp; and from PY is 1591 bp. *NcZNT1* DNA contains four exons and three introns in all these accessions, with a coding region of 1227 bp, which translates into 408 amino acids (Fig. 8). We also performed a predicted protein comparison of NcZNT1 between LC, PY and AtZIP4 from *A. thaliana*, which showed there are no stop codons or frameshifts suggesting not the first (used by Milner et al., 2012), but the second ATG is used for translation of these coding sequences (Fig. 9).

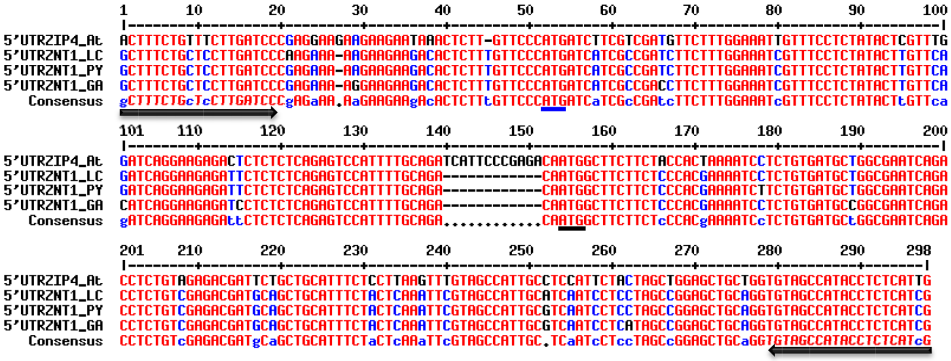

Fig. 7: Comparison of 5′ *NcZNT1* cDNA between *N. caerulescens* accessions La Calamine, Prayon, and Ganges. The translational start site (ATG) is underlined blue (used as start codon in current study). The second ATG, used as a start codon by Milner et al (2012), is underlined black. Forward and reverse primer pairs used for the amplification of this 5′ region are shown as arrows. The alignment was performed by MultAlin software (Multiple sequence alignment by Florence Corpet; http://multalin.toulouse.inra.fr/multalin).

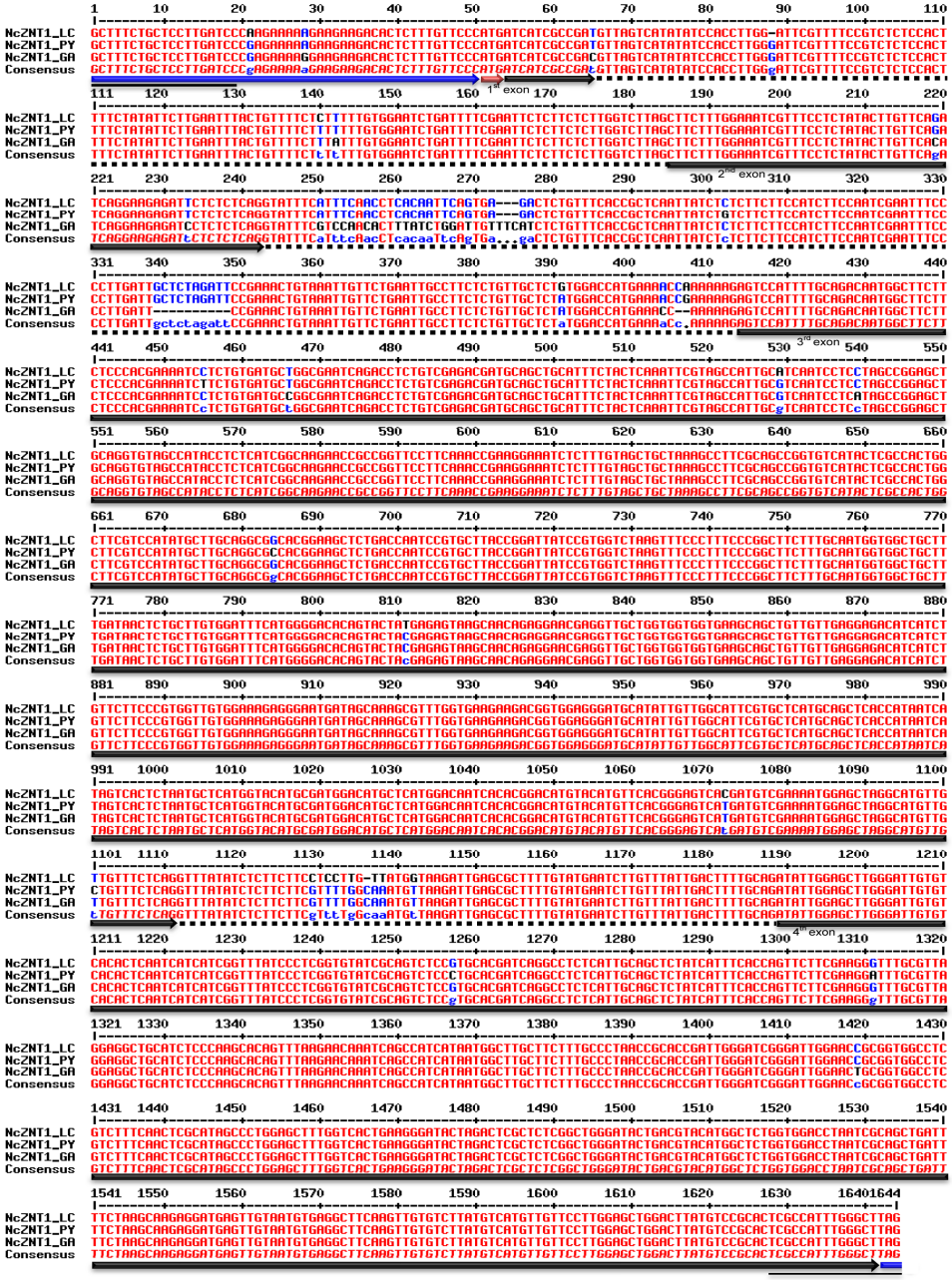

Fig. 8: Comparison of *NcZNT1* DNA sequences between *N. caerulescens* accessions La Calamine, Prayon, and Ganges. NcZNT1 DNA fragment were amplified from La

Calamine (LC), Prayon (PY), and Ganges (GA) by using the primer pairs shown as black lines (___). Blue arrow indicates part of 5′UTR sequences. Red arrow is the translational start site (ATG) and blue line is the translational stop site (TAG). Four exons were shown in black arrows and three introns were indicated by dotted lines. The alignment was performed by MultAlin software (Multiple sequence alignment by Florence Corpet; http://multalin.toulouse.inra.fr/multalin.

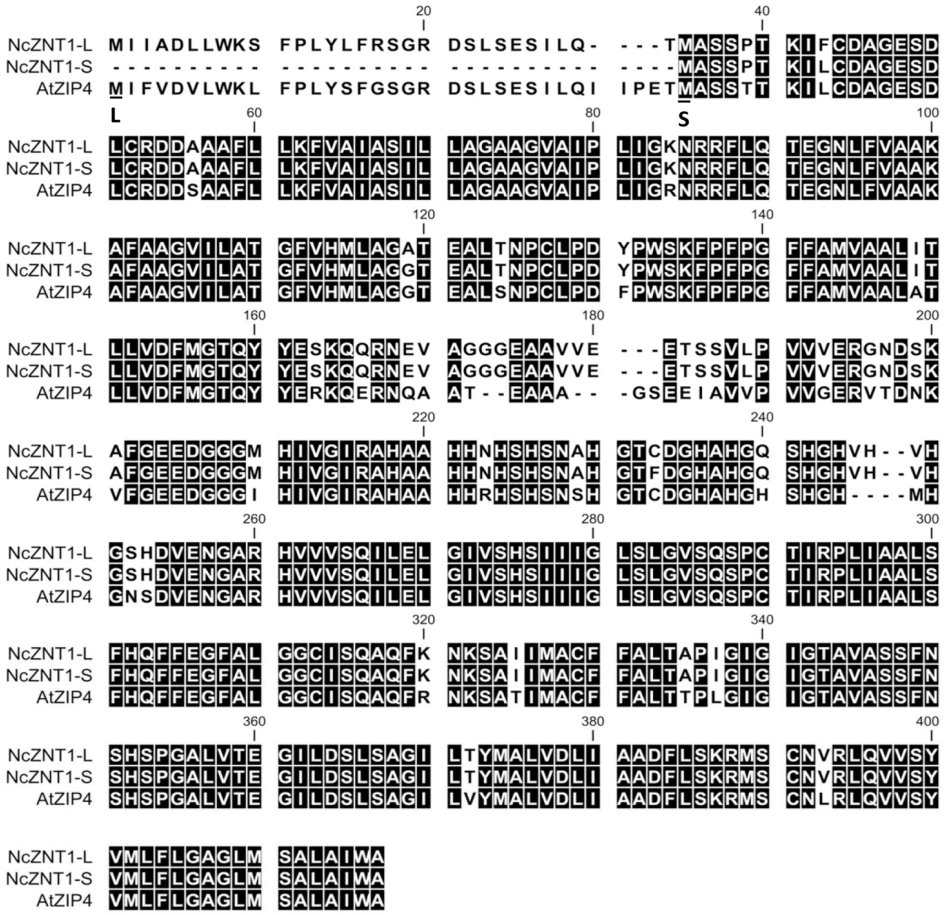

Fig. 9: Comparison of predicted protein sequences of NcZNT1 between *N. caerulescens* accessions La Calamine (Long; L), Prayon (Short; S) and *A. thaliana ZIP4*. The first amino acid Methionine (M) (used as first amino acid in current study) is underlined by letter L. The second Methionine (M) (used as first amino acid by Milner et al., 2012) at position 35 is underlined by letter S. The alignment was performed by MultAlin software (Multiple sequence alignment by Florence Corpet; http://multalin.toulouse.inra.fr/multalin.

DISCUSSION

Understanding plant metal hypertolerance and hyperaccumulation has drawn a lot of interest to unravel its adaptive evolution and to apply this knowledge for phytoremediation and biofortification purposes. A number of genes were analysed for their role in metal tolerance and accumulation. Previous studies have shown the possible role of a ZIP family member gene *NcZNT1* from *N. caerulescens* as a Zn and Cd uptake transporter (Pence et al., 2000; Assunção et al., 2001). Furthermore, it was reported that *NcZNT1* is predominantly expressed in roots and less in shoots in *N. caerulescens* compared to non-accumulator *T. arvense*, but expression of this gene is barely responsive to changes in Zn supply (Assunção et al., 2001). Only at very high Zn concentrations, the expression is somewhat reduced (Pence et al., 2000; van de Mortel et al., 2006). *AtZIP4* is the orthologue of *NcZNT1* in *A. thaliana,* which is also known to be expressed in root and shoot tissues mainly under Zn deficiency (Grotz et al., 1998; van de Mortel et al., 2006). In the present study, *NcZNT1* gene was found to be highly expressed in both shoots and roots of *N. caerulescens* under Zn deficiency and repressed under Zn excess. This elucidates the important role of this gene in Zn transport. We consider our analysis to be more reliable because we performed a quantitative *NcZNT1* gene expression analysis while a less quantitative RNA blot analysis was carried out in the previous study (Assunção et al., 2001). Recently, *NcZNT1* was found to be highly expressed under Zn deficient conditions and under Cd exposure, particularly in root tissues (Milner et al., 2012). These observations point out that NcZNT1 is a Zn and Cd uptake transporter mainly in root tissues. Previously, It was found that NcZNT1 is able to efficiently transport Zn and to a lesser extent Cd, in yeast system (Pence et al., 2000). However, Milner and colleagues (2012) suggest that NcZNT1 can only transport Zn but not Cd, which is contradictory to what was previously published (Pence et al., 2000). Our transgenic *A. thaliana* lines expressing *pro35S::NcZNT1* accumulated higher Zn and Cd than WT, which is consistent with a Zn and Cd transport ability of NcZNT1. Thus the Zn and Cd responsiveness of *NcZNT1* expression, and its Zn and Cd accumulation in transgenic *pro35S::NcZNT1* expressing *A. thaliana* still suggest it to be a Zn and Cd transporter. The Zn and Cd transport ability of

NcZNT1 is in agreement with it homologues TjZNT1, which was able to transport Zn, Cd and even Mn (Mizuno et al., 2005). The yeast cells expressing *NjZNT1* had a higher Ni tolerance. Our transgenic also lines exhibited enhanced shoot Mn accumulation in Zn excess, which urges to analyse the Mn transport ability of NcZNT1 in future studies.

It is known that under Zn and Cd excess, Fe uptake is compromised in *A. thaliana* (van de Mortel., 2006). As some of the known Fe responsive genes can transport Zn and Cd (Korshunova et al., 1999) they could possibly play a role in indirect Zn and Cd accumulation. Since we found a reduced Fe accumulation in our transgenic lines exposed to Cd excess, we analysed the gene expression of known Fe transporters to find their possible role in indirect Zn and Cd accumulation in our transgenic lines. Fe deficiency responsive genes like *AtbHLH100 AtIRT1, AtIRT2* and *AtFRO2* were highly upregulated in both transgenic and WT lines, although with some differences, under Zn and Cd excess clearly showing that these lines were experiencing Fe deficiency (Fig. 4, Table 1). Furthermore, higher expression of these known transporters could possibly mediate Zn and Cd accumulation. Particularly AtIRT1 was previously shown to transport Zn and Cd in addition to Fe in root epidermal cells (Eide et al. 1996; Korshunova et al. 1999; Rogers et al. 2000; Vert et al. 2002). Thus there is likely to be some indirect Zn and Cd uptake in *pro35S::NcZNT1 A. thaliana* lines due to upregulation of Fe deficiency responsive machinery. These observations of upregulation of Fe transporters and Cd accumulation in the transgenic lines are in agreement with the previously known Cd uptake by up regulation of *NcIRT1* in *N. caerulescens* under Fe deficient conditions (Lombi et al., 2002). However, as NcZNT1 is a known Cd transporter (Pence et al., 2000), we consider it to be a major player in the Cd accumulation in our transgenic *A. thaliana* lines since WT line also had the higher expression of Fe transporters but it could not accumulate enhanced Cd (Fig. 4, Table 1; Fig. 3 C).

Most Zn and Cd metals are stored in vacuoles. Members of the CDF protein family are involved in the sequestration of metals into vacuoles. Some CDF family members, like *AtMTP1*, *PtdMTP1*, *AtMTP3* and *TgMTP1*, were shown to cause increased Zn tolerance and accumulation when ectopically or

heterologously expressed in *A. thaliana* (van der Zaal et al., 1999; Blaudez et al., 2003; Arrivault et al., 2006; Gustin et al., 2009), 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 or as buffer in case of Zn deficiency. Since expression of *AtMTP1* was found to be higher in the shoots and roots of *pro35S::NcZNT1* expressing plants under excess Zn and Cd, this clearly illustrates the important role of this gene in the detoxification of Zn metal in these conditions. Thus the enhanced Zn tolerance exhibited by *NcZNT1* expressing line is likely be due to vacuolar sequestration of metals mediated by *AtMTP1* since it is vacuolar membrane localized transporter (Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004). Vacuolar sequestration of Cd has been reported to confer Cd hypertolerance in *N. caerulescens* (Ueno et al., 2011) by HMA3 gene (Heavy Metal ATPase 3). In order to see if *AtHMA3* could also have the higher expression explaining the Cd tolerance in our transgenic lines, we analysed its expression but we did not find any differential expression of this gene in *pro35S::NcZNT1* expressing lines compared to WT. This leads to the conclusion that enhanced Cd tolerance exhibited by our transgenic lines is not mediated by AtHMA3. Phytochelatins (PCs), metallothioneins (MTs), amino acids and organic acids are compounds that can chelate metals and thus play a role in detoxification of metals in plants (Ernst et al., 1992). It was reported that phytochelatins (PCs), which can detoxify Cd, had a long distance root to shoot transport together with Cd in *A. thaliana* (Gong et al., 2003). Since our transgenic lines had higher Cd accumulation in shoots but not in roots, PCs might be involved in Cd tolerance and long distance Cd transport. However, this needs to be done in future to elucidate the role of PCs in Cd tolerance and accumulation in our transgenic lines.

Previously, it was reported that the *AtZIP4* promoter has little activity under sufficient Zn supply but strongly induces transcription in response to Zn deficiency (van de Mortel., 2006), which agreed with the first study about this transporter (Grotz et al., 1998). Both *proAtZIP4::GUS* and *proNcZNT1::GUS* constructs, when used in *A. thaliana*, showed GUS expression in endodermis and pericycle in roots and also in leaves, trichomes and even flowers after exposure to Zn deficiency (Talukdar and Aarts, unpublished data). This similar

expression pattern of *AtZIP4* and *NcZNT1* in *A. thaliana* demonstrated important roles of these genes in Zn uptake in these organs. Furthermore, the promoter analysis lead to the identification of two palindromic cis- regulatory elements which were same in both *NcZNT1* and *AtZIP4* promoters. The deletion of these elements resulted in reduction in GUS activity in *A. thaliana* (Talukdar and Aarts, unpublished data). Our group has published these cis elements called Zinc Deficiency Response Element (ZDRE), RTGTCGACAY, present in *AtZIP4* promoter which are the binding sites for basic-region leucinezipper (bZIP) transcription factors *bZIP19* and *bZIP23* (Assunção et al., 2010). These transcription factors regulate a set of target genes as a response to Zn deficiency as these genes contained ZDRE in their promoters. The *AtZIP4* promoter contains two ZDRE, at -246 to -236bp and at -118 to -108bp, and *NcZNT1* as well, at -189 to -179 bp and -107 to -97bp (Talukdar and Aarts, unpublished data). The conserved Zn deficiency responsive elements (ZDRE) were reported for *AtZIP4* and *NcZNT1*, whereas few other sets of cis elements were identified in few other micronutrient responsive promoters (Assunção et al., 2010; Kobyashi et al., 2003). We have cloned and expressed both promoters in *N. caerulescens* roots and have found that like in *A. thaliana*, both *AtZIP4* and *NcZNT1* promoter activities are induced under Zn deficiency in *N. caerulescens* roots, but repressed by Zn supply. This implies that a similar regulatory mechanism is involved in regulating these promoters in response to Zn. However, there are also differences. The *NcZNT1* promoter showed stronger GUS expression than *AtZIP4* promoter in *N. caerulescens* at low Zn supply, and the former promoter is also active at higher Zn supply levels, while the *AtZIP4* promoter is not (Fig. 5). This differential expression of *NcZNT1* and *AtZIP4* in *N. caerulescens* compared to their similar expression in *A. thaliana* points out that there is likely to be an additional *cis*-element present in the *NcZNT1* promoter recognized by a *N. caerulescens* specific transcription factor, to ensure the higher promoter activity even at higher Zn supply levels compared to *AtZIP4* promoter. Recently, another putative cis- region of *NcZNT1* promoter, upstream of the known ZDRE, was identified, which was not influenced by the Zn supply of *N. caerulescens* and could possibly be involved in higher

expression of this gene (Milner et al., 2012). From this information and due to the presence of similar known ZDRE elements in *NcZNT1* and *ATZIP4* promoters, with similar expression in *A. thaliana* but different expression pattern in *N. caerulescens*, we can say that a new cis element might have evolved in *NcZNT1* promoter and probably a new cis-trans interaction has also evolved. Of course, only altering the cis element, so that it recruits an existing transcription factor, would be sufficient for hyperexpression in *N. caerulescens*. However, it would not be unlikely that the regulation of this *N. caerulescens* transcription factor is also different from *A. thaliana*. A remarkable characteristic of Zn hyperaccumulators is the high expression of Zn homeostasis genes (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006, 2008). In non-hyperaccumulators these genes are mainly induced upon Zn deficiency and *bZIP19* and *bZIP23* are the known regulators of Zn deficiency responsive genes. Therefore, the transcription factors controlling the Zn deficiency response are likely to be important regulators of hyperaccumulation traits. It will be interesting to look for *bZIP19* and *bZIP23* in *N. caerulescens* in order to understand the altered regulation of Zn hyperaccumulation related genes.

The *proNcZNT1::eGFP* expression in pericycle cells in *N. caerulescens* and *proAtZIP4::eGFP* expression in pericycle and cortical cells in *A. thaliana* implies their role in Zn uptake in root endodermal and pericycle cells (Fig. 6). Their lack of expression in epidermis is unexpected for transporters involved in root uptake from the soil. For instance, the *AtIRT1* gene involved in Fe-uptake from the soil, was found to be localised in the epidermis of *A. thaliana* roots (Vert et al., 2002). We propose the role of *NcZNT1* and *AtZIP4* in Zn transport from pericycle cells into cells associated with xylem loading, ultimately for long distance transport, rather than their direct involvement in Zn uptake from the soil. During Zn deficiency, creating a strong loading of apoplastic Zn into the stele may be sufficient and no additional, epidermal Zn uptake and subsequent symplastic transport to the stele may be needed (Kramer, 2012). *NcZNT1* and *AtZIP4* expression in stele of older root tissues with less mineral uptake, would prevent Zn leakage and will ensure Zn availability for xylem loading and ultimately Zn supply to the shoot tissues. The role of these genes, particularly of

NcZNT1 in root tissues enabling long distance metal transport is consistent with the known shoot metal hyperaccumulation controlled by root processes in *N. caerulescens* (de A Guimaraes et al., 2009).

Recently, *NcZNT1* was proposed to be involved in Zn uptake in root tissues and long distance transport (Milner et al., 2012). Our data is consistent with the role of *NcZNT1* in keeping higher influx into cells associated with xylem loading for shoot translocation but rejects its involvement in Zn uptake in root tissues. Milner et al. (2012) have also carried out the functional analysis of the *NcZNT1* gene in yeast and in *A. thaliana*. Their *pro35S::NcZNT1* expressing *A. thaliana* lines were sensitive to excess Zn but not to Cd. A major difference in both studies which might explain some of the difference lies in the fact that they have used a shorter *NcZNT1* cDNA from accession Prayon (*NcZNT1-PR*), missing the first ATG start codon thus resulting in N terminal truncated protein missing 30 amino acids (Fig. 7-9). We have isolated and cloned the full length *NcZNT1* cDNA from accession La Calamine (*NcZNT1-LC*), which population is only some 30 km distant from Prayon (Assunção et al., 2001). The same ATG start codon is found for the *NcZNT1/AtZIP4* orthologue of *A. lyrata* (GenBank acc. no. XM_002892566). Omitting the first ATG of a cDNA is expected to influence its functional analysis and also tissue localization as performed by Milner et al. (2012). It is known from literature that N terminal can affect localization of proteins. It was reported that the deletion of 30 amino acids in N terminal of RGS4 protein, a GTPase activating protein, results in loss of plasma membrane localization (Srinivasa et al., 1998). Although, the functional analysis of N terminal of NcZNT1 for plasma membrane localization has not been performed, it is plausible that the truncated *NcZNT1* protein may be mislocalized. This is consistent with the fact that *NcZNT1* expressing *A. thaliana* had higher sensitivity to excess Zn (Milner et al., 2012) while we have observed Zn tolerance and accumulation in our *pro35S::NcZNT1* expressing *A. thaliana* lines. The possible reason could be the mislocalization of NcZNT1 protein to the organelles which are susceptible to excess metals. The analysis performed by using the shorter version of *NcZNT1* are thus questionable. Therefore, we consider our data to be more reliable; however, we suggest

analysing both *NcZNT1-LC* and N terminal truncated *NcZNT1-PR* cDNAs together, in future, to find out their possible functional differences.

ACKNOWLEDGEMENTS

This research was supported by the Higher Education Commission of Pakistan (HEC), and the EU-METALHOME project (HPRN-CT-2002-00243) (ST, MA). We would like to thank Dr. R. Jefferson, CAMBIA, Australia, for providing us with the CAMBIA1301 vector. We are grateful to Dr. Erik Limpens, Molecular Biology, Wageningen University, the Netherlands for the kind provision of the pKGWFS-RR vector. We thank Henk Kieft and Dr. Norbert de Ruijter, Plant Cell Biology, Wageningen University, The Netherlands for their help in microscopic analysis. We are grateful to Dr. Maarten Koornneef for critical reading and suggestions for the manuscript preparation.

Table 2: Primers used for q-PCR analysis.

Primer	**Primer Sequence (5'-3')**
Forward primer for *NcZNT1*	GATCTTCGTCGATGTTCTTTGG
Reverse primer for *NcZNT1*	TGAGAGGTATGGCTACACCAGCAGC
Forward primer for *Clathrin*	AGCATACACTGCGTGCAAAG
Reverse primer for *Clathrin*	TCGCCTGTGTCACATATCTC
Forward primer for *AtUBP6*	GAAAGTGGATTACCCGCTG
Reverse primer for *AtUBP6*	CTCTAAGTTTCTGGCGAGGAG
Forward primer for *AtIRT1*	AAGCTTTGATCACGGTTGG
Reverse primer for *AtIRT1*	TTAGGTCCCATGAACTCCG
Forward primer for *AtIRT2*	ATGGCTACTACCAAGCTCGTC
Reverse primer for *AtIRT2*	CTAGACCGGACATCATAGCG
Forward primer for *AtFRO2*	CTTGGTCATCTCCGTGAGC
Reverse primer for *AtFRO2*	AAGATGTTGGAGATGGACGG
Forward primer for *AtBHLH100*	AAGTCAGAGGAAGGGGTTACA
Reverse primer for *AtBHLH100*	GATGCATAGAGTAAAAGAGTCGCT
Forward primer for *AtFRD3*	CGAGTTGCATCTCTTCTTCCT
Reverse primer for *AtFRD3*	TGATAACGGTCTCTCGAACA
Forward primer for *AtMTP1*	ACGGCCATGACCATCACAATC
Reverse primer for *AtMTP1*	TGCTTGTCCTCTCCATGACCA
Forward primer for *AtYSL3*	GAATTGAGAGACTAGTTTATTC
Reverse primer for *AtYSL3*	CGAGTTTTTACTTTTTGTGTAGCG
Forward primer for *AtNRAMP3*	ACAATGGGAGTCTCATTCGC
Reverse primer for *AtNRAMP3*	ATGCAACCCACAACTCCAAC
Forward primer for *AtHMA4*	ATGGCGTTACAAAACAAAG
Reverse primer for *AtHMA4*	GAGATTTGGTTTTACTGCTCTGAGC
Forward primer for *AtHMA3*	TTAAAGCTGGAGAAAGTATACCGA
Reverse primer for *AtHMA3*	GCTAGAGCTGTAGTTTTCACCT

CHAPTER 3

Expressing a vacuolar metal transporter NcMTP1 of *Noccaea caerulescens* enhances Zn and Cd tolerance and accumulation in *Arabidopsis thaliana*

Zeshan Hassan[1], Valeria Ochoa[1], Miki Kawachi[2], Christiane Noronha Fernandes[1], Henk Schat[3], Ute Krämer[2] and Mark G.M. Aarts[1]

[1]Laboratory of Genetics, Wageningen University and Research Centre, Droevendaalsesteeg1, 6708 PB, Wageningen, The Netherlands

[2]Plant Physiology Department, Ruhr University, D-44801, Bochum, Germany

[3]Ecology and Physiology of Plants, Faculty of Biology, Free Universiteit, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

ABSTRACT

- This study aimed at performing the functional analysis of the *NcMTP1* gene from the metal hyperaccumulator species *Noccaea caerulescens* regarding its ability to act in the transport of Zn and Cd and in comparison to the *AtMTP1* orthologue from *Arabidopsis thaliana*.
- Transport activity of NcMTP1 was analysed by expressing it in yeast. *pro35S::NcMTP1* and *pro35S::AtMTP1* transformed *A. thaliana* and root *NcMTP1* knockdown *N. caerulescens* plants were analysed for the function of the *NcMTP1* gene *in planta*. Variation in copy number of *NcMTP1* was analysed in various *N. caerulescens* accessions. In situ *NcMTP1* promoter analysis was performed.
- In yeast, both NcMTP1 and AtMTP1 transported Zn and probably Cd. Expression of *NcMTP1* enhanced Zn and Cd tolerance and accumulation when expressed in *A. thaliana* while the *AtMTP1* over-expressing line showed only tolerance and accumulation of Zn, but not Cd. Down-regulation of *NcMTP1* in *N. caerulescens* roots enhanced shoot Zn and lowered root Cd concentrations. The copy number of *NcMTP1* varies between 1 and 2 in various *N. caerulescens* accessions. Various putative *cis* regulatory elements were identified in the promoter of *NcMTP1* which might play a role in regulation *NcMTP1* expression.
- We conclude that *NcMTP1* is involved in Zn and probably Cd sequestration in root cell vacuoles in *Noccaea caerulescens*. Identified putative cis regulatory elements in the promoter of NcMTP1 might play a role in its enhanced expression in *N. caerulescens* and need further verification. Expression of *NcMTP1* in *A. thaliana* contributes to enhanced Zn and Cd tolerance and accumulation of these metals, probably through Zn and Cd compartmentalization into the vacuole. The accumulation of these metals is most likely to be supplemented by the activity of Fe deficiency responsive transporters. Furthermore *NcMTP1* is involved This knowledge can pave the way to engineer plants expressing *NcMTP1* with enhanced Zn and Cd tolerance and accumulation, which are useful for phytoremediation.

INTRODUCTION

Micronutrients are essential elements required for proper functioning of various processes within an organism (Chapter 1). They play a key role in proper physiological and metabolic functioning of plants (Ramesh et al., 2004). One of these important micronutrients is zinc (Zn). This micronutrient acts as a cofactor for various Zn metalloproteins. These proteins are Zn dependent, like Zn finger containing transcription factors and more than 400 enzymes (Clarke and Berg, 1998). Zn is a structural or catalytic component in hydrolytic enzymes and DNA binding proteins like RNA polymerase (Guerinot and Eide, 1999; Broadley et al., 2007). Despite their essentiality, Zn^{2+} ions can cause toxicity when accumulated in excess. Surplus Zn can bind to proteins and cofactors and cause toxicity (Eide, 2003). In plants, an excess of Zn leads to the inhibition of root growth, decreased photosynthetic rates and chlorosis (Marchner, 1995). Cd is an element which can enter the plant via Zn or Fe uptake mechanisms due to its chemical resemblance to these minerals (Pence et al., 2000). Its toxicity is caused by damaging the DNA repair machinery, by disturbing nutrient uptake and by reducing photosynthesis in plants (Banerjee and Flores-Rozas, 2005; Sanita di Toppi and Gabbrielli, 1999). Chlorosis, root tip browning, growth inhibition and ultimately death of the plants are visual symptoms of Cd toxicity (Kahle, 1993). Fe is involved in cell wall synthesis and the production of ethylene and chlorophyll and in addition its plays a role in the electron transport chain. Fe deficiency results in intervenal chlorosis and necrotic lesions (Bell and Dell, 2008). Fe deficiency is known to trigger accumulation of other metals like Zn and Cd most likely due to the broad substrate range of Fe transporters (Vert et al., 2002; Korshunova et al., 1999). It is thus essential for a plant to maintain a suitable micronutrient balance to deal with increased or reduced mineral concentrations.

There are some fascinating plant species which hyperaccumulate Zn and Cd and store them in their shoot parts. These metal hyperaccumulators have the selective advantage of pioneering new niches and to confer defence against herbivory (Boyd et al. 2002). These plants can accumulate metals at 100-1000 folds higher levels than normal plants (Baker et al., 2000; McGrath et al.,

2002). *Noccaea caerulescens* (J. & C. Presl) F.K. Meyer, formerly known as *Thlaspi caerulescens* J. & C. Presl is one of the most important plant metal hyperaccumulators known by time. It can accumulate about 30,000 mg Zn kg^{-1} dry weight (DW) in its shoots (Baker et al., 1994; Baker et al., 2000), however under control conditions; there is a significant difference of Zn hyperaccumulation among different populations (Lombi et al., 2000; Assunção et al., 2003b; Roosens et al., 2003). *N. caerulescens* belongs to the Brassicaceae family and shares about 88.5% coding region sequence identity with *A. thaliana* (Rigola et al., 2006). Apart from Zn hyperaccumulation, this is one of the few species to also hyperaccumulate Cd, together with *Arabidopsis halleri* and *Sedum alfredii* (Xiong et al., 2004). By understanding the molecular mechanism of heavy metal tolerance and accumulation, we can use the knowledge to engineer crops useful for "biofortification" and high biomass plant species useful for "phytoremediation" purposes.

A wealth of knowledge is generated by analysing the function of various genes shown or hypothesized to be involved in Zn tolerance and accumulation traits (Chapter 1). Most of these genes were found by comparative transcriptomic analysis between metal accumulators and non-accumulators and by genetically mapping these metal tolerance and accumulation traits (van de Mortel et al. 2006; Shahzad et al., 2010; Assunção et al 2003a). Members of the Cation Diffusion Facilitator (CDF) family were shown to sequester metals into vacuoles (Lin and Aarts, 2012). *N. caerulescens Metal Tolerance Protein 1* (*NcMTP1*) is a member of the CDF gene family in *N. caerulescens* (Assunção et al., 2001), which is an orthologue of *AtMTP1* of *A. thaliana* (previously known as *ZAT*; van der Zaal et al., 1999). *NcMTP1* is constitutively higher expressed in *N. caerulescens* than in the related non-hyperaccumulator *Thlaspi arvense* (Assunção et al., 2001). AtMTP1 was shown to be localized to the vacuolar membrane and its overexpression enhanced Zn tolerance and accumulation in *A. thaliana*, while its promoter activity was highest in young leaves and root tips (Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004; van der Zaal et al., 1999). In the Zn/Cd hyperaccumulator *A. halleri* there are several copies of the *AhMTP1* gene, distributed over four genetic loci,

which seem to have contributed to the higher expression of this gene (Shahzad et al., 2010). Such might also be the case in other metal hyperaccumulating species with high expression of this gene, since copy number expansion is a known phenomenon governing higher expression of metal accumulation related genes (Hanikenne et al., 2008). The *MTP1* gene from *Noccaea goesingense* was shown to be involved in Zn tolerance and accumulation via a systemic Zn deficiency response (Gustin et al., 2009). The authors also provided evidence for the localization of NgMTP1 at the vacuolar membrane. All these studies describe the important role of NgMTP1 as an important transporter involved in metal transport into the vacuole. Based on its similarity to AtMTP1 and NgMTP1, also NcMTP1 it is predicted to localize to the vacuolar membrane and to transport Zn into the vacuole. In *N. caerulescens*, shoot and root tissues play different roles in metal tolerance and accumulation. Reciprocal shoot and root grafting experiments performed between *N. caerulescens* and non-accumulator *Microthlaspi perfoliatum* have shown that Zn hyperaccumulation is mainly controlled by root tissues in *N. caerulescens*, while shoot processes control Zn hypertolerance (Guimaraes et al., 2009). The authors speculated that the shoot driven Zn hypertolerance might be controlled mainly by *NcMTP1*.

Although many *MTP1* genes were cloned from various plant species, functional analysis of this important gene in metal tolerance and accumulation from *N. caerulescens* was lacking. The present study aims at analysing the Zn and Cd transport ability of NcMTP1 and to analyse its role in Zn and Cd tolerance and accumulation by knocking down expression in *N. caerulescens* roots, by expressing the gene in *A. thaliana* under control of the CaMV 35S promoter and by comparing these transgenics to plants transformed with a *pro35S::AtMTP1* construct. The hypothesis of the present study was that NcMTP1 is able to transport Zn and Cd and is involved in both Zn and Cd tolerance, in contrast to AtMTP1, which is involved in Zn, but not in Cd, tolerance and accumulation.

MATERIALS AND METHODS

Heterologous expression of *NcMTP1* cDNA in yeast

The *NcMTP1*-LC cDNA was amplified by PCR from the plasmid pEZR (H)-LN containing the *NcMTP1* cDNA (see below), using primers 5′-AAGGAATTCATGGAGTCTTCAAGTCCCC-3′ (forward) and 5′-AAGGTCGACTCAGCGCTCGATTTGTACGG-3′ (reverse). The DNA fragment was then inserted into the EcoRI-SalI site of the URA3-marked, high copy yeast expression vector pKT10 (Tanaka et al., 1990). Plasmids were introduced into the Zn/Co/Cd-sensitive mutant *zrc1 cot1 ycf1* (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0; zrc1::natMX3; cot1::kanMX; ycf1::LEU2; Kawachi et al., 2012) of yeast (*Saccharomyces cerevisiae*) strain BY4741 by the lithium acetate/single-strand DNA polyethylene glycol transformation method (Gietz et al., 1995). Positive Ura+ colonies were selected on HC-U plates, and the presence of NcMTP1 proteins in yeast was confirmed by immunoblotting using an anti-MTP1 antibody (epitope: DVTEQLLDKSKTQVA) as described by Kawachi et al. (2008).

Yeast metal tolerance assays

The yeast strains that expressed the empty vector, *NcMTP1*-LC or *AtMTP1* were pre-cultured in HC-U liquid medium at 30 °C for 16 h (Kawachi et al., 2008). Pre-cultured cells were diluted to an OD600 to 0.1 and 5 µl-aliquots were spotted onto HC-U plates containing added $ZnCl_2$, $CoCl_2$ or $CdCl_2$ at concentrations as indicated. Plates were incubated at 30 °C for 3 d, and photographs were taken.

Development of binary constructs

Binary constructs were developed following Sambrook et al. (1989). In order to develop the *pro35S::NcMTP1* construct, a 1352-bp *NcMTP1* cDNA (GenBank accession No. AF275750) fragment was digested with BamHI and XbaI from a previously identified cDNA clone (Assunção et al. 2001). This NcMTP1 fragment was ligated into pEZR(H)-LN, (Dr. Gert de Boer, Ehrhardt laboratory, Dept. of Plant Biology, Carnegie Institution of Washington, USA) which was developed by ligating pCambia1300 (http://www.cambia.org /daisy/bios/585.html) and the cassette from pEZS-LN (David Ehrhardt,

Stanford University; http://deepgreen.stanford.edu/). The expression construct contained the hygromycin resistance gene *HPT* as selection marker. The construct was verified by sequencing. To generate a *NcMTP1* RNAi construct, a 336-bp fragment from *pro35S::NcMTP1* (as mentioned above) was PCR amplified using primers shown in Table 4. The PCR fragment of *NcMTP1* was cloned into pENTR™/D-TOPO vector (Invitrogen). The vector was digested with ScaI and EcoRV and sequenced to confirm the presence of the desired fragment. Afterwards, the LR recombination reaction using the Gateway® LR ClonaseTM Enzyme Mix (InvitrogenTM) was carried out to create an expression clone (in pK7GWIW2-RR ; Limpens et al., 2004) with DsRed used as a fluorescent marker to detect transformation. The destination construct was confirmed by sequencing.

Plant transformations

The *pro35S::NcMTP1* construct was transformed into *A. thaliana* (accession Columbia) as described by Clough and Bent (1998). The transformed T1 seedlings were selected in vitro in ½ strength MS plates (Murashige and Skoog, 1962; Duchefa Biochemie B.V., Haarlem, The Netherlands) supplemented with 20 mg L^{-1} Hygromycin B (Duchefa Biochemie, Haarlem, The Netherlands) as selection marker in a climate cell (25°C day; 16 h day with illumination at a light intensity of 120 µmol m^{-2} s^{-1}). 10 independent transformed plants were selected and propagated until the homozygous T3 generation. Three lines with highest expression (based on semi-quantitative RT-PCR; data not shown) were used in experimentation. Seeds of homozygous *pro35S::AtMTP1* expressing *A. thaliana* (accession C24) and wild type C24 lines were kindly provided by Dr. Bert van der Zaal, University of Leiden (van der Zaal et al., 1999).

The *NcMTP1* RNAi construct was transformed into *N. caerulescens* roots by *Agrobacterium rhizogenes* using a modification to the *Medicago truncatula* root transformation method as described by Limpens et al., (2004). Sterilized seeds of *N. caerulescens* (La Calamine) were grown in vertical ½ strength MS plates (Murashige and Skoog, 1962; Duchefa Biochemie B.V., Haarlem, The Netherlands), pH 5.8 at 24 °C (16/8 hr light/darkness). After seven days, roots

were cut from the seedlings at the hypocotyl-root boundary. A dot of *A. rhizogenes* culture (strain MSU440, harbouring the *NcMTP1* RNAi construct) was applied to the cut surface and kept for 5 day at 20°C /15 °C (day/night, 12 hours light). Next, the seedlings were grown in vertical ½ strength MS plates supplemented with 200 mg L^{-1} Timetin (Ticarcillin disodium mixture 15:1 & potassium clarulanate; Duchefa Biochemie BV, Haarlem, The Netherlands). Non-transformed roots (as determined using a Leica MZ FLIII Fluorescence Stereo Microscope) were cut off once a week and seedlings were grown for 3 weeks until full transformed roots were emerged and the complete root system was transgenic. Same cutting procedure was performed with WT roots to be used as control.

Plant growth conditions

To determine Zn tolerance and accumulation, nine plants per genotype of three independently transformed *pro35S::NcMTP1* lines, one Col WT, one *pro35S::AtMTP1* and one C24 WT *A. thaliana* line were grown on modified ½ Hoagland's nutrient solution, which supplied sufficient Zn (2 µM $ZnSO_4$), for two weeks. After two weeks, all lines were exposed to the same nutrient solution to which excess Zn (60 µM $ZnSO_4$) was added, for another three weeks. The same number of plants per genotype was kept in sufficient Zn media as control. To determine Cd tolerance and accumulation, nine of the above mentioned *A. thaliana* plants per genotype were exposed to excess Cd (2 µM $CdSO_4$). Control lines were kept growing in sufficient Zn media. All lines were grown in the same hydroponic trays, each containing about 9L of nutrient solution. In all experiments the nutrient solution was refreshed twice a week. 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 14 h/d; 75% RH). After five weeks, shoot and root dry weights were recorded and the tissues were kept separately for the metal accumulation assay.

RNAi *NcMTP1* root knockdown *N. caerulescens* plants (KD) and wild-type (WT) plants were grown in modified ½ Hoagland's nutrient solution, containing 2 µM $ZnSO_4$ for two weeks. Afterwards, KD and WT plants were

transferred to the same nutrient solution, but supplemented with excess Zn (1000 µM $ZnSO_4$) or excess Cd (50 µM $CdSO_4$ + 100 µM $ZnSO_4$) for another five weeks. The media was refreshed once a week. 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 14 h/d; 75% RH). At the end of the experiment, plants were analysed for chlorophyll fluorescence (FluoroCam 700MF, as described by Baker (2008)) and samples were kept for gene expression and metal accumulation analysis. To determine the *NcMTP1* copy number in different *N. caerulescens* accessions: La Calamine (B), Plombières (B), Prayon (B), Ganges (Saint-Laurent-le-Minier; F), Monte Prinzera (I), Saint-Félix-de-Pallières (F), Durfort (F), Lellingen (LUX), Moravia (Cz), Pontaut (ES), Clough (UK), Saint-Julien-Molin-Molette (F); and *Noccaea praecox* (Slovenia), plants were grown in ½ Hoagland's medium containing 2 µM $ZnSO_4$ for five weeks in a climate chamber (20/15°C day/night temperatures; 250 µmoles light m^{-2} s^{-1} at plant level during 14 h/d; 75% RH). At the end of this period, samples were kept for DNA isolation.

Root and shoot metal accumulation assay

Zn, Cd, Fe, and Mn concentrations were determined in dried shoot and root tissues using Atomic Absorption Spectroscopy as described by Assunção et al. (2003b).

RNA and DNA isolation and quantitative PCR

Quantitative RT-PCR (qRT-PCR) was carried out to determine the expression of known metal transporter genes in *pro35S::NcZNT1*, Col WT, *pro35S::AtMTP1* and C24 WT *A. thaliana* lines exposed to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) or excess Cd (2 µM $CdSO_4$). Similarly, gene expression was determined for *NcMTP1*, *NcIRT1*, *NcZNT2*, *NcHMA3* and *NcNRAMP3* in *NcMTP1* KD and WT plants grown in sufficient Zn (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) or excess Cd (50 µM $CdSO_4$ + 100 µM $ZnSO_4$). Three biological samples per genotype per treatment and two technical repeats were used for the qRT-PCR analysis. The Qiagen RNeasy® easy mini kit was used to isolate

total RNA from these lines. A NanoDrop 2000 (Thermo Fisher Scientific) was used to measure RNA quantity and quality in the samples. Only samples of good RNA quantity and quality were used. 1 µg total RNA was used to synthesize cDNA by using the iScriptTM cDNA Synthesis Kit (Bio-Rad).

For *NcMTP1* copy number analysis, genomic DNA was isolated from the *N. caerulescens* accessions and *N. praecox* indicated under ***Plant growth conditions*** by using the Qiagen DNeasy® kit. DNA quantity and quality was analysed with the NanoDrop 2000 as described above. *A. thaliana Ubiquitin-specific protease 6 (AtUBP6*, At1g51710) was used as cDNA normalization reference gene for analysis of *AtBHLH100*, *AtIRT1*, *AtIRT2*, *AtFRO2*, *AtHMA4*, *AtNRAMP3*, *AtYSL3*, *AFRD3*, *AtMTP1* and *NcMTP1* expression in all *A. thaliana* lines. *NcTubulin* was used as the reference gene for expression analysis in *N. caerulescens*. *NcTubulin* was also used as single copy marker gene for the *NcMTP1* copy number analysis. Table 4 shows the primers for all the genes used in this analysis. Normfinder (http://www.leonxie.com/referencegene.php) was used to confirm the stability of reference genes (Hellemans et al., 2007). A total reaction volume of 10 µL was used for q(RT-)PCR comprising of 5 µL of iQ SYBR Green supermix, 5 pmol of forward and reverse primers, and 2 µL of 10 times diluted cDNAs/DNA (for cDNA corresponding to 5 ng/µL RNA) by using the iQTMSYBR® Green Supermix kit (Bio-Rad). PCR conditions were set to 3 min at 95°C, followed by 40 cycles of 10 sec at 95°C and 1 min at 60°C. The CFX96TM Real-Time Detection System (Bio-Rad) was used to detect fluorescent signals. There were no unspecific products and primer-dimers formed as revealed by analysing melting curves. The $2^{-\Delta\Delta Ct}$ method as described by Livak and Schmittgen, (2001) was used to calculate relative transcript levels (RTL). The expression of all above mentioned genes in shoots of plants grown at Zn supply (2 µM $ZnSO_4$) was set to RTL=1 and used to calibrate other expression values. Heat maps representing q-PCR data were developed by using "BAR Heat Mapper Plus Tool" (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_heatmapper_plus.cgi).

Bioinformatics analysis of *MTP1* promoters from *N. caerulescens*, *A. halleri*, *A. lyrata* and *A. thaliana*

The *NcMTP1* sequence from accession Ganges (GenBank acc. nr. AY999083) was compared against preliminary assemblies of an *N. caerulescens* (accession Ganges) genome sequence (Y.-F. Lin and M.G.M. Aarts, unpublished) to identify a scaffold containing the *NcMTP1* gene, including promoter sequence. DNA sequence analysis was performed using CLC Main Workbench 6.7.1 (www.CLCbio.com). The *NcMTP1* genomic DNA sequence was compared to the non-redundant GenBank database (www.ncbi.nlm.nih.gov/) to find similar sequences from other Brassicaceae species. The sequences selected were the ones that included the *MTP1* gene and its upstream and downstream sequences containing promoter and flanking genes. All these sequences were compared against the *N. caerulescens* scaffold to determine the putative length of the *NcMTP1* promoter. The promoter sequences from *N. caerulescens*, *A. thaliana*, *A. lyrata*, and *A. halleri* were aligned and analysed to find *cis*-regulatory elements. A motifs search was performed with Regulatory Sequence Analysis Tool (RSAT, rsat.ulb.ac.be/) and also by using the database of Plant Cis-acting Regulatory DNA Elements (www.dna.affrc.go.jp/PLACE/). The alignment of the motifs was performed by using the Readseq version 2.1.30 online tool (www.ebi.ac.uk/cgi-bin/readseq.cgi). Various *cis*-acting elements were identified and potential sequences were compared to known *cis*-elements.

Statistical analysis

Where needed, data were analysed for significance at $p<0.05$ by using Student's t-test, two-way ANOVA and ANOVA (Least Significance Difference) in the SPSS v. 12 software package for MS Windows.

RESULTS

NcMTP1 expression complements Zn deficiency and confers Cd sensitivity in yeast

To determine the metal transport functionality of NcMTP1 to and compare this to the well-examined AtMTP1 protein, we have expressed *NcMTP1* and

ATMTP1 into the *zrc1 cot1 ycf1* triple mutant, hypersensitive to Zn, Co and Cd, by mutation of their respective Zn, Co and Cd vacuolar transporter genes (Kawachi et al., 2012). Mutant and transgenic yeast were grown on a range of media with increasing Zn, Cd and Co concentrations. *AtMTP1* complemented the Zn sensitivity of the *zrc1 cot1 ycf1* mutant (Fig. 1 A), confirming previous findings for *AtMTP1* (Desbrosses-Fonrouge et al., 2005), but *NcMTP1* only slightly reduced the Zn sensitivity. *NcMTP1* expression has no effect on Co sensitivity and it makes the mutant even more Cd sensitive than the mutant. To examine this further, the single Cd-hypersensitive *ycf1* mutant (Li et al., 1996) was transformed with a *NcMTP1* expressing construct and compared to wild-type yeast and an empty vector transformed mutant (Fig. 1 B), which confirms the enhanced sensitivity to Cd. Thus in yeast, NcMTP1 appears to transport Zn

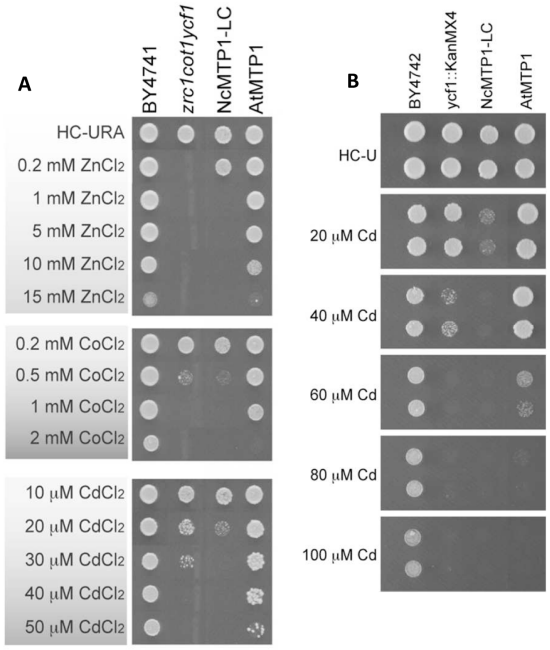

Fig. 1: Complementation assay of Zn Cd and Co hypersensitivity of the *ycf1 zrc1 cot1* yeast mutant by *NcMTP1-LC* and *AtMTP1*. Pre-cultures of wild-type (BY4741) yeast and the *ycf1 zrc1 cot1* yeast mutant containing an empty vector (*zrc1cot1ycf1*), the *NcMTP1*-LC or *AtMTP1* plasmid (Kawachi et al., 2008) were diluted to an OD600 of 0.1 and spotted in 5 µl-aliquots onto HC-URA plates containing different $ZnCl_2$, $CoCl_2$ and $CdCl_2$ concentrations (A); or $CdCl_2$ at concentrations as indicated (B). Plates were incubated at 30 °C for 3 d, before photographs were taken.

but less efficiently than AtMTP1, and is unable to transport Co or Cd. Alternatively, the protein may not be targeted to the correct membrane in this heterologous species, which will not allow a proper conclusion to be drawn from this experiment.

NcMTP1 expression enhances tolerance to high Zn exposure in *A. thaliana* and leads to higher Zn accumulation

To study the response to excess Zn, nine plants per line in three replicates were used for three homozygous transgenic lines of *pro35S::NcMTP1*, one Col WT line, one homozygous line expressing *pro35S::AtMTP1* along with its C24 WT line grown on modified half Hoagland's hydroponic media provided with sufficient Zn (2 µM $ZnSO_4$) and excess Zn (60 µM $ZnSO_4$). Both *pro35S::NcMTP1* and *pro35S::AtMTP1* lines had very high expression of the respective transgene relative to the housekeeping gene *AtUBP6* used as reference and also much higher than the endogenous *AtMTP1* expression levels (Fig. 2 A,B).

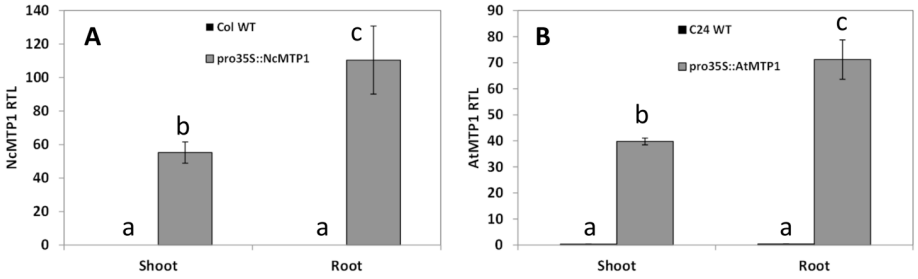

Fig. 2: (A) Quantitative reverse transcriptase PCR (qRT-PCR) analysis of *NcMTP1* expression in *pro35S::NcMTP1* expressing *A. thaliana* (Col WT) grown in sufficient Zn (2 µM $ZnSO_4$) for four weeks. (B) *AtMTP1* expression in *pro35S::AtMTP1* expressing *A. thaliana* (C24 WT) grown in sufficient Zn (2 µM $ZnSO_4$) for four weeks. The *MTP1* expression was normalized to the *AtUBP6* housekeeping gene in *pro35S::NcMTP1 and pro35S::AtMTP1* lines. Different letters indicate the significant difference (p value < 0.05 ANOVA, Least Significance Difference) in gene expression (mean ± SE of four replicates).

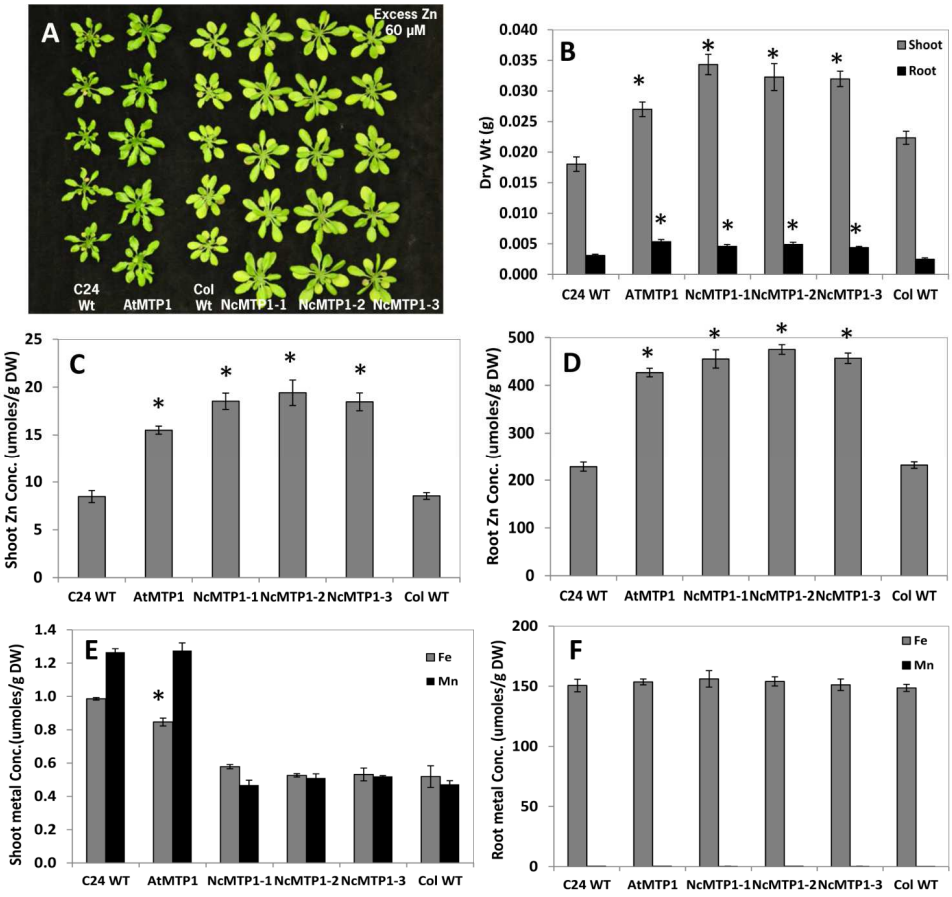

Fig. 3: The phenotypic response of transgenic *pro35S::NcMTP1*, its Col wild-type (Col WT) and *pro35S::AtMTP1* and its C24 wild-type (C24 WT) *A. thaliana* lines to excess Zn. Three independently transformed *pro35S::NcMTP1* lines (NcMTP1-1, NcMTP1-2, NcMTP1-3) and their Col WT and one independently transformed *pro35S::AtMTP1* (AtMTP1) and its C24 WT were grown hydroponically on half Hoagland's media with excess Zn (60 µM $ZnSO_4$) for four weeks. (A) Visible phenotypes of *pro35S::NcMTP1* and Col WT and *pro35S::AtMTP1* and C24 WT plants grown on Zn excess medium; (B) Dry shoot and root weight; (C) Zn concentration (µmoles/g DW) in shoots; (D) and in roots; (E) Fe and Mn concentrations (µmoles/g DW) in shoots; (F) and in roots. Photographs were taken at the end of the 4th week after seed sowing. * indicates a significant difference (at $p < 0.05$ in two-way ANOVA) between transgenic and respective wild type line (mean ± SE of four replicates).

When challenged with excess Zn, not only rosettes of transgenics were larger than their wild-type controls (Fig. 3 A), but the transgenic lines also had higher dry shoot and root weights (Fig. 3 B). This increased tolerance had a positive effect on the Zn concentration in the shoots and roots of both the *NcMTP1* and *AtMTP1* expression lines (Fig. 3 C,D). In general, the *pro35S::NcMTP1* lines showed slightly higher Zn concentrations in shoots compared to the *pro35S::AtMTP1* transgenic line. When plants were grown on sufficient Zn (2 µM $ZnSO_4$), the Zn concentration in roots was significantly higher in the transgenic lines for both constructs, but not in shoots (data not shown). Expression of *NcMTP1* or *AtMTP1* had hardly any detectable effect on plant Fe or Mn concentrations, when compared to wild-type plants. Only the *pro35S::AtMTP1* line had a significantly lower Fe concentration in shoot tissues compared to the C24 WT line (Fig. 3 E, F).

NcMTP1, but not _AtMTP1_, over-expression enhances Cd tolerance and accumulation in _A. thaliana_ grown in hydroponics media

Previous work on over-expression of *AtMTP1* (*ZAT*) showed no effect on Cd accumulation or tolerance in *A. thaliana* (van der Zaal et al., 1999). Nevertheless, since expression of *NcMTP1* and *AtMTP1* affected Cd tolerance of yeast, lines expressing *NcMTP1* and *AtMTP1* were exposed to excess Cd. Three independently transformed *pro35S::NcMTP1* lines were compared to the same *pro35S::AtMTP1* line (*pro35S::AtZAT*) used by van der Zaal et al. (1999) on modified half Hoagland's hydroponic media containing sufficient Zn (2 µM $ZnSO_4$) and media containing excess Cd (2 µM $CdSO_4$ + 2 µM $ZnSO_4$). Since the *pro35S::AtMTP1* line obtained from van der Zaal was generated in the C24 background, but the *pro35S::NcMTP1* lines were made in the Col-0 background, both wild-types were included as respective controls. The *pro35S::NcMTP1* lines showed increased Cd tolerance compared to the control plants, with larger rosette sizes, less chlorosis and significantly higher shoot and root dry weights (Fig. 4 A,B). Also the Cd concentrations in the shoot and root samples of *pro35S::NcMTP1* transgenic lines were significantly higher than in the control (Fig. 4 C,D). We confirmed the previous findings from van de Zaal et al. (1999),

that the transgenic *pro35S::AtMTP1* lines did not show enhanced tolerance to excess Cd, nor enhanced Cd accumulation. Both *pro35S::NcMTP1* and *pro35S::AtMTP1* lines showed higher Zn concentrations in their roots compared to wild-type lines, but not in shoots (Fig. 4 C, D). Cd exposure also had an effect on Fe and Mn concentrations, again only in the *pro35S::NcMTP1* lines, which showed a significant reduction in shoot and root Fe and shoot Mn concentrations compared to Col WT (Fig. 4 E, F).

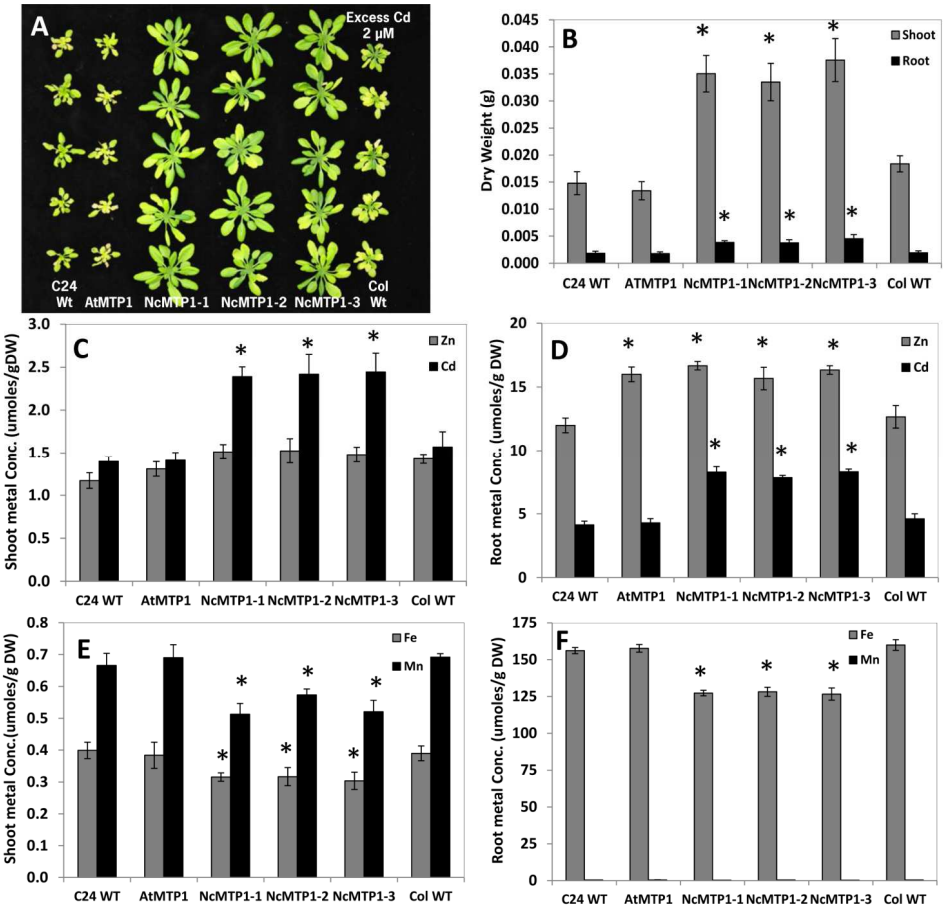

Fig. 4: The phenotypic response of transgenic *pro35S::NcMTP1* its Col wild-type (Col WT) and *pro35S::AtMTP1* and its C24 wild-type (C24 WT) *A. thaliana* lines to excess Cd. Three independently transformed *pro35S::NcMTP1* lines (NcMTP1-1, NcMTP1-2, NcMTP1-3) and their Col WT and one independently transformed *pro35S::AtMTP1*

(AtMTP1) and its C24 WT were grown hydroponically on half Hoagland's media with excess Cd (2 µM $CdSO_4$) for four weeks. (A) Visible phenotype of *pro35S::NcMTP1* and Col WT and *pro35S::AtMTP1* and C24 WT plants grown on Cd excess medium; (B) Dry shoot weight; (C) Zn and Cd concentration (µmoles/g DW) in shoots; (D) and in roots; (E) Fe and Mn concentrations (µmoles/g DW) in shoots; (F) and in roots. Photographs were taken at the end of the 4th week after seed sowing. * indicates a significant difference (at $p < 0.05$ in two-way ANOVA) between transgenic and respective wild type line (mean ± SE of four replicates).

Expression of *NcMTP1/AtMTP1* alters the expression of endogenous metal homeostasis genes in *A. thaliana*

To examine if the observed effect on transgenic plant phenotypes could be solely attributed to the *MTP1* expression, we also determined the effect of excess Zn and Cd exposure on the expression level of 10 known metal transporter genes (*AtIRT1, AtIRT2, AtBHLH100, AtFRO2, AtZIP4, AtHMA3, AtHMA4, AtNRAMP3, AtYSL3* and *AtFRD3*) in the *pro35S::NcMTP1* and *pro35S::AtMTP1* transgenic lines, when grown hydroponically on ½ Hoagland's media supplemented with sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$).

Expression of *AtIRT1*, encoding a soil Fe^{2+} uptake transporter (Korshunova et al., 1999), in shoots and roots of all *A. thaliana* lines was induced by excess Zn and Cd exposure (Fig. 5, 6; Table 1, 2). This indeed confirmed the induction of a Fe deficiency response by Zn and Cd exposure (Lombi et al., 2002), probably due to competition of Zn and Cd with Fe. Zn excess exposure induced more *AtIRT1* expression than Cd exposure, most likely due to the much higher molarity of Zn exposure compared to Cd exposure. Expression of *pro35S::NcMTP1* or *pro35S::AtMTP1* suppressed root *AtIRT1* expression in plants grown under control conditions (2 µM $ZnSO_4$). Only *pro35S::AtMTP1*, and not *pro35S::NcMTP1* expression, led to higher expression of *AtIRT1* in roots of plants grown at excess Zn and Cd (Fig. 5, 6; Table 1, 2). Almost the same effect was found for expression of *AtIRT2*, encoding another soil Fe^{2+} uptake transporter (Vert et al., 2009) (Fig. 5, 6; Table 1, 2). In this case, *AtIRT2* transcript levels were extra induced in both *pro35S::NcMTP1* as well as *pro35S::AtMTP1* lines when exposed to Cd. Expression of *AtFR02*, encoding for a root Fe reductase, is another marker for Fe deficiency in plants (Connolly et

al., 2003). Like *AtIRT1* and *AtIRT2*, it was highly induced in plants grown under Zn excess (Fig. 5, 6; Table 1, 2). The effect of *pro35S::NcMTP1* or *pro35S::AtMTP1* expression was less obvious for *AtFRO2* compared to the other two Fe deficiency markers, with only the *pro35S::NcMTP1* line showing significant reduction in *AtFRO2* expression upon excess Zn exposure.

AtBHLH100 is a transcription factor normally found to be induced by Fe deficiency in both roots and shoots (Vorwieger et al., 2007). Its expression was highly induced under excess Zn and Cd exposure, when compared to sufficient Zn, but this gene was much less responsive in C24 than in Col (Fig. 5, 6; Table 1, 2). The expression of *pro35S::NcMTP1* or *pro35S::AtMTP1* had a similar effect though, generally reducing expression of *AtBHLH100*. *AtFRD3* encodes a MATE-like citrate efflux transporter, involved in Zn and Fe translocation to the shoot. It is normally induced by Zn and Fe deficiency (Talke et al., 2006). Its expression level was decreased in the *pro35S::NcMTP1* expressing line in shoot tissues under excess Cd and also reduced in root tissue under sufficient Zn condition (Fig. 5; Table 1). In shoot tissue under Zn excess, *pro35S::NcMTP1* line exhibited enhanced *AtFRD3* transcript levels. *pro35S::AtMTP1* lines showed a differential expression pattern. An increased *AtFRD3* transcript levels was found in this line in shoot tissues in sufficient Zn and excess Cd conditions and same was the case in root tissues under sufficient Zn and excess Cd conditions (Fig. 6; Table 2). *AtFRD3* expression was decreased in the *pro35S::AtMTP1* line under excess Zn in shoot while there was no difference in root issues compared to C24 WT line.

AtZIP4 encodes a Zn^{2+} uptake transporter expressed in roots and shoots, normally induced by Zn deficiency (Assunção et al., 2010). Expression of *pro35S::NcMTP1* reduced the expression of *AtZIP4* under nearly all conditions except in roots uder excess Zn where both transgenic and Col WT lines had reduced expression. In case of Cd exposure its expression in roots of *pro35S::NcMTP1* was induced compared to the Col WT line (Fig. 5; Table 1). This was different for *pro35S::AtMTP1* expression, which led to enhanced *AtZIP4* expression under all conditions but was same in shoot under Cd excess (Fig. 6; Table 2). *AtYSL3* was up-regulated in all conditions in p*ro35S::NcMTP1*

line compared to Col WT (Fig. 5; Table 1). Similarly, *pro35S::AtMTP1* line also showed either higher *YSL3* expression in roots or similar in shoots, compared to C24 WT line in all conditions(Fig. 6; Table 2). Transcript levels of *AtNRAMP3* were reduced in the *pro35S::NcMTP1* line in shoots and root tissues under all treatments which means a sufficient Zn availability in these tissues under given treatments (Fig. 5; Table 1). Similarly, there was a down-regulation of *AtNRAMP3* in *pro35S::AtMTP1* line in shoot tissues in all treatments and also in root tissue under sufficient Zn condition (Fig. 6; Table 2). Excess Zn treatment showed enhanced *AtNRAMP3* expression in *pro35S::AtMTP1* line in root tissue while there was no difference in root tissue under excess Cd treatment compared to C24 WT. *AtHMA4* expression levels were found to be lower in *pro35S::NcMTP1* line in all treatments (Fig. 5; Table 1). *pro35S::ATMTP1* line showed reduced *AtHMA4* transcript levels under sufficient Zn and excess Cd conditions in shoot tissues, however, there was no *AtHMA4* differential expression between *ATMTP1* and C24 WT lines in rest of treatments (Fig. 6; Table 2). In case of *AtHMA3,* transcript levels were found to be lowered in *pro35S::NcMTP1* line in all treatments (Fig. 5; Table 1). Similarly, *AtHMA3* expression was reduced in *pro35S::AtMTP1* line in all treatments in shoot tissues while there was no difference in root tissues (Fig. 6; Table 2).

RNAi-mediated down regulation of *NcMTP1* in *N. caerulescens* roots enhanced Zn accumulation in shoot but reduced Cd accumulation in roots

The increased expression of *NcMTP1* in *N. caerulescens* compared to closely related non-hyperaccumulator species (Assunção et al., 2001), suggests its important function in either metal tolerance or accumulation of this species. To investigate this, we examined a set of knock-down (KD) plants, generated by RNAi-mediated silencing of the *NcMTP1* gene in roots upon *A. rhizogenes*-mediated transformation of *N. caerulescens*. Plants were compared to non-transformed WT plants, all grown hydroponically in three different metal supply regimes i.e. supplemented with Zn supply (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$ + 100 µM $ZnSO_4$), comparable to field-like exposures at La Calamine site (Assunção et al., 2003b). Upon excess

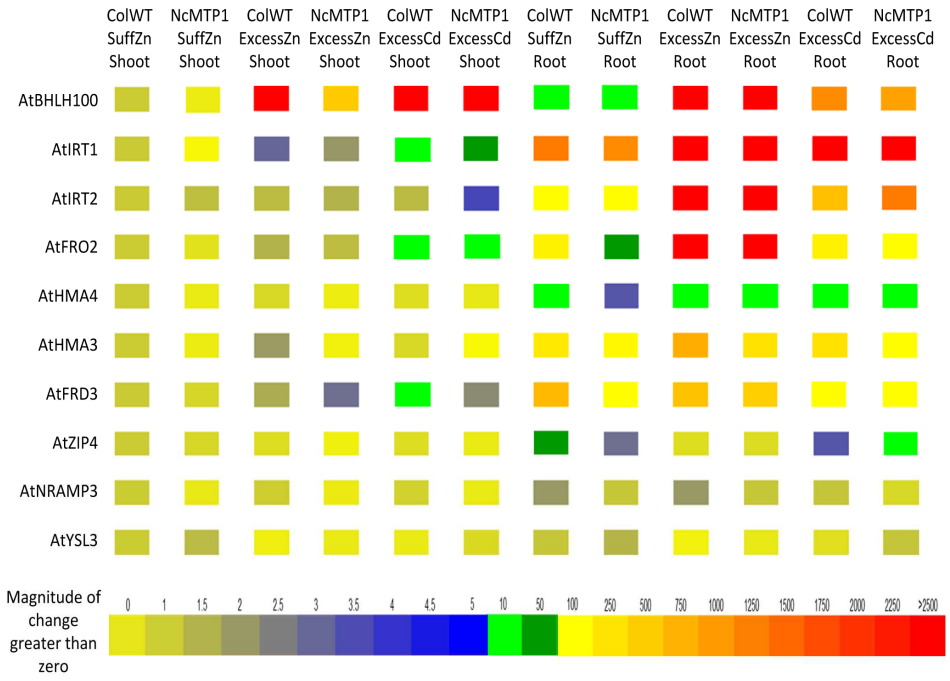

Fig. 5: Heat map representing relative gene expression analysis of known metal transporter genes *AtBHLH100*, *AtIRT1*, *AtIRT2*, *AtFRO2*, *AtHMA4*, *AtHMA3*, *AtFRD3*, *AtZIP4*, *AtNRAMP3* and *AtYSL3* in shoot and root of *pro35S::NcMTP1* and Col wild-type *A. thaliana* lines in response to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in Col wild-type in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data. NcMTP1 represents *pro35S::NcMTP1* line, ColWT is for Col wild-type and "suff" represents sufficient.

	ColWT SuffZn Shoot	NcMTP1 SuffZn Shoot	ColWT ExcessZn Shoot	NcMTP1 ExcessZn Shoot	ColWT ExcessCd Shoot	NcMTP1 ExcessCd Shoot	ColWT SuffZn Root	NcMTP1 SuffZn Root	ColWT ExcessZn Root	NcMTP1 ExcessZn Root	ColWT ExcessCd Root	NcMTP1 ExcessCd Root
AtBHLH100	b 1.0 ±0.1	a 0.1 ±0.0	f 2760.2 ±361.6	de 517.9 ±76.8	h 13045.6 ±595.3	g 4899.9 ±346.5	c 5.5 ±0.7	c 7.4 ±0.9	g 5377.0 ±760.1	f 2568.7 ±551.0	e 1127.2 ±66.2	de 916.7 ±77.1
AtIRT1	b 1.0 ±0.2	a 0.2 ±0.0	d 3.0 ±0.5	c 2.0 ±0.0	e 5.9 ±1.5	f 15.6 ±1.3	g 1286.2 ±90.9	g 1135.3 ±114.0	k 81903.6 ±15916.6	j 63885.5 ±5769.4	i 11650.8 ±2095.5	h 7808.7 ±420.8
AtIRT2	a 1.0 ±0.2	a 1.3 ±0.1	a 1.3 ±0.1	c 1.5 ±0.1	a 1.3 ±0.0	b 3.6 ±0.5	c 18.3 ±2.2	c 13.1 ±0.9	g 3661.4 ±494.2	f 2966.8 ±199.3	d 596.8 ±121.2	e 1493.6 ±44.7
AtFRO2	a 1.0 ±0.1	a 0.6 ±0.1	a 1.5 ±0.2	a 1.3 ±0.1	b 5.7 ±0.1	c 7.4 ±0.8	de 122.5 ±8.2	d 87.8 ±15.2	g 6018.4 ±665.2	f 4268.0 ±183.2	de 129.4 ±5.7	e 171.7 ±4.1
AtHMA4	c 1.0 ±0.1	a 0.4 ±0.0	b 0.7 ±0.1	a 0.3 ±0.0	b 0.6 ±0.1	a 0.4 ±0.0	gh 9.5 ±0.9	d 4.0 ±0.3	h 10.0 ±0.6	ef 6.3 ±0.1	fg 7.8 ±1.0	de 5.6 ±0.4
AtHMA3	b 1.0 ±0.1	a 0.4 ±0.0	c 2.0 ±0.1	a 0.3 ±0.0	b 0.7 ±0.1	a 0.1 ±0.0	f 206.8 ±2.0	d 60.3 ±12.1	h 792.2 ±113.9	g 282.4 ±10.5	g 283.6 ±36.7	e 128.8 ±4.9
AtFRD3	a 1.0 ±0.2	a 0.8 ±0.1	ab 1.6 ±0.1	c 2.8 ±0.3	d 6.2 ±0.6	bc 2.3 ±0.2	h 671.2 ±60.1	e 163.4 ±19.3	g 597.7 ±4.1	f 466.4 ±40.0	e 114.4 ±23.2	e 102.1 ±3.3
AtZIP4	c 1.0 ±0.1	b 0.8 ±0.1	b 0.7 ±0.0	a 0.3 ±0.0	b 0.6 ±0.1	a 0.4 ±0.0	f 37.0 ±9.3	d 2.8 ±0.1	b 0.6 ±0.1	b 0.7 ±0.0	d 3.3 ±0.2	e 5.8 ±0.4
AtNRAMP3	bc 1.0 ±0.1	a 0.4 ±0.0	bc 1.0 ±0.1	a 0.4 ±0.0	bc 0.9 ±0.1	a 0.4 ±0.0	d 2.0 ±0.1	c 1.1 ±0.1	d 2.0 ±0.3	c 1.1 ±0.1	c 1.1 ±0.1	b 0.7 ±0.0
AtYSL3	d 1.0 ±0.1	e 1.3 ±0.0	ab 0.3 ±0.0	ab 0.4 ±0.0	ab 0.4 ±0.0	c 0.7 ±0.0	d 1.1 ±0.1	e 1.4 ±0.0	ab 0.3 ±0.0	b 0.4 ±0.0	c 0.6 ±0.1	d 1.1 ±0.0

Table 1: Relative gene expression analysis of known metal transporter genes *AtBHLH100, AtIRT1, AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtFRD3, AtZIP4, AtNRAMP3* and *AtYSL3* in shoot and root of *pro35S::NcMTP1* and Col wild-type *A. thaliana* lines in response to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in Col wild-type in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data. NcMTP1 represents *pro35S::NcMTP1* line, ColWT is for Col wild-type and "suff" represents sufficient. Different letters indicate the significant difference in gene expression of the respective gene between lines grown in given treatments ($p<0.05$, ANOVA, Least Significant Difference) (mean ± SE of 4 replica).

Zn exposure, KD plants showed some slight chlorosis of rosette leaves and even further reduced shoot growth (Fig. 7 A). Fv/Fm (or (Fm–Fo)/Fm, where Fm is the maximal fluorescence measured during the first saturation pulse after dark adaption and Fo is the dark adapted initial minimum fluorescence) is a measurement of photosystem II efficiency, a parameter to monitor stress in plants (Baker, 2008), was measured in KD and WT plants.

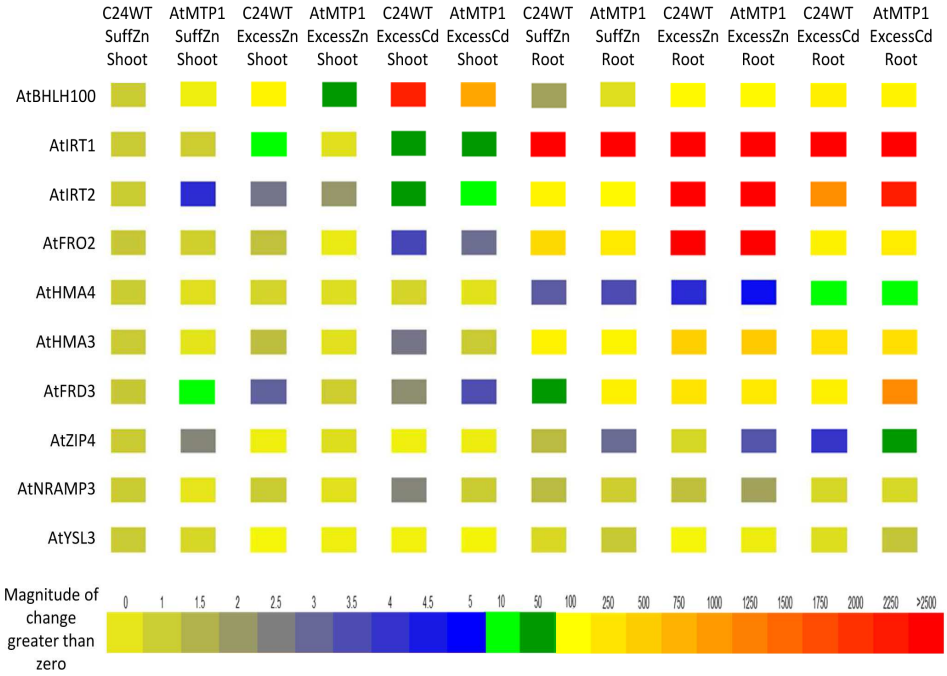

Fig. 6: Heat map representing relative gene expression analysis of known metal transporter genes *AtBHLH100, AtIRT1, AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtFRD3, AtZIP4, AtNRAMP3* and *AtYSL3* in shoot and root of *pro35S::AtMTP1* and C24 wild-type *A. thaliana* lines in response to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in C24 wild-type in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data. AtMTP1 represents *pro35S::AtMTP1* line, C24WT is for C24 wild-type and "suff" represents sufficient.

	C24WT SuffZn Shoot	AtMTP1 SuffZn Shoot	C24WT ExcessZn Shoot	AtMTP1 ExcessZn Shoot	C24WT ExcessCd Shoot	AtMTP1 ExcessCd Shoot	C24WT SuffZn Root	AtMTP1 SuffZn Root	C24WT ExcessZn Root	AtMTP1 ExcessZn Root	C24WT ExcessCd Root	AtMTP1 ExcessCd Root
	b	a	ef	d	h	g	c	ab	d	d	f	e
AtBHLH100	1.0 ±0.1	0.0 ±0.0	140.9 ±18.5	41.4 ±3.7	2180.2 ±358.1	867.9 ±239.1	1.8 ±0.2	0.6 ±0.1	51.9 ±4.3	58.4 ±9.6	148.0 ±10.7	112.2 ±4.4
	a	a	b	a	d	c	f	e	i	j	g	h
AtIRT1	1.0 ±0.2	1.0 ±0.1	8.5 ±2.0	0.6 ±0.1	31.4 ±5.1	19.2 ±1.0	12179.5 ±3930.6	3860.2 ±256.8	146965.3 ±28443.8	246722.9 ±10259.1	29576.8 ±2538.9	66813.4 ±10780.1
	a	c	bc	b	d	c	e	e	h	h	f	g
AtIRT2	1.0 ±0.1	4.1 ±0.2	2.7 ±0.7	2.0 ±0.3	12.7 ±1.4	5.7 ±0.6	98.6 ±43.8	53.1 ±4.2	4475.8 ±429.6	3439.5 ±665.6	1094.3 ±85.1	2209.7 ±305.0
	b	b	b	a	c	c	d	d	e	e	d	d
AtFRO2	1.1 ±0.3	0.9 ±0.2	1.2 ±0.4	0.4 ±0.0	3.6 ±0.4	2.8 ±0.3	354.7 ±87.1	197.7 ±17.0	3242.3 ±90.9	2979.6 ±281.0	122.3 ±13.0	181.7 ±33.4
	c	a	bc	ab	b	a	d	d	e	e	f	g
AtHMA4	1.0 ±0.1	0.6 ±0.0	0.8 ±0.1	0.7 ±0.0	0.8 ±0.0	0.5 ±0.0	3.2 ±0.4	3.5 ±0.4	4.2 ±0.2	4.7 ±0.4	6.9 ±0.2	9.4 ±1.8
	b	a	b	a	c	b	d	d	f	f	e	e
AtHMA3	1.0 ±0.2	0.5 ±0.1	1.3 ±0.2	0.6 ±0.1	2.7 ±0.2	1.0 ±0.1	115.1 ±35.9	102.5 ±12.6	462.8 ±17.0	511.9 ±23.5	296.6 ±11.2	312.1 ±33.2
	a	c	b	a	b	b	d	e	e	e	e	f
AtFRD3	1.1 ±0.3	8.6 ±0.8	3.1 ±0.9	1.0 ±0.1	2.2 ±0.4	3.5 ±0.4	17.1 ±1.1	133.7 ±37.6	255.9 ±60.9	205.9 ±71.0	133.3 ±14.7	1130.9 ±290.5
	ab	bcd	a	ab	a	a	abc	cd	ab	d	d	e
AtZIP4	1.0 ±0.2	2.4 ±0.6	0.3 ±0.0	0.6 ±0.0	0.3 ±0.1	0.3 ±0.1	1.3 ±0.0	2.9 ±0.4	0.8 ±0.1	3.3 ±0.2	4.0 ±0.3	12.7 ±2.0
	bcd	a	bcd	a	f	bcd	d	bc	cd	e	ab	ab
AtNRAMP3	1.0 ±0.1	0.5 ±0.0	1.0 ±0.1	0.6 ±0.0	2.4 ±0.3	1.0 ±0.1	1.3 ±0.1	1.0 ±0.1	1.2 ±0.1	1.8 ±0.1	0.8 ±0.0	0.8 ±0.1
	de	cd	a	a	a	a	cd	de	a	ab	bc	e
AtYSL3	1.0 ±0.0	0.8 ±0.1	0.2 ±0.0	0.3 ±0.0	0.3 ±0.1	0.2 ±0.0	0.7 ±0.1	1.0 ±0.1	0.2 ±0.0	0.3 ±0.1	0.6 ±0.1	1.1 ±0.3

Table 2: Relative gene expression analysis of known metal transporter genes *AtBHLH100, AtIRT1*, *AtIRT2, AtFRO2, AtHMA4, AtHMA3, AtFRD3, AtZIP4, AtNRAMP3* and *AtYSL3* in shoot and root of *pro35S::AtMTP1* and C24 wild-type *A. thaliana* lines in response to sufficient Zn (2 µM $ZnSO_4$), excess Zn (60 µM $ZnSO_4$) and excess Cd (2 µM $CdSO_4$) after two weeks of metal exposure. Expression is relative to the shoot in C24 wild-type in sufficient Zn treatment, used as RTL 1. *AtUBP6* (At1g51710) was used as housekeeping gene to normalize the data. AtMTP1 represents *pro35S::AtMTP1* line, C24WT is for C24 wild-type and "suff" represents sufficient. Different letters indicate the significant difference in gene expression of the respective gene between lines grown in given treatments ($p<0.05$, ANOVA, Least Significant Difference) (mean ± SE of 4 replica).

KD plants had lower Fv/Fm compared to WT (Fig. 7 B), indicating reduced photosynthesis efficiency and probably leading to higher oxidative stress. Down-regulation of *NcMTP1* enhanced the accumulation of Zn in shoots when exposed to excess Zn in comparison to WT plants, but had no discernible effect

on root Zn accumulation, which was very high at excess Zn exposure (Fig. 8 A,B). KD plants grown in excess Cd showed significantly enhanced Cd accumulation in roots, but had hardly any increase in Cd accumulation in shoots (Fig. 8 C). In contrast, Fe accumulation in roots was enhanced in KD plants, while only at Zn supply (2µM ZnSO4), the shoot Fe concentration was reduced in KD plants compared to WT plants (Fig. 8 D,E). Mn were higher in shoots of WT plants compared to KD in Zn supply (2µM ZnSO4), while there was no difference in roots, however, both KD and Wt plants had higher Mn in roots at excess Zn treatment (Fig. 8 F,G).

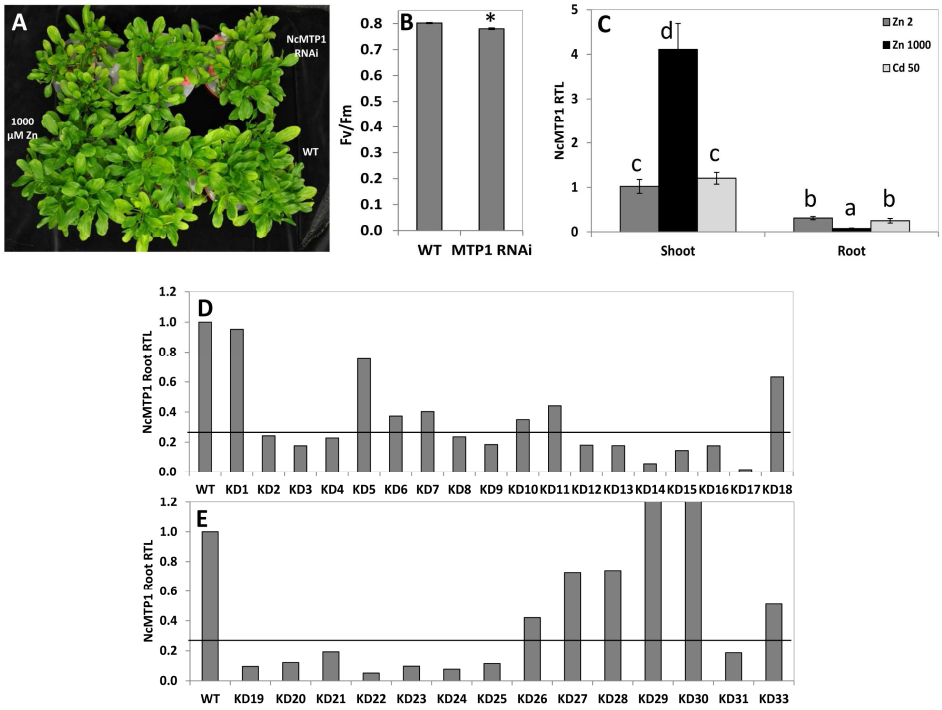

Fig. 7: Phenotypic response of *NcMTP1* RNAi knocked down (KD) and wild-type (WT) *N. caerulescens* plants to excess Zn, Fv/Fm of KD and WT *N. caerulescens* plant exposed to excess Zn and quantitative reverse transcriptase PCR (qRT-PCR) analysis of *NcMTP1* expression in KD and WT *N. caerulescens* plants. (A) Visible phenotype of KD and WT *N. caerulescens* plants grown on Zn excess medium (1000 µM $ZnSO_4$) for four weeks (B) Fv/Fm of KD and WT *N. caerulescens* plants grown on Zn excess medium (1000 µM $ZnSO_4$) for four weeks. (C) Relative expression (relative to shoot expression in WT in Zn supply (2µM $ZnSO_4$) treatment, used as RTL 1) of *NcMTP1* in WT plants grown under Zn supply (2 µM $ZnSO_4$) for four weeks. (D) Expression of *NcMTPI* in KD and WT *N. caerulescens* plants grown in ½ Hoagland's nutrient solution with Zn supply (2 µM

$ZnSO_4$) for one week. The black line represents the threshold of 0.25 which was chosen to select KD plants. *NcTubulin* was used as housekeeping gene to normalize the gene expression data. * in panel "B" indicates the significant difference (p value < 0.05, Student's t-test) in Fv/Fm between KD and WT *N. caerulescens* plants. Different letters in panel "C" indicate the significant difference between WT in different treatments ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

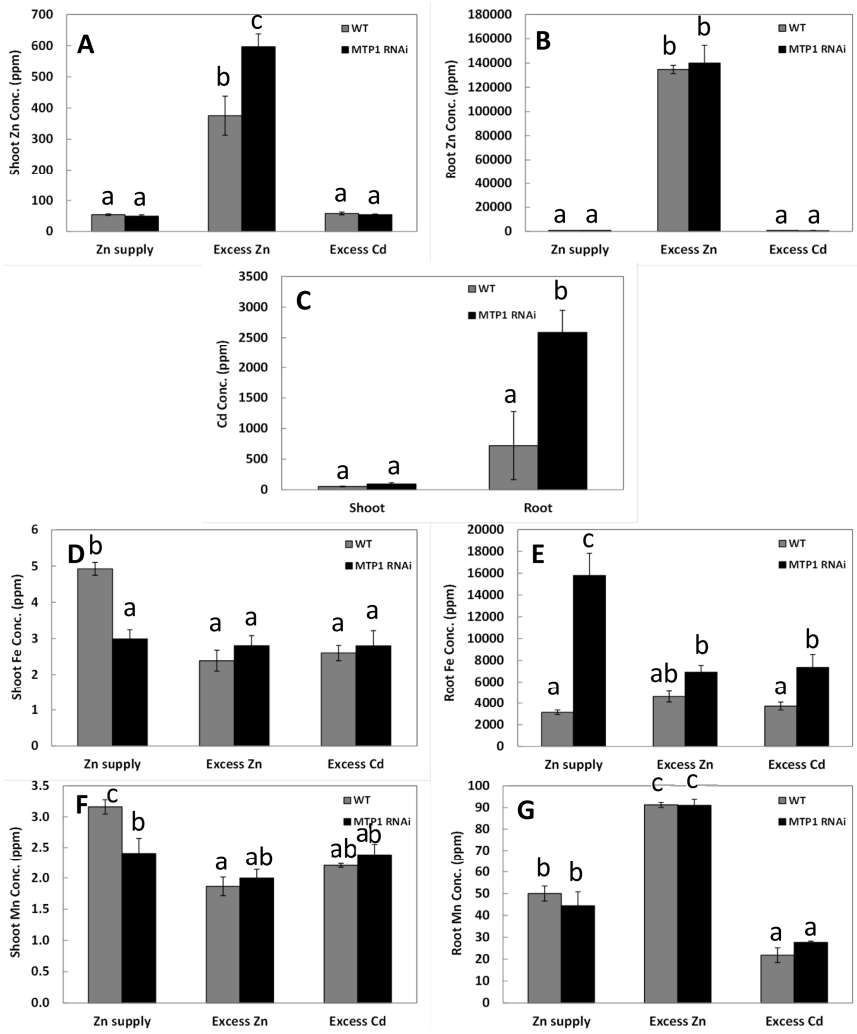

Fig. 8: The metal accumulation of *MTP1* KD and WT *N. caerulescens* in response to Zn supply (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$-100 µM $ZnSO_4$) after five weeks of metal exposure. (A) Zn concentration (ppm) in shoots (B) and in roots (C) Cd concentration (ppm) in shoots and roots (D) Fe concentration (ppm) in

shoots (E) and in roots (F) Mn concentration (ppm) in shoots (G) and in roots. Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of 4 replica).

Silencing of *NcMTP1* in *N. caerulescens* alters the expression of other metal homeostasis genes

First, we performed the gene expression of *NcMTP1* in WT *N. caerulescens* plants grown under Zn supply (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$ + 100 µM $ZnSO_4$). In general, expression of *NcMTP1* was higher in shoots than in roots (Fig. 7 C). There was about four fold higher expression in shoot in excess Zn. Root expression was lowered in excess Zn. *NcMTP1* was downregulated in the roots of KD plants which confirmed that the RNAi construct worked (Fig. 7 D,E). Furthermore, we analysed the expression of six known metal transporter genes in *NcMTP1* KD and WT plants grown in Zn supply (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$ + 100 µM $ZnSO_4$) to see if they play a role in the observed differential Zn, Cd and Fe accumulation. The transcript levels of *NcIRT1* (involved in Fe uptake), *NcHMA3* (involved in vacuolar Cd sequestration), *NcNRAMP3* (involved in Fe remobilization), *NcZNT1* (involved in Zn and Cd uptake; Chapter 2), *NcZNT2* (involved in Zn uptake) and *NcMTP1* were analysed (Fig. 9; Table 3). Expression of *NcMTP1* in shoot tissues was analysed and was found to be same in KD as in WT with higher expression under Zn excess (Fig. 9; Table 3). The *NcMTP1* expression was reduced in KD plants than WT in Cd excess. Expression of *NcIRT1* was same in shoots of KD and WT while in roots of KD plants, it was lowered in Zn supply (2µM $ZnSO_4$) (Fig. 9; Table 3). Expression of *NcHMA3* was found to be significantly lower in shoots of KD plants in excess Zn exposure and in roots upon excess Cd treatment (Fig. 9; Table 3). *NcNRAMP3* exhibited a significantly down regulation in roots of KD plants under excess Cd but had similar shoot expression compared to WT (Fig. 9; Table 3). *NcZNT1* expression was found to be un altered between KD and Wt plants (Fig. 9; Table 3). KD and WT had similar expression of *NcZNT2* in shoots while KD shoots showed lower *NcZNT2* expression in roots in Cd excess condition compared to WT plants (Fig. 9; Table 3).

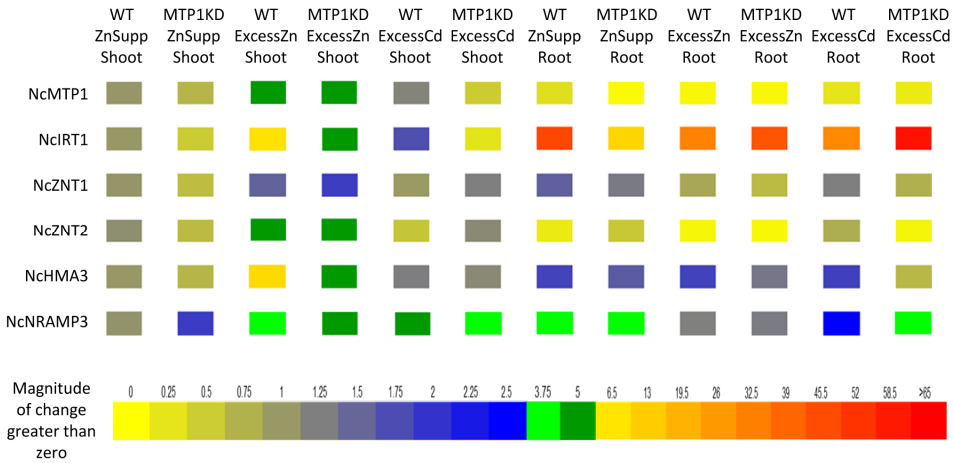

Fig. 9: Heat map representing relative gene expression analysis of known metal transporter genes *NcMTP1, NcIRT1, NcZNT1, NcZNT2, NcHMA3* and *NcNRAMP3* in shoot and root of *NcMTP1* RNAi and wild-type WT *N. caerulescens* in response to Zn supply (2 µM $ZnSO_4$), excess Zn (1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$-100 µM $ZnSO_4$) after five weeks of metal exposure. Expression is relative to the shoot in wild-type in 2 µM $ZnSO_4$ treatment, used as RTL 1. *NcTubulin* was used as housekeeping gene to normalize the data. MTP1KD represents *NcMTP1* RNAi (knockdown), WT is for wild-type and "supp" represents supply.

	WT ZnSupp Shoot	MTP1KD ZnSupp Shoot	WT ExcessZn Shoot	MTP1KD ExcessZn Shoot	WT ExcessCd Shoot	MTP1KD ExcessCd Shoot	WT ZnSupp Root	MTP1KD ZnSupp Root	WT ExcessZn Root	MTP1KD ExcessZn Root	WT ExcessCd Root	MTP1KD ExcessCd Root
NcMTP1	cd **1.0** ±0.2	cd **0.7** ±0.2	f **4.1** ±0.6	f **3.9** ±0.4	e **1.2** ±0.1	cd **0.5** ±0.1	c **0.3** ±0.0	a **0.0** ±0.0	a **0.1** ±0.0	a **0.1** ±0.0	bc **0.3** ±0.1	b **0.2** ±0.0
NcIRT1	ab **1.0** ±0.1	ab **0.5** ±0.2	c **7.2** ±3.5	bc **4.6** ±1.3	ab **1.7** ±0.9	a **0.3** ±0.1	de **46.8** ±19.0	cd **10.7** ±5.4	de **31.6** ±10.1	d **43.5** ±13.3	de **29.9** ±8.0	d **60.4** ±26.3
NcZNT1	abc **1.0** ±0.2	a **0.6** ±0.1	cd **1.5** ±0.2	d **1.9** ±0.4	abc **1.0** ±0.2	abcd **1.2** ±0.3	cd **1.6** ±0.3	cd **1.3** ±0.4	abc **0.9** ±0.1	ab **0.7** ±0.1	abcd **1.2** ±0.3	abc **0.8** ±0.2
NcZNT2	b **1.1** ±0.3	b **0.7** ±0.2	c **6.1** ±2.8	c **6.0** ±1.9	b **0.6** ±0.1	b **1.1** ±0.4	a **0.2** ±0.1	b **0.5** ±0.2	a **0.1** ±0.0	a **0.1** ±0.0	b **0.8** ±0.1	a **0.1** ±0.0
NcHMA3	a **1.0** ±0.1	a **0.7** ±0.1	d **9.2** ±1.3	c **5.6** ±0.7	ab **1.3** ±0.1	ab **1.2** ±0.0	ab **1.8** ±0.6	ab **1.6** ±0.4	ab **1.8** ±0.3	ab **1.3** ±0.2	b **1.9** ±0.3	a **0.7** ±0.1
NcNRAMP3	a **1.1** ±0.2	abcd **1.9** ±0.2	de **3.4** ±1.3	e **4.3** ±0.7	de **3.9** ±0.5	abcd **2.6** ±0.7	abde **3.1** ±0.3	bde **3.1** ±0.6	ab **1.2** ±0.0	abc **1.3** ±0.2	f **6.7** ±0.8	de **3.5** ±0.6

Table 3: Relative gene expression analysis of known metal transporter genes *NcMTP1, NcIRT1, NcZNT1, NcZNT2, NcHMA3* and *NcNRAMP3* in shoot and root of *NcMTP1* RNAi and wild-type *N. caerulescens* in response to Zn supply (2 µM $ZnSO_4$), excess Zn

(1000 µM $ZnSO_4$) and excess Cd (50 µM $CdSO_4$-100 µM $ZnSO_4$) after five weeks of metal exposure. Expression is relative to the shoot in wild-type in 2 µM $ZnSO_4$ treatment, used as RTL 1. *NcTubulin* was used as housekeeping gene to normalize the data. MTP1KD represents *NcMTP1* RNAi (knockdown), WT is for wild-type and "supp" represents supply. Different letters indicate the significant difference in gene expression of the respective gene between lines grown in given treatments ($p<0.05$, ANOVA, Least Significant Difference) (mean ± SE of 4 replica).

Different *N. caerulescens* accessions have one or two copies of *NcMTP1*

To estimate the copy number of *NcMTP1* among various *N. caerulescens* accessions, genomic DNA was isolated from leaf tissue for a Quantitative Real Time (qRT-PCR). In accession Ganges (GA), screening the preliminary assembly of the genomic sequence only revealed one copy, thus we normalized the Ct value of *NcMTP1* of all accessions with GA, which means the *NcMTP1* copy number in GA was defined as one. This means we found one *NcMTP1* copy in accessions Durfort, Monte Prinzera, St. Julien, Lellingen, Pontaut, Clough and in *N. praecox*, while there were two *NcMTP1* copies in Plombières, La Calamine and Prayon (Fig. 10). We have used *NcTubulin*, a known single copy gene not involved in metal tolerance and accumulation and we used the ratio of *NcMTP1* and *NcTubulin* for normalization in this analysis, which also confirmed that it indeed had a single copy in all above mentioned accessions (Fig. 10).

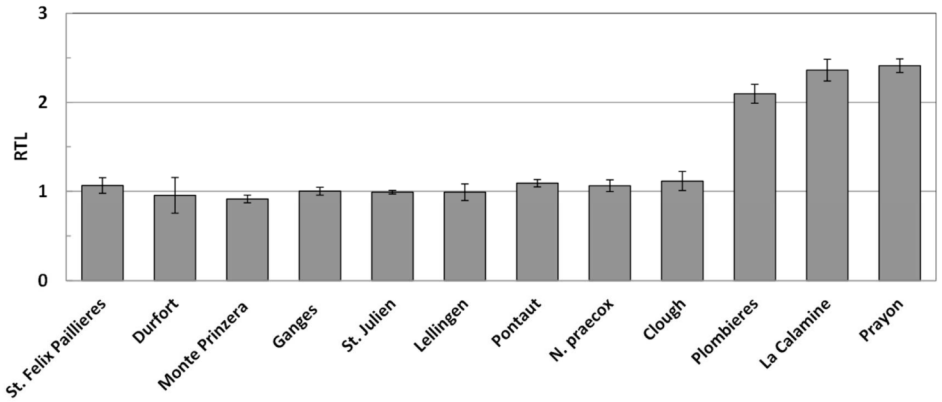

Fig. 10: *NcMTP1* copy number analysis by relative gene expression on DNA in different *N. caerulescens* accessions grown hydroponically in Zn supply (2 µM $ZnSO_4$) after five weeks. The *NcMTP1* copy number was calculated relative to the ratio of *MTP1/aTubulin* in different accessions.

Bioinformatics analysis of *MTP1* promoters from *N. caerulescens*, *A. halleri*, *A. lyrata* and *A. thaliana*

The putative *MTP1* promoter regions from *N. caerulescens*, *A. halleri, A. lyrata* and *A. thaliana* were compared as presented in Fig. 11. The following *MTP1* sequences were used: *A. halleri* partial BAC clone 7G24 (acc. no. FN428855), *A. lyrata* ARALYscaffold_4 (acc. no. GL348716) and *A. thaliana chromosome* 2 (region: 19235100-19239500) to identify and compare the Brassicaceae *MTP1* promoter regions. This analysis showed the presence of four different motifs, which mainly formed clusters and followed a common pattern. These motif clusters were represented as B1 and B2 in the promoter scheme. B1 was characterized as a 6-bp palindromic sequence (P1: TTCGAA) at the 3′ site. In *A. halleri* a 10-bp palindromic sequence (TCTTCGAAGA) was found which showed the same core sequence as P1. A second palindromic sequence P2 (AACGT) and also the dehydration response element (DRE) were present only in the hyperaccumulator species, All the promoters showed one G-box, light and abscisic acid responsive element (LRE and ABRE). An auxin responsive element (AUX) was present in all the promoters except in *A. thaliana*. The *N. caerulescens* promoter was the only one that contained an enhancer sequence (E-box). *A. halleri, A. thaliana* and *A. lyrata* contained the metal response element b (MREb) inside the *MTP1* coding sequence. MREb was also present in *N. caerulescens*, at the same position, but with a difference of one nucleotide. In all promoters, a putative TATA-box was identified which was close to the 5′ UTR. The 5′ UTR in *N. caerulescens* and *A. thaliana* was identified by aligning the corresponding transcript sequences. In *A. halleri and A. lyrata* the 5′ UTR was predicted based on alignment with the *A. thaliana* 5′ UTR. B3 was a region highly similar between *A. thaliana, A. halleri,* and *A. lyrata*, however, few internal deletions, mainly in *A. halleri*, were present which caused differences inside this region. This analysis showed putative cis- regulatory elements which might play a role in the differential transcriptional regulation of *NcMTP1* between accumulator (*N. caerulescens*: *A. halleri*) and non-accumulator (*A. thaliana*: *A. lyrata*).

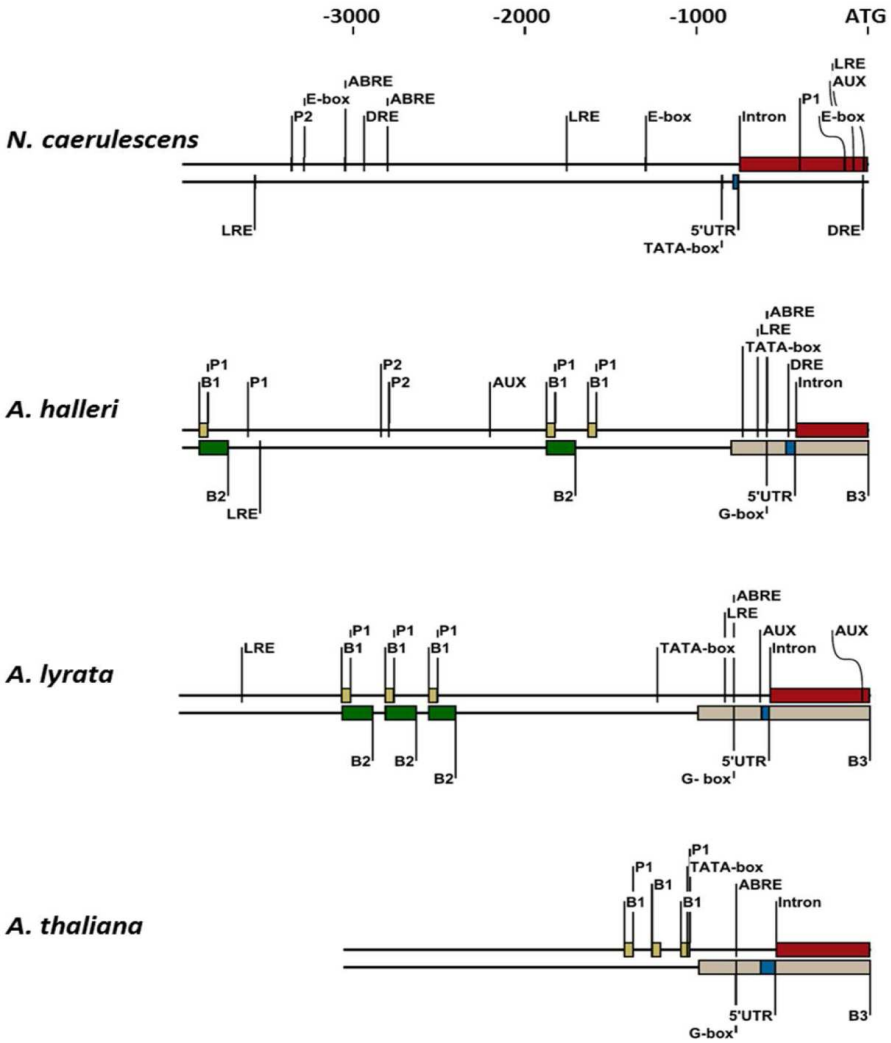

Fig. 11: Scheme of the MTP1 promoter in two metal hyperaccumulators species *N. caerulescens*, *A. halleri* and two non-accumulator spices *A. lyrata* and *A. thaliana* all belonging to the family Brasicaceae. B1 (yellow, sequence with identity 90% between and within A. halleri and A. lyrata, and identity 80% with *A. thaliana*). B2 (green, sequence with identity >80% between *A. halleri* and *A. lyrata*), B3 (grey, sequence with identity 80% between *A. halleri*, *A. lyrata* and *A. thaliana*, excluding the internal deletions), ABRE (absicic acid responsive element), AUX (auxin responsive element), DRE (dehydration response element), E-box (enhancer), G-box and LRE (light responsive element), MREb (metal responsive element), P1 and P2 (Palindromic sequence 1 and 2 respectively).

DISCUSSION

In the current study, we report that *NcMTP1* is involved in Zn and Cd tolerance and accumulation. We have shown that this member of the CDF family gene encodes a protein that can transport Zn and probably Cd in yeast and its expression enhances metal tolerance and accumulation in *A. thaliana*. *NcMTP1* knockdown (KD) in roots of *N. caerulescens* plants caused enhanced shoot Zn and root Cd accumulation. Furthermore, there are one to two copies of this gene in various *N. caerulescens* accessions. Moreover, putative cis regulatory elements were identified in the promoter of *NcMTP1* gene in *N. caerulescens*.

Previously, Assunção et al. (2001) and van de Mortel et al. (2006) reported that *NcMTP1* is constitutively expressed in *N. caerulescens* compared to the non-accumulators *T. arvense* and *A. thaliana*. Its particular higher shoot expression suggested it to be an important gene that is involved in Zn tolerance and accumulation in *N. caerulescens*. Our quantitative expression data of *NcMTP1* in *N. caerulescens* suggest that *NcMTP1* is a Zn transporter expressed mainly in shoot tissues which probably can transport Cd. Since orthologues *NgMTP1* and *AtMTP1* were reported to transport Zn and Cd in yeast system (Persans et al., 2001; Kawachi et al., 2012), we also determined the Zn and Cd transport activity of NcMTP1 compared to AtMTP1. The Zn complementation in mutant yeast exhibited by NcMTP1 and AtMTP1 confirmed that both proteins are able to transport Zn into yeast vacuolar compartments and thus keep the cellular machinery protected from the damaging effect of excess Zn. However, complementation of Cd hypersensitivity by AtMTP1 demonstrated that this protein can also transport Cd when expressed in yeast. NcMTP1 expression in Cd mutant yeast showed higher sensitivity when grown on excess Cd. This could mean that probably, NcMTP1 is able to transport Cd but its transport activity is too low to see phenotypic effect on Cd complementation assay in yeast. In order to confirm this assumption, Cd content measurements in the yeasts expressing NcMTP1 should be performed and the higher Cd content in these yeasts would confirm the ability of NcMTP1 to transport Cd. This assumption is further supported by the evidence that our *NcMTP1* expressing *A. thaliana* lines displayed tolerance to excess Cd. Our results are in

agreement with the previous known Zn and Cd transport abilities of AtMTP1 and NgMTP1 and will further open the discussion about the Cd transport ability of NcMTP1 (Persans et al., 2001; Kawachi et al., 2012; Desbrosses-Fonrouge et al., 2005).

In order to understand the role of *NcMTP1* in planta, we constitutively expressed this gene in *A. thaliana* (Col background) and compared to already published *AtMTP1* expressing *A. thaliana* (C24background) exposed to excess Zn and Cd (van der zaal et al., 1999). AtMTP1 was reported to be localized to vacuolar membrane and its expression enhanced Zn tolerance and accumulation in *A. thaliana* (van der Zaal et al., 1999). Our *AtMTP1* expressing lines showed higher Zn tolerance and accumulation (Fig. 3 B-D) which confirmed the previously known involvement of this gene in Zn tolerance and accumulation (van de Zaal et al., 1999). *pro35S::NcMTP1* lines also exhibited higher Zn tolerance and accumulation, which revealed the involvement of NcMTP1 in these processes. In view of the localization of other CDF family orthologous proteins like AtMTP1, AtMTP3 and PtdMTP1 to the plant vacuolar membrane, and the similar effect of *NcMTP1* expression on Zn tolerance and accumulation in *A. thaliana*, we assume that also NcMTP1 is localized to the vacuolar membrane, effluxing Zn into the vacuole. The sequestration of Zn into vacuolar compartments is the most likely reason of Zn tolerance and accumulation conferred by *NcMTP1* expression in *A. thaliana*. Similar results were also found in previous studies ectopically expressing MTP1 proteins like AtMTP1, NgMTP1 and PtdMTP1 in *A. thaliana*, which resulted in enhanced Zn tolerance and accumulation thus showing that their normal function is to create a sink for Zn in the vacuoles of plant cells in case of intracellular Zn excess (Blaudez et al., 2003; Arrivault et al., 2006; Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004). 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 vacuoles (Delhaize et al., 2003). 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), similar as we found for the expression of *NcMTP1*. Recently, it was reported that shoot vacuolar membrane was the site where NgMTP1 was strongly accumulated in *N. goesingense* (Gustin et., 2009). This protein enhanced Zn accumulation and tolerance when expressed in *A. thaliana*, most probably due to the role it plays in vacuolar Zn transport. The authors also showed that the MTP1 protein of *N. goesingense* can enhance accumulation of Zn in shoot and root tissues when specifically expressed in shoot and root respectively in *A. thaliana*. Furthermore, shoot specific expression of this protein enhanced shoot Zn accumulation by initiating a systemic Zn deficiency response. Zn was found to be sequestered predominantly in the epidermal vacuoles in the leaves of *N. 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). All these observations and the higher shoot expression of *NcMTP1* in *N. caerulescens* under excess Zn point out that *NcMTP1* plays a role in shoot Zn detoxification by vacuolar sequestration in epidermal cells. Furthermore, creating a strong metal sink in the vacuoles can possibly play a role in the accumulation of extra metals in *N. caerulescens*.

A novel observation was that *pro35S::NcMTP1 A. thaliana* lines were more tolerant and accumulated higher Cd levels in shoot and root tissues compared to Col WT line. Interestingly, the transgenic *pro35S::AtMTP1* expressing line and its C24 WT did not show any Cd tolerance and accumulation when grown in the same experiment, as was also reported previously with the same lines by van der Zaal et al. (1999). We initially could not rule out that there might be a background affect for *pro35S::NcMTP1* (Col) and *pro35S::AtMTP1* (C24) lines, as Col carries a mutant allele of *AtHMA3* with a single base pair deletion, making it non-functional, while the gene is expected to still function properly in C24 (Wong and Cobbett, 2009; Morel et al., 2009). However, this means that Col is expected to have a higher sensitivity to Cd exposure compared to C24, in the absence of a proper AtHMA3 protein shuttling Cd into the vacuole in roots. In contrast, we found similar Cd toxicity symptoms in Col compared to C24 at the high Cd we exposed plants to, and

concluded that the *AtHMA3* mutation is not the reason for the observed effect of *NcMTP1* expression (Fig. 3 A). The Cd tolerance and accumulation data of the *pro35S::NcMTP1* line means that Cd is most likely taken up through NcMTP1. We consider that the improved Zn and Cd tolerance and accumulation in *pro35S::NcMTP1* lines is most probably due to detoxification of Zn and Cd in vacuolar compartments, effluxing these metals from the cytoplasm into the vacuole.

N. 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 *NcMTP1* cDNA, is very tolerant to Cd, but is a poor Cd accumulator. This in contrast to several other accessions from the south of France, which are both Cd tolerant and Cd accumulating (Assunção et al., 2003b; Reeves et al., 2001). A very high and constitutive Zn tolerance and inducible Cd tolerance was found at the cellular level in Ganges, a southern France *N. caerulescens* accession, accompanied with enhanced Zn sequestration in the vacuoles (Marqués et al., 2004). Cd sequestration was not analysed. Several studies were 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 Cd tolerant *N. caerulescens* accessions (Cobbett and Goldsbrough, 2002; Ebbs et al., 2002). Clearly 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). The correlation between Cd tolerance and Cd accumulation in *A. thaliana* upon expression of *NcMTP1* gene, together with Zn and Cd transport activity of this protein in yeast system means that NcMTP1 is likely be able to either transport Cd directly or affects the plant in such a way that Cd is transported via Fe deficiency responsive transporters into plant cells. Enhanced Cd tolerance is a consequence of the ability to detoxify more Cd than WT plants, e.g. by storing it in vacuoles. This characteristic is not known for

other plant MTP proteins, also not for *AhMTP1* from *A. halleri*, which is also a Cd tolerant species. However in nature, *A. halleri* is barely Cd hyperaccumulating (Macnair et al., 1999), which may explain the evolution of the Cd transport function of NcMTP1 in this species.

It is known that when *A. thaliana* is exposed to excess Cd, the Zn concentration in roots increases substantially, to similar levels as seen upon exposure to excess Zn (van de Mortel et al., 2006; van de Mortel et al., 2008). Thus, one also expects this to cause Zn excess problems in roots and because plants try to translocate Zn to shoots which cause Zn toxicity. We found that the Zn concentration in roots of *NcMTP1* and *AtMTP1* (over) expressing transgenic lines exposed to excess Cd was higher compared to their respective wild-type lines (Fig. 4 D). Our gene expression data confirmed that under excess Zn and excess Cd, *A. thaliana* switched on its Fe uptake mechanism, including the expression of *AtIRT1, AtIRT2, AtFRO2* and *AtbHLH100* (Fig. 5, 6; Table 1, 2). Furthermore, the Fe concentration data of *pro35S::NcMTP1* lines under Cd stress confirmed that these plants were likely to be experiencing Fe shortage (Fig. 5; Table 1). Since IRT1 also transports Zn and Cd, next to Fe (Eide et al. 1996; Korshunova et al. 1999; Rogers et al. 2000; Vert et al. 2002), and these probably compete with Fe, we expected that Zn and Cd levels in roots also go up, which was indeed the case (Fig. 3, 4). In order to deal with especially increased Zn levels, the *pro35S-NcMTP1* and *-AtMTP1* plants offered a sink for Zn which might allowed these lines to further grow and to translocate more Zn into shoot. In case of Cd exposure, the Cd transport by *NcMTP1* expressing plants would have protected them from Cd stress due to vacuolar Cd sequestration but this was not the case in Col WT, *pro35S::AtMTP1* and C24 WT lines. These observations showed that there is very likely an indirect Zn and Cd transport due to the expression of *AtIRT1*, *AtIRT2*, *AtBHLH100* and *AtFRO2*. This indirect Zn and Cd uptake has contributed towards Zn and Cd accumulation as exhibited by enhanced Zn and Cd accumulation in *pro35S::NcMTP1* lines.

One characteristic of hyperaccumulator species is to have a higher concentrations of Zn in the shoots than in the roots, while in non

hyperaccumulators, the higher Zn concentration is in the roots (Shen et al., 1997). *Thlaspi arvense* (non-accumulator) had 2.5 more times higher Zn in root cells vacuoles than in *N. caerulescens*. In *N. caerulescens* roots, a small fraction of the Zn was reported to be present in the vacuole, so there was more Zn available for loading into the xylem vessels (Lasat et al., 1998). In shoots of *N. caerulescens,* Zn accumulation occurs mainly in the vacuoles of epidermal cells, but also substantially in mesophyll cells (Küpper et al., 1999). AtMTP1 and NgMTP1 were shown to localize to vacuolar membranes involved in Zn vacuolar sequestration (Desbrosses-Fonrouge et al., 2005; Kobae et al., 2004; Gustin et., 2009). Therefore *NcMTP1* is a good candidate to be responsible for shoot metal hypertolerance and accumulation. A stable transformation system in *N. caerulescens* is cumbersome, but we can transform its roots by *A. rhizogenes* mediated hairy root transformation system. So we analysed the essentiality of NcMTP1's in the roots. The chimeric *NcMTP1* KD plants we generated were a good tool to analyse the function of this gene in the roots. The *NcMTP1* gene was significantly higher expressed in shoots than in roots in the WT *N. caerulescens* plants, in low Zn, excess Zn and excess Cd treatments (Fig. 7 A), which is in agreement with previous observations (Assunção et al., 2001). The *NcMTP1* expression was mainly enhanced in shoots under excess Zn supply (2µM $ZnSO_4$) which is in line with the general higher *NcMTP1* expression in *N. caerulescens* populations from calamine soil with excess Zn compared to populations originating from low Zn serpentine or normal soils (Assunção et al., 2001). A significantly higher Zn accumulation in the shoots of *NcMTP1* KD plants in excess Zn treatment was found compared with the WT *N. caerulescens*. We think that this is due to reduced Zn sequestration into vacuoles of root cells, ultimately leaving more Zn available for shoot translocation. *NcMTP1* KD plants were smaller in size and had reduced photosynthesis (Fv/Fm) upon excess Zn treatment (Fig. 7 A,B), suggesting that the higher Zn concentrations found in their shoot tissues rendered KD plants more stressed than WT (Fig. 8 A). We also exposed our KD and WT plants to excess Cd. The *NcHMA3* gene which is known to be involved in Cd sequestration into the vacuole (heavy metal ATPase 3; (Ueno et al., 2011), was found to be down regulated in roots of

KD plants under excess Cd (Fig. 9; Table 3). This is in agreement with the higher Cd accumulated in the roots of KD plants which were disturbed in root Cd sequestering into vacuoles probably due to reduced expression of NcMTP1. Thus our results showed that *NcMTP1* could also be instrumental in Cd uptake in the roots, considering that fact that *NcMTP1* KD plant roots accumulated a significantly lower Cd concentration compared to WT. This difference in Cd concentration was not detected in the shoot probably because the La Calamine accession we used is not a very good Cd hyperaccumulator (Assunção et al., 2008). These results confirmed a very important role of NcMTP1 in influx of excess Zn and Cd into root vacuoles in *N. caerulescens*. The involvement of NcMTP1 in Cd accumulation in *N. caerulescens* is a novel observation so far not found for other MTPs.

NcMTP1 copy number analysis indicated that there are 1-2 copies of the *NcMTP1* gene in various *N. caerulescens* accessions (Fig. 10). An increase in copy number is a known phenomenon in metal accumulation related genes, particularly for *NcHMA3*, NcHMA4, *AhHMA4* and *AhMTP1*, which allows hyperaccumulators to enhance the expression of these important metal transporters. (Ueno et al., 2011; O´Lochlainn et al., 2012; Craciun et al., 2012; Hannikenne et al., 2008; Shahzad et al., 2010). La Calamine, having two copies of *NcMTP1*, is a Zn hyperaccumulator originally from a calamine ore waste, enriched in Zn, Cd and Pb, at La Calamine, Belgium while MP, having one copy of NcMTP1, is a Ni but not Zn hyperaccumulator originated from a Ni-enriched serpentine soil at Monte Prinzera, Italy. However, Ganges (GA), Durfort and Pontaut are exceptions in the present analysis which showed only one copy of *NcMTP1*. GA is a known Zn and Cd tolerant and hyperaccumulator accession (Assunção et al., 2003b). This means in GA, the higher expression of *NcMTP1* may not be due to copy no. expansion, but probably by a differential regulation driven by cis or trans acting regulatory factors. Further expression analysis of *NcMTP1* in all these *N. caerulescens* accessions will enable to find the correlations between its copy no. and gene expression. Apart from conferring higher expression, copy number increase also provides raw material useful for adaptation and plasticity (Ohno, 1970; Zhang, 2003). This can lead to non-

functionalization, neo-functionalization or sub-functionalization of the duplicated gene (Shahzad et al., 2010). Furthermore, copy number increases also allow plants the ability to relax the transcriptional pressure on one gene to perform at its best and will make the plant less vulnerable to stress conditions or accidental mutations, especially when a gene is constantly highly expressed. Further research is needed to figure out the effect of copy number variation of *NcMTP1* on metal tolerance and accumulation traits in various *N. caerulescens* accessions.

Another important phenomenon which leads to the differential expression in genes in accumulators and no accumulators is modifications in cis- and trans- regulatory elements present on the promoters of metal responsive genes (Hanikenne et al., 2008; Assunção et al., 2010). We performed an in silico analysis of *NcMTP1* promoters from hyperaccumulators *N. caerulescens* and *A. halleri* compared to non accumulators A. *lyrata* and *A. thaliana* (Fig. 11). Interestingly, a palindromic motif P2 (AACGT) was found to be present only in the hyperaccumulator *N. caerulescens* and *A. halleri*. This palindromic sequence could be interesting for further verification to find its role in the regulation of *MTP1* gene. Two palindromic motifs were reported in the promoter of *AtZIP4* gene in *A. thaliana* (Assuncão et al., 2010). Two basic-region leucinezipper (bZIP) transcription factors *bZIP19* and *bZIP23* could bind to these motifs and regulate the expression of *AtZIP4* and downstream genes involved in the adaptation to Zn deficiency. Furthermore, MTP1 promoters of both hyperaccumulators also had a dehydration response element (DRE) which was not found in the non-accumulators. *DREB1A* and *DREB2A* transcription factors binding to the DRE elements can regulate the response to low temperature and water deficiency in *A. thaliana* (Liu et al., 1998). Abiotic stress responsive genes were reported to be higher expressed in *N. caerulescens* compared to *A. thaliana* (van de Mortel, et al., 2006). At the moment, the response of the *MTP1* gene from *N. caerulescens* and *A. halleri* to abiotic stress is unknown, however further research might solve this. There is a cross talk between metals and stress signalling pathways like oxidative stress and drought stress probably via H_2O_2 and hormones like ABA, which might

produce signals about the cellular homeostasis (Polle and Schützendübel, 2003). Combination of various abiotic stresses can have antagonistic responses in plants (Mittler, 2006). As plants need resources like Zn, Fe, Cu etc in order to cope with various forms of stresses by production of antioxidants, it is plausible that plants produce a harmonized response of maintaining resources while dealing with abiotic stress conditions. The role of Zn uptake, for instance via MTP1, might play a role in this scenario. B3 sequence is highly similar between *A. thaliana*, *A. halleri*, and *A. lyrata*, the differences inside the sequence are the product of internal deletions mainly in *A. halleri*. *A. halleri, A. thaliana* and *A. lyrata* contain a metal response element b (MREb) inside the MTP1 coding sequence. MREb was also present in *N. caerulescens*, in the same position, but with one nucleotide different. Metal-responsive transcription factor 1 (MTF-1), is known to bind to metal responsive elements (MRE) in the promoter of metallothionein gene, which are cysteine rich proteins that can detoxify Zn and Cd (Zhang et al., 2003). The authors suggested that Zn can regulate MTF-1 expression upon heavy metal stress, thus a role of Zn transporter MTP1 in this regulation is plausible but needs future verification. In conclusion, this analysis revealed the presence of interesting cis regulatory elements which to be mutated to find their role in MTP1 regulation.

A major objective in studying metal hyperaccumulators is to identify the mechanisms that confer metal tolerance and accumulation to these species. Constitutively elevated expression of MTP1-like genes was reported to occur in the metal hyper- accumulators species *N. caerulescens, N. 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, our observations for enhanced Zn and Cd tolerance exhibited by *NcMTP1* expression point out that vacuolar sequestration of Zn and Cd results in tolerance and accumulation and Fe deficiency responsive machinery plays a role in indirect Zn and Cd accumulation. This vacuolar sequestration phenomenon was reported to be a

key mechanism in metal tolerance in *N. caerulescens* and *NcMTP1* is most probably involved in exactly this function (Küpper et al., 1999; Küpper et al., 2000). A novel observation for the function of *NcMTP1* was that the expression also has an effect on Cd tolerance and accumulation and resistance to low Fe nutrition when expressed in *A. thaliana*. All these observations lead us to propose that NcMTP1 is involved in Zn and Cd hypertolerance in shoot tissues. Furthermore, we think that down regulation of *NcMTP1* gene in roots plays a role in long distance Zn translocation into shoots and Cd transport in root tissues. The *NcMTP1* RNAi data proposed the role of *NcMTP1* in Zn and Cd sequestration into vacuoles of roots cells under exposure conditions, which would save the roots from the drastic effects of these metals. All our observations make *NcMTP1* a candidate gene that could be useful to further understand the metal physiology and possibly its use can enhance the phytostabilization and/ or phytoextraction potential of high biomass plant species useful for phytoremediation.

ACKNOWLEDGEMENTS

This research was supported by the Higher Education Commission of Pakistan (HEC), and the EU-METALHOME project (HPRN-CT-2002-00243) (ST, MA). The vector pEZR (H)-LN was kindly provided by Dr. Gert de Boer, formerly at the Ehrhardt laboratory, Dept. of Plant Biology, Carnegie Institution of Washington, USA. We thank Dr. Bert van der Zaal for providing the seeds of the *pro35S::AtMTP1* line and the C24 WT and Corrie Hanhart for help in growing plant material. We are thankful to Pádraic Flood and Jeremy Harbinson for their help in chlorophyll fluorescence measurements and to Mohamed El Soda for help in statistical analysis. We are grateful to Prof. Dr. Maarten Koornneef for critical reading and suggestions for the manuscript preparation.

Table 4: Primers used for q-PCR analysis.

Primer	Primer Sequence (5'-3')
Forward primer for *AtUBP6*	GAAAGTGGATTACCCGCTG
Reverse primer for *AtUBP6*	CTCTAAGTTTCTGGCGAGGAG
Forward primer for *AtIRT1*	AAGCTTTGATCACGGTTGG
Reverse primer for *AtIRT1*	TTAGGTCCCATGAACTCCG
Forward primer for *AtIRT2*	ATGGCTACTACCAAGCTCGTC
Reverse primer for *AtIRT2*	CTAGACCGGACATCATAGCG
Forward primer for *AtFRO2*	CTTGGTCATCTCCGTGAGC
Reverse primer for *AtFRO2*	AAGATGTTGGAGATGGACGG
Forward primer for *AtBHLH100*	AAGTCAGAGGAAGGGGTTACA
Reverse primer for *AtBHLH100*	GATGCATAGAGTAAAAGAGTCGCT
Forward primer for *AtFRD3*	CGAGTTGCATCTCTTCTTCCT
Reverse primer for *AtFRD3*	TGATAACGGTCTCTCGAACA
Forward primer for *AtZIP4*	GATCTTCGTCGATGTTCTTTGG
Forward primer for *AtZIP4*	TGAGAGGTATGGCTACACCAGCAGC
Forward primer for *AtYSL3*	GAATTGAGAGACTAGTTTATTC
Reverse primer for *AtYSL3*	CGAGTTTTTACTTTTTGTGTAGCG
Forward primer for *AtNRAMP3*	ACAATGGGAGTCTCATTCGC
Reverse primer for *AtNRAMP3*	ATGCAACCCACAACTCCAAC
Forward primer for *AtHMA4*	ATGGCGTTACAAAACAAAG
Reverse primer for *AtHMA4*	GAGATTTGGTTTTACTGCTCTGAGC
Forward primer for *NcTubulin*	ACTTGGTCCCTTACCCGAGAATCC
Reverse primer for *NcTubulin*	CATGGAAGCTGGCTCGAAAGC
Forward primer for *NcMTP1*	CAAAGCTCGAAAAGGGTTTGCTCG
Reverse primer for *NcMTP1*	GTTGAGCACCATATCTGCATCTGC
Forward primer for *NcIRT1*	GTACCATTAAAGGACTCATCGCAGC
Reverse primer for *NcIRT1*	AGGATGCAACCGCCAAGACC
Forward primer for *NcHMA3*	ATGTCTCAAGGGCCTACAATGATGG
Reverse primer for *NcHMA3*	CCCTGAGATCCCCATTGAAATACC
Forward primer for *NcNRAMP3*	TGTCACGGCTCGAGAACGATCG
Reverse primer for *NcNRAMP3*	GATCCCTCTCGTTGTCGTCTTCG
Forward primer for *NcZNT1*	ATCCTCTGTGATGCTGGCGAATC
Reverse primer for *NcZNT1*	AAGGCTTTAGCAGCTACAAAGAGATTTCC
Forward primer for *NcZNT2*	GTTGCTGCTTTGATCACTC
Reverse primer for *NcZNT2*	CTCATCGTTCCTTTCTTCC

CHAPTER 4

Separate and combined expression of NcZNT1 and NcMTP1 metal transporters improves the Zn and Cd phytoremediation potential of *Nicotiana tabacum*

Zeshan Hassan[1], Ann Cuypers[2], Henk Schat[3], Jaco Vangronsveld[2] and Mark G.M. Aarts[1]

[1]Laboratory of Genetics, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands

[2]Hasselt University, Environmental Biology, Agoralaan Building D, 3590, Diepenbeek, Belgium

[3]Ecology and Physiology of Plants, Faculty of Biology, Free University, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

ABSTRACT

- The present study aimed at the functional analysis of expressing the *NcZNT1* and *NcMTP1* zinc transporter genes from the metal hyperaccumulator *Noccaea caerulescens* in *Nicotiana tabacum* cultivar SR1 to enhance the metal tolerance and accumulation potential of this high biomass crop species for phytoremediation purposes.
- Functional analysis of *NcZNT1* and *NcMTP1* was performed by expressing single *pro35S::NcZNT1, pro35S::NcMTP1* and double *pro35S::NcZNT1* + *pro35S::NcMTP1* constructs in *N. tabacum*. Metal accumulation, photosynthetic capacity, cellular redox state and lipid peroxidation of the transgenic lines were compared to wild-type line to assess the enhanced metal tolerance and accumulation.
- Single *pro35S::NcZNT1, pro35S::NcMTP1* and double *pro35S::NcZNT1* + *pro35S::NcMTP1 N. tabacum* lines displayed enhanced Zn and Cd excess tolerance. These lines also exhibited higher Zn and Cd accumulation in both treatments. Double transgenic lines had even higher Zn and Cd accumulation compared to single transgenic and wild-type lines. Chlorophyll fluorescence and redox enzyme measurements showed that transgenic lines were better protected against Zn and Cd excess. These results proposed that both *NcZNT1* and *NcMTP1* are involved in Zn and Cd tolerance and accumulation and it is possible to enhance the metal tolerance and accumulation potential of high biomass *N. tabacum*, potentially useful for phytoremediation purposes.

INTRODUCTION

Various metals are essential for the plants as micronutrients, such as Zn, Fe, Cu, Mn and Ni, but they become toxic when present in elevated concentrations in the soil (Epstein and Bloom, 2005). Toxicity caused by excess Zn in the cells is due to binding of the cations to inappropriate sites in proteins or co-factors (Eide, 2003). Zn toxicity in plants is characterized by chlorosis in young leaves, most likely via competition with Fe and Mg, resulting in growth reduction (Marschner, 1995). Therefore, the essential but also potentially toxic nature of Zn necessitates precise control mechanisms in plants. The optimal Zn concentration in cells is controlled in a process called Zn homeostasis, by which plants balance Zn uptake, intracellular compartmentalization and partitioning to the various organs (Clemens et al., 2002). Cadmium (Cd) is not an essential element, but an important environmental pollutant and a potent toxicant in plants. It does not have a known biological function in plants but it can enter the plants via Fe, Ca and Zn uptake transporters (Zhu et al., 2012; Pence et al., 2000). Its toxicity damages the DNA repair machinery, disturbs nutrient uptake and causes a reduction in photosynthesis in plants (Banerjee and Flores-Rozas, 2005; Sanita di Toppi and Gabbrielli, 1999). Kahle (1993), reported chlorosis, root tip browning, growth inhibition and ultimately death of the plants due to Cd toxicity. Cd has a very high affinity for sulfhydryl (thiol) groups and through binding with these functional groups of e.g. enzymes or structural proteins it causes metabolic disruptions (Sharma & Dietz, 2009; Vangronsveld and Clijsters, 1994). Many micronutrients are cations and are essential components of metalloproteins, as cofactor in the enzymatic catalysis and multiple other cellular processes (Cuypers et al., 2009). As a bivalent cation, Cd can either replace elements that are essential components of metalloproteins (as cofactor in the enzymatic catalysis and multiple other cellular processes (Cuypers et al., 2009) or interfere with their uptake and in this way exert its toxic action.

It is known for some time that Zn and Cd ions can inactivate the PSII reaction centre or even seize the flow of electrons in water splitting site in isolated thylakoid membranes (Molins et a., 2012; Kojima et al. 1987; Prasad et al., 1991; Singh et al., 1991). Another important underlying mechanism of Zn

and Cd induced damage is oxidative stress, a process in which the cellular redox balance between pro- and antioxidants is disturbed in favor of the former. Furthermore, Zn can initiate the enhanced production of Reactive Oxygen Species (ROS), which are known cellular damaging species in plants (Cuypers et al 2011; Smeets et al., 2005; Cuypers et al., 1999). Although Cd is not redox-active and therefore unable to directly induce reactive oxygen species (ROS) production, multiple studies report elevated ROS levels when organisms are exposed to Cd (Cuypers et al., 2009). In order to deal with this Zn and Cd induced oxidative damage, plants have evolved antioxidant defense systems (Foyer and Noctor, 2005; Mittler et al., 2004). Antioxidants like peroxidases (POD), superoxide dismutases (SOD), and catalases (CAT) helps the plants in keeping the cellular redox balance (Cuypers et al., 2011; Cuypers et al., 2002). Peroxidases are known for scavenging H_2O_2 and organic peroxides produced under metal stress and the role of cell wall localized peroxidases in lignification is known (Cuypers et al., 2002). Cell expansion is reduced by lignification which is a known mechanism to cope with metals stress (Smeets et al., 2005). Zn and Cd stress could also cause lipid peroxidation, which is an indication of plasma membrane damage. This response is known to be due to ROS production (Cuypers et al 2011). Thus by determining chlorophyll fluorescence (PSII efficiency), redox enzymes activities, and lipid peroxidation, we might understand the biochemical responses of Zn and Cd stress in plants.

Soil contamination with metals is an environmental problem throughout the world, as these elements can be toxic even in low concentrations (Rascio and Navari-Izzo, 2011) and can persist in soils for centuries (Voglar and Lestan, 2012). Conventional methods of remediation like leaching, solidification, vitrification, electrokinetical treatment, chemical oxidation or reduction, excavation and off-side treatment are expensive, disturb soil structure and fertility and are limited to relatively small areas (Baker et al., 1994; McGrath et al., 1997; Luo et al., 2000; Zhao et al., 2000). For quite some time, use of plants to contain or even clean these contaminated soils has been proposed as a cheap and environmental friendly alternative (Brooks et al., 1998; Lahner et al., 2003; McGrath and Zhao, 2003). The term phytoremediation denotes using plants to remove or to contain environmental pollutants and has been used as a

potential treatment method of various metal contaminated sites worldwide (Salt et al., 1995). There are numerous plant species found in nature that have evolved metal tolerance and accumulation properties, which can result in an over 100 fold higher metal concentration in their above ground tissues than non-accumulators (Chaney, 1997; Reeves and Baker, 2000). Currently, the application of metal phytoremediation is limited to the use of natural metal hyperaccumulators like *Berkheya coddii* (Ni), *Alyssum bertolonii* (Ni) and *Noccaea caerulescens* (Zn/Cd). *N. caerulescens* is one of the most important metal hyperaccumulators known by time, as it can accumulate about 30,000 mg Zn kg^{-1} dry weight (DW) in its shoots (Baker *et al*., 1994; Baker *et al*., 2000). However, under control conditions, there is a significant difference of Zn hyperaccumulation between different populations of this plant (Baker *et al*., 1994; Meerts and Van Isacker, 1997; Lombi *et al*., 2000; Assuncão *et al*., 2003b; Roosens *et al*., 2003). It belongs to the Brassicaceae family with about 88.5% coding region sequence homology with *A. thaliana* (Rigola et al., 2006). Apart from Zn hyperaccumulation, some populations of this plant species also hyperaccumulate Cd and together with *Arabidopsis halleri*, *Noccaea praecox* and *Sedum alfredii* (Bert et al., 2002; Vogel-Mikus et al., 2005; Xiong et al., 2004) it is one of the few known Cd hyperaccumulator species. Self-fertility, sufficient seed setting, high natural genetic variation, and its close relatedness to *A. thaliana* are some of the many characteristics of this metal hyperaccumulator, which make it a suitable model plant to understand the molecular mechanisms involved in metal tolerance and accumulation (Peer *et al*., 2006; Assuncão *et al*., 2003a).

Low biomass production and slow growth are the major limiting factors in the use of natural metal hyperaccumulator plants for phytoremediation purposes. Due to the much lower biomass production of hyperaccumulator species in comparison with non-accumulators ones, there is no economic added value. Moreover, many generations of growing a hyperaccumulator species are needed and in fact many years to remediate a contaminated soil (Chaney et al., 2005). Therefore, we might need some alternative technology to perform efficient phytoremediation. Genetic modification of plants can help us to get rid of these constraints in two different

ways. A first is to improve the growth and biomass production of known metal hyper-accumulators by genetic engineering. The second and most interesting alternative is to genetically equip a high biomass producing plant species with metal tolerance and accumulation related genes. By understanding the molecular mechanisms involved in metal tolerance and hyperaccumulation, we may solve this problem. Genes responsible for these properties could be transferred into a high biomass producing plant species like for instance *N. tabacum*, thus reducing the number of croppings needed to remediate a particular area. *N. tabacum* is a moderately metal tolerant species compared to many non-accumulators and has many advantages that make it a suitable plant species useful for phytoextraction i.e. a high biomass production and fast growth rate, modest nutrient requirements and easy to harvest, with several cropping systems developed (Sarret et al., 2006). By further enhancing its metal tolerance and accumulation potential, we might use it for field phytoremediation purposes.

Proteins belonging to the ZRT-, IRT- like (ZIP) family play an important role in metal uptake within a plant (Fox and Guerinot, 1998). In *N. caerulescens, NcZNT1* was the first Zn transporter gene identified by using functional complementation of a yeast Zn uptake defective mutant and was reported to mediate high affinity Zn uptake and low affinity Cd uptake (Pence et al., 2000). We have found that *NcZNT1* localizes to plasma membrane when expressed in cowpea protoplasts (Chapter 2). Expression of *AtZIP4*, orthologue of *NcNT1* from *A. thaliana*, is not in the root epidermis, which would be expected for an uptake transporter, but instead in the root endodermis and pericycle (Milner and Kochian, 2008; chapter 3). The same was seen for the *NcZNT1* gene (chapter 3), suggesting that enhancing endodermis-specific metal uptake by tissue specific expression of metal uptake transporters would be the target for modification of Zn/Cd/Ni root uptake capacity. *NcZNT1* was observed to be highly expressed in roots and at lower level in shoots under normal and elevated as well as Zn deficient conditions (Assunção et al., 2001; Van de Mortel, 2006). We have demonstrated that expression of *pro35S::NcZNT1* confers higher Zn and Cd accumulation when expressed in *A. thaliana* (Chapter 2). It was thus concluded that this gene is involved in keeping higher metal

concentrations in the pericycle in *N. caerulescens* roots, which ensures the availability of these metals for long distance transport via xylem loading, as performed by HMA4/HMA2 proteins. The *NcZNT1* gene can be exploited as a tool to enhance Zn and Cd accumulation in plants useful for phytoremediation.

Members of the CDF protein family are the transporters involved in the sequestration of metals into vacuoles. CDF family members, like *AtMTP1* and *NgMTP1*, were shown to cause increased Zn tolerance and accumulation when ectopically or heterologously expressed in *A. thaliana* (van der Zaal et al., 1999; Gustin et al., 2009), 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 or to create a Zn reservoir to moderate the effects of Zn deficiency. *MTP1*-like genes have previously been reported to be higher expressed in *N. caerulescens, N. goesingense* and *A. halleri*, compared with non-accumulators *Thlaspi arvense, A. thaliana* and *Brassica juncea* (Assunção et al., 2001; Persans et al., 2001; Dräger et al., 2004). *NcMTP1* is a prominent member of the CDF gene family in *N. caerulescens*. It is orthologous to *AtMTP1* of *A. thaliana* (van der Zaal et al., 1999) but showing constitutively higher expression when compared to the non-hyperaccumulator *T. arvense* (Assunção et al., 2001). This indicates the involvement of *NcMTP1* expression in Zn hypertolerance and hyperaccumulation. Previously we have illustrated that a *pro35S::NcMTP1* construct enhances both Zn and Cd tolerance in *A. thaliana* (Chapter 3). *NcMTP1* is involved in cation influx into vacuole thus conferring Zn and Cd tolerance and the expression of this gene is a useful tool to modulate vacuolar cation sequestration supporting phytoremediation.

The present study was aimed at analyzing the applicability of *NcMTP1* and *NcZNT1* expression in Zn and Cd tolerance and accumulation in *N. tabacum* and thereby enhancing the phytoremediation potential of this high biomass species by the combined constitutive expression of both of these genes. The newly acquired properties could be useful for phytoremediation purposes.

MATERIALS AND METHODS

Development of binary constructs

The cloning of *pro35S::NcZNT1* is described in Chapter 2 while *pro35S::NcMTP1* as described in Chapter 3, was used as expression construct.

Plant transformation and growth conditions

pro35S::NcZNT1 and *pro35S::NcMTP1* constructs were transformed separately into *N. tabacum*, wild-type (WT) accession Streptomycin Resistance1 (SR1), by the *Agrobacterium tumefaciens* mediated leaf disc method as described by Horsch et al. (1985). T_0 *pro35S::NcZNT1* transformed seedlings were selected on ½ MS agar plates (Murashige and Skoog, 1962) supplemented with 50 mg L^{-1} kanamycin while, (Duchefa Biochemie, Haarlem, The Netherlands) 20 mg L^{-1} hygromycin B was used to select *pro35S::NcMTP1* transformed T_0 seedlings. 50 independently transformed plants were tested for *NcZNT1* and *NcMTP1* expression by semi-quantitative RT-PCR (data not shown). Three lines with the highest transgene expression for both constructs were used for experimentation. Plants were cultured in on ½ MS agar plates (Murashige and Skoog, 1962) supplemented with 20 g L^{-1} sugar, pH 5.8, incubated in a climate-controlled growth cabinet (25°C 16/8 hr, light/darkness with illumination at a light intensity of 120 µmol m^{-2} s^{-1}).

In order to obtain double transgenic *N. tabacum* plants expressing both *pro35S::NcZNT1* and *pro35S::NcMTP1* constructs, the *pro35S::NcMTP1* line with the highest *NcMTP1* expression (based on semi-quantitative RT-PCR; data not shown) was further transformed with the pro35S::*NcZNT1* as described above. All T_0 transgenic and WT lines were multiplied by tissue culture to generate sufficient plant material for experimentation. This method had advantages of keeping T_0 lines without growing them to get homozygous lines to save time and to avoid gene silencing.

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

Leaves of 10 independently transformed *pro35S::NcZNT1*, *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* and WT Tobacco T_0 plants were used to isolate total RNA by Trizol (Invitrogen, Carlsbad, CA, USA) as described by the

manufacturers protocol. In order to check the quality and quantity, total RNA was run on agarose gel electrophoresis and measured spectrophotometrically. RNA was treated with DNAse to get rid of any DNA molecules (MBI Fermentas, St. Leon-Rot, Germany). cDNA was synthesized from 1 µg of total RNA using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo (dT) primer (Invitrogen, Carlsbad, CA, USA). PCR was performed on cDNA's using Tubulin (EU938079) gene with forward primer 5'-ATGGCAGACGGTGAGGATATTCA-3' and reverse primer 5'-GCCTTTGCAATCCACATCTGTTG-3' used as a constitutively expressed control gene. *NcZNT1* was amplified by using forward primer 5'-ATCCTCTGTGATGCTGGCGAATC-3' and reverse primer 5'-AAGGCTTTAGCAGCTACAAAGAGATTTCC-3'. *NcMTP1* was amplified by forward primer 5'-CCCAAGCTTACCCAAAAAAAGAGATCGAATT-3' and reverse primer 5'-CTTTGTCGACCGCTCGATTTGTACGGTTACA-3'. PCR primers were designed spanning an intron to differentiate between amplifications from genomic DNA and cDNA. Three plants per *pro35S::NcZNT1, pro35S::NcMTP1 and pro35S::NcZNT1 + pro35S::NcMTP1* genotypes with highest level of transgenes expression were chosen for further experiments.

Metal exposure

To determine the metal tolerance and accumulation of three independently transformed *pro35S::NcZNT1, pro35S::NcMTP1 and pro35S::NcZNT1 + pro35S::NcMTP1 N. tabacum* lines, nine plants for each line and one control *N. tabacum* wild-type (WT) line were grown hydroponically in modified ¼ strength Hoagland's nutrient solution containing 2 µM $ZnSO_4$ (sufficient Zn) and 100 µM $ZnSO_4$ (excess Zn) in three separate experiments performed at three different time points. In the first experiment, three independently transformed *pro35S::NcZNT1* lines and one WT line were grown in the above given treatment for five weeks. In the second experiment, three independently transformed *pro35S::NcMTP1* lines and one WT line were grown for five weeks and in the third experiment, one of the most Zn excess tolerant *pro35S::NcZNT1* and *pro35S::NcMTP1* lines, three independently transformed *pro35S::NcZNT1 +*

pro35S::NcMTP1 lines and WT lines were grown in above mentioned treatments for about 4.5 weeks. For each treatment, the transgenic and control lines were grown in pots containing 700 mL nutrient solution. 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 the first week, plants were grown in 2 µM $ZnSO_4$ (sufficient Zn) and for the rest of the period, they were exposed to excess Zn. same no of transgenic and WT lines were kept in sufficient Zn for the whole experiments as controls. The nutrient medium was refreshed every week. Each hydroponics experiment was repeated twice at different time points keeping all the growth conditions the same.

To determine the response of *pro35S::NcZNT1, pro35S::NcMTP1 and pro35S::NcZNT1 + pro35S::NcMTP1 N. tabacum* lines to excess Cd, the transgenic and WT lines were grown hydroponically on modified ¼ Hoagland's solution containing excess Cd in three independent experiments performed at three different time points. In the first experiment, three independently transformed *pro35S::NcZNT1* lines and one WT line were tested in 8 µM $CdSO_4$ (excess Cd) for five weeks. In the second experiment, three independently transformed *pro35S::NcMTP1* lines and one WT line were tested in 8 µM $CdSO_4$ (excess Cd) for five weeks. In the final experiment, above mentioned *pro35S::NcZNT1, pro35S::NcMTP1* and *pro35S::NcZNT1 + pro35S::NcMTP1* lines and one WT line were tested in 5 µM $CdSO_4$ (excess Cd) for 4.5 weeks, because in a preliminary experiment, it was found that 5 µM Cd treatment displayed a more differential tolerance phenotypes among the transgenic lines than 8 µM Cd treatment. For each treatment, the transgenic and control lines were grown in pots containing about 700 mL nutrient solution. 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 the first week, plants were grown in 2 µM $ZnSO_4$ (sufficient Zn) and for the rest of the period in the given excess Cd treatments. Same no of transgenic and WT lines were kept in sufficient Zn for the whole experiments as controls. The nutrient medium was refreshed every week. Each hydroponics experiment was repeated twice at different time point keeping all the growth conditions the same.

Phenotypic analysis

Fresh, first fully expanded leaves of all *N. tabacum* lines were dark adapted for 20 minutes and chlorophyll fluorescence was measured by using a FluoroCam 700MF, as described by Baker (2008). Dried root and shoot tissues were analysed spectroscopically for Zn, Cd, Fe and Mn minerals at the end of the experiments as described by Assunção et al. (2003b). Frozen leaf and root samples were homogenized and thiobarbituric acid reactive metabolites (TBA) and enzymes activities were measured separately as described by Cuypers et al. (2011).

Statistical analysis

Where needed, data were analysed for significance at $p<0.05$ by using two-way ANOVA and ANOVA (Least Significance Difference) in the SPSS v. 12 software package for MS Windows.

RESULTS

Single and the combined expression of *NcZNT1* and *NcMTP1* conferred Zn tolerance and accumulation in *N. tabacum*

To investigate Zn tolerance and accumulation, a set of three independently transformed *pro35S::NcZNT1* lines and one WT line (Fig. 1) and a set of three independently transformed *pro35S::NcMTP1* lines and one WT line (Fig. 2) and one *pro35S::NcZNT1* line, one *pro35S::NcMTP1* line and three independently transformed *pro35S::NcZNT1+pro35S::NcMTP1* lines together with the WT line (Fig. 3) were exposed to excess Zn (100 µM $ZnSO_4$) and also to sufficient Zn (2 µM $ZnSO_4$) as control.. These lines were chosen on the basis of highest transgenes expression (data not shown). All lines displayed similar healthier phenotype when grown in sufficient Zn condition (data not shown). Double transgenic lined had lower Zn accumulation in shoots than rest of the lines while both *pro35S::NcZNT1 and pro35S::NcZNT1+pro35S::NcMTP1* lines had higher root Zn concentrations compared to *pro35S::NcMTP1* and WT lines (data not shown). In case of excess Zn grown conditions, ll the transgenic lines were greener and larger in size whereas WT lines had more chlorosis and were

smaller in size (Fig. 1 A; Fig. 2 A: Fig 3 A). The *pro35S::NcMTP1* and double transgenic lines were greener than *pro35S::NcZNT1* and WT lines while *pro35S::NZNT1* showed less chlorosis in leaves compared to WT but higher than the double transgenic lines and hence has an intermediate Zn tolerance phenotype (Fig. 3 A). Zn tolerance phenotype in the double transgenic lines was because of the *pro35S::NcMTP1* background as it was as much greener as was the *pro35S::NcMTP1* line. Shoot and root dry weights (DW) of all the transgenic lines were significantly higher than their respective WT lines (Fig. 1 B, C; Fig. 2 B, C: 3 B, C). The *pro35S::NcMTP1* and double transgenic lines exhibited the highest dry biomass while *pro35S::NcZNT1* displayed a phenotype intermediate between WT and the other transgenic lines (Fig. 3 B, C). In general, the phenotype and biomass data showed that all transgenic lines were more tolerant to Zn excess as compared to WT. The Zn concentration in the shoot tissues of all the transgenic lines grown on Zn excess was significantly higher as compared to WT (Fig. 1 D; Fig. 2 D; 3 D). The *pro35S::NcZNT1* and double transgenic lines possessed higher shoot Zn accumulation than *pro35S::NcMTP1.* Thus double transgenic lines displayed a combination of higher Zn tolerance conferred by *pro35S::NcMTP1* and higher shoot Zn accumulation conferred by *pro35S::NcZNT1.* The root Zn concentration also showed a similar trend i.e. higher root Zn concentration in transgenic lines than WT lines (Fig. 1 E; Fig. 2 E; Fig. 3 E). In this case, *pro35S::NcZNT1* exhibited the highest root Zn accumulation compared to the other transgenic lines (Fig. 3 E). On the basis of shoot and root Zn concentration and respective dry weights, total plant Zn content was estimated. Overall, all transgenic line had significantly higher total plant Zn content than the WT (Fig. 1 F; Fig. 2 F; Fig. 3 F). The ***pro35S::****NcZNT1* line had the lowest while double transgenic lines showed the highest total Zn content and the *pro35S::NcMTP1* line remained the intermediate (Fig. 3 F). These results indicated that the combination expression of *NcZNT1* and *NcMTP1* (double transgenic lines) can further enhance the Zn accumulation compared to single *NcZNT1* and *NcMTP1* expression in *N. tabacum*. Fe and Mn concentrations were found to be higher in shoots but lower in the roots of all the transgenic lines (Fig. 4 C, D).

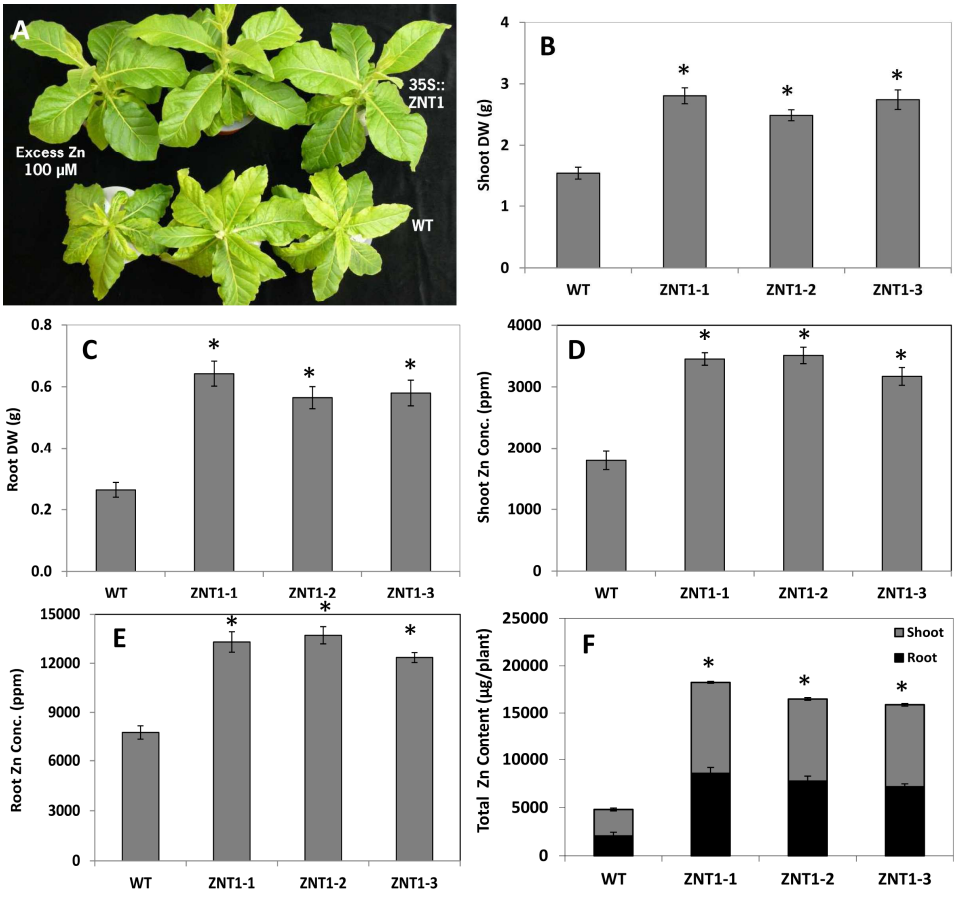

Fig. 1: Phenotypic analysis of three independent *pro35S::NcZNT1* lines (ZNT1-1, ZNT1-2, ZNT1-3) and one wild-type (WT) *N. tabacum* line exposed to 100 µM $ZnSO_4$ (excess Zn) in ¼ Hoagland's nutrient media (A) *pro35S::NcZNT1* transformed *N. tabacum* lines compared to WT. (B) Shoot (C) and root dry weight (D) Zn concentration (in ppm) in shoots (E) and in roots (F); estimated total plant Zn content (µg/plant) after growing for five weeks. The photograph was taken after 35 days since transplantation. * indicate significant difference between transgenic and WT line at $p<0.05$ in a two way ANOVA (mean ± SE of 4 replica).

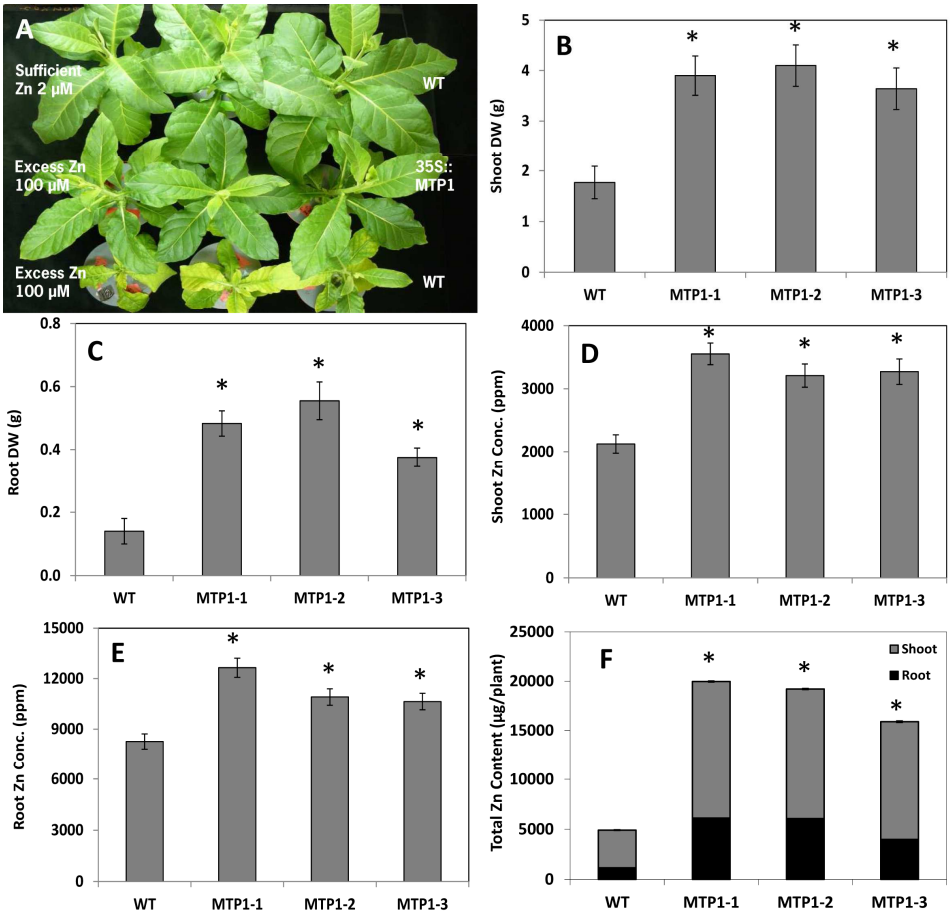

Fig. 2: Phenotypic analysis of three independent *pro35S::NcMTP1* lines (MTP1-1, MTP1-2, MTP1-3) and one wild-type (WT) *N. tabacum* line exposed to 100 µM $ZnSO_4$ (excess Zn) in ¼ Hoagland's nutrient media (A) *pro35S::NcMTP1* transformed *N. tabacum* lines compared to WT. (B) Shoot dry weight (DW) (C) and Root dry weight (D) Zn concentration in shoots (ppm) (E) and in roots (F) estimated total plant Zn content (µg/plant) after growing for five weeks. The photograph was taken after 35 days since transplantation. * indicate significant difference between transgenic and WT line at $p<0.05$ in a two way ANOVA (mean ± SE of 4 replica).

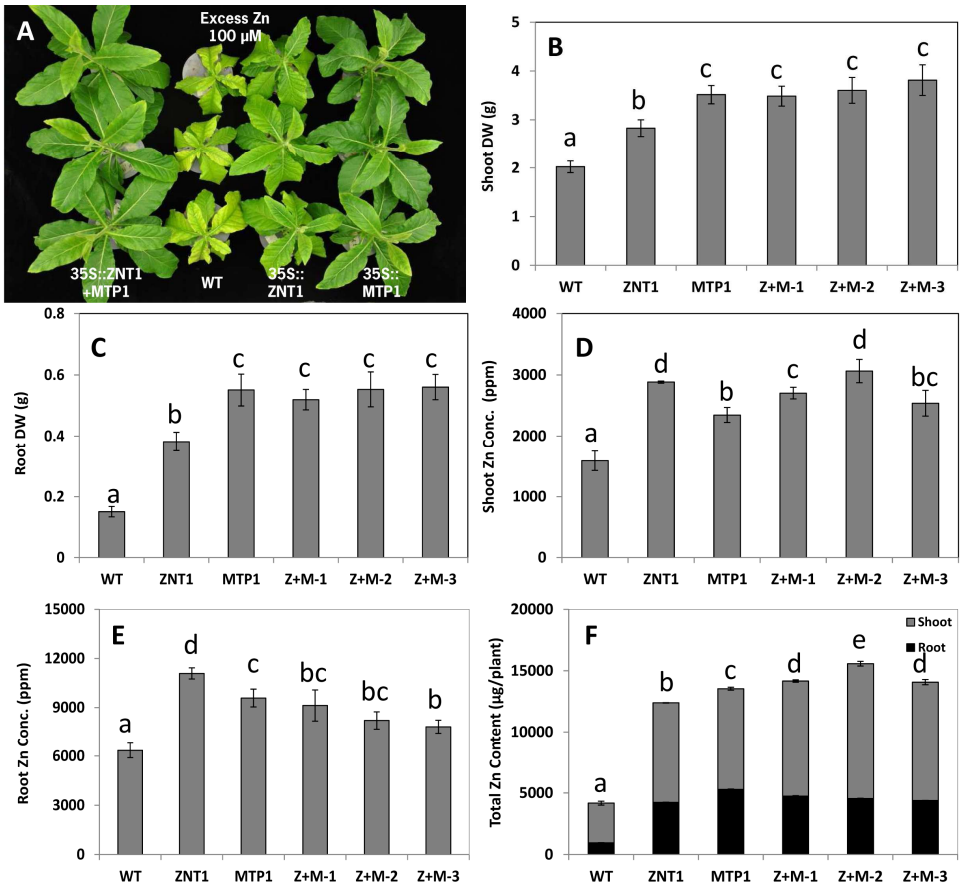

Fig. 3: Phenotypic analysis of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) and one wild-type (WT) *N. tabacum* line exposed to 100 µM $ZnSO_4$ (excess Zn) in ¼ Hoagland's nutrient media (A) *pro35S::NcZNT1* line, *pro35S::NcMTP1* line, three *pro35S::NcZNT1* + *pro35S::NcMTP1* lines compared to WT. (B) Shoot dry weight (DW) (C) and Root dry weight (D) Zn concentration in shoots (ppm) (E) and in roots (F) estimated total plant Zn content (µg/plant) after growing for 4.5 weeks. The photograph was taken after 33 days since transplantation. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significane Difference) (mean ± SE of 4 replica).

Cd tolerance and accumulation was enhanced by single and combined expression of *NcZNT1* and *NcMTP1* in *N. tabacum*

Expression of *NcZNT1* in *A. thaliana* previously led to increased Cd tolerance and accumulation. Therefore, the three independently transformed *pro35S::NcZNT1* tobacco lines and one WT line (Fig. 5) and the three independently transformed *pro35S::NcMTP1* lines and one WT line (Fig. 6) which were exposed to excess Zn, were also exposed to excess Cd (8 μM $CdSO_4$) in two separate experiments. In the third experiment, one *pro35S::NcZNT1* line, one *pro35S::NcMTP1* line and three independently transformed *pro35S::NcZNT1+pro35S::NcMTP1* lines together with WT line (Fig. 7) were exposed to excess Cd (5 μM $CdSO_4$). All transgenic lines looked greener and healthier than the WT line which showed severe chlorosis in leaves (Fig. 5 A; Fig. 6 A; Fig. 7 A). However, there was some variation in the phenotype among the transgenic lines. *pro35S::NcZNT1* and double transgenic lines looked similar in phenotype, with prominent intervenal chlorosis, while this was not seen in the *pro35S::NcMTP1* line (Fig. 7 A). This suggests that the Cd tolerance phenotype in the double transgenic lines was due to the *pro35S::NcZNT1* background, and not due to the *pro35S::NcMTP1* background. All transgenic lines had higher tolerance to excess Cd and had larger and greener shoots than the WT lines (Fig. 5 A; Fig. 6 A; Fig. 7 A). The shoot and root dry weights were significantly higher in all the transgenic lines compared to their respective WT lines (Fig. 5 C; Fig. 6 B, C; Fig. 7 B, C).

Shoot and root Cd concentrations were also higher in the *pro35S::NcZNT1* lines (Fig. 5 D, E; Fig. 7 D), but only higher in shoot of *pro35S::NcMTP1*, while roots had the same Cd concentration as the WT line (Fig. 7 D). Double transgenic lines showed even higher shoot Cd accumulation than *pro35S::NcZNT1*, which was intermediate, and *pro35S::NcMTP1*, which had the least Cd accumulation among the transgenic lines (Fig. 7 D). The root Cd concentration exhibited a different trend. Where *pro35S::NcZNT1* accumulated significantly more Cd, double transgenic lines accumulated less Cd, while *pro35S::NcMTP1* had similar root Cd accumulation compared to the WT line (Fig. 5 E; Fig. 6 E; Fig. 7 D). Double transgenic lines had same shoot Cd concentrations as in their roots, which means a higher Cd translocation to the

shoot because rest of the lines had more Cd concentrations in their roots than shoot. In shoots, Zn concentration was higher but lowered in the roots of all the transgenic lines compared to the WT (Fig. 7 E). All transgenic lines also had higher total plant Cd content than the WT line (Fig. 5 F; Fig. 6 F; Fig. 7 F). One of the double transgenic lines exhibited significantly higher total Cd accumulation than *pro35S::NcZNT1*, which was intermediate, and *pro35S::NcMTP1*, which had the lowest Cd accumulation among the transgenic lines. These analysis showed that, indeed, the single *pro35S::NcZNT1* and *pro35S::NcMTP1* and their combination (double transgenic) can further enhance the accumulation of Cd in *N. tabacum*. Fe and Mn concentrations were found to be higher in shoots but lower in roots of the transgenic lines (Fig. 8 C, D).

***NcZNT1* and *NcMTP1* expressing *N. tabacum* lines showed enhanced chlorophyll fluorescence under Zn and Cd stress**

Fv/Fm (or (Fm–Fo)/Fm, where Fm is the maximal fluorescence measured during the first saturation pulse after dark adaption and Fo is the dark adapted initial minimum fluorescence) is the measure of maximum quantum efficiency of photosystem II photochemistry (Baker, 2008l) and was determined based on dark adapted chlorophyll fluorescence. This analysis was performed to get an indication of photosynthetic capacity of *pro35S::NcZNT1, pro35S::NcMTP1 and pro35S::NcZNT1* + *pro35S::NcMTP1* and wild-type (WT) lines exposed to excess Zn (100 µM $ZnSO_4$) and Cd (5 µM $CdSO_4$). We have found Fv/Fm to be higher in all the transgenic lines compared to the WT line when exposed to excess Zn (Fig. 4 A, B). This illustrated the severe damage in the photosynthesis machinery of the WT line caused by excess Zn while transgenic lines were clearly healthier. Another noticeable point was that we can capture the slight differences in images of Fv/Fm, being qualitative data, in a better way than the quantitative data itself. Overall, this analysis showed a better chlorophyll fluorescence and confirmed the increased heavy metal tolerance of transgenic lines compared to the WT lines in conditions of excess Zn. Cd exposed transgenic lines also exhibited significantly higher Fv/Fm than the WT line (Fig. 8 A, B). WT lines had severe damage in photosystem II, however, all the transgenic lines although suffering from excess Cd, had clearly higher Fv/Fm.

***NcZNT1* and *NcMTP1* expressing *N. tabacum* lines had differential lipid peroxidation under Zn and Cd stress**

Lipid peroxidation, which reflects plasma membrane damage, was measured by determining thiobarbituric acid reactive metabolites (TBA) in *pro35S::NcZNT1, pro35S::NcMTP1, pro35S::NcZNT1+pro35S::NcMTP1* and WT lines exposed to excess Zn (100 µM $ZnSO_4$) and excess Cd (5 µM $CdSO_4$). Zn exposure significantly increased TBA content in the shoots of *pro35S::NcMTP1* and double transgenic lines but reduced in the *pro35S::NcZNT1* line (Fig. 4 E). On the other hand, root TBA content were significantly lower in the transgenic lines, where *pro35S::ZNT1* and *pro35S::NcMTP1* lines even showed significantly lower content of TBA compared to double transgenic lines (Fig. 4 F). In case of excess Cd, transgenic lines had significantly higher TBA content in shoots than the WT lines (Fig. 8 E). However, the content of TBA in the roots were found to be significantly lower in the transgenic lines, where the *pro35S::ZNT1* and double transgenic lines had even significantly lower content of TBA compared to the *pro35S::NcMTP1* line (Fig. 8 F).

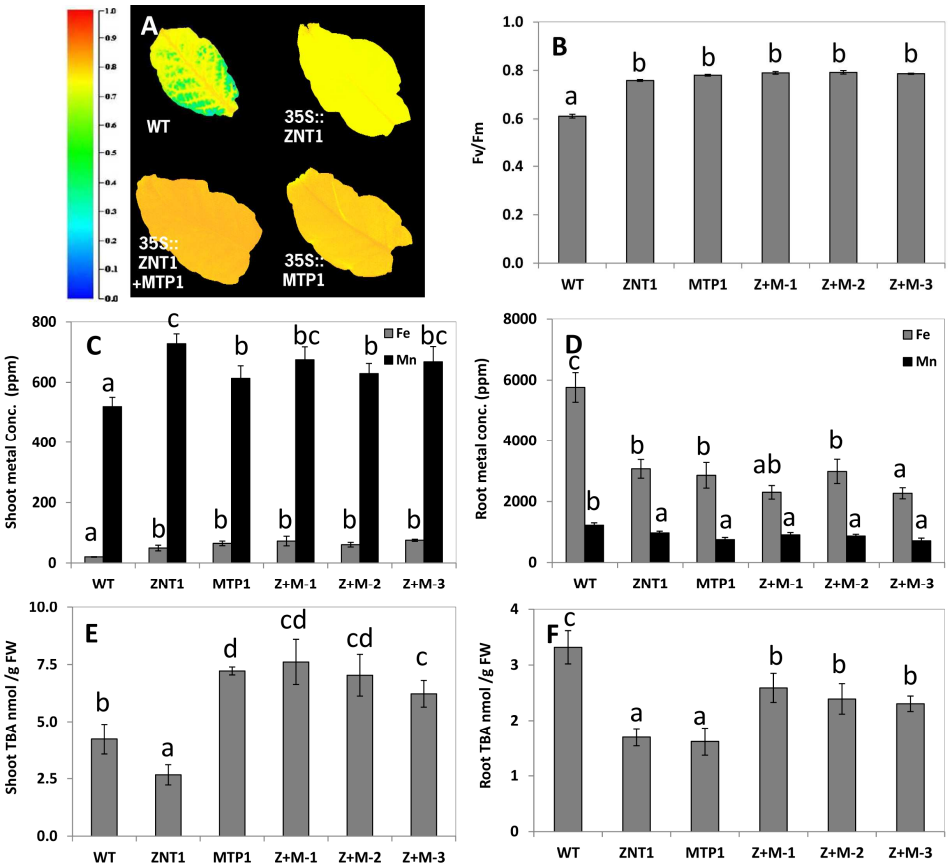

Fig. 4: Fv/Fm (Chlorophyll fluorescence), Fe and Mn concentration and Lipid peroxidation (thiobarbituric acid reactive metabolite (TBA) analysis of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown on ¼ Hoagland's media supplemented with 100 µM $ZnSO_4$ (excess Zn) (A) Chlorophyll fluorescence image of transgenic and WT lines (B) Fv/Fm of transgenic and WT lines (C) Fe and Mn concentrations in shoots (ppm) (D) and in roots (E) TBA contents in shoots (nmoles/g Fresh weight (FW)) (F) and in roots after growing for 4.5 weeks. The photograph was taken after 33 days since transplantation. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significance Difference) (mean ± SE of 4 replica).

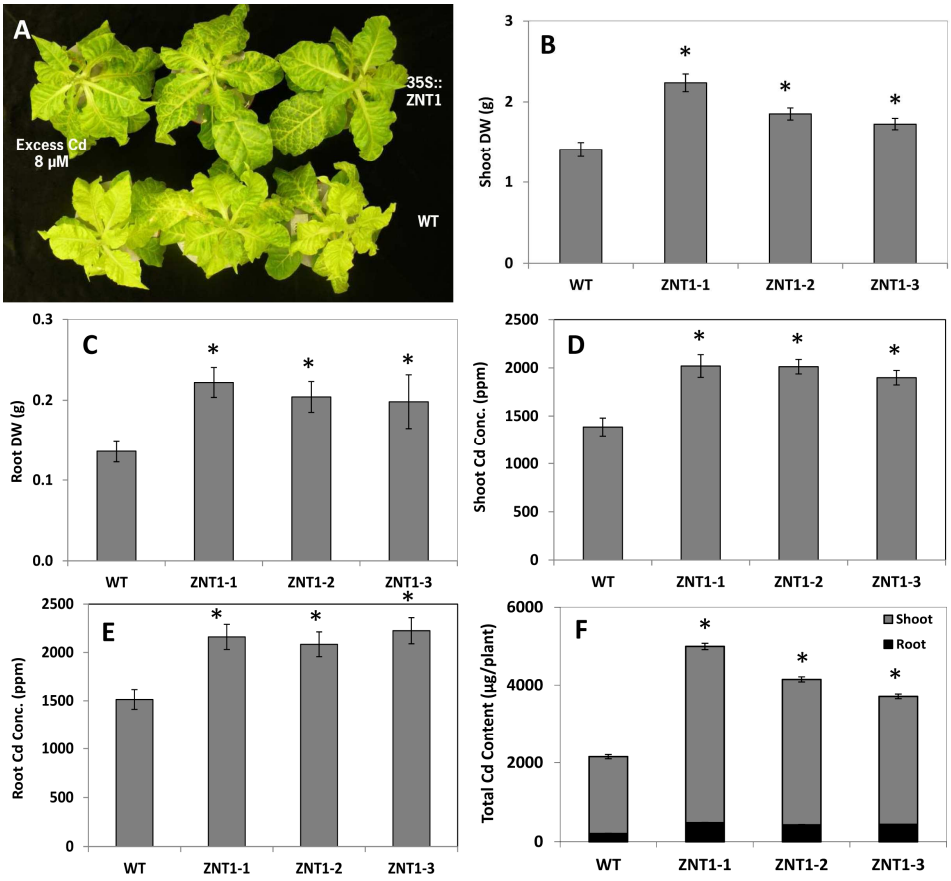

Fig. 5: Phenotypic analysis of three independent *pro35S::NcZNT1* lines (ZNT1-1, ZNT1-2, ZNT1-3) and one wild-type (WT) *N. tabacum* line exposed to 8 µM $CdSO_4$ (excess Cd) in ¼ Hoagland's nutrient media (A) *pro35S::NcZNT1* transformed *N. tabacum* lines compared to WT (B) Shoot dry weight (DW) (C) and Root dry weight (D) Cd concentration in shoots (ppm) (E) and in roots (F) estimated total plant Cd content (µg/plant) after growing for five weeks. The photograph was taken after 35 days since transplantation. * indicate significant difference between transgenic and WT line at $p<0.05$ in a two way ANOVA (mean ± SE of 4 replica).

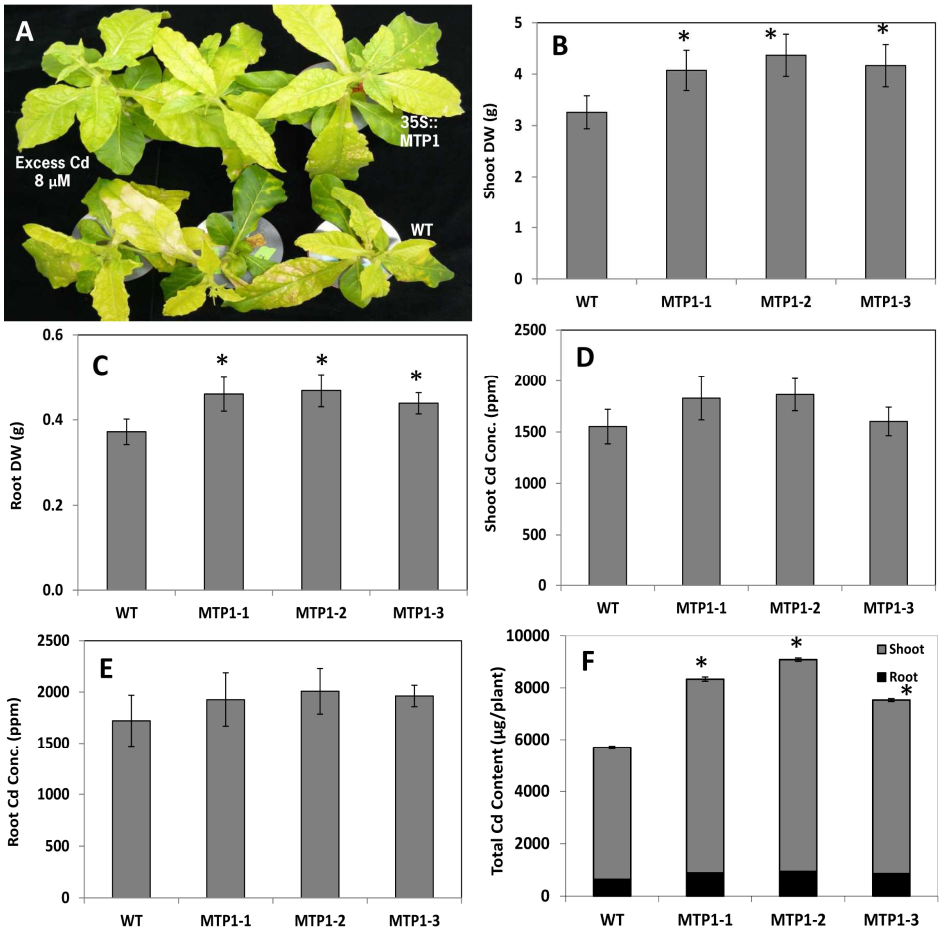

Fig. 6: Phenotypic analysis of three independent *pro35S::NcMTP1* lines (MTP1-1, MTP1-2, MTP1-3) and one wild-type (WT) *N. tabacum* line exposed to 8 µM $CdSO_4$ (excess Cd) in ¼ Hoagland's nutrient media (A) *pro35S::NcMTP1* transformed *N. tabacum* lines compared to WT. (B) Shoot dry weight (DW) (C) and Root dry weight (D) Cd concentration in shoots (ppm) (E) and in roots (F) estimated total plant Cd content (µg/plant) after growing for five weeks. The photograph was taken after 35 days since transplantation. * indicate significant difference between transgenic and WT line at $p<0.05$ in a two way ANOVA (mean ± SE of 4 replica).

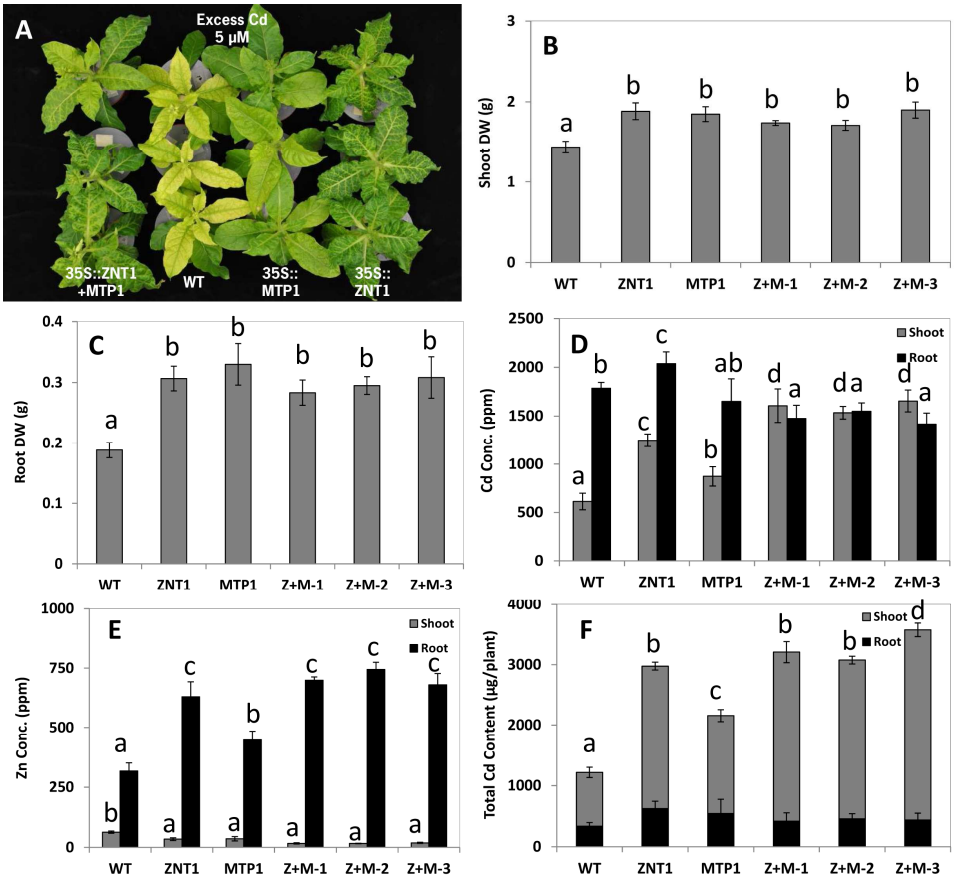

Fig. 7: Phenotypic analysis of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1+pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) and one wild-type (WT) *N. tabacum* line exposed to 5 µM $CdSO_4$ (excess Cd) in ¼ Hoagland's nutrient media (A) *pro35S::NcZNT1* line, *pro35S::NcMTP1* line, three *pro35S::NcZNT1* + *pro35S::NcMTP1* lines compared to WT grown (B) Shoot dry weight (DW) (C) and Root dry weight (D) Cd concentration in shoots and in roots (ppm) (E) Zn concentration in shoots and in roots (ppm) (F) estimated total plant Cd content (µg/plant) after growing for 4.5. The photograph was taken after 33 days since transplantation. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significance Difference) (mean ± SE of 4 replica).

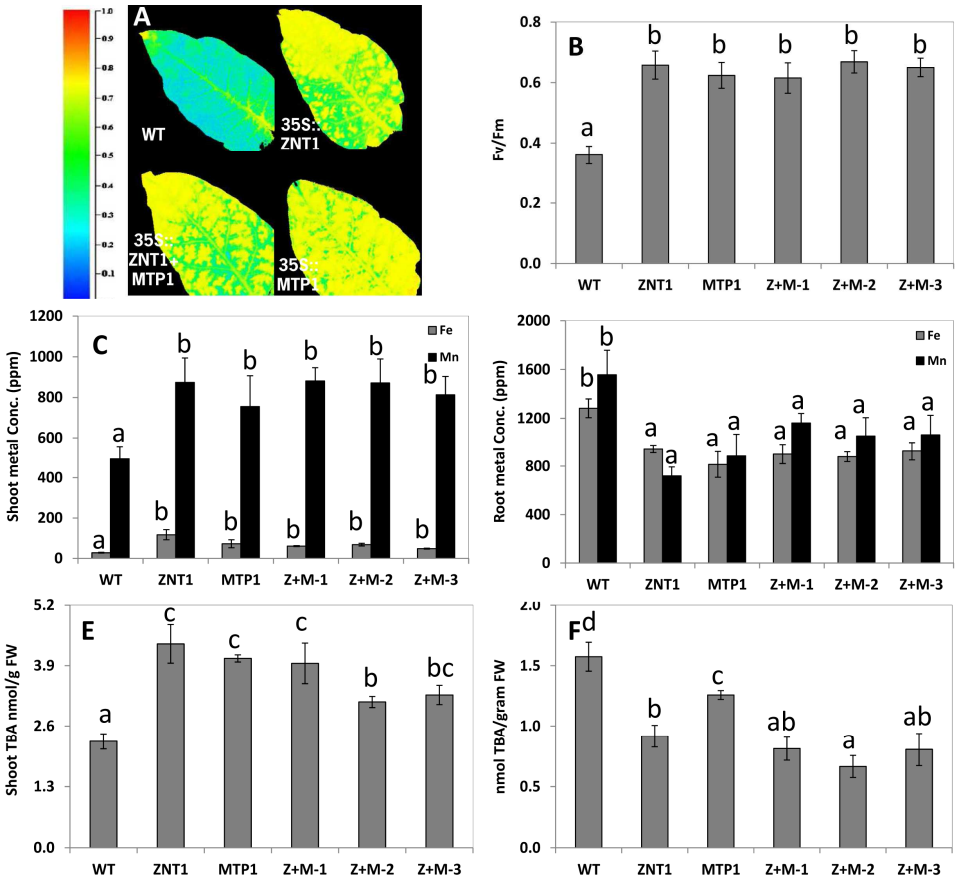

Fig. 8: Fv/Fm (Chlorophyll fluorescence), Fe and Mn concentration and Lipid peroxidation (thiobarbituric acid reactive metabolite (TBA)) measurements of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown on ¼ Hoagland's nutrient media supplemented with 5 µM $CdSO_4$ (excess Cd) (A) Chlorophyll fluorescence image of transgenic and WT lines (B) Fv/Fm of transgenic and WT lines (C) Fe and Mn concentrations in shoots (ppm) (D) and in roots (E) TBA contents in shoots (nmoles/g Fresh weight (FW)) (F) and in roots after growing for 4.5 weeks. The photograph was taken after 33 days since transplantation. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significance Difference) (mean ± SE of 4 replica).

***NcZNT1* and *NcMTP1* expressing *N. tabacum* lines showed differential cellular redox enzymes activities under Zn and Cd stress**

Enzymes involved in anti-oxidative defense such as syringaldazine peroxidase (SPOD), ascorbate peroxidase (APOD), guaiacol peroxidase (GPOD), catalases (CAT) and superoxide dismutases (SOD) were investigated in the *pro35S::NcZNT1, pro35S::NcMTP1*, *pro35S::NcZNT1+pro35S::NcMTP1* and WT lines exposed to excess Zn (100 µM $ZnSO_4$) and Cd (5 µM $CdSO_4$). Significantly lower activities of SPOD, GPOD, CAT and SOD than the WT line were found in roots and shoots of all transgenic lines upon Zn exposure conditions, with an exception of root SOD, which were the same (Fig. 9 A-D, G-J). The lowered shoot and root SOD and CAT activities indicate better scavenging systems for free Zn than in the WT line. All transgenic lines exhibited significantly higher root APOD levels but significantly lower in shoots compared to the WT line (Fig. 9 E, F). All these data suggest that in the transgenic lines the antioxidant system was either shut down, as a consequence of severe to Zn toxicity, or was not required; the latter hypothesis being more plausible since the transgenic lines showed fewer signs of Zn toxicity than WT (Fig. 3 A).

Cd exposure led to the increased shoot SPOD and GPOD levels in the transgenic lines, and to lower levels in roots (Fig. 10 A, D). This showed that the transgenic lines responded differently to excess Zn compared to excess Cd. APOD levels were also significantly lower in roots of the transgenic lines (Fig. 10 E, F). Shoot and root SOD activities were significantly lower in the transgenic lines suggesting better scavenging of free Cd than in the WT line (Fig. 10 G, H). CAT levels were similar in all the lines and only significantly lower in roots of the *pro35S::NcMTP1* line (Fig. 10 I, J).

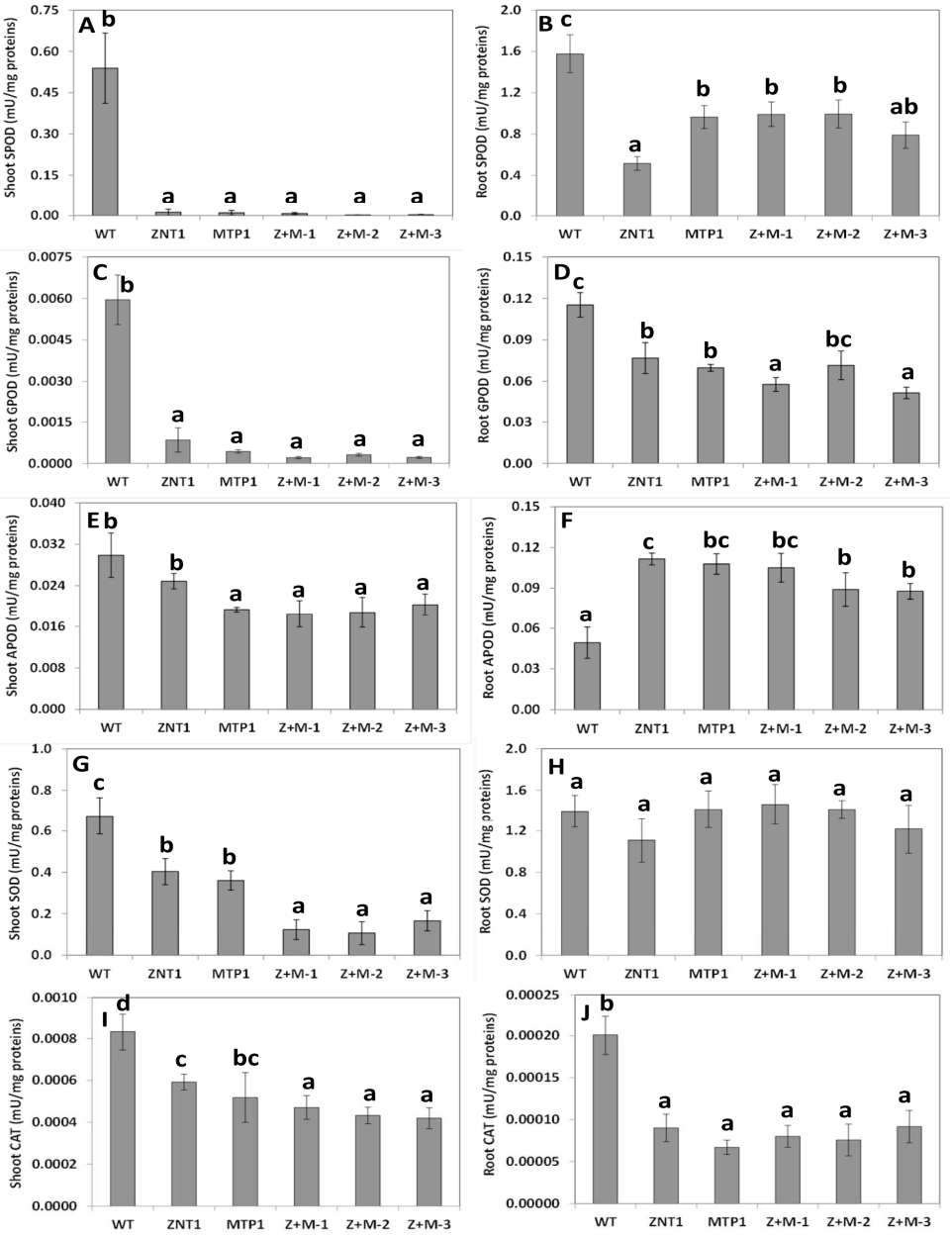

Fig. 9: Redox enzyme activities of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown on

¼ Hoagland's nutrient media supplemented with 100 µM $ZnSO_4$ (excess Zn) (A) Syringaldazine peroxidase (SPOD) activity (mU/mg Proteins) in shoot (B) and in roots (C) Guaiacol peroxidase (GPOD) activity (mU/mg Proteins) in shoot (D) and in roots (E) Ascorbate peroxidase (APOD) activity (mU/mg Proteins) in shoot (F) and in roots (G) Superoxide dismutases (SOD) activity (mU/mg Proteins) in shoot (H) and in roots (I) Catalases (CAT) activity (mU/mg Proteins) in shoot (J) and in roots after growing for 4.5 weeks. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significance Difference) (mean ± SE of 4 replica).

DISCUSSION

Phytoremediation is an emerging technology which could potentially be used to solve the problem of metal contamination in polluted soils (Brooks et al., 1998; Lahner et al., 2003; McGrath and Zhao, 2003). In order to develop a viable genetically modified organism (GMO) based phytoremediation technology, we need to understand the molecular mechanisms underlying metal tolerance and accumulation traits in plants and ultimately transfer the genes controlling these mechanisms into high biomass producing plant species. Various candidate genes have been cloned and functionally analysed. Previously, we found enhanced Zn and Cd tolerance and accumulation in *A. thaliana* lines expressing *NcZNT1* or *NcMTP1* (Chapter 2, 3). This urged us to transfer these traits to *N. tabacum*, which has much higher biomass than Arabidopsis and is a known crop, to explore the possibilities to accumulate Zn and Cd at sufficiently high levels to be useful for phytoremediation. In the current study, we indeed show that high expression of *NcZNT1* and *NcMTP1* separately and in combination, confers enhanced Zn and Cd tolerance and accumulation to *N. tabacum*. This is very interesting for phytostabilization and phytoextraction purposes. Tobacco is an attractive species to be used for phytoremediation purposes, because of its high yield (up to 170 ton $hectare^{-1}$), extended root system and the low cost of production (Schillberg et al., 2003). It is a moderately metal tolerant plant species compared to many metal non-accumulators and has many advantages that make it a suitable plant species useful for phytoextraction i.e. high biomass

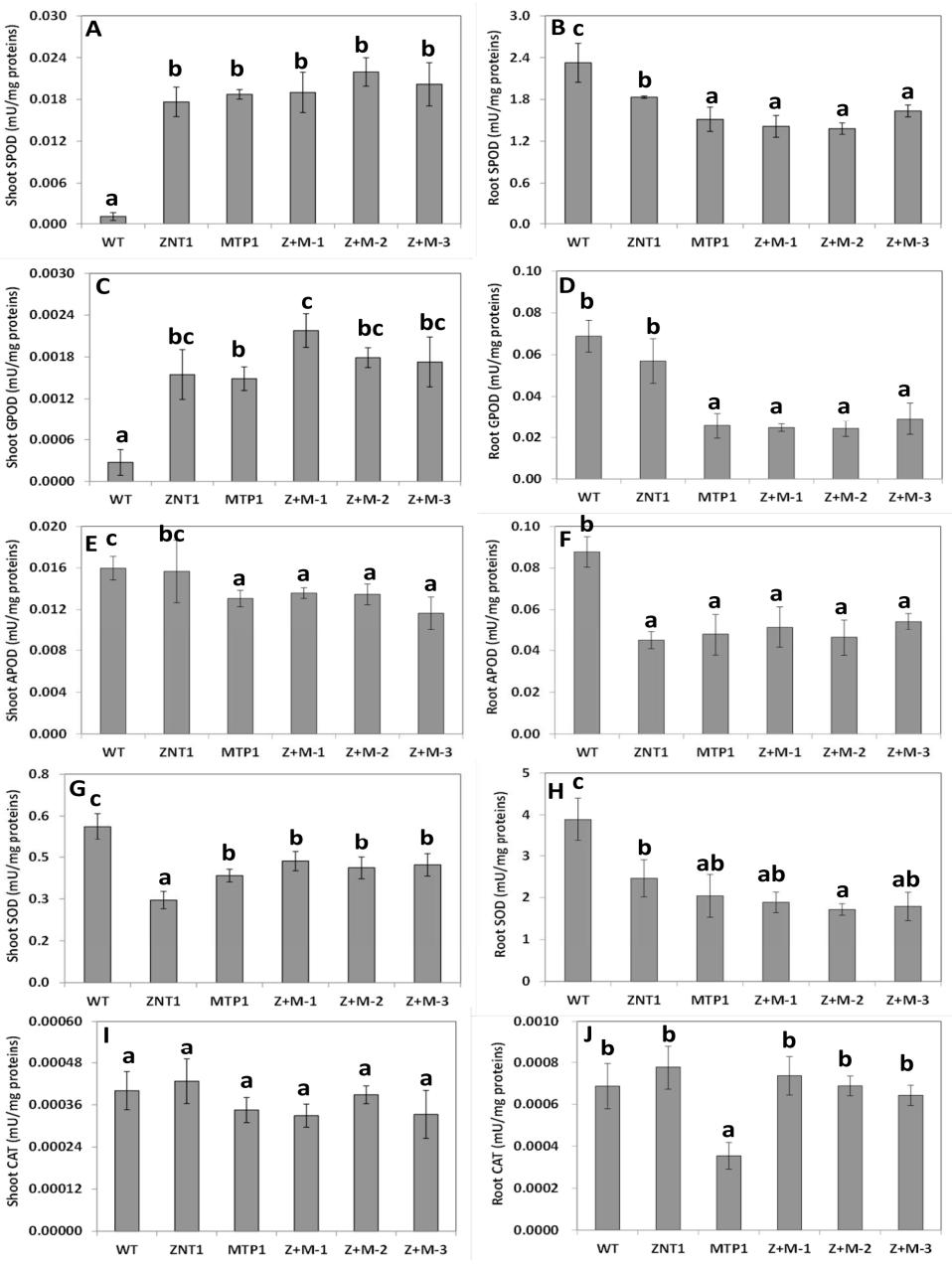

Fig. 10: Redox enzyme activities of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1) and three independently transformed *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown on *140*

¼ Hoagland's nutrient media supplemented with 5 µM $CdSO_4$ (excess Cd) (A) Syringaldazine peroxidase (SPOD) activity (mU/mg Proteins) in shoot (B) and in roots (C) Guaiacol peroxidase (GPOD) activity (mU/mg Proteins) in shoot (D) and in roots (E) Ascorbate peroxidase (APOD) activity (mU/mg Proteins) in shoot (F) and in roots (G) Superoxide dismutases (SOD) activity (mU/mg Proteins) in shoot (H) and in roots (I) Catalases (CAT) activity (mU/mg Proteins) in shoot (J) and in roots after growing for 4.5 weeks. Different alphabets indicate significant difference between lines at $p<0.05$ in ANOVA (Least Significance Difference) (mean ± SE of 4 replica).

fast growth rate, modest nutrient requirements and easy harvesting (Sarret et al., 2006). Furthermore, it has other advantages like high seed rate (1 million seeds/plant) useful for planting large areas, easy transformation and regeneration system, its non-food crop nature and self-pollination which can reduce transgene outcrossing (Hussein et al 2007). These properties make it a promising species for phytoremediation and our transgenic *N. tabacum* lines have additional traits of enhanced metal tolerance and accumulation which makes them interesting to be analysed for these purposes.

The present study has further strengthened our previous suggestions that *NcZNT1* is involved in Zn and Cd uptake and *NcMTP1* is involved in conferring Zn and Cd detoxification by vacuolar metal sequestration (Chapter 2, 3). The enhanced Zn and Cd tolerance and accumulation exhibited by *NcZNT1* and *NcMTP1* expression in *N. tabacum* is consistent with the transport ability for these metals in yeast systems (Pence et al., 2000; Chapter 2). Although the Cd transport activity of NcZNT1 has recently been revoked by Milner et al. (2012). Important to note here is that Milner et al. (2012) have accidentally cloned and used a truncated version of the *NcZNT1* gene from *N. caerulescens* accession Prayon, which lacks the first two exons, unlike the *NcZNT1* cDNA from the La Calamine accession, which we have used (Chapter 2). Thus the Zn and Cd metal transport of NcZNT1 should be further analysed by using full length cDNA. Single gene transformations have been shown to improve metal tolerance and accumulation in plants. However in metal hyperaccumulator species, these traits are polygenic and we likely need more than one genes to transform into high biomass plant species to further enhance these traits (Chapter 1). To date, there is little knowledge about testing multiple genes to confer Zn and Cd tolerance and accumulation in high biomass species suitable

for phytoremediation. We hypothesized that the combined expression of *NcZNT1* and *NcMTP1* will further enhance these properties in high biomass *N. tabacum*. Double transgenic lines exhibited significantly higher total plant Zn and Cd concentrations when tested on Zn and Cd excess respectively, which confirmed our hypothesis. We consider the role of *NcZNT1* expression in conferring higher metal uptake in the roots and shoots of these plants since this gene is known as a plasmamembrane localized metal transporter involved in enhanced metal influx into pericycle cells thus keeping them available for xylem loading and ultimately shoot translocation (Chapter 2; Milner et al., 2012). *NcMTP1* expression offered a sink in the vacuoles of root and shoot for metal detoxification in the double transgenic lines as the expression of this gene conferred Zn and Cd tolerance in *A. thaliana* (Chapter 3). This is consistent with previous studies where *AtMTP1* and *NgMTP1* were shown to cause increased Zn tolerance and accumulation when ectopically or heterologously expressed in *A. thaliana* (van der Zaal et al., 1999; Gustin et al., 2009). Particularly, even higher Zn and Cd accumulation in shoots was found in the double transgenic lines compared to single *NcZNT1* and *NcMTP1* lines, demonstrating that a polygenic transformation approach was superior to single transformation in enhancing the Zn/Cd tolerance and accumulation of *N. tabacum*.

Roots are the first plant organs, coming into direct contact with the excess metals. In WT *N. tabacum* plants, root biomass was lower compared to all transgenic lines which means that Zn and Cd toxicity has led to the impairment of root metabolism, which in turn could harm the cellular functions and could restrict the movement of metals to the shoot tissues. This reduces the translocation of nutrients and Zn and Cd metals into shoot tissues. Since our single and double transgenic lines accumulated higher Zn in roots than in their shoots, this is consistent with the known retention of Zn in non hyperaccumulators since there was a 2.5 fold higher Zn held in the root vacuoles in non-accumulator *Thlaspi arvense* than *N. caerulescens* (Lasat et al., 1998). Because of *NcMTP1* expression, enhanced Zn sequestration in roots occurred which contributed to higher tolerance of excess Zn in *pro35S::NcMTP1* and *pro35S::NcZNT1 + pro35S::NcMTP1* lines than *pro35S::NcZNT1* and WT lines which lack this gene. Another evidence that *pro35S::NcZNT1* and WT lines

were suffering from Zn toxicity comes from their relatively higher leaf chlorosis and reduced biomass compared to *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* lines under Zn excess. This implies the important role of *NcMTP1* in Zn detoxification by vacuolar sequestration. Same was the case in Cd excess, since Zn accumulation in roots of transgenic lines were higher. This means that these lines were able to deal with this higher Zn by vacuolar sequestration, except for *pro35S::NcZNT1,* which allowed them to grow further under Cd excess conditions. Thus Zn together with Cd in Cd exposure was the problem for these lines and transgenic lines were able to cope with this compared to WT. It is known that excess Zn and Cd can suppress Fe accumulation in *A. thaliana* (van de Mortel et al., 2006). This is probably due to the competition among these metals which share same transporters (Korshunova et al., 1999). Since all transgenic lines exhibited lowered Fe accumulation in roots but higher in shoots, this implies the disturbance in Fe homeostasis and probably upregulation of Fe deficiency responsive genes in roots. We have demonstrated that Fe deficiency genes were upregulated in roots of *A. thaliana* lines under excess Zn and Cd (Chapter 2, 3). This is consistent with the known involvement of epidermally expressed Fe deficiency responsive *IRT1* and *IRT2* genes in Zn and Cd uptake apart from Fe transport. (Korshunova et al., 1999; Vert et al., 2002; Vert et al., 2001). *N. caerulescens* accumulated higher Cd concentration under Fe deficiency by the upregulation of *NcIRT1* (Lombi et al., 2002). *Pisum sativum* exhibited enhanced Cd accumulation under Fe deficient conditions (Cohen et al., 1998; Cohen et al., 2004). Thus all these observations imply that indirect Zn and Cd uptake, apart from a direct uptake by *NcZNT1* and *NcMTP1*, in roots was happening in our transgenic lines by the Fe transporters and this is also is in agreement with the higher Fe accumulation in shoots of our transgenic lines. However, we need to perform the gene expression analysis of Fe deficiency responsive genes to verify this phenomenon. The higher Zn and Cd tolerance and accumulation in roots of *pro35S::NcZNT1* expressing lines can be explained by the higher Zn and Cd uptake by Fe transporters which could be further mobilized by *NcZNT1* in cells around xylem tissues and ultimately loaded into xylem. Xylem loading can result in shoot translocation and tolerance to these metals in root tissues

(Hanikenne et al., 2008). In that study, the authors showed that impaired root to shoot Zn/Cd translocation and tolerance was resulted by knocking down the expression of *HMA4* in A. *halleri.* The lowered Cd accumulation in roots but enhanced in shoots of double transgenic lines compared to both single *NcZNT1* and *NcMTP1* expressing lines illustrate an enhanced translocation of Cd in shoots, mediated by xylem metal loading. This physiological process seem to be enhanced in these double transgenic lines which is known to be mediated by the *HMA4* gene (Hanikenne et al, 2008) and this property is promising for phytoextraction purposes. Thus the *HMA4* gene from *N. tabacum* can play a role in higher Cd but lowered Zn translocation in our double transgenic lines since most of the Zn was retained in roots. This property of xylem loading of metals is higher in accumulators than in non-accumulators (Lasat et al., 1996). Furthermore, phytochelatins, which can detoxify Cd, were reported to be translocated from root to shoot along with Cd (Gong et al., 2003). This might also be the case in our transgenic lines since they had higher Cd in shoots and were tolerant to Cd. Once metals reach the shoot tissues, they are sequestered in vacuoles of leaf cells and are stored mostly in epidermal cells in hyperaccumulators since these cells lack chloroplasts and hence are less prone to photosynthetic damage caused by toxic metal ions (Kupper et al., 1999; Vogeli-Lange and Wagner, 1990). As our pro35S*::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* lines were equipped with a vacuolar metal sink, offered by *NcMTP1,* these lines were better protected by Zn and probably Cd, in shoot tissues. That is why the *pro35S::NcZNT1* line exhibited higher leaf chlorosis compared to rest of the transgenic lines under Zn excess. Recently, Ueno et al., (2011) have demonstrated that HMA3, which is a tonoplast localized transporter, sequesters Cd into leaf cells vacuoles and is thus responsible for Cd hypertolerance in *N. caerulescens*. This gene might be involved in shoot Cd tolerance in our transgenic lines. In conclusion, *NcZNT1* and *NcMTP1* expression enhanced Zn and Cd tolerance and accumulation and indirect transport of Zn and Cd is plausible, mediated by Fe transporters.

It is known that Zn and Cd can inactivate the PSII reaction centre or even seize the flow of electrons in the water splitting site in isolated thylakoid membranes (Molins et a., 2012; Kojima et al. 1987; Prasad et al., 1991; Singh et

al., 1991). The enhanced chlorophyll fluorescence in *pro35S::NcZNT1, pro35S::NcMTP1* and *pro35S::NcZNT1 + pro35S::NcMTP1 N. tabacum* lines supported our hypothesis that the maximum quantum efficiency of photosystem II photochemistry was higher in these lines probably by detoxification of symplastic Zn and Cd due to sequestration into the vacuoles. It is known that excess Fe can induce lipid peroxidation (Repetto et al., 2010). There was enhanced Fe accumulation in shoots which correlates with the higher shoot TBA (a measure of lipid peroxidation) content. Furthermore, excess Zn and Cd in shoot may also be involved in initiating this response but since our transgenic lines displayed tolerance to these metals probably due to vacuolar sequestration, we suggest a role of excess Fe in initiating this response. This is consistent with the fact that lipid peroxidation in phospholipid liposomes was initiated by the oxidation of Fe^{2+} or reduction of Fe^{3+} (Repetto et al., 2010). Fe concentrations were decreased in the roots of transgenic lines, possibly explaining the decrease of lipid peroxidation in these organs. Nevertheless, it is important to investigate the specific localization of Fe, Zn, and Cd in these transgenic lines to further investigate their role in plasma membrane damage.

Zn and Cd exposure has been known to initiate Reactive Oxygen Species (ROS) production, which are known cellular damaging species in plants (Cuypers et al 2011; Cuypers et al., 1999). In order to cope with this damage, plants have evolved anti-oxidant defense system (Foyer and Noctor, 2005; Mittler et al., 2004). Our data regarding SPOD, GPOD, APOD, SOD, and CAT indicated an overall reduction in the activities of these enzymes suggesting that there was no need to activate these defense systems as all the transgenic lines were better protected against these metals than WT. Another explanation might be that in transgenic lines, the antioxidant defence system had collapsed due to excess of metals; however, this seems implausible since transgenic lines had higher metal tolerance as supported by higher biomass and higher chlorophyll fluorescence data and were not showing enhanced signs of metal toxicity when compared to WT. Previously, reduced effects on activities of the anti-oxidative defense enzymes by Zn were proposed in case of vacuolar sequestration and compartmentalization of this metal (Cuypers et al., 2002). Our data are in agreement with this and further suggests that Cd sequestration have a

comparable effect as Zn. However, there was a higher difference in the activities of SPOD and GPOD under Zn and Cd excess compared to WT. Very high activities of these peroxidases under Cd stress suggest that the transgenic lines may be undergoing lignification, which is a known mechanism to detoxify Cd (Cuypers et al., 2002). This phenomenon was found to be happening in the root endodermis, and thus prevented Cd translocation to the shoot (Schreiber et al. 1999). *N. caerulescens* exhibited a higher expression of lignin/suberin related genes and it was consistent with an extra endodermal layer, composed of secondary cell walls saturated with lignin/suberin, covering the outer side of the endodermal layer (van de Mortel., 2006; Broadly et al., 2007). This layer is missing in the non-accumulators and is hypothesized to inhibit the higher metal efflux from vascular tissues thus keeping it available for shoot translocation. Our SPOD and GPOD data also correlates with the relatively low biomass of the WT line under Zn but not under Cd stress as cell wall lignification limits the expansion of cells. This enhanced lignification was found to be important in order to adapt to stress and correlated with the lower leaf growth of *Phaseolus vulgaris* upon Cd exposure (Smeets et al., 2005). An alternative hypothesis is that these enhanced SPOD and GPOD activities in transgenic lines would increase the binding capacity in the cell walls for extracellular Cd. Investigating the subcellular localization of Cd, Zn and Fe could enable us to figure this out.

In conclusion, we have demonstrated that it is possible to engineer the high biomass plant species *N. tabacum* for improved Zn and Cd tolerance and accumulation by expressing *NcZNT1* and *NcMTP1* genes from metal hyperaccumulator *N. caerulescens*. Furthermore, we have shown that combined expression of these genes can have an additive effect on the enhanced phytoremediation potential of Tobacco. However, this metal accumulation is still less compared to the 100 fold higher Zn and Cd accumulation in *N. caerulescens* compared to non-accumulators. This leads us to conclude that 35S expression of these genes is not sufficient to mimic *N. caerulescens* like hyperaccumulation phenotype in *N. tabacum*. Nevertheless, this enhanced Zn and Cd tolerance/accumulation in a high biomass crop species like tobacco is very important from the perspective of the development of a GMO based phytoremediation technology. It has been proposed earlier that field oriented

phytoremediation is a more complicated process compared to lab tested methods because of several factors affecting metal uptake i.e. degree and depth of metals pollution, bioavailability of metals, soil properties, pH, irrigation, fertilization etc. (Hussein et al 2007; McGrath et al., 2005). Further and more in depth research is needed to test the phytoremediation potential of our engineered *N. tabacum* lines, preferentially by conducting field trials.

ACKNOWLEDGEMENTS

This research was supported by the Higher Education Commission of Pakistan (HEC), the graduate school Experimental Plant Sciences WUR and COST (European Cooperation in Science and Technology) Action FA0905, STSM grant. We would like to thank Ann Wijgaerts, Els Keunen and Marijke Jozefczak for their help in TBA and enzymes analysis. We are grateful to Pádraic Flood and Jeremy Harbinson for their help in chlorophyll fluorescence measurements and Mohamed El Soda for support in statistical analysis. We are thankful to Dr. Maarten Koornneef for critical reading and suggestions for the preparation of this manuscript.

CHAPTER 5

Single and double *NcZNT1* and *NcMTP1* expression in *Nicotiana tabacum* displayed enhanced Zn and Cd phytoremediation potential in metal contaminated field soil

Zeshan Hassan[1], Ann Cuypers[2], Jaco Vangronsveld[2] and Mark G.M. Aarts[1]

[1]Laboratory of Genetics, Wageningen University and Research Centre, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands

[2]Hasselt University, Environmental Biology, Agoralaan Building D, 3590, Diepenbeek, Belgium

ABSTRACT

- The aim of the current study was to analyse and evaluate the metal tolerance and accumulation potential of *pro35S::NcZNT1, pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* expressing *N. tabacum* lines on their suitability for phytoremediation purposes when grown in an original metal contaminated field soil.
- Six metal contaminated soils with varying levels of Zn and Cd pollution were collected from different sites in NE-Belgium. Wild-type *N. tabacum* lines were grown on these soils to identify the best candidate soil exhibiting metal toxicity in wild-type plants useful to test the transgenic lines. *pro35S::NcZNT1, pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S ::NcMTP1* expressing *N. tabacum* lines were tested in a Zn and Cd contaminated soil and plant morphology, dry biomass, metal content, chlorophyll content, carotenoid content, and lipid peroxidation of the transgenic lines were compared to wild-type in order to assess the metal tolerance and accumulation.
- Single *pro35S::NcZNT1, pro35S::NcMTP1,* and double *pro35S::NcZNT1* + *pro35S::NcMTP1 N. tabacum* lines exhibited increased Zn and Cd tolerance when exposed to metal contaminated field soil. Chlorophyll a, chlorophyll b and carotenoid contents and estimations for lipid peroxidation revealed that the transgenic lines were better protected against Zn and Cd excess. These lines, particularly *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1*, also displayed enhanced total plant Zn, total plant Cd and shoot Mg accumulation. These results confirm that through genetic engineering, it is possible to improve the Zn and Cd tolerance and accumulation of high biomass species *N. tabacum* suitable for phytoremediation purposes.

INTRODUCTION

Zinc (Zn) is an essential micronutrient for plants, playing an important role in several physiological processes. At elevated concentrations, it induces toxicity and impaired growth in plants. In excess, this trace element can shift the plant physiological equilibrium by local competition with other divalent cations like Mn, Fe, Cu, and Mg. For example, it can displace Mg in Rubisco resulting in a loss of activity of this enzyme, which leads to the generation of oxygen radicals. Many enzymes can be inactivated by Zn binding to O, N and S atoms (Tewari et al., 2008). The effects of Zn toxicity are inhibition of root growth, leaf chlorosis, interference with the Calvin cycle, inhibition of photosynthesis (Mateos-Naranjo et al., 2008) and nutrient imbalances (Cambrollé et al., 2012). When Zn concentrations exceed around 300 mg kg^{-1} DW of plant tissues, it will lead to necrosis and ultimately plant death (Chapter 1).

Cadmium (Cd) is an important environmental pollutant and a potent toxicant to plants. Even though Cd is not an essential nutrient for plants, it can be taken up by the roots and translocated to the leaves because of the low Cd affinity of Zn and Fe transporters. In plants, excess Cd can cause many physiological, biochemical, structural and morphological changes (Liu et al., 2012). As Cd has a high affinity to sulfhydryl (thiol) groups, which are functional domains of enzymes and structural proteins, it causes disruptions in metabolic processes (Sharma & Dietz, 2009; Vangronsveld and Clijsters, 1994). It can also replace essential elements e.g. components of metalloproteins or interfere with their uptake and cause toxicity (Cuypers et al., 2009). These toxic changes are associated with disturbance of uptake and transport of water and nutrients, photosynthesis and respiration, loss of membrane function, chromosomal aberrations as well as cell cycle and division alterations (Arasimowicz-Jelonek et al., 2011). Most of the plants showed Cd toxicity symptoms at leaf concentrations of about 5-10 mg Cd kg^{-1} DW (Vamerali et al., 2010). The most notorious symptoms of Cd toxicity are leaf rolling and chlorosis, root necrosis, stunted growth, decreased reproducibility and eventually death (Hu et al., 2009; Shi et al., 2010).

Increasing environmental contamination with toxic metals is a growing risk for the ecological and health associated hazards for the global human

community (Sharma and Agrawal, 2005). These elements can be toxic even at lower concentrations (Rascio and Navari-Izzo, 2011) and can persist in soils for centuries and more (Voglar and Lestan, 2012). The most common metals in industrial effluents are Cd, Zn, Cr, and Ni (Sun et al., 2010; Gusiatin and Klimiuk, 2012). The risk of their long term presence in soils increases by lowered soil buffering capacity which causes leaching of these metals into ground water and ultimately cause water pollution (Sun et al., 2010; Gusiatin and Klimiuk, 2012). Metal contaminated soils cause injury to soil organisms and reduce crop yield. In agricultural soils, Zn concentrations are found normally around 1-100 mg Zn kg^{-1} dry soil, but in highly contaminated areas, concentrations of more than 1000 mg Zn kg^{-1} dry soil were recorded (Tewari et al., 2008; Jain et al., 2010). Cd from soil enters efficiently into edible plants, which are reported to be the major source of human Cd intake (Clemens et al, 2012). In case of soil Cd levels toxic for plant uptake and ultimately dangerous for human health, the highest values of 50 mg Cd kg^{-1} dry soil were recorded while 0.2-1.0 mg Cd kg^{-1} dry soil are the background values found in various soils (Clemens et al 2012; UNEP, 2008). Soil metal contamination is caused by the disposal of municipal wastes, use of irrigation water containing industrial effluents, agricultural use of sewage sludge residues from metalliferous mining, livestock manure, the use of Zn and phosphate fertilizers, atmospheric deposition of industrial emission, land filling of industrial wastes, mining and the smelting industry (Solti et al., 2008).

Soil remediation can be performed by conventional physical or chemical remediation methods. These methods like leaching, solidification, vitrification, electrokinetical treatment, chemical oxidation or reduction, excavation and off-side treatment are expensive, disturb soil structure and fertility and are limited to relatively small areas (Baker et al., 1994; McGrath et al., 1997; Luo et al., 2000; Zhao et al., 2000; Wu et al., 2010). However, a more environmentally friendly technology to treat contaminated soils is phytoremediation, which involves the use of plants to remove metals or to make them immobile to reduce further environmental pollution. This is a cheap alternative technology carried out *in situ* avoiding the transport of large amounts of soil (Vangronsveld et al., 2009; Vamerali et al., 2010; Varun et al., 2012). Phytoremediation comprises of several

types with phytostabilization and phytoextraction being the most generally used (Vamerali et al., 2010). Phytostabilization does not remove the contaminants, but reduces their mobility and bioavailability in the soil thus making metals less hazardous for human health and environment. This practice helps to improve the chemical and biological characteristics of the soil by the establishment of a green canopy. The metals can be stored in roots and have a low translocation to shoots. On the contrary, phytoextraction uses plants to remove metals from the soil by accumulating them in harvestable biomass (Vamerali et al., 2010; Varun et al., 2012), which can then be processed and used to produce for instance bioenergy or even to recover metals useful for industry (Brooks et al., 1998). Indeed, economic valorisation of the biomass produced will improve the economic feasibility of phytoremediation (Vassilev et al., 2004). The potential of a plant to extract metals is influenced by the mobility and availability of metals in soil and plants. Therefore, to assess the plant's phytoremediation potential, one should consider the BioAbsorption Coefficient (BAC; metal concentration in shoot/metal content in soil), the BioConcentration Factor (BCF; metal concentration in root/metal content in soil), and the Translocation Factor (TF; metal concentration in shoot/metal content in root). Plants showing a high BCF and a low TF are promising for phytostabilization whereas plants with BAC and TF values higher than 1 are promising for phytoextraction (Varun et al., 2012; Mcgrath and Zhao, 2003). As a prerequisite, plants used for phytoremediation purposes should be able to tolerate and survive under high metals concentrations (Vamerali et al., 2010).

There are numerous plant species which have evolved different strategies to successfully deal with elevated soil metal concentrations, one of them is the metal hypertolerance-hyperaccumulation strategy in which metals are taken up into root tissues and mostly translocated to the shoot tissues where they are sequestered into vacuoles (Lin and Aarts, 2012). These metal hypertolerant-hyeraccumulator plants can accumulate over 100-fold higher metal concentrations than non-accumulator plants, which makes them interesting for the removal of metals from polluted soils. One well-known model metal hypertolerant-hyperaccumulator species is *N. caerulescens* (J. & C. Presl) F.K. Meyer, formerly known as *Thlaspi caerulescens* J. & C. Presl (Krämer,

2010). This species is widely spread in central Europe and is used as a model species for the physiological understanding of metal hypertolerance and hyperaccumulation (Lasat et al., 1996; Assunaco et al., 2003b; Vamerali et al., 2010; Visioli et al., 2012). *N. caerulescens* is able to tolerate and accumulate over 10,000 mg Zn kg^{-1} DW while in most other plant species the toxicity threshold is 150-200 mg kg^{-1} DW (Vamerali et al., 2010). In the case of Cd, *N. caerulescens* was able to tolerate and accumulate over 100 mg Cd kg-$^{-1}$ DW. The highest Cd concentrations reported in nature in *N. caerulescens* shoots were 3600 mg kg^{-1} DW and when grown on hydroponics, values up to 10,000 mg kg^{-1} DW were recorded (Liu et al., 2008).

Low biomass production and slow growth are the major limiting factors in the use of natural metal hyperaccumulators, including *N. caerulescens,* for the above mentioned purposes, due to little economic added value compared to fast growing, high biomass species. Moreover, many generations of growing a hyperaccumulator species are needed and in fact many years to remediate contaminated soils. Therefore, improvements are needed. Genetic modification of plants could help to get rid of these constraints in two different ways. A first option is to improve the growth and biomass production of known metal hyper-accumulators by genetic engineering. The second and most interesting alternative is to genetically equip high biomass producing plant species with new traits for metal tolerance and accumulation. By understanding the molecular mechanisms involved in metal tolerance and hyperaccumulation, we may provide the required gene. Such genes for example *NcZNT1* and *NcMTP1* could be transferred into a high biomass producing plant species like Tobacco (*Nicotiana tabacum*). It is a moderately metal tolerant species compared to many non-accumulators (Sarret et al., 2006), it is a known crop, for which efficient cultivation methods and breeding tools are available and by further enhancing its metal tolerance and accumulation potential by expressing *NcZNT1* and *NcMTP1* genes, we might utilize this species for phytoremediation purposes.

The present study was aimed at verifying the metal tolerance and accumulation potential of *pro35S::NcZNT1, pro35S::NcMTP1 and pro35S::NcZNT1* + *pro35S::NcMTP1* expressing *N. tabacum* lines as previously observed in hydroponic culturing, using soil from a metal-contaminated field

(Chapter 4). The hypothesis of the present study was that these transgenic lines will exhibit enhanced properties of metal tolerance and accumulation when grown in metal contaminated soil, which facilitates phytoremediation purposes. Using an original metal-contaminated soil is a more reliable way to test the phytoremediation potential than by using hydroponic culture or artificially metal-spiked soils, due to different metal bioavailability and physical properties of these systems (Megharaj and Naidu, 2003).

MATERIALS AND METHODS

Development of binary constructs and plant transformations

The development of binary constructs is described in chapter 2 (*pro35S::NcZNT1*) and 3 (*pro35S::NcMTP1*). Wild-type *N. tabacum* was transformed with these constructs as described in chapter 4, in this thesis.

Testing SR1 wild-type *N. tabacum* in six metal contaminated soils

In order to select a suitable soil for this experiment, we preferred soil that would represent the soil at sites that was contaminated by several toxic metals over a period of several years. Therefore we preferred not to use regular agricultural soil in which metals were artificially mixed prior to the experiment, which often shows very different bioavailability characteristics when compared to soil from contaminated sites (Megharaj and Naidu, 2003). In addition, the soil should induce metal toxicity symptoms in the SR1 (Streptomycin Resistance 1 cultivar) wild-type (WT) *N. tabacum* plants used for generating the transgenic lines. We chose North East-Belgium as region to collect different soils for this analysis. Zn smelters are active in these areas since the end of the 19th century (http://enfo.agt.bme.hu/drupal/sites/default/files/ARuttensPhytorem.pdf).
Six different metal contaminated soils (mainly containing Zn and Cd; Vangronsveld et al., 2009) were collected from 0-20 cm depth and the WT *N. tabacum* plants were grown in them. These soils were named O1, O2, O3, (Overpelt, Belgium), B1 (Balen, Belgium), L1 and LW (Lommel, Belgium). Soil samples were analysed for the elemental concentrations by using inductively coupled plasma–atomic emission spectrometry (ICP-AES; Perkin-Elmer, 1100B,

USA) as described by Cuypers et al. (2011). 1-L pots were filled with 800 g of well mixed, sieved and air dried soil. The pH of the soils was determined by using a pH meter (WTW multi 197i) and electrical conductivity (EC) was recorded with an EC meter (WTW LF 537). Seeds of WT *N. tabacum* were sterilized and geminated on ½ MS agar plates (Murashige and Skoog, 1962) without sugar, pH 5.8, and incubated in a climate controlled growth cabinet (25°C 16/8 h, light/darkness with illumination at a light intensity of 120 µmol m^{-2} s^{-1}). Seven-days-old seedlings were transplanted to the metal-contaminated-soil-filled pots. Nine WT plants were grown in each contaminated soil for four weeks in a greenhouse (18 °C, 16/8 h light/darkness, with illumination at a light intensity of 200 µmol m^{-2} s^{-1} and 65 % relative humidity). Pots were irrigated with 1/8 Hoagland's nutrient media once per week in the first and the third week. They were watered with normal tap water once per week for the rest of the period. Metal toxicity effects were evaluated by measuring the shoot and the root dry weights (DW). Samples for soil and plant metal analysis were kept.

Testing transgenic Tobacco lines in O3 metal contaminated soil

To determine the metal tolerance and accumulation potential of *pro35S::NcMTP1, pro35S::NcZNT1*, *pro35S::NcZNT1* + *pro35S::MTP1* and SR1 (WT) *N. tabacum* lines, they were grown in metal contaminated O3 soil (Overpelt, Belgium). Based on previous experiments (Chapter 3), the most tolerant line for each of the transgenic lines (containing either one of the *pro35S::NcMTP1* or *pro35S::NcZNT1* constructs (single transformants) or both (double transformants)) and one WT line were grown in pots filled with O3 soil, prepared as described above. The experiment was set up using nine plants per genotype grown in O3 and control soil (normal greenhouse soil) in greenhouse (18 °C, 16/8 h light/darkness, with illumination at a light intensity of 200 µmol m^{-2} s^{-1} and 65 % relative humidity). At the end of the experiment, samples for chlorophyll a, chlorophyll b, carotenoid, lipid peroxidation, shoot and root metal analysis were collected. Shoot and root dry weights were recorded.

Plant phenotypic analysis

Dried soil, shoot and root samples were analysed for the elemental concentrations by using ICP-AES (Perkin-Elmer, 1100B, USA) as described by Cuypers et al. (2011). Chlorophyll a, chlorophyll b and carotenoid contents were measured as described by Lichtenthaler and Wellburn (1983). Frozen shoot and root samples were homogenized and TBA was measured as described by Cuypers et al. (2011).

Statistical analysis

Where needed, data were analysed for significance at $p<0.05$ by using Student's t-test, two-way ANOVA and ANOVA (Least Significance Difference) in the SPSS v. 12 software package for MS Windows.

RESULTS

Wild-type *N. tabacum* displayed different responses to six metal contaminated soils

Wild-type (WT) *N. tabacum* plants were grown in six different metal contaminated soils to select a suitable metal-toxic soil which could be used for testing the transgenic lines. WT plants grown in LW soil did grow much better and had a higher shoot and root biomass (Fig. 1 A-D) than other soil-grown plants. Soil collected from Overpelt (O1, O2 and O3) significantly affected the growth of the WT plants, which displayed reduced shoot and root biomass (Fig. 1 A-D) compared to plants grown on LW soil. The decrease in biomass can be summarized as follows LW>L1>B1>O3>O2>O1. pH and electrical conductivity (EC) of LW and B1 soils were similar. However in comparison, O1, O2, O3 and L1 exhibited significantly higher pH and EC (Fig. 1 E, F). From WT *N. tabacum* dry biomass, pH and EC data of the soils, O3 was considered to be suitable soil in causing metal toxicity to WT plants and it was used in further experiments.

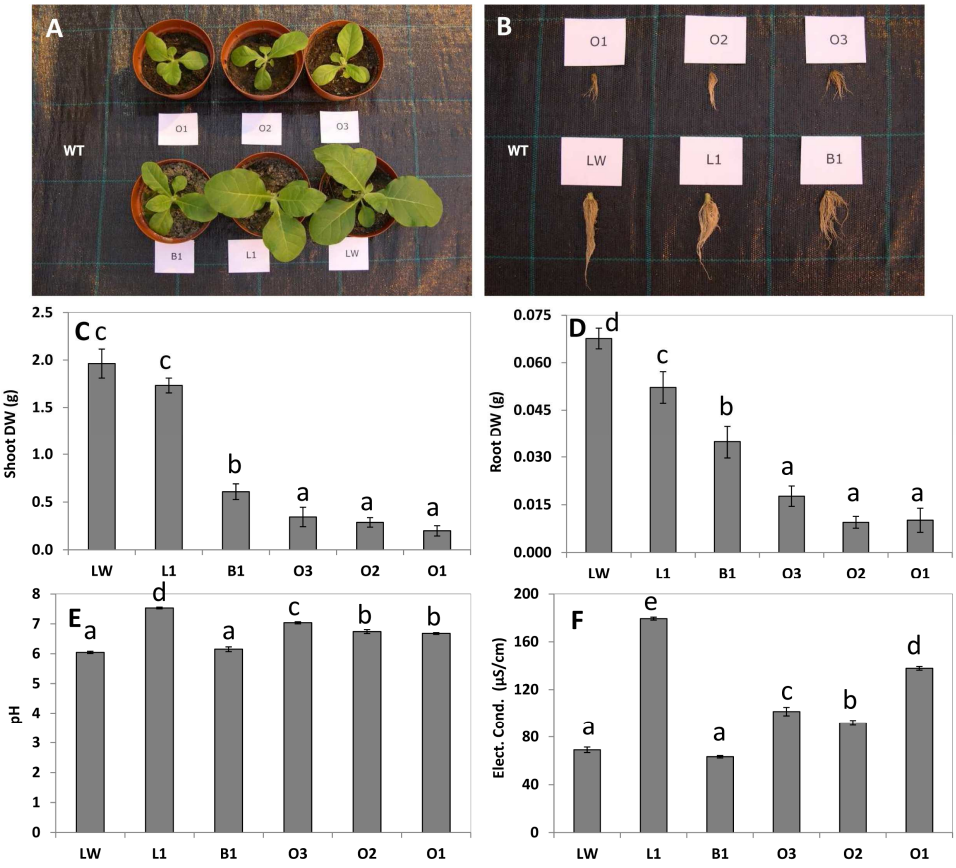

Fig. 1: Phenotypic analysis of wild-type (WT) *tabacum* plants exposed to six different metal contaminated soils named O1, O2, O3, (Overpelt, Belgium) B1 (Balen, Belgium), L1, LW (Lommel, Belgium), pH and electrical conductivities of these soils. (A) Shoot phenotype of WT plants grown on different soils. (B) Root phenotype. The photographs of WT plants were taken 28 days after transplantation of the seedlings to the contaminated soil. (C) Shoot dry weight (g) (D) and root dry weight (g), (E) pH of soils, and (F) electrical conductivity of soils. Plants were grown for four weeks. Different letters indicate the significant difference between WT plants grown in different soils ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

The O3 soil contained much higher Zn and Cd concentration than the non-contaminated control soil

The metal-contaminated O3 soil exhibited up to 656.71 mg kg^{-1} DW compared to 5.8 mg Zn kg^{-1} DW control soil (about 113 times higher Zn) which is

extremely high compared to normal agricultural soils (Fig. 2 A; Tewari et al., 2008; Jain et al., 2010). The control soil did not contain any detectable amount of Cd, while the O3 soil contained up to 2.11 ppm of Cd. This is considered a moderately high level of Cd pollution (Fig. 2 B; Clemens et al., 2012). Total Fe and Mn levels were significantly reduced in the O3 soil (Fig. 2 C, D). The O3 soil had a slightly higher pH while it had similar electrical conductivity as the control soil (Fig. 2E, F). These observations confirmed a very high Zn and Cd pollution of the O3 soil and thus a suitable substrate to use for analysis of the transgenic tobacco lines.

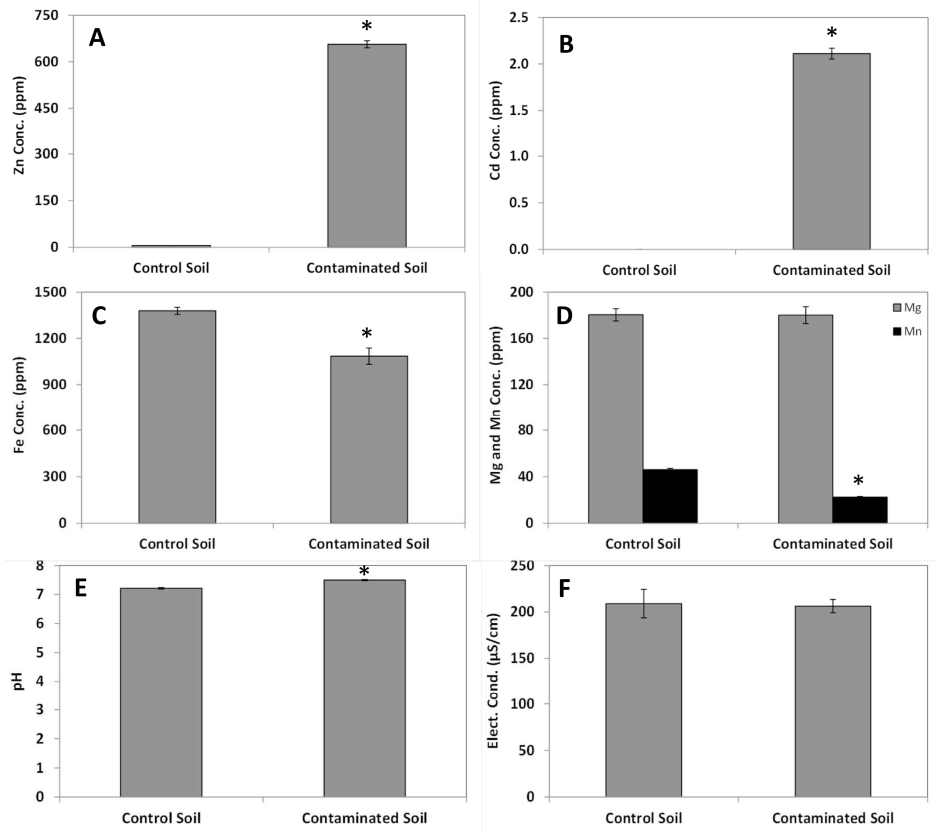

Fig. 2: Metal concentrations, pH and electrical conductivities of contaminated soil O3 (Overpelt, Belgium) and control soil (A) Zn concentrations (ppm) (B) Cd concentrations (ppm) (C) Fe concentrations (ppm) (D) Mg and Mn concentrations (ppm) (E) pH (F) Electrical conductivity. * indicate significant difference between contaminated O3 and control soil ($p<0.05$, Student's t-test) (mean ± SE of four replicates).

Zn and Cd tolerance and accumulation was enhanced by the single and combined expression of *NcZNT1* and *NcMTP1* in *N. tabacum*

Since the *pro35S*::*NcZNT1, pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* expressing *N. tabacum* lines displayed enhanced Zn and Cd tolerance and accumulation when grown in a hydroponic system (Chapter 4), we further analyzed the metal tolerance and accumulation in these lines when grown in the metal-contaminated O3 soil. Transgenic lines grown in O3 soil, particularly the *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines, were more tolerant to metal exposure, with larger and less chlorotic leaves than the WT (Fig. 3 A). Shoot and root dry weights of *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines were significantly higher than WT (Fig. 3 B). The *pro35S::NcZNT1* line did not exhibit any difference in the dry biomass compared to WT, however, it had less leaf chlorosis compared to WT (Fig. 3 A, B). There was no significant difference in shoot and root Zn accumulation between the transgenic and WT lines (Fig 3 C). However, due to their higher biomass, the *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines accumulated significantly more total Zn per plant than the WT line (Fig. 3 D). In case of Cd accumulation, there was no significant difference in Cd concentration between transgenic and WT lines but the *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines exhibited a higher total plant Cd content than the WT line (Fig. 3 E, F). The calculations of total metal removal and estimated time needed to clean up these metals from O3 soil is summarized in Table 1. These calculations were done for shoot tissues since shoot is the harvestable part of a tobacco crop. It means that it will take 110 generations of *pro35S::NcMTP1* line compared to 176 of WT in order to reduce Zn pollution levels to half in O3 soil. The Cd pollution can easily be reduced from 2.11 mg kg^{-1} to 1.05 mg kg-1, which is present in normal agricultural soils (Clemens et al., 2012), in 14 generations compared to 28 generations of WT (Table 1). Enhanced shoot Mg concentrations were recorded in the transgenic lines when grown in O3 soil, although root Mg concentrations were not different from the WT line except for *pro35S::NcZNT1* line (Fig. 4 A, B). Fe concentrations were lower in the transgenic lines (Fig. 4 C), but this was only significant for shoots, while there was significantly more Mn in the roots of *pro35S::NcZNT1* +

pro35S::MTP1 plants compared to the other lines (Fig. 4 F).

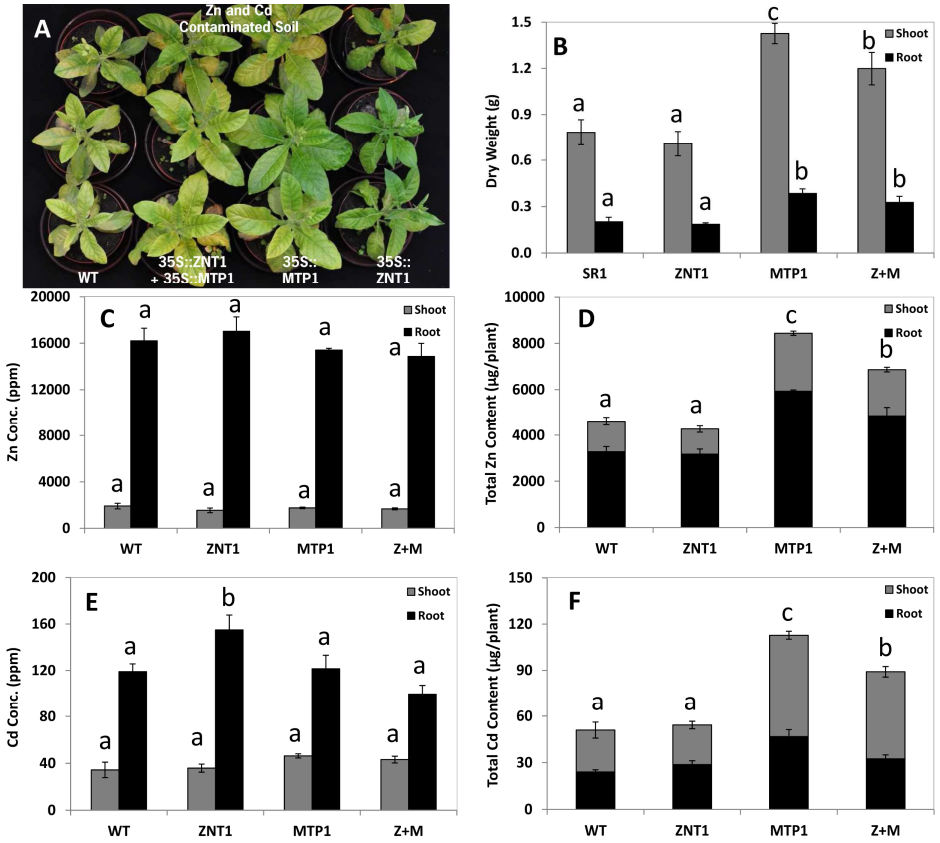

Fig. 3: Phenotypic analysis of one *pro35S::NcZNT1* line (ZNT1), one *pro35S::NcMTP1* line (MTP1), one *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M) compared to one wild-type (WT) *N. tabacum* line grown in metal contaminated O3 soil. (A) Transgenic lines compared to WT. The photograph was taken after 28 days after transplantation (B) Shoot and root dry weight (g) (C) Zn concentration in shoots and roots (ppm) (D) Total plant Zn content (µg/plant) (E) Cd concentration in shoots and roots (ppm) (F) Total plant Cd content (µg/plant) after growing for four weeks on the metal contaminated O3 soil. Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

We also calculated the BioAbsorption Coefficient (BAC; metal concentration in shoot/metal concentration in soil), the BioConcentration Factor (BCF; metal concentration in root/metal concentration in soil), and the Translocation Factor (TF; metal concentration in shoot/metal content in root) as summarised in Table 2. The Cd BAC factors of *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* were higher compared to *pro35S::NcZNT1* and WT lines. The *pro35S::NcZNT1* line had a higher Cd BCF compared to rest of the lines. The Cd TFs of *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines were higher than *pro35S::NcZNT1* and WT lines.

Table 1: Estimated Zn/Cd phytoremediation potential of *pro35S::NcMTP1* (MTP1) line compared to wild-type (WT) *N. tabacum.*

	Shoot metal Conc. (mg kg^{-1})		Shoot dry biomass (g)		Shoot metal removal in one generation (mg/shoot)		No. of generations to reduce Zn/Cd pollution to half	
	WT	**MTP1**	**WT**	**MTP1**	**WT**	**MTP1**	**WT**	**MTP1**
Zn	1922.73	1674.53	0.78	1.43	1.49	2.39	176	110
Cd	34.33	43.11			0.03	0.06	28	14

Table 2: BioAbsorption Coefficient (BAC), BioConcentration Factor (BCF) and Translocation Factor (TF) of *pro35S::NcZNT1* line (ZNT1), *pro35S::NcMTP1* line (MTP1), *pro35S::NcZNT1+ pro35S::NcMTP1* line (ZNT1+MTP1) compared to wild-type (WT) *N. tabacum.*

	BAC Shoot Conc./Soil Conc. (mg kg^{-1})		BCF Root Conc./Soil Conc. (mg kg^{-1})		TF Shoot Conc./Root Conc. (mg kg^{-1})	
	Zn	**Cd**	**Zn**	**Cd**	**Zn**	**Cd**
WT	2.93	16.27	24.67	56.38	0.12	0.29
ZNT1	2.36	16.98	25.94	73.46	0.09	0.23
MTP1	2.68	21.95	23.47	57.56	0.11	0.38
ZNT1+MTP1	2.55	20.43	22.64	47.15	0.11	0.43

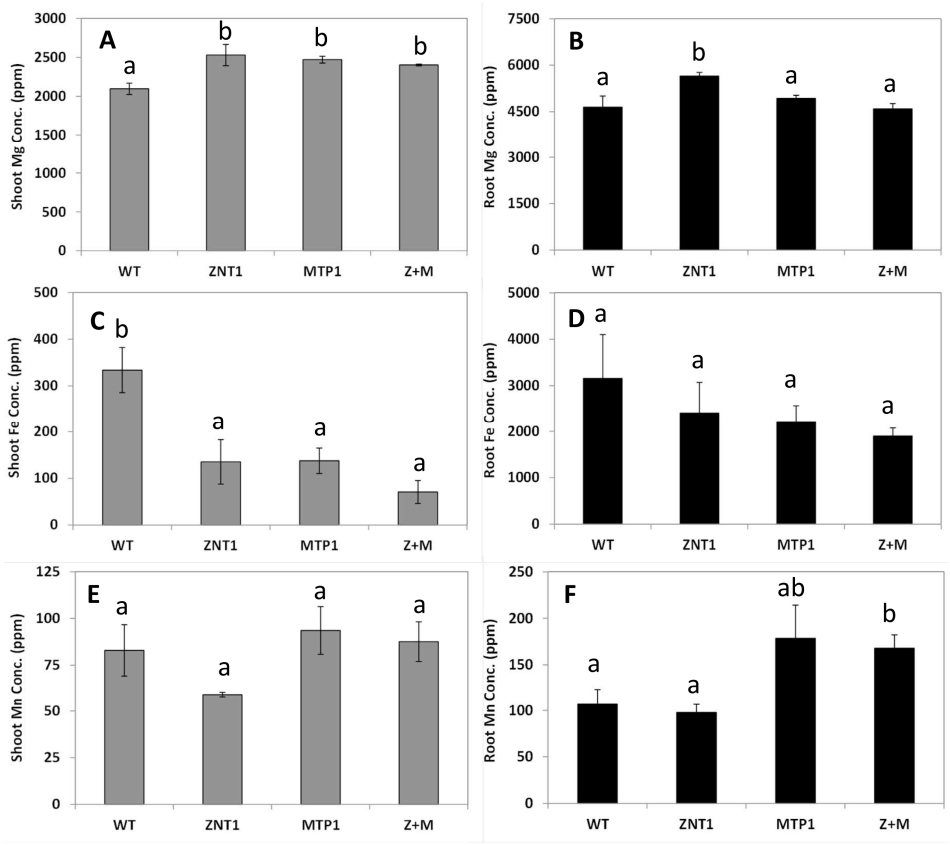

Fig. 4: Metal concentrations of one *pro35S::NcZNT1* line (ZNT1), one *pro35S::NcMTP1* line (MTP1), one *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M) compared to one wild-type (WT) *N. tabacum* line grown in O3 metal contaminated soil. (A) Mg concentration in shoot (ppm) (B) and in root (C) Fe concentration in shoot (ppm) (D) and in root (E) Mn concentration in shoot (ppm) (F) and in root in plants after growing for four weeks in O3 metal contaminated soil. Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

In case of control soil grown conditions, all lines had similar shoot and root dry weights, except for the *pro35S::NcZNT1* + *pro35S::MTP1* line, which exhibited lowered root dry weight (Fig. 5 A, B) and in general looked a lot much greener than when compared to O3 soil grown lines There was no significant difference in shoot Zn accumulation between the transgenic and WT lines (Fig 3C). In roots, *pro35S::NcZNT1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines accumulated significantly higher Zn compared to *pro35S::NcMTP1* and WT lines (Fig. 5 C). This root accumulation is consistent with the higher root Zn accumulation

exhibited by these lines when grown in hydroponic system (Chapter 4). All transgenic and WT lines had similar shoot Mg accumulation except for *pro35S::NcZNT1* + *pro35S::MTP1* line which accumulated higher Mg in shoots compared to rest of the lines (Fig. 5 D). In roots, *pro35S::NcZNT1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines accumulated significantly higher Zn

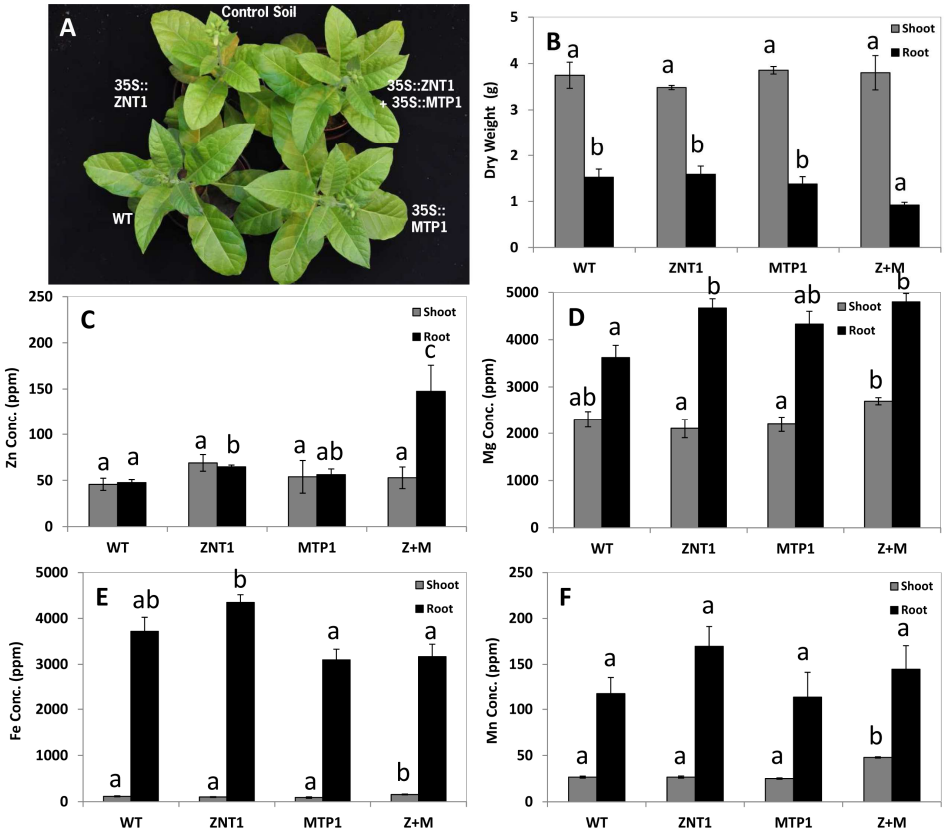

Fig. 5: Phenotypic analysis of one *pro35S::NcZNT1* line (ZNT1), one *pro35S::NcMTP1* line (MTP1), one *pro35S::NcZNT1* + *pro35S::NcMTP1* lines (Z+M) and one wild-type (WT) *N. tabacum* line grown in normal greenhouse soil (control soil). (A) Phenotype of transgenic and WT lines. The photograph was taken after 28 days since transplantation (B) Shoot and root dry weight (g) (C) Zn concentration in shoots and roots (ppm) (D) Mg concentration in shoots and roots (ppm) (E) Fe concentration in shoots and roots (ppm) (F) Mn concentration in shoots and roots (ppm) after growing for four weeks in O3 metal contaminated soil. Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

compared to *pro35S::NcMTP1* and WT lines (Fig. 5 D). This root Mg data is correlated with Zn accumulation of these lines in roots (Fig. 5C). *pro35S::NcZNT1* + *pro35S::MTP1* was the only transgenic line having higher Fe and Mn concentrations in shoot than WT while there was no difference in Fe and Mg concentration in roots of all lines (Fig. 5 E, F).

Single and double *NcZNT1* and *NcMTP1* expressing *N. tabacum* lines exhibited enhanced chlorophyll a, chlorophyll b and carotenoid content

Since excess metals can inhibit photosynthesis due to reduced chlorophyll synthesis, the measure of chlorophyll a, and chlorophyll b provides a good criteria to determine cellular metal toxicity (Ernst, 1980). Furthermore, metal toxicity can initiate the production of hazardous reactive oxygen species (ROS) in plants and the lipophilic antioxidants like carotenoids provide one of the detoxification mechanisms for ROS and they are also important pigments to enhance the efficiency of chlorophyll (Polle and Rennenberg, 1994). So, we measured chlorophyll a, chlorophyll b and carotenoids in our transgenic lines in order to determine cellular metal toxicity and ROS detoxification. All transgenic lines had significantly higher chlorophyll a, chlorophyll b and carotenoids compared to the WT line (Fig. 6 A-C) grown in the metal-contaminated O3 soil. This reflected the higher damage to the photosynthetic machinery and lowered ROS detoxification in WT plants caused by Zn and Cd excess while transgenic lines were better in dealing with metal toxicity. However, all lines had similar chlorophyll a, chlorophyll b and carotenoids when grown in control soil confirming no metal toxicity (Fig. 7 A-C).

***NcMTP1* expressing *N. tabacum* line showed lowered lipid peroxidation**

Lipid peroxidation, which is a measure of plasma membrane damage caused due to metal toxicity (Cuypers et al., 2011), was analyzed by measuring thiobarbituric acid (TBA) concentrations in the *pro35S::NcZNT1, pro35S::NcMTP1, pro35S::NcZNT1* + *pro35S::MTP1* and WT lines grown in the O3 soil and control soil. There were significantly lower TBA concentrations in the shoots of the *pro35S::NcMTP1* line compared to the other lines (Fig. 6 D), indicating a better protection of the plasma membrane in this line. There was no

difference in TBA among lines grown in control soil (Fig. 7 D).

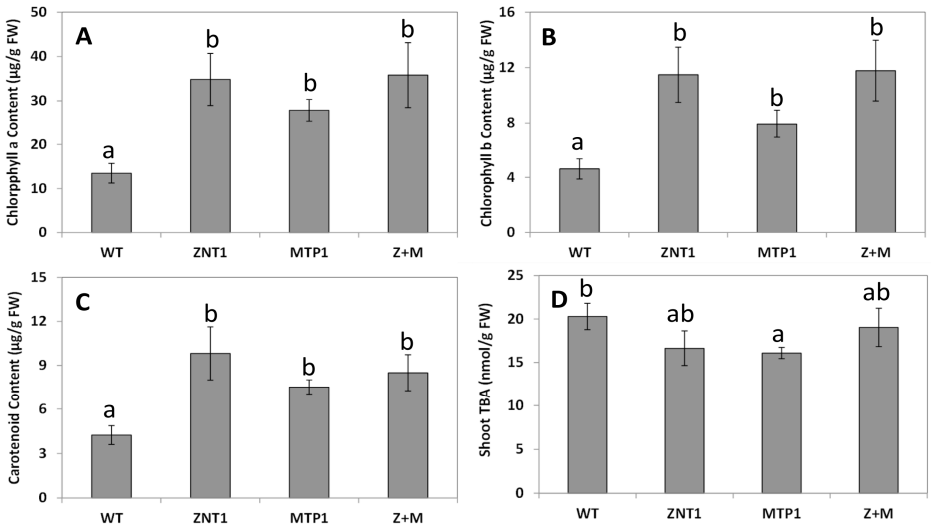

Fig. 6: Chlorophyll a, chlorophyll b, carotenoid and shoot Lipid peroxidation (thiobarbituric acid reactive metabolite (TBA)) contents of one *pro35S::NcZNT1* line (ZNT1), one *pro35S::NcMTP1* line (MTP1), one *pro35S::NcZNT1* + *pro35S::NcMTP1* line (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown in O3 metal contaminated soil. (A) Chlorophyll a content (µg g^{-1} fresh weight) (B) Chlorophyll b content (µg g^{-1} fresh weight) (C) Carotenoid content (µg g^{-1} fresh weight) (D) shoot TBA concentrations (nmoles g^{-1} fresh weight) in plants grown for four weeks in O3 metal contaminated soil. Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

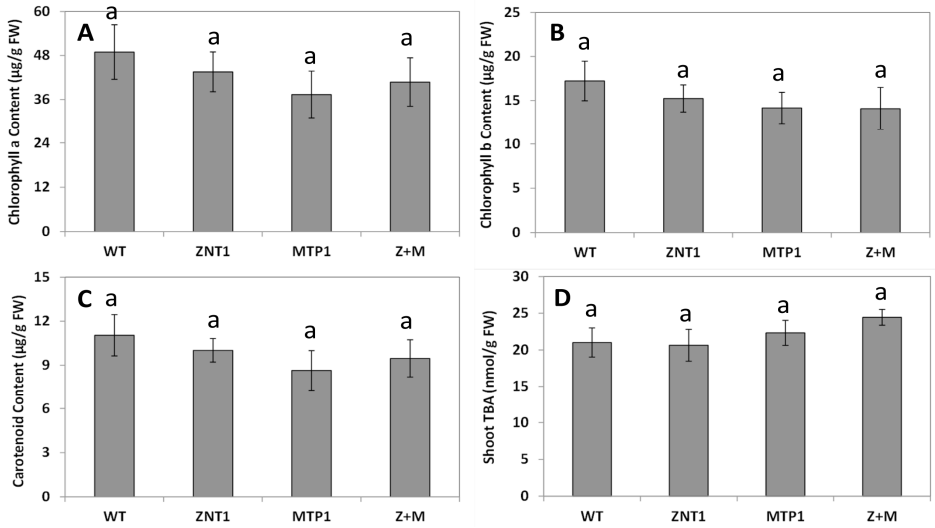

Fig. 7: Chlorophyll a, chlorophyll b, carotenoid and shoot Lipid peroxidation (thiobarbituric acid reactive metabolite (TBA)) contents of one *pro35S::NcZNT1* line (ZNT1), *one pro35S::NcMTP1* line (MTP1), one *pro35S::NcZNT1* + *pro35S::NcMTP1* line (Z+M-1, Z+M-2, Z+M-3) compared to one wild-type (WT) *N. tabacum* line grown in control soil. (A) Chlorophyll a content (µg/g Fresh weight) (B) Chlorophyll b content (µg/g Fresh weight) (C) Carotenoid content (µg g^{-1} Fresh weight) (D) shoot TBA concentrations (nmoles g^{-1} Fresh weight) in plants grown for four weeks in control soil Different letters indicate the significant difference between lines ($p<0.05$, ANOVA, Least Significance Difference) (mean ± SE of four replicates).

DISCUSSION

The biggest limitation in the use of natural hyperaccumulators for phytoremediation is that the biomass production of these species is often not sufficient to remediate soil in a few croppings, but instead several years are required. The phytoremediation time may be reduced if higher biomass producing species, such as tobacco, corn, maiden grass (*Miscanthus)*, willow or poplar, are engineered to express genes that enhance their metal tolerance and hyperaccumulation traits. The present study aimed at analysing the potential for enhanced Zn and Cd tolerance and accumulation of single *pro35S::NcZNT1, pro35S::NcMTP1* and double *pro35S::NcZNT1* + *pro35S::NcMTP1* expressing *N.*

tabacum lines in soil collected from a metal-contaminated site. Since most of the analysis of metal tolerance and accumulation are carried out in hydroponic systems or metal-spiked soil, which does not represent the contaminated soil characteristics in the field (Megharaj and Naidu, 2003), our study is unique as we have tested our transgenic *N. tabacum* lines in an original metal-contaminated soil.

The metal contaminated soil (O3) used in this study contained extremely high Zn (up to 656.71 mg kg-1 DW) and moderately high Cd concentrations (up to 2.11 mg kg-1 DW) (Fig. 2 A, B) compared to regular agricultural soils, which contain 1-1000 mg Zn kg-1 DW and 0.2-1.0 mg Cd kg-1 DW (Jain et al., 2010; Clemens et al., 2012). The slightly higher pH of the O3 soil is likely to result in a reduced metal solubility and bioavailability and consequently to lower metal uptake than would be expected based on total soil metal concentrations (McBride et al, 1997). Metal tolerance as reflected in the higher shoot and root dry weights of *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::MTP1* lines grown in O3 soil reflected their ability to deal with Zn and Cd toxicity. These lines also accumulated higher total plant Zn and Cd content compared to wild type which illustrates that they are promising for phytoremediation. The interesting aspect of this study was that our best performing line, *pro35S::NcMTP1*, showed the ability to reduce the Cd pollution level of O3 soil from 2.11 mg kg-1 to 1.05 mg kg-1 (which lies in the normal range in agricultural soils; Clemens et al., 2012) in only 14 generations compared to the 28 generations for WT (Table 1). The could save lot of time to remediate Cd compared to WT. Furthermore, this line also had the highest Cd BioAbsorption (BAC; 21.95) and Translocation Factor (TF; 0.38) (Table 2) among all lines. All these properties demonstrate this line to be the best suitable for Cd phytoremediation among all the tested lines. Previously, a BAC factor of less than one was reported for many non-accumulators, which make them inappropriate for phytoextraction because it could take more than 100 generations to reduce the pollution levels to half (McGrath and Zhao, 2003). The authors calculated that the crops producing 10 ton hectare^{-1} with a BAC around 20 will take less than 10 generation to reduce metal pollution to half. *N. tabacum* was found to generate a biomass of about 8.4 ton hectare^{-1} when grown in very

marginal metal polluted field near by our soil sampling site in NE Belgium (Ruttens and Vangronsveld, unpublished data; http://enfo.agt.bme.hu/drupal/sites/default/files/ARuttensPhytorem.pdf). If we assume that our *pro35S::NcMTP1* line will produce the same biomass, than it can reduce the Cd pollution in O3 soil to half in less than 10 generations, since it showed a BAC of 21.95. This makes it very promising for phytoextraction of Cd. However in case of Zn, it will take about 110 generations to reduce the Zn pollution level to half (from 656.7 mg Zn kg^{-1} to 328.35 mg Zn kg^{-1}) compared to 176 generations needed for the WT lines to do this job. While assuming that the decrease is following a mathematical pattern, we can predict that it will probably take another 110 generations to bring the Zn pollution close to 100 mg Zn kg^{-1} (found in normal soils). Thus this line does not seem to be as good for Zn phytoremediation as for Cd. However, we should also take into account that in this experiment, *N. tabacum* lines were only grown for four weeks, while *N. tabacum* is an annual summer crop with a reported biomass of 170 ton $hectare^{-1}$ in best agronomic conditions while 8.4 ton $hectare^{-1}$ in contaminated soils (Schillberg and Emans, 2003; Ruttens and Vangronsveld, unpublished data). So it will take less time to remediate Zn and Cd as estimated for our four week grown plants. *N. tabacum* was found to be better phytoextractor than maize, rapeseed and sunflower (Ruttens and Vangronsveld, unpublished data). Furthermore it was suggested that *N. tabacum* could be useful for phytoremediation of low to moderate Cd pollution. In light of these facts, our transgenic *pro35S::NcMTP1 N. tabacum* line is promising for field oriented Cd phytoextraction in moderately polluted soils. Further experiments with using various pollution levels can determine the maximum potential of this line for phytoextraction purposes.

Another noticeable aspect was the differential metal tolerance and accumulation exhibited in the transgenic lines in hydroponics (Chapter 4) compared to contaminated soil. Metal accumulation was found to be higher in all transgenic lines when grown in hydroponic system than in the contaminated O3 soil. Hydroponics is a good system being used in a large majority of metal-related research activities as it is excellent for dedicated phenotypic studies, under optimum pH, to understand physiological processes and new gene

functions. However, it does not represent actual field conditions, where generally metals are mixed and less available than in hydroponics. We have observed that contaminated O3 soil had mixed pollution of very high Zn and Cd concentrations and had a higher pH (above 7; Fig. 2 E) which is known to contribute to reduced metal bioavailability (Megharaj and Naidu, 2003; McBride et al, 1997). Furthermore, in contaminated soils metal are less mobile due to other factors like physico-chemical properties of the soil, organic content, chemical forms of metals, ageing of metals and soil biota (Megharaj and Naidu, 2003). All these factors reduce the metal bioavailability compared to a hydroponic system. Thus the more reliable way of testing the actual phytoremediation potential of plants is to use contaminated soil rather than a hydroponic system, although the latter will probably give a good clue as to the theoretical potential, and is generally easier to use in a laboratory set-up.

Previously, we have shown that our double transgenic line had even higher Zn and Cd accumulation than single transgenic lines in hydroponic conditions (Chapter 4). This led us to conclude that the combined expression of *NcZNT1* and *NcMTP1* has an additive effect on metal accumulation. However, this was not confirmed in the contaminated soil grown experiment where *pro35S::NcMTP1* was the better performing line both in terms of biomass and total plant metal content. Since metal accumulation in the double transgenic was found to be higher in the hydroponic system, we consider that Zn and Cd may not be highly bioavailable in the polluted O3 soil compared to hydroponics, as explained above, which could have led to reduce metal accumulation in double transgenic line. This is consistent with the higher pH of O3 soil (pH above 7; Fig. 2 F) compared to optimum pH in hydroponics system (5.8; Chapter 4) and the high Zn uptake in the roots of this line when grown in control soil with lower pH then the contaminated soil (Fig. 5 C). Thus we conclude that in contrast to the hydroponic experiments, the expression of both *NcZNT1* and *NcMTP1* transporters is not conferring higher metal accumulation in polluted soil system. The *pro35S::NcZNT1* line did not have a higher biomass than WT when grown in O3 soil. On the other hand, there was significant improvement in root Cd accumulation in this line compared to *pro35S::NcMTP1* and WT lines when grown in O3 soil and higher root Zn in control soil, which

confirmed its previously known ability to accumulate Zn and Cd (Chapter 3, 4). The possible reason of its lowered growth could be the higher Zn and Cd uptake in inappropriate tissues, which the plant was unable to store in vacuolar compartments. This could lead to affect its root metabolism since roots are the first tissues to be contact with metals. This ultimately could affect shoot and the overall metal toxicity resulted in reduced growth. This means that this line may not be useful for phytoremediation in highly contaminated soils since there would be no sink i.e. in the vacuoles, for the accumulated metals for detoxification.

Lipid peroxidation is caused due to the production of ROS upon metal toxicity which causes oxidative damage to plasma membranes since these membranes are the primary target of metal action (Cuypers et al 2011). The *pro35S::NcMTP1* line had a lowered lipid peroxidation compared to WT and other transgenic lines. This means less plasma membrane oxidative damage was caused by excess Zn and Cd, probably due to vacuolar sequestration of these metals in this line. Also this line together with all transgenic lines had significantly higher chlorophyll a, chlorophyll b and carotenoid content when grown in O3 soil (Fig. 6 A-C), displaying less damage to the photosynthetic apparatus. This correlates very well with the higher shoot Mg concentrations in all the transgenic lines (Fig. 4 A). There was also enhanced Mg in roots of *pro35S::NcZNT1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* lines grown in control soil (Fig. 5 D). Mg is a well-known central constituent of chlorophyll molecules and thus essential for photosynthesis (Knoop et al, 2005). Zn and Cd have similar ionic radii and may displace Mg ions in chlorophyll and Rubisco, thus rendering them inactive (Kupper et al., 1998; Van Assche et al., 1986). Therefore the enhanced Mg accumulation could have prevented part of the Mg displacement in chlorophyll and Rubisco and subsequently reduce the photosynthetic damage, contributing to reduced chlorosis in the leaves of all transgenic lines (Fig. 3 A). There are two possible ways to increase Mg in these lines, one would be the direct Mg transport by NcZNT1 and NcMTP1 proteins, which is not known to occur, or it would be due to the increased transport by regular Mg transporters. The latter seems more plausible. It was previously found that Cd tolerance in *Brassica rapa* is enhanced by enhanced Mg supply

(Kashema & Kawai, 2007). In contrast, Mg starvation alleviated Cd toxicity in *A. thaliana* probably through low Mg dependent enhanced anti-oxidative capacity (Hermans et al., 2011). Further experiments are therefore needed to determine the expression of known Mg transporter genes involved in this enhanced shoot Mg uptake. It is known for quite some time that Mg and Fe have an antagonistic behaviour in plants (Agarwala & Mehrotra, 1984). Our transgenic lines had a lower Fe accumulation but higher Mg accumulation in shoot tissues, which is consistent with these studies. Also Hermans et al. (2010) reported an increase in Fe concentration in *A. thaliana* when grown in Mg deficient conditions. Since transgenic lines also had markedly lower Fe in shoot tissues, it is plausible that Fe uptake transporters like IRT1 would be upregulated and could contribute to Zn and Cd transport. *IRT1* was found to be highly expressed in *pro35S::NcZNT1* and *pro35S::NcMTP1* expressing *A. thaliana* lines exposed to Zn and Cd excess and could be responsible for the increased Zn and Cd transport (Chapter 2, 3). All these observations and previous reports suggest complex interactions among different metals, which is not straightforward to understand without additional research into the molecular mechanisms underlying these interactions.

There is little knowledge about enhancing Zn, and more importantly Cd, tolerance and accumulation in *N. tabacum* by genetic engineering while testing these traits in metal contaminated field soil. Previously, expression of a mammalian metallothionein (MT) gene in *N. tabacum* increased its resistance to high Cd but the plants were tested in a MS-based agar-medium system and not in contaminated soil (Pan et al. 1994). Transgenic *N. tabacum* expressing CAX2, a vacuolar Ca^{2+}/H^{+} antiporter from *A. thaliana*, had a higher Cd and Mn accumulation and Mn tolerance when grown in a hydroponic system (Hirschi et al., 2000). There was a higher accumulation of Cu but not Cd when *N. tabacum* was transformed with the yeast metallothionein gene *CUP1* (Thomas et al., 2003). Enhanced tolerance to Zn in *N. tabacum* was also reported by the single and combined expression of glyoxalase I and II, however this this study was again not conducted in metal contaminated field soil (Singla-Pareek et al., 2006). Increased mercury accumulation and tolerance by enhanced volatilization was reported upon the expression of bacterial *merA* and *merB* genes in *N. tabacum*

grown in hydroponics (Hussein et al., 2007). All these studies were conducted in either MS agar-media or hydroponic systems and not validated under contaminated soil grown conditions. Thus, our testing of transgenic *N. tabacum* lines in an original metal-contaminated soil appears rather unique. Unfortunately, the higher total plant metal content exhibited by *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* is still little compared to the 100 fold higher Zn and Cd accumulation in *N. caerulescens* compared to non-accumulators. This leads us to conclude that CaMV35S promoter mediated expression of these genes is not appropriate to approach *N. caerulescens*-like hyperaccumulation phenotypes in *N. tabacum*.

The data from the current study demonstrate that it is possible to engineer the high biomass plant species *N. tabacum* for enhanced Zn and Cd tolerance and accumulation when grown in toxic metal contaminated soil, by expressing the *NcMTP1* and *NcZNT1* genes from *N. caerulescens*. It is encouraging for us to further analyse these lines in actual field conditions which would be useful for Zn and Cd phytoextraction. Particularly, the 14 generations of *pro35S::NcMTP1* required to remediate Cd from O3 soil make it suitable to grow in field trials. *N. tabacum* was proposed as suitable species for phytoremediation of soils with pollution in 0-30 cm depth (Vangronsveld et al, 2009). It has various advantageous like moderate tolerance to metals, particularly to Cd, high yield (up to 170 ton hectare^{-1}), extensive root system and low costs of cultivation (Schillberg et al., 2003; Cramer et al., 1999). This species has a fast growth rate, low nutrient requirements and easy harvest (Sarret et al., 2006). Its non-food crop nature and its self-pollination will reduce any risk of transgene contamination of nearby crop varieties (Hussein et al., 2007), especially in Western Europe, where tobacco is no longer a widely grown crop. The transgenic tobacco lines which we have developed have the additional advantages of higher biomass production and increased Zn and Cd tolerance and accumulation when grown in metal contaminated soil. This enables them to be promising for phytoremediation from economical point of view.

ACKNOWLEDGEMENTS

This research was supported by the Higher Education Commission of Pakistan (HEC), graduate school Experimental Plant Sciences and COST (European Cooperation in Science and Technology) "Action FA0905" "STSM" grant. We would like to thank Ann Wijgaerts for help in pigment and TBA analysis and support in growing and harvesting of plant material. We are grateful to Mohamed El Soda for assistance in statistical analysis. We are thankful to Dr. Maarten Koornneef for critical reading and suggestions for the preparation of this manuscript.

CHAPTER 6

GENERAL DISCUSSION

Metals like Zn, Fe, Cu, Mn and Ni are essential micronutrients required for the proper functioning of various physiological and metabolic processes in plants (Ramesh et al., 2004). Other metals like Cd, Hg and Pb have no known biological function in plants. Cd is dangerous for humans as it is also a carcinogenic metal. Since it is widely distributed in the environment and used in industry, it currently ranks 7th on US-Agency for Toxic Substances and Disease Registry (ATSDR) priority list of hazardous substances (http://www.atsdr.cdc.gov/cercla/07list.html). It can enter the food chain and can reach humans via foods derived from Cd polluted plants (Clemens et al., 2012). Exposure to the elevated levels of these metals causes toxicity in plants. The result of Zn and Cd toxicity is leaf chlorosis and growth reduction in plants and prolonged or extreme exposure ultimately leads to death of the plant. A very high concentration of over 1,000 mg Zn kg^{-1} dry soil was reported in certain polluted areas while most of the agricultural soils contain 10-100 mg Zn kg^{-1} dry soil as background levels (Audet and Charest 2006; Marschner, 1995). In case of Cd, toxic concentrations of about 50 mg Cd kg^{-1} of dry soil were recorded in polluted soils while normally soils contain less than 1.0 mg Cd kg^{-1} dry soil (Clemens et al 2012; UNEP, 2008). Where do these elevated levels of metals come from? Many human activities like mining, smelting, atmospheric deposition of industrial emissions, land filling of industrial wastes, the use of irrigation water containing industrial effluents, the use of phosphate and manure as fertilizers are causing the increase in soil metal pollution (Solti et al., 2008). This enhanced pollution can be a threat for crop yields, disturb ecosystems and can eventually cause serious human health hazards (Sharma and Aragwal, 2005). How to cope with these contaminants and to reduce their threat? There are various conventional soil remediation methods like excavation with off–side treatment, leaching, vitrification, electrokinetical treatment and chemical oxidation and reduction but they are expensive, disturb soil structure and fertility and are limited to be used in small areas (McGrath et al., 1997; Luo et al., 2000; Zhao et al., 2000; Wu et al., 2010). This has forced the researchers to develop suitable alternatives for soil remediation.

From some time, the use of plants to remediate metal contaminated soils is proposed, as this is a cheap and environmental friendly method (Brooks

et al., 1998; McGrath and Zhao, 2003). This emerging technology is known as phytoremediation and can involve the use of metal tolerant plants to contain a polluted soil from further dispersal of metals by runoff, leaching and erosion (phytostabilization) (Chapter 1). Another type of phytoremediation utilizes metal tolerant accumulator plants to extract metals from the soils into their harvestable parts (phytoextraction). The concentrated metals can then be recovered, either for proper and controlled disposal, or for re-use along with the non-conventional advantage of biofuel production. However, these technologies can only become practically feasible if fast growing; high biomass species equipped with enhanced metal tolerance and accumulation properties are identified or developed and utilized.

There are few metal hyperaccumulator plant species found in nature with 100 fold higher Zn and Cd uptake into their shoot compared to non-accumulators, without exhibiting toxicity symptoms (Chapter 1). Slow growth and low biomass production are the major limitations for the exploitation of natural hyperaccumulators for phytoremediation, because it can take many croppings and in fact years to efficiently remediate a piece of polluted soil. So what could be the possible solution for these limitations? Genetic engineering has opened new technological horizons which could be useful to cope with this problem. One way of dealing with this issue is to enhance the biomass production of these natural hyperaccumulators. Since biomass is a complex multigenic trait (Robins et al., 2007) and difficult to engineer, the most interesting alternative will be to genetically modify high biomass producing crop species with genes conferring higher metal tolerance and accumulation, which are believed to be relatively few in number (Chapter 1).

Noccaea (previously called *Thlaspi*) *caerulescens* (J.&C. Presl) F.K. Meyer is a metal hypertolerant and hyperaccumulator species with tremendously enhanced ability of shoot accumulation of Zn (30 g kg^{-1} DW) Cd (2.7 g kg^{-1} DW) and Ni (4 g kg^{-1} DW) (Reeves and Brooks, 1983; McGrath et al., 1993; Brown et al., 1995; Lombi et al., 2000). This species is used as a model to unravel the molecular mechanisms underlying metal hypertolerance and hyperaccumulation because of its close relatedness to the known plant model species *Arabidopsis thaliana* (88% sequence identity in coding regions (van de

Mortel et al., 2006; Rigola et al., 2006), and its favourable characteristics like diploid nature, small chromosome number (n=7), self-compatibility and high fecundity (Assunção et al., 2003; Rigola et al., 2006; Milner and Kochian, 2008). Furthermore, there lies a substantial natural variation in its different accessions for these traits which can be exploited for the identification of underlying genes (Alonso-Blanco et al., 2009). Comparative transcriptomic analysis of *N. caerulescens* with non-accumulators *A. thaliana and Thlaspi arvense* was performed, which resulted in the identification of numerous candidate genes involved in metal hyperaccumulation (van de Mortel et al., 2006; Hammond et al., 2006). A cumbersome stable transformation system is a limitation for the rapid understanding of gene functions in this species. Nevertheless, an efficient root transformation system is available for *N. caerulescens* (Chapter 2) and the ability to express its genes in related heterologous systems like *A. thaliana*, are useful tools to perform functional analysis of its hyperaccumulation related genes. The generation and screening of mutants is a useful approach for functional analysis using forward genetics (from mutant to gene) now that next generation sequencing technologies can be used to speed up the cloning of the mutated genes (Beckmann et al., 2012; Hartwig et al., 2012) and this may open new insights in understanding metal hypertolerance and hyperaccumulation in *N. caerulescens*. As mentioned earlier, understanding the molecular basis of metal hypertolerance and hyperaccumulation in *N. caerulescens* and other heavy metal accumulators such as *Arabidopsis halleri* (McGrath and Zhao, 2003) is essential for the development of a viable GMO based phytoremediation technology as it provides the genes and regulatory elements that are essential to properly transfer the metal hyperaccumulation and/or hypertolerance traits to transgenic high biomass plants.

The present study was aimed at the functional analysis of two previously identified Zn transporter genes, *NcZNT1* and *NcMTP1* from *N. caerulescens*, for phytoremediation purposes. The major questions which I addressed were:

1. Are *NcZNT1* and *NcMTP1* involved in Zn and Cd tolerance and accumulation and what is their role in these processes in *N. caerulescens*?

2. Can the obtained knowledge about these genes be utilized for practical implications in phytoremediation?

The analysis of these genes confirmed their involvement in Zn and Cd tolerance and accumulation and enlightened their role in *N. caerulescens*. Furthermore, expression of *NcZNT1* and *NcMTP1* genes in high biomass *Nicotiana tabacum* enhanced the phytoremediation potential of this crop species. As we took a further step of analysing the engineered transgenic *N. tabacum* lines in an original metal contaminated field soil, the positive results will open future possibilities of their utilization for phytoremediation.

The functional analysis of *NcZNT1* and *NcMTP1* genes was performed by expressing them into *A. thaliana* and *N. tabacum*, by knocking down the expression of *NcMTP1* in *N. caerulescens* and finally by promoter and copy number analysis. The choice of *N. tabacum* as test crop for phytoremediation was made because it is a crop species which can be easily transformed with high biomass, fast growth and already moderate tolerance to metal exposure (Sarret et al., 2006), which previously tempted others to propose it as a suitable candidate for phytoremediation of metals occurring in 0-30 cm soil depth in contaminated soils (Vangronsveld et al., 2009).

In natural hyperaccumulators, the first step in metal accumulation is root metal uptake. The metals are thereafter loaded into the xylem to be transported into shoot tissues which are the ultimate sites of metal storage. In chapter 2 of this thesis, we have demonstrated the role of *NcZNT1* in cellular Zn uptake into root stele of *N. caerulescens* and that its constitutively high expression in *A. thaliana* enhanced Zn and Cd accumulation. Since *NcZNT1* is a plasma membrane localized transporter of Zn, and probably Cd, this gene can be utilized to improve the metal accumulation in high biomass plants useful for phytoremediation. One striking characteristic of Zn hyperaccumulators is the high expression of Zn homeostasis genes (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006; Talke et al., 2006; van de Mortel et al., 2006, 2008). In non-hyperaccumulators these genes are mainly induced upon Zn deficiency. Therefore, the transcription factors controlling the Zn deficiency response in plants are likely to be important regulators of hyperaccumulation traits. From the differential regulation of *NcZNT1* and its homologue *AtZIP4* (from *A.*

thaliana) in *A. thaliana* and *N. caerulescens*, we can deduce the presence of a new *cis* element, apart from already known *cis* elements (Assução et al., 2010) in the *NcZNT1* promoter. It is likely that a transcription factor (TF) from *N. caerulescens* can bind to this *cis* element in the *NcZNT1* promoter and thus control the hyperexpression of the *NcZNT1* gene. In principle, only altering the *cis* element, so that it recruits an existing TF, would be sufficient for the higher expression of *NcZNT1* in *N. caerulescens*. However, it would not be unlikely that the transcriptional regulation of *N. caerulescens* TFs is also different from *A. thaliana*. This will further lead to the experimental testing of the above mentioned hypothesis that activation or changes in the expression of TFs, rather than an altered expression of their target genes, have resulted in the evolution of metal hypertolerance and hyperaccumulation. As *bZIP19* and *bZIP23* TFs, which regulate the expression of several Zn deficiency related genes, were identified by the *AtZIP4* promoter analysis in *A. thaliana* (Assunção et al., 2010), the search for these TFs in *N. caerulescens* and their functional analysis might shed light on their involvement in regulating the high expression of *NcZNT1*. An alternative to the use of *N. caerulescens* specific regulatory elements is the use of constitutive promoters like CaMV 35S, although these will lack the fine-tuning of the endogenous promoter (in terms of tissue specific and inducible expression). The latter approach was pursued in this study.

Once the metals are taken up by the hyperaccumulators, they need to be stored in proper organelles for detoxification. Our analysis of *NcMTP1* in chapter 3, illustrate the involvement of this gene in Zn and Cd accumulation and most interestingly their effect on metal tolerance when expressed constitutively at a high level in *A. thaliana*. Sequestering the metals into vacuoles can confer tolerance as exhibited by *NcMTP1* and this mechanism is essential to engineer high biomass species for phytoremediation. This tolerance phenomenon permits the plant to continue its growth under elevated metal exposure and ultimately result in higher biomass production, which is an essential property for the plants utilized for phytoremediation. Another interesting observation was the increase in Zn accumulation in shoot tissues of *N. caerulescens* upon knocking down *NcMTP1* expression in roots. *N. caerulescens* is known to have reduced retention of metals in root tissues

compared to non-accumulators which plays a role in enhanced shoot translocation (Lasat et al. 1998). This indicates that further lowered accumulation of metals in root vacuoles increases metal availability for shoot translocation in *N. caerulescens*. It emphasizes that if we want to increase the shoot metal concentrations in plants for phytoremediation, we need to engineer them with low metals retention capacity in root tissues. The *in silico* analysis of the *NcMTP1* promoter led to the identification of *cis* regulatory elements, which could be involved in the regulation of *NcMTP1*. Further deletion and point mutation analysis of these elements are needed to disclose their role, if any, in *NcMTP1* regulation and subsequent identification of the transcription factors controlling the expression of this gene. The analysis of the *NcMTP1* promoter + GUS/GFP reporter constructs in *N. caerulescens* might reveal the tissues specific localization of this gene, which will further help to understand its role in metal tolerance and accumulation.

As some of the known Fe responsive genes can transport Zn and Cd next to Fe (Vert et al., 2002; Korshunova et al., 1999) they could possibly play a role in indirect Zn and Cd accumulation. Previously, Cd uptake was reported due to the up regulation of *NcIRT1* in *N. caerulescens* under Fe deficient conditions (Lombi et al., 2002). There was also a seven fold higher root Cd influx in *Pisum sativum* seedlings when grown under Fe deficient compared to Fe sufficient conditions (Cohen et al., 1998: 2004). We have observed the upregulation of Fe deficiency responsive genes like *AtBHLH100, AtIRT1, AtIRT2, and AtFRO2* in our *NcZNT1* and *NcMTP1* expressing *A. thaliana* lines grown in Zn and Cd excess (Chapter 2, 3). This implies the involvement of these Fe responsive genes in higher Zn and Cd accumulation due to their broad metal specificities (Vert et al., 2002; Korshunova et al., 1999). Thus these Fe deficiency responsive genes could also be useful for enhancing Zn and Cd accumulation in plants for phytoremediation. From this knowledge we can also deduce that the problem of Cd accumulation in food crops could be decreased by supplementing them with Fe fertilizers since Fe transporters have more affinity for Fe, they will not be highly expressed and their indirect effect on Cd accumulation will be reduced.

From the functional analysis of *NcZNT1* and *NcMTP1* genes as described in Chapter 2 and 3, I hypothesized that the combined expression of both genes might further increase metal accumulation and tolerance properties in high biomass plants useful for phytoremediation. This led us to engineer *N. tabacum* having separate and combined expression of the *NcZNT1* and *NcMTP1* genes (Chapter 4). *NcZNT1* expressing *N. tabacum* lines had higher Zn and Cd accumulation while *NcMTP1* expressing lines accumulated more Zn and Cd and showed improved tolerance to these metals higher than *NcZNT1* and wild-type (WT) line when tested in hydroponic system. The combined expression of both genes could further enhance Zn and Cd accumulation and tolerance compared to single transgenic and WT line. This study confirmed that it is possible to increase Zn and Cd tolerance and accumulation by expressing *NcZNT1* and *NcMTP1*. These acquired properties could be useful for field oriented phytoremediation purposes.

To our knowledge, almost all the studies targeted at engineering *N. tabacum* with higher metal tolerance and accumulation, used optimal hydroponic growth conditions for the analysis of these traits, while they were hardly ever tested in metal contaminated field soil (Pan et al. 1994; Hirschi et al., 2000; Thomas et al., 2003; Singla-Pareek et al., 2006; Hussain et al., 2007). The only exception I could find refers to the transformation of *Nicotiana glauca*, a relative of *N. tabacum*, with the *Phytochelatin Synthase 1* gene from wheat (*TaPCS1)* that confers enhanced Zn and Pb accumulation when grown in soil from a Cu, Zn and Pb mine (Marinez et al., 2006). Since metals are more bioavailable, a hydroponics system allows excellent conditions for phenotypic studies to understand the physiological processes and gene functions. However, in actual field soils a mix of toxic metals is generally found, which are often less bioavailable than when supplied in hydroponics (Chapter 1). This lack of analysing the potent phytoremediator plants in a soil system is a limitation to the assessment of their phytoremediation potential. Chapter 5 documents our efforts to evaluate six different metal contaminated soils and the performance of our transgenic *N. tabacum* lines (described in chapter 4) in a contaminated soil system. The results demonstrated that the *NcMTP1* and *NcZNT1* + *MTP1* lines were better protected than non-transgenic plants against

the excess of Zn and Cd present in the contaminated soil O3, and that they had an enhanced potential for phytoremediation. Particularly the *NcMTP1* expressing line had the potential to reduce the Cd pollution from 2.11 mg kg^{-1} to 1.05 kg^{-1} (which is close to an acceptable level in agricultural soils; Clemens et al., 2012) in only 14 generations. This estimate is based on the biomass and metal accumulation after four weeks of growth. Since *N. tabacum* is an annual summer crop with a maximum reported biomass of 170 ton $hectare^{-1}$ in best agronomic conditions (Schillberg et al., 2003) while it could yield at least 8.4 ton $hectare^{-1}$ when grown in very marginal high polluted soils (Ruttens and Vangronsveld, unpublished data). Thus we are certain that our *pro35S::NcMTP1* transgenic line will be able to remediate Cd from polluted O3 soil in less than 14 generations when grown for longer time in field conditions. This line can be useful for remediation of medium polluted soils with metals existing in the top soil layer of 30 cm, where they are accessible for *N. tabacum* roots. The *NcZNT1* expressing line had the same biomass as that of the WT line but was significantly reduced compared to *NcMTP1* and *NcZNT1* + *NcMTP1* expressing lines. This clearly illustrates that this line suffered from metal toxicity due to lack of a metal detoxification system and did not accumulate higher concentration of metals although it had higher Cd in roots, which confirmed its Cd uptake ability. The poor performance might be due to the reduced bioavailability of metals in the polluted soil which did not allow efficient metal uptake in this line. This underlines that efforts to improve phytoremediation potential should also consider improving the metal detoxification system . Since our double transgenic lines had higher Zn and Cd accumulation in an hydroponic system (Chapter 4) than in polluted soil, compared to single *NcMTP1* line, we conclude that the expression of both NcZNT1 and NcMTP1 transporters is not giving better phytoremediation results than expression of NcMTP1 alone. This study also urges for further analysis of our transgenic *N. tabacum* lines in other soil metal pollution levels to discover the pollution level limits at which they can still be efficiently utilized. Interestingly, the transgenic lines accumulated higher Mg levels in shoots which correlated with enhanced chlorophyll a, chlorophyll b and carotenoid contents and ultimately less photosynthetic damage than the WT line. Mg is a well-known central

constituent of chlorophyll molecules and thus essential for photosynthesis (Knoop et al, 2005). Zn and Cd have similar ionic radii and may displace Mg ions in chlorophyll and Rubisco, thus rendering them inactive (Küpper et al., 1998; Van Assche et al., 1986). Therefore the enhanced Mg accumulation could have prevented part of the Mg displacement in chlorophyll and Rubisco and subsequently reduce the photosynthetic damage, contributing to reduced chlorosis in the leaves of all transgenic lines (Fig. 3 A). It was reported that Cd tolerance is enhanced by supply of Mg in *Brassica rapa* (Kashema & Kawai, 2007). Thus we can deduce that the fertilization of Mg in food crops might alleviate metals, particularly Cd, toxicity.

Although we have demonstrated that the CaMV35S promoter mediated expression of *NcZNT1* and *NcMTP1* has resulted in enhanced tolerance and accumulation, this is still low compared to the 100-fold higher metal accumulation in *N. caerulescens* (Chapter 1). The next question would be on how can we further enhance the phytoremediation capacity of *N. tabacum* by using the information generated in the current thesis? We propose that the utilization of tissue specific expression of these genes might lead to a much higher metal accumulation in *N. tabacum*. If *NcZNT1* is expressed in endodermal and pericylce cells of *N. tabacum*, as it is expressed in *N. caerulescens*, it will allow more metals to be available for xylem loading and ultimately shoot translocation will be higher. However, a proper expression will be needed as lower expression of this gene may not be helpful in accumulating higher metals while higher expression might lead to toxicity in associated cells due to higher expression. Thus a proper expression will keep a gradient for metals for shoot translocation. Mathematical modelling predicted that an activator-inhibitor model for bZIP transcription factors can outperform an activator model to provide robust and stable homeostasis of metals (Claus and Krauser, 2012). This means that only higher expression of bZIP transcription factors may not maintain a stable homeostasis instead tight regulation is needed to do the proper job. This also means that a sink for metals is needed to maintain a gradient for higher accumulation. Leaf cell vacuoles, particularly in the leaf epidermal cells, are the sinks for Zn and Cd in *N. caerulescens*. Epidermal cells may be preferred, as they mostly lack chloroplasts which are easily

compromised by high metal concentrations, however, mesophyll cells also contained high metal accumulation (Vogeli-Lange and Wagner, 1990; Küpper et al., 1999). Therefore, we suggest to express the *NcMTP1* gene at high levels especially in shoot epidermal cells of *N. tabacum*, to further enhance accumulation. Such tissue specific expression might create the required sink for toxic metals, and thus contribute towards higher shoot accumulation. We also recommend that a reduced *NcMTP1* expression in *N. tabacum* root tissues will favour the metal translocation to shoot tissues (Chapter 3), but a basal metal tolerance mechanism would be needed for the protection of root cells from metal toxicity. The combined expression of *NcZNT1* and *NcMTP1* under the control of their own promoters is also worth trying. As we have illustrated, single and also double transgenes expression enhanced Zn and Cd tolerance and accumulation in *N. tabacum* (Chapter 4) but not in soil (Chapter 5), but most of the Zn and much Cd was retained in the roots of our transgenic lines. It is likely to assume additional genes are needed to create a gradient to further improve shoot translocation of these metals. We propose to use the tissue specific expression of *HMA4* gene, which is involved in xylem loading of metals for shoot translocation ('O Lochlainn et al., 2011; Hanikenne et al., 2008), into these transgenic background lines. However, it should be taken into account that too much or too little expression of this gene might result in excess metals in the cells where HMA4 is expressed (and killing them) or have no effect. Thus a proper expression of the *HMA4* gene, to maintain the required metal gradient in the root is recommended. This will further enhance the shoot accumulation of metals which is essential for easy harvesting of the biomass. The *NcHMA3* gene (*Heavy Metal ATPase 3*; Ueno et al., 2011) was reported to be involved in Cd sequestration into the vacuole. This is also an interesting candidate to be expressed in shoot epidermal cells to enhance shoot Cd tolerance and accumulation in *N. tabacum*. Further expressing any transcription factors (TFs) found in *N. caerulescens* which control the expression of various metal responsive genes will be very interesting to also try in *N. tabacum*. Since regulation of gene expression in *N. caerulescens* appears to be crucial for metal hyperaccumulation (Chapter 2), by using newly identified TFs, we might mimic this phenotype in *N. tabacum* while using only few transgenes.

Next to the engineering challenges, public acceptance of genetically modified (GM) plants will be a major limiting factor for the development of a GMO based phytoremediation technology. Acceptance of GMOs will definitely boost the development of these innovative solutions to remediate and exploit the metal polluted areas. There are various concerns about growing GMOs from an ecological and economical point of view, since there are intellectual property rights associated with their use. There are likely to be specific concerns about using transgenic *N. tabacum* in the field. These include gene escape to nearby wild varieties which might cause environmental issues. *N. tabacum* is used for smoking and it is known that about 50% of the Cd present in a smoker's body is due to smoking (Clemens et al., 2012). Thus out-crossing of metal accumulation genes into *N. tabacum* varieties used for smoking will pose an even higher threat to smokers' health. However, *N. tabacum* is self-pollinated, and without any further modifications, the transgene outflow will generally be low. Furthermore, this phenomenon can be decreased by using chloroplast transformation system in *N. tabacum*, since chloroplast are inherited maternally (Ruf et al., 2007) but this might affect the targeting of metal transporter proteins to the proper membranes because they will be expressed in chloroplasts (Marchis et al., 2012). Nevertheless, chloroplasts transformation might be useful for expressing genes involved in synthesis of organic acids that are known metal chelators such as histidine (Kramer et al., 1996), nicotianamine, citrate, malate or oxalate (Senden et al., 1995) that could reduce the metal toxicity effect on photosynthesis (Molins et a., 2012; Kojima et al. 1987; Prasad et al., 1991; Singh et al., 1991). Another interesting alternative will be to engineer already developed cytoplasmic male sterile *N. tabacum* lines with *NcZNT1* and *NcMTP1* (Ruiz and Daniell, 2005). This will certainly reduce the transgene flow to a great extent since transgenic seed will only be made on the mother plants, that can be controlled, and not on nearby cultivated tobacco crop varieties. In addition, we propose to use low nicotine *N. tabacum* varieties for phytoremediation, which are much less attractive for illegal harvesting.

NcZNT1 and *NcMTP1* could also be transformed into high biomass tree species like poplar and willow, which have even higher biomass than *N. tabacum*. They are fast growing and have a very deep root system, accessing

much deeper pollutions than tobacco (up to several meters deep). However, the efforts should be targeted to store the metals into the wood of these trees rather than in leaves, because the metal containing leaves would need to be collected before they are dispersed in autumn. This means the harvesting of the leaves should be done at the end of summer rather than in autumn/winter along with additional nutrient fertilisation to avoid growth arrest. Finally, valorisation of the biomass produced from the plants grown on polluted soils is essential to add economic feasibility to phytoremediation and give an incentive to farmers to apply it. The enhanced metal tolerance properties can be transferred into a range of other species which can be grown in these contaminated soils with additional economical products such as oil, fibre, fragrance and biofuel production (Schwitzguébel et al. 2002; Vangronsveld et al., 2009; Chapter 1). There is debate over the increasing arable areas being utilized for the production of biofuel crops which is replacing food crops and ultimately threatening the food production and possible increase in food prices (Cassman and Liska, 2007). High biomass plant species grown in polluted soils, especially if these are marginal for agriculture, can be safely used for biofuel production and hence can reduce this serious concern since the arable soils will remain to be available for food production.

REFERENCES

Aarts, M., Koncz, C., Pereira, A., 2000. Transposon and T-DNA mutagenesis, in: Wilson, Z. (Ed.), Arabidopsis, a practical approach Oxford University press, London.

Agarwala, S., Mehrotra, S., 1984. Iron-magnesium antagonism in growth and metabolism of radish. Plant and Soil 80, 355-361.

Arasimowicz-Jelonek, M., Floryszak-Wieczorek, J., Gwóźdź, E.A., 2011. The message of nitric oxide in cadmium challenged plants. Plant Science 181, 612-620.

Arrivault, S., Senger, T., Krämer, U., 2006. The Arabidopsis metal tolerance protein AtMTP3 maintains metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply. Plant Journal 46, 861-879.

Assunção, A., Bleeker, P., ten Bookum, W., Vooijs, R., Schat, H., 2008. Intraspecific variation of metal preference patterns for hyperaccumulation in *Thlaspi caerulescens*: evidence from binary metal exposures. Plant and Soil 303, 289-299.

Assunção, A.G.L., Da CostaMartins, P., De Folter, S., Vooijs, R., Schat, H., Aarts, M.G.M., 2001a. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant, Cell and Environment 24, 217-226.

Assunção, A.G.L., Herrero, E., Lin, Y.-F., Huettel, B., Talukdar, S., Smaczniak, C., Immink, R.G.H., van Eldik, M., Fiers, M., Schat, H., Aarts, M.G.M., 2010. *Arabidopsis thaliana* transcription factors *bZIP19* and *bZIP23* regulate the adaptation to zinc deficiency. Proceedings of the National Academy of Sciences 107, 10296-10301.

Assunção, A.G.L., Martins, P.D.C., De Folter, S., Vooijs, R., Schat, H., Aarts, M.G.M., 2001b. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator Thlaspi caerulescens. Plant, Cell & Environment 24, 217-226.

Assunção, A.G.L., Schat, H., Aarts, M.G.M., 2003a. *Thlaspi caerulescens*, an attractive model species to study heavy metal hyperaccumulation in plants. New Phytologist 159, 351-360.

Assunção, A.G.L., Ten Bookum, W.M., Nelissen, H.J.M., Vooijs, R., Schat, H., Ernst, W.H.O., 2003b. A cosegregation analysis of zinc (Zn) accumulation and Zn tolerance in the Zn hyperaccumulator *Thlaspi caerulescens*. New Phytologist 159, 383-390.

Audet, P., Charest, C., 2006. Effects of AM colonization on "wild tobacco" plants grown in zinc-contaminated soil. Mycorrhiza 16, 277-283.

Baker, A., Brooks, R., 1989. Terrestrial higher plants which hyperaccumulate metallic elements. A review of their distribution, ecology and phytochemistry. Biorecovery 1, 81-126.

Baker, A.J.M., McGrath, S.P., Reeves, D.R., Smith, J.A.C., 2000. Metal hyperaccumulator plants: A review of the ecology and physiology of the biological resource for phytoremediation of metal polluted soils, in: Terry, N., Banuelos, G. (Eds.), Phytoremediation of contaminated soils and water. CRC Press LLC, Boca Raton, FL, USA, pp 171-188.

Baker, A.J.M., Reeves, R.D., Hajar, A.S.M., 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J.& C.Presl (Brassicaceae). New Phytologist 127, 61-68.

Baker, N.R., 2008. Chlorophyll fluorescence: A probe of photosynthesis in vivo, Annual Review of Plant Biology. Annual Reviews, Palo Alto, pp 89-113.

Banerjee, S., Flores-Rozas, H., 2005. Cadmium inhibits mismatch repair by blocking the ATPase activity of the MSH2-MSH6 complex. Nucleic Acids Research 33, 1410-1419.

Bauer, P., Ling, H.-Q., Guerinot, M.L., 2007. *Fit*, the *Fer*-like iron deficiency induced transcription factor in Arabidopsis. Plant Physiology and Biochemistry 45, 260-261.

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

Beckmann, B.M., Hoch, P.G., Marz, M., Willkomm, D.K., Salas, M., Hartmann, R.K. 2012. A pRNA-induced structural rearrangement triggers 6S-1 RNA release from RNA polymerase in *Bacillus subtilis*. The EMBO Journal 31, 1727-1738.

Bell, R.W., Dell, B., 2008. Micronutrients for Sustainable Food, Feed, Fibre and Bioenergy Production. International Fertilizer Industry Association (IFA) Paris, France.

Bert, V., Macnair, M.R., De Laguerie, P., Saumitou-Laprade, P., Petit, D., 2000. Zinc tolerance and accumulation in metallicolous and nonmetallicolous populations of *Arabidopsis halleri* (Brassicaceae). New Phytologist 146, 225-233.

Blaudez, D., Kohler, A., Martin, F., Sanders, D., Chalot, M., 2003. Poplar Metal Tolerance Protein 1 Confers Zinc Tolerance and Is an Oligomeric Vacuolar Zinc Transporter with an Essential Leucine Zipper Motif. Plant Cell 15, 2911-2928.

Blossfeld, S., Perriguey, J., Sterckeman, T., Morel, J.-L., Lösch, R., 2010. Rhizosphere pH dynamics in trace-metal-contaminated soils, monitored with planar pH optodes. 330, 173-184.

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

Broadhurst, C.L., Chaney, R.L., Angle, J.S., Maugel, T.K., Erbe, E.F., Murphy, C.A., 2004. Simultaneous hyperaccumulation of nickel, manganese, and calcium in Alyssum leaf trichomes. Environmental Science and Technology 38, 5797-5802.

Broadley, M.R., White, P.J., Hammond, J.P., Zelko, I., Lux, A., 2007. Zinc in plants: Tansley review. New Phytologist 173, 677-702.

Brooks, R.R., Chambers, M.F., Nicks, L.J., Robinson, B.H., 1998. Phytomining. Trends in plant science 3, 359-362.

Brooks, R.R., Lee, J., Reeves, R.D., Jaffre, T., 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. Journal of Geochemical Exploration 7, 49-57.

Brown, S., Chaney, R., Angle, J., Baker, A., 1995. Zinc and cadmium uptake by hyperaccumulator *Noccaea caerulescens* grown in nutrient solution. Soil Science Scosiety of American Journal 59, 125-133.

Cambrollé, J., Mancilla-Leytón, J.M., Muñoz-Vallés, S., Luque, T., Figueroa, M.E., 2012. Zinc tolerance and accumulation in the salt-marsh shrub *Halimione portulacoides*. Chemosphere 86, 867-874.

Chaney, R.L., 1993. Zinc Phytotoxicity, in: Robson, A.D. (Ed.), Zinc in soils and plants. Kluwer Dordrecht.

Chaney, R.L., Malik, M., Li, Y.M., Brown, S.L., Brewer, E.P., Angle, J.S., Baker, A.J.M., 1997. Phytoremediation of soil metals. Current Opinion in Biotechnology 8, 279-284.

Chaney, R.L., Scott Angle, J., McIntosh, M.S., Phytoextraction Associates LLC, B., Reeves, R.D., Yin-Ming, L., Brewer, E.P., Kuang-Yu, C., Synkowski, E.C., Leigh Broadhurst, C., Wang, S., Roseberg, R.J., Perner, H., Baker, A.J.M., 2005. Using hyperaccumulator plants to phytoextract soil Ni and Cd. Zeitschrift fuer Naturforschung 60, 190-198.

Clarke, N.D., Berg, J.M., 1998. Zinc fingers in *Caenorhabditis elegans*: Finding families and probing pathways. Science 282, 2018-2022.

Claus, J., Krauser, A. 2012. Modeling regulation of zinc uptake via ZIP transporters in yeast and plant roots. PLoS One 7, e37193.

Clemens, S., Aarts, M.G.M., Thomine, S., Verbruggen, N., 2012. Plant science: the key to preventing slow cadmium poisoning. Trends in Plant Science doi.org/10.1016/j.tplants.2012.08.003.

Clemens, S., Palmgren, M.G., Krämer, U., 2002. A long way ahead: Understanding and engineering plant metal accumulation. Trends in Plant Science 7, 309-315.

Clough, S.J., Bent, A.F., 1998. Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant Journal 16, 735-743.

Cobbett, C., Goldsbrough, P., 2002. Phytochelatins and metallothioneins: Roles in heavy metal detoxification and homeostasis. Annual Review of Plant Biology 53, 159-182.

Cohen, C.K., Fox, T.C., Garvin, D.F., Kochian, L.V., 1998. The role of iron-deficiency stress responses in stimulating heavy-metal transport in plants. Plant Physiology 116, 1063-1072.

Cohen, C.K., Garvin, D.F., Kochian, L.V., 2004. Kinetic properties of a micronutrient transporter from *Pisum sativum* indicate a primary function in Fe uptake from the soil. Planta 218, 784-792.

Colangelo, E.P., Guerinot, M.L., 2004. The essential basic helix-loop-helix protein *FIT1* is required for the Iron deficiency response. The Plant Cell Online 16, 3400-3412.

Colangelo, E.P., Guerinot, M.L., 2006. Put the metal to the petal: metal uptake and transport throughout plants. Current Opinion in Plant Biology 9, 322-330.

Connolly, E.L., Campbell, N.H., Grotz, N., Prichard, C.L., Guerinot, M.L., 2003. Overexpression of the *FRO2* ferric chelate reductase confers tolerance to growth on low Iron and uncovers posttranscriptional control. Plant Physiology 133, 1102-1110.

Connolly, E.L., Fett, J.P., Guerinot, M.L., 2002. Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation. Plant Cell 14, 1347-1357.

Costello, L.C., Liu, Y., Franklin, R.B., Kennedy, M.C., 1997. Zinc inhibition of mitochondrial aconitase and its importance in citrate metabolism of prostate epithelial cells. Journal of Biological Chemistry 272, 28875-28881.

Craciun, A.R., Meyer, C.-L., Chen, J., Roosens, N., De Groodt, R., Hilson, P., Verbruggen, N., 2012. Variation in *HMA4* gene copy number and expression among *Noccaea caerulescens* populations presenting different levels of Cd tolerance and accumulation. Journal of Experimental Botany 63, 4179-4189.

Cramer, C.L., Boothe, J.G., Oishi, K.K., 1999. Transgenic plants for therapeutic proteins: Linking upstream and downstream strategies, Current Topics in Microbiology and Immunology, pp. 95-118.

Curie, C., Cassin, G., Couch, D., Divol, F., Higuchi, K., Le Jean, M., Misson, J., Schikora, A., Czernic, P., Mari, S., 2009. Metal movement within the plant: contribution of nicotianamine and yellow stripe 1-like transporters. Annals of Botany 103, 1-11.

Cuypers, A., Karen, S., Jos, R., Kelly, O., Els, K., Tony, R., Nele, H., Nathalie, V., Suzy, V.S., Frank, V.B., Yves, G., Jan, C., Jaco, V., 2011. The cellular redox state as a modulator in cadmium and copper responses in *Arabidopsis thaliana* seedlings. Journal of Plant Physiology 168, 309-316.

Cuypers, A., Smeets, K., Vangronsveld, J., 2010. Heavy metal stress in plants, Plant Stress Biology. Wiley-VCH Verlag GmbH & Co. KGaA, pp 161-178.

Cuypers, A., Vangronsveld, J., Clijsters, H., 1999. The chemical behaviour of heavy metals plays a prominent role in the induction of oxidative stress. Free Radical Research 31, S39-S43.

Cuypers, A., Vangronsveld, J., Clijsters, H., 2002. Peroxidases in roots and primary leaves of *Phaseolus vulgaris* Copper and Zinc Phytotoxicity: A comparison. Journal of Plant Physiology 159, 869-876.

Dahmani-Muller, H., van Oort, F., Balabane, M., 2001. Metal extraction by *Arabidopsis halleri* grown on an unpolluted soil amended with various metal-bearing solids: a pot experiment. Environmental Pollution 114, 77-84.

De Marchis, F., Pompa, A., Bellucci, M., 2012. Plastid Proteostasis and Heterologous Protein Accumulation in Transplastomic Plants. Plant Physiology 160, 571-581.

Delhaize, E., Kataoka, T., Hebb, D.M., White, R.G., Ryan, P.R., 2003. Genes encoding proteins of the cation diffusion facilitator family that confer manganese tolerance. Plant Cell 15, 1131-1142.

Desbrosses-Fonrouge, A.-G., Voigt, K., Schröder, A., Arrivault, S., Thomine, S., Krämer, U., 2005a. *Arabidopsis thaliana* MTP1 is a Zn transporter in the vacuolar membrane which mediates Zn detoxification and drives leaf Zn accumulation. FEBS Letters 579, 4165-4174.

DiDonato, R.J., Roberts, L.A., Sanderson, T., Eisley, R.B., Walker, E.L., 2004. Arabidopsis Yellow Stripe-Like2 (YSL2): a metal-regulated gene encoding a plasma membrane transporter of nicotianamine–metal complexes. The Plant Journal 39, 403-414.

Dräger, D.B., Desbrosses-Fonrouge, A.G., Krach, C., Chardonnens, A.N., Meyer, R.C., Saumitou-Laprade, P., Krämer, U., 2004. Two genes encoding *Arabidopsis halleri* MTP1 metal transport proteins co-segregate with zinc tolerance and account for high *MTP1* transcript levels. Plant Journal 39, 425-439.

Durrett, T.P., Gassmann, W., Rogers, E.E., 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. Plant Physiology 144, 197-205.

Ebbs, S., Lau, I., Ahner, B., Kochian, L., 2002. Phytochelatin synthesis is not responsible for Cd tolerance in the Zn/Cd hyperaccumulator *Thlaspi caerulescens* (J. & C. Presl). Planta 214, 635-640.

Eide, D., Broderius, M., Fett, J., Guerinot, M.L., 1996. A novel iron-regulated metal transporter from plants identified by functional expression in yeast. Proceedings of the National Academy of Sciences of the United States of America 93, 5624-5628.

Eide, D.J., 2003. Multiple regulatory mechanisms maintain zinc homeostasis in *Saccharomyces cerevisiae*. Journal of Nutrition 133, 1532S-1535S.

Epstein, E., 1972. Mineral nutrition of plants : principles and perspectives. [s.n.], New York [etc.].

Epstein, E., Bloom, A.J., 2005. Mineral nutrition of plants: principles and perspectives. Sinauer, Sunderland, MA.

Ernst, W.H.O., 1980. Biochemical aspects of cadmium in plants, in: Nriagu, E.J.O. (Ed.), Cadmium in the Environment. Wiley and Sons, New York, pp 639–653.

Ernst, W.H.O., Verkleij, J.A.C., Schat, H., 1992. Metal tolerance in plants. Acta Botanica Neerlandica 41, 229-248.

Fox, T.C., Guerinot, M.L., 1998. Molecular biology of cation transport in plants. Annual Review of Plant Physiology and Plant Molecular Biology 49, 669-696.

Foyer, C.H., Noctor, G., 2005. Oxidant and antioxidant signalling in plants: a re-evaluation of the concept of oxidative stress in a physiological context. Plant, Cell & Environment 28, 1056-1071.

Freeman, J.L., Persans, M.W., Nieman, K., Albrecht, C., Peer, W., Pickering, I.J., Salt, D.E., 2004. Increased Glutathione Biosynthesis Plays a Role in Nickel Tolerance in Thlaspi Nickel Hyperaccumulators. The Plant Cell Online 16, 2176-2191.

Gendre, D., Czernic, P., Conéjéro, G., Pianelli, K., Briat, J.-F., Lebrun, M., Mari, S., 2007. *TcYSL3*, a member of the YSL gene family from the hyper-accumulator *Thlaspi caerulescens*, encodes a nicotianamine-Ni/Fe transporter. The Plant Journal 49, 1-15.

Genon, J., Hepcee, N., Duffy, J., Delvaux, B., Hennebert, P., 1994. Iron toxicity and other chemical soil constraints to rice in highland swamps of Burundi. Plant and Soil 166, 109-115.

Gerendás, J., Polacco, J.C., Freyermuth, S.K., Sattelmacher, B., 1999. Significance of nickel for plant growth and metabolism. Journal of Plant Nutrition and Soil Science 162, 241-256.

Ghasemi, R., Ghaderian, S.M., Krämer, U., 2009. Interference of nickel with copper and iron homeostasis contributes to metal toxicity symptoms in the nickel hyperaccumulator plant *Alyssum inflatum*. New Phytologist 184, 566-580.

Gietz, R.D., Schiestl, R.H., 1994. Transforming yeast with DNA. Methods in Molecular and Cellular Biology 5, 255-269.

Gong, J.M., Lee, D.A., Schroeder, J.I., 2003. Long-distance root-to-shoot transport of phytochelatins and cadmium in Arabidopsis. Proceedings of the National Academy of Sciences of the United States of America 100, 10118-10123.

Grill, E., Winnacker, E.L., Zenk, M.H., 1985. Phytochelatins: the principal heavy-metal complexing peptides of higher plants. Science (New York, N.Y.) 230, 674-676.

Grotz, N., Fox, T., Connolly, E., Park, W., Guerinot, M.L., Eide, D., 1998. Identification of a family of zinc transporter genes from Arabidopsis that respond to zinc deficiency. Proceedings of the National Academy of Sciences of the United States of America 95, 7220-7224.

Grotz, N., Guerinot, M.L., 2002. Limiting nutrients: an old problem with new solutions? Current Opinion in Plant Biology 5, 158-163.

Guerinot, M.L., 2000. The ZIP family of metal transporters. Biochimica et Biophysica Acta (BBA) - Biomembranes 1465, 190-198.

Guerinot, M.L., Eide, D., 1999. Zeroing in on zinc uptake in yeast and plants. Current Opinion in Plant Biology 2, 244-249.

Guerinot, M.L., Yi, Y., 1994. Iron: Nutritious, Noxious, and Not Readily Available. Plant Physiology 104, 815-820.

Guimarães, M.D.A., Gustin, J.L., Salt, D.E., 2009. Reciprocal grafting separates the roles of the root and shoot in zinc hyperaccumulation in *Thlaspi caerulescens*. New Phytologist 184, 323-329.

Gusiatin, Z.M., Klimiuk, E., 2012. Metal (Cu, Cd and Zn) removal and stabilization during multiple soil washing by saponin. Chemosphere 86, 383-391.

Gustin, J.L., Loureiro, M.E., Kim, D., Na, G., Tikhonova, M., Salt, D.E., 2009. MTP1-dependent Zn sequestration into shoot vacuoles suggests dual roles in Zn tolerance and accumulation in Zn-hyperaccumulating plants. Plant Journal 57, 1116-1127.

Hagemann, R., 2002. Milestones in plastid genetics of higher plants. Prog. Bot., 1-51.

Hall, J.L., 2002. Cellular mechanisms for heavy metal detoxification and tolerance. Journal of Experimental Botany 53, 1-11.

Hammond, J.P., Bowen, H.C., White, P.J., Mills, V., Pyke, K.A., Baker, A.J.M., Whiting, S.N., May, S.T., Broadley, M.R., 2006. A Comparison of the *Thlaspi caerulescens* and *Thlaspi arvense* Shoot Transcriptomes. New Phytologist 170, 239-259.

Hanikenne, M., Talke, I.N., Haydon, M.J., Lanz, C., Nolte, A., Motte, P., Kroymann, J., Weigel, D., Krämer, U., 2008. Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of *HMA4*. Nature 453, 391-395.

Hänsch, R., Mendel, R.R., 2009. Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl). Current opinion in plant biology 12, 259-266.

Hartwig, B., James, G.V., Konrad, K., Schneeberger, K., Turck, F. 2012. Fast isogenic mapping-by-sequencing of EMS-induced mutant Bulks. Plant Physiology doi:10.1104/pp.112.200311.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 8.

Herbette, S., Taconnat, L., Hugouvieux, V., Piette, L., Magniette, M.L., Cuine, S., Auroy, P., Richaud, P., Forestier, C., Bourguignon, J., Renou, J.P., Vavasseur, A., Leonhardt, N., 2006. Genome-wide transcriptome profiling of the early cadmium response of Arabidopsis roots and shoots. Biochimie 88, 1751-1765.

Hermans, C., Chen, J., Coppens, F., Inzé, D., Verbruggen, N., 2011. Low magnesium status in plants enhances tolerance to cadmium exposure. New Phytologist 192, 428-436.

Hermans, C., Vuylsteke, M., Coppens, F., Cristescu, S.M., Harren, F.J.M., Inzé, D., Verbruggen, N., 2010. Systems analysis of the responses to long-term magnesium deficiency and restoration in *Arabidopsis thaliana*. New Phytologist 187, 132-144.

Hirschi, K.D., Korenkov, V.D., Wilganowski, N.L., Wagner, G.J., 2000. Expression of Arabidopsis *CAX2* in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiology 124, 125-133.

Horsch, R.B., Fry, J.E., Hoffmann, N.L., Eichholtz, D., Rogers, S.G., Fraley, R.T., 1985. A simple and general method for transferring genes into plants. Science 227, 1229-1230.

Hu, Y., Ge, Y., Zhang, C., Ju, T., Cheng, W., 2009. Cadmium toxicity and translocation in rice seedlings are reduced by hydrogen peroxide pretreatment. Plant Growth Regulation 59, 51-61.

Hussain, D., Haydon, M.J., Wang, Y., Wong, E., Sherson, S.M., Young, J., Camakaris, J., Harper, J.F., Cobbett, C.S., 2004. P-type ATPase heavy metal transporters with roles in essential zinc homeostasis in Arabidopsis. Plant Cell 16, 1327-1339.

Hussein, H.S., Ruiz, O.N., Terry, N., Daniell, H., 2007. Phytoremediation of mercury and organomercurials in chloroplast transgenic plants: Enhanced root uptake, translocation to shoots, and volatilization. Environmental Science and Technology 41, 8439-8446.

Jain, R., Srivastava, S., Solomon, S., Shrivastava, A.K., Chandra, A., 2010. Impact of excess zinc on growth parameters, cell division, nutrient accumulation, photosynthetic pigments and oxidative stress of sugarcane (saccharum spp.). Acta Physiologiae Plantarum 32, 979-986.

Kahle, H., 1993. Response of roots of trees to heavy metals. Environmental and Experimental Botany 33, 99-119.

Kashem, M.A., Kawai, S., 2007. Alleviation of cadmium phytotoxicity by magnesium in Japanese mustard spinach. Soil Science and Plant Nutrition 53, 246-251.
Kasuga, M., Liu, Q., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K., 1999. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nature biotechnology 17, 287-291.
Kawachi, M., Kobae, Y., Kogawa, S., Mimura, T., Krämer, U., Maeshima, M., 2012. Amino acid screening based on structural modeling identifies critical residues for the function, ion selectivity and structure of Arabidopsis MTP1. FEBS Journal 279, 2339-2356.
Kawachi, M., Kobae, Y., Mimura, T., Maeshima, M., 2008. Deletion of a histidine-rich loop of AtMTP1, a vacuolar Zn 2+/H+ antiporter of *Arabidopsis thaliana*, stimulates the transport activity. Journal of Biological Chemistry 283, 8374-8383.
Kawachi, M., Kobae, Y., Mori, H., Tomioka, R., Lee, Y., Maeshima, M., 2009. A mutant strain *Arabidopsis thaliana* that lacks vacuolar membrane zinc transporter MTP1 revealed the latent tolerance to excessive zinc. Plant and Cell Physiology 50, 1156-1170.
Kerkeb, L., Mukherjee, I., Chatterjee, I., Lahner, B., Salt, D.E., Connolly, E.L., 2008. Iron-induced turnover of the Arabidopsis Iron regulated transporter1 metal transporter requires lysine residues. Plant Physiology 146, 1964-1973.
Kim, D., Gustin, J.L., Lahner, B., Persans, M.W., Baek, D., Yun, D.J., Salt, D.E., 2004. The plant CDF family member TgMTP1 from the Ni/Zn hyperaccumulator *Thlaspi goesingense* acts to enhance efflux of Zn at the plasma membrane when expressed in *Saccharomyces cerevisiae*. Plant Journal 39, 237-251.
Kim, S., Takahashi, M., Higuchi, K., Tsunoda, K., Nakanishi, H., Yoshimura, E., Mori, S., Nishizawa, N.K., 2005. Increased nicotianamine biosynthesis confers enhanced tolerance of high levels of metals, in particular nickel, to plants. Plant & cell physiology 46, 1809-1818.
Klatte, M., Schuler, M., Wirtz, M., Fink-Straube, C., Hell, R., Bauer, P., 2009. The analysis of Arabidopsis nicotianamine synthase mutants reveals functions for nicotianamine in seed iron loading and iron deficiency responses. Plant Physiology 150, 257-271.
Knoop, V., Groth-Malonek, M., Gebert, M., Eifler, K., Weyand, K., 2005. Transport of magnesium and other divalent cations: Evolution of the 2-TM-GxN proteins in the MIT superfamily. Molecular Genetics and Genomics 274, 205-216.
Kobae, Y., Uemura, T., Sato, M.H., Ohnishi, M., Mimura, T., Nakagawa, T., Maeshima, M., 2004. Zinc transporter of *Arabidopsis thaliana* AtMTP1 is localized to vacuolar membranes and implicated in zinc homeostasis. Plant and Cell Physiology 45, 1749-1758.
Kobayashi, T., Nakayama, Y., Itai, R.N., Nakanishi, H., Yoshihara, T., Mori, S., Nishizawa, N.K., 2003. Identification of novel cis-acting elements, *IDE1* and *IDE2*, of the barley *IDS2* gene promoter conferring iron-deficiency-inducible, root-specific expression in heterogeneous tobacco plants. The Plant Journal 36, 780-793.
Koch, M., Al-Shehbaz, I., 2009. Molecular systematics and evolution of "wild" crucifers (Brassicaceae or Cruciferae), in: Gupta, S. (Ed.), Biology and Breeding of Crucifers. CRC Press/Taylor & Francis Group, Boca Raton, pp 1-19.
Koch, M., Mummenhoff, K., 2001. Thlaspi s.str. (Brassicaceae) versus Thlaspi s.l.: Morphological and anatomical characters in the light of ITS nrDNA sequence data. Plant Systematics and Evolution 227, 209-225.
Kojima, Y., Hiyama, T., Sakurai, H., 1987. Effect of mercurials on iron sulfur centers of PS I of *Anacystis nidulans*, in: Biggins, J. (Ed.), Progress in Photosynthetic Research. Nijhoff/Junk, The Hague.
Kondo, N., Imai, K., Isobe, M., Goto, T., Murasugi, A., Wada-Nakagawa, C., Hayashi, Y., 1984. Cadystin a and b, major unit peptides comprising cadmium binding peptides induced in a fission yeast separation, revision of structures and synthesis. Tetrahedron Letters 25, 3869-3872.
Korshunova, Y.O., Eide, D., Clark, W.G., Guerinot, M.L., Pakrasi, H.B., 1999. The IRT1 protein from *Arabidopsis thaliana* is a metal transporter with a broad substrate range. Plant Molecular Biology 40, 37-44.

Krämer, U., 2005. Phytoremediation: novel approaches to cleaning up polluted soils. Current opinion in biotechnology 16, 133-141.
Krämer, U., 2010. Metal Hyperaccumulation in Plants. Annual Review of Plant Biology 61, 517-534.
Krämer, U., Chardonnens, A.N., 2001. The use of transgenic plants in the bioremediation of soils contaminated with trace elements. Applied microbiology and biotechnology 55, 661-672.
Kramer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M., Smith, J.A.C., 1996. Free histidine as a metal chelator in plants that accumulate nickel. Nature 379, 635-638.
Krämer, U., Cotter-Howells, J.D., Charnock, J.M., Baker, A.J.M., Smith, J.A.C., 1996. Free histidine as a metal chelator in plants that accumulate nickel. Nature 379, 635-638.
Kramer, U., Smith, R.D., Wenzel, W.W., Raskin, I., Salt, D.E., 1997. The Role of Metal Transport and Tolerance in Nickel Hyperaccumulation by *Thlaspi goesingense* Halacsy. Plant Physiology 115, 1641-1650.
Krämer, U., Talke, I.N., Hanikenne, M., 2007. Transition metal transport. FEBS Letters 581, 2263-2272.
Kumar, S., Tamura, K., Nei, M., 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in bioinformatics 5, 150-163.
Kupper, H., Jie Zhao, F., McGrath, S.P., 1999. Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. Plant Physiology 119, 305-312.
Küpper, H., Kochian, L.V., 2010. Transcriptional regulation of metal transport genes and mineral nutrition during acclimatization to cadmium and zinc in the Cd/Zn hyperaccumulator, *Thlaspi caerulescens* (Ganges population). The New phytologist 185, 114-129.
Küpper, H., Küpper, F., Spiller, M., 1998. In situ detection of heavy metal substituted chlorophylls in water plants. Photosynthesis Research 58, 123-133.
Küpper, H., Lombi, E., Zhao, F.J., McGrath, S.P., 2000. Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis halleri* Planta 212, 75-84.
Küpper, H., Lombi, E., Zhao, F.J., Wieshammer, G., McGrath, S.P., 2001. Cellular compartmentation of nickel in the hyperaccumulators *Alyssum lesbiacum*, *Alyssum bertolonii* and *Thlaspi goesingense*. Journal of Experimental Botany 52, 2291-2300.
Küpper, H., Zhao, F.J., McGrath, S.P., 1999. Cellular compartmentation of zinc in leaves of the hyperaccumulator *Thlaspi caerulescens*. Plant Physiology 119, 305-311.
Lahner, B., Gong, J., Mahmoudian, M., Smith, E.L., Abid, K.B., Rogers, E.E., Guerinot, M.L., Harper, J.F., Ward, J.M., McIntyre, L., Schroeder, J.I., Salt, D.E., 2003. Genomic scale profiling of nutrient and trace elements in *Arabidopsis thaliana*. Nature biotechnology 21, 1215-1221.
Lasat, M.M., Baker, A.J.M., Kochian, L.V., 1996. Physiological characterization of root Zn2+ absorption and translocation to shoots in Zn hyperaccumulator and nonaccurnulator species of Thlaspi. Plant Physiology 112, 1715-1722.
Lasat, M.M., Baker, A.J.M., Kochian, L.V., 1998. Altered Zn compartmentation in the root symplasm and stimulated Zn absorption into the leaf as mechanisms involved in Zn hyperaccumulation in *Thlaspi caerulescens*. Plant Physiology 118, 875-883.
Lasat, M.M., Kochian, L.V., 2000. Physiology of Zn hyperaccumulation in *Thlaspi caerulescens*, in: Terry, N., Banuelos, G. (Ed.), Phytoremediation of Contaminated Soils and Water. CRC Press LLC, Boca Raton, FL, pp 159-169.
Leeper, G.W., 1978. Managing the heavy metals on the land. Marcel Dekker, New York.
Li, Y., Dhankher, O.P., Carreira, L., Lee, D., Chen, A., Schroeder, J.I., Balish, R.S., Meagher, R.B., 2004. Overexpression of phytochelatin synthase in Arabidopsis leads to enhanced arsenic tolerance and cadmium hypersensitivity. Plant & cell physiology 45, 1787-1797.
Li, Z.S., Szczypka, M., Lu, Y.P., Thiele, D.J., Rea, P.A., 1996. The yeast cadmium factor protein (YCF1) is a vacuolar glutathione S-conjugate pump. The Journal of biological chemistry 271, 6509-6517.
Liang, H.M., Lin, T.H., Chiou, J.M., Yeh, K.C., 2009. Model evaluation of the phytoextraction potential of heavy metal hyperaccumulators and non-hyperaccumulators. Environmental pollution (Barking, Essex : 1987) 157, 1945-1952.

Lichtenthaler, H.K., Wellburn, A.R., 1983. Determination of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 11, 591-592.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling, T., Geurts, R., 2004. RNA interference in *Agrobacterium rhizogenes*-transformed roots of Arabidopsis and *Medicago truncatula*. Journal of Experimental Botany 55, 983-992.

Lin, Y.F., Aarts, M.G.M., 2012. The molecular mechanism of zinc and cadmium stress response in plants. Cellular and Molecular Life Sciences 69, 3187-3206.

Ling, H.Q., Koch, G., Bäumlein, H., Ganal, M.W., 1999. Map-based cloning of chloronerva, a gene involved in iron uptake of higher plants encoding nicotianamine synthase. Proceedings of the National Academy of Sciences of the United States of America 96, 7098-7103.

Liu, C.H., Huang, W.D., Kao, C.H., 2012. The decline in potassium concentration is associated with cadmium toxicity of rice seedlings. Acta Physiologiae Plantarum 34, 495-502.

Liu, M.Q., Yanai, J., Jiang, R.F., Zhang, F., McGrath, S.P., Zhao, F.J., 2008. Does cadmium play a physiological role in the hyperaccumulator *Thlaspi caerulescens*? Chemosphere 71, 1276-1283.

Liu, Q., Kasuga, M., Sakuma, Y., Abe, H., Miura, S., Yamaguchi-Shinozaki, K., Shinozaki, K., 1998. Two Transcription Factors, DREB1 and DREB2, with an EREBP/AP2 DNA Binding Domain Separate Two Cellular Signal Transduction Pathways in Drought- and Low-Temperature-Responsive Gene Expression, Respectively, in Arabidopsis. The Plant Cell Online 10, 1391-1406.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25, 402-408.

Lombi, E., Tearall, K.L., Howarth, J.R., Zhao, F.-J., Hawkesford, M.J., McGrath, S.P., 2002. Influence of Iron Status on Cadmium and Zinc Uptake by Different Ecotypes of the Hyperaccumulator *Thlaspi caerulescens*. Plant Physiology 128, 1359-1367.

Lombi, E., Zhao, F.J., Dunham, S.J., McGrath, S.P., 2000. Cadmium accumulation in populations of *Thlaspi caerulescens* and *Thlaspi goesingense*. New Phytologist 145, 11-20.

Lombi, E., Zhao, F.J., McGrath, S.P., Young, S.D., Sacchi, G.A., 2001. Physiological evidence for a high-affinity cadmium transporter highly expressed in a *Thlaspi caerulescens* ecotype. The New phytologist 149, 53-60.

Luo, Y.M., Christie, P., Baker, A.J.M., 2000. Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of *Noccaea caerulescens* grown in a Zn/Cd contaminated soil. Chemosphere 41, 161-164.

Ma, J.F., Ueno, D., Zhao, F.J., McGrath, S.P., 2005. Subcellular localisation of Cd and Zn in the leaves of a Cd-hyperaccumulating ecotype of *Thlaspi caerulescens*. Planta 220, 731-736.

Macnair, M.R., Bert, V., Huitson, S.B., Saumitou-Laprade, P., Petit, D., 1999. Zinc Tolerance and Hyperaccumulation are Genetically Independent Characters. Proceedings: Biological Sciences 266, 2175-2179.

Marquès, L., Cossegal, M., Bodin, S., Czernic, P., Lebrun, M., 2004. Heavy metal specificity of cellular tolerance in two hyperaccumulating plants, *Arabidopsis halleri* and *Thlaspi caerulescens*. New Phytologist 164, 289-295.

Marschner, H., 1986. Mineral nutrition of higher plants, 2nd edn. Academic, 2nd ed, Orlando, FL.

Marschner, H., 1995. Mineral nutrition of higher plants, 2nd ed. Academic Press, New York, NY, USA.

Martínez, M., Bernal, P., Almela, C., Vélez, D., García-Agustín, P., Serrano, R., Navarro-Aviñó, J., 2006. An engineered plant that accumulates higher levels of heavy metals than *Thlaspi caerulescens*, with yields of 100 times more biomass in mine soils. Chemosphere 64, 478-485.

Mateos-Naranjo, E., Redondo-Gómez, S., Cambrollé, J., Luque, T., Figueroa, M.E., 2008. Growth and photosynthetic responses to zinc stress of an invasive cordgrass, *Spartina densiflora*. Plant Biology 10, 754-762.

McBride, M., Sauvé, S., Hendershot, W., 1997. Solubility control of Cu, Zn, Cd and Pb in contaminated soils. European Journal of Soil Science 48, 337-346.

McGrath, S.P., Lombi, E., Gray, C.W., Caille, N., Dunham, S.J., Zhao, F.J., 2006. Field evaluation of Cd and Zn phytoextraction potential by the hyperaccumulators *Thlaspi caerulescens* and *Arabidopsis halleri*. Environmental Pollution 141, 115-125.

McGrath, S.P., Shen, Z.G., Zhao, F.J., 1997. Heavy metal uptake and chemical changes in the rhizosphere of *Thlaspi caerulescens* and *Thlaspi ochroleucum* grown in contaminated soils. Plant and Soil 188, 153-159.

McGrath, S.P., Sidoli, C.M.D., Baker, A.J.M., Reeves, R.D., 1993. The potential for the use of metal-accumulating plants for the in situ decontamination of metal-polluted soils, in: Eijsackers, H.J.P., Hamers, T. (Ed.), Integrated Soil and Sediment Research: a Basis for Proper Protection. . Kluwer Dordrecht, The Netherlands, pp 673–677.

McGrath, S.P., Zhao, F.J., 2003. Phytoextraction of metals and metalloids from contaminated soils. Current opinion in biotechnology 14, 277-282.

McGrath, S.P., Zhao, J., Lombi, E., 2002. Phytoremediation of metals, metalloids, and radionuclides. Advances in Agronomy 75, 1-56.

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

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

Mei, H., Cheng, N.H., Zhao, J., Park, S., Escareno, R.A., Pittman, J.K., Hirschi, K.D., 2009. Root development under metal stress in *Arabidopsis thaliana* requires the H +/cation antiporter CAX4. New Phytologist 183, 95-105.

Milner, M.J., Craft, E., Yamaji, N., Koyama, E., Ma, J.F., Kochian, L.V., 2012. Characterization of the high affinity Zn transporter from *Noccaea caerulescens*, NcZNT1, and dissection of its promoter for its role in Zn uptake and hyperaccumulation. New Phytologist 195, 113-123.

Milner, M.J., Kochian, L.V., 2008. Investigating Heavy-metal Hyperaccumulation using *Thlaspi caerulescens* as a Model System. Annals of Botany 102, 3-13.

Mittler, R., 2006. Abiotic stress, the field environment and stress combination. Trends in plant science 11, 15-19.

Mittler, R., Vanderauwera, S., Gollery, M., Van Breusegem, F., 2004. Reactive oxygen gene network of plants. Trends in Plant Science 9, 490-498.

Mizuno, N., Nosaka, S., Mizuno, T., Horie, K., Obata, H., 2003. Distribution of Ni and Zn in the leaves of *Thlaspi japonicum* growing on ultramafic soil. Soil Science and Plant Nutrition 49, 93-97.

Mizuno, T., Usui, K., Horie, K., Nosaka, S., Mizuno, N., Obata, H., 2005. Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni2+-transport abilities. Plant Physiology and Biochemistry 43, 793-801.

Montanini, B., Blaudez, D., Jeandroz, S., Sanders, D., Chalot, M., 2007. Phylogenetic and functional analysis of the Cation Diffusion Facilitator (CDF) family: Improved signature and prediction of substrate specificity. BMC Genomics 8.

Morel, M., Crouzet, J., Gravot, A., Auroy, P., Leonhardt, N., Vavasseur, A., Richaud, P., 2009. AtHMA3, a P1B-ATPase allowing Cd/Zn/Co/Pb vacuolar storage in Arabidopsis. Plant Physiology 149, 894-904.

Moulis, J.-M., Thévenod, F., 2010. New perspectives in cadmium toxicity: an introduction. BioMetals 23, 763-768.

Murashige, T., Skoog, F., 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15, 473-497.

Nap, J.P., Dirkse, W.G., Louwerse, J., Onstenk, J., Visser, R., Loonen, A., Heidekamp, F., Stiekema, W.J., 1992. Analysis of the region in between two closely linked patatin genes: class II promoter activity in tuber, root and leaf. Plant Molecular Biology 20, 683-694.

Narváez-Vásquez, J., Pearce, G., Ryan, C.A., 2005. The plant cell wall matrix harbors a precursor of defense signalling peptides. Proceedings of the National Academy of Sciences of the United States of America 102, 12974-12977.

Ó Lochlainn, S., Bowen, H.C., Fray, R.G., Hammond, J.P., King, G.J., White, P.J., Graham, N.S., Broadley, M.R., 2011. Tandem Quadruplication of in the Zinc (Zn) and Cadmium (Cd) Hyperaccumulator *Noccaea caerulescens*. PLoS ONE 6, e17814.

Ohno, S., 1970. Evolution by gene duplication. Springer-Verlag, Berlin; New York.

Oomen, R.J.F.J., Wu, J., Lelièvre, F., Blanchet, S., Richaud, P., Barbier-Brygoo, H., Aarts, M.G.M., Thomine, S., 2009. Functional characterization of *NRAMP3* and *NRAMP4* from the metal hyperaccumulator *Thlaspi caerulescens*. New Phytologist 181, 637-650.

Ouerdane, L., Mari, S., Czernic, P., Lebrun, M., Łobiński, R., 2006. Speciation of non-covalent nickel species in plant tissue extracts by electrospray Q-TOFMS/MS after their isolation by 2D size exclusion-hydrophilic interaction LC (SEC-HILIC) monitored by ICP-MS. Journal of Analytical Atomic Spectrometry 21, 676-683.

Palmgren, M.G., Clemens, S., Williams, L.E., Krämer, U., Borg, S., Schjørring, J.K., Sanders, D., 2008. Zinc biofortification of cereals: problems and solutions. Trends in Plant Science 13, 464-473.

Pan, A., Yang, M., Tie, F., Li, L., Chen, Z., Ru, B., 1994. Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants. Plant Molecular Biology 24, 341-351.

Papoyan, A., Kochian, L.V., 2004. Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATPase. Plant Physiology 136, 3814-3823.

Peer, W.A., Mahmoudian, M., Freeman, J.L., Lahner, B., Richards, E.L., Reeves, R.D., Murphy, A.S., Salt, D.E., 2006. Assessment of plants from the Brassicaceae family as genetic models for the study of nickel and zinc hyperaccumulation. New Phytologist 172, 248-260.

Peer, W.A., Mamoudian, M., Lahner, B., Reeves, R.D., Murphy, A.S., Salt, D.E., 2003. Identifying model metal hyperaccumulating plants: Germplasm analysis of 20 brassicaceae accessions from a wide geographical area. New Phytologist 159, 421-430.

Pence, N.S., Larsen, P.B., Ebbs, S.D., Letham, D.L.D., Lasat, M.M., Garvin, D.F., Eide, D., Kochian, L.V., 2000. The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proceedings of the National Academy of Sciences of the United States of America 97, 4956-4960.

Persans, M.W., Nieman, K., Salt, D.E., 2001. Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*. Proceedings of the National Academy of Sciences of the United States of America 98, 9995-10000.

Pianelli, K., Mari, S., Marquès, L., Lebrun, M., Czernic, P., 2005. Nicotianamine over-accumulation confers resistance to nickel in *Arabidopsis thaliana*. Transgenic Research 14, 739-748.

Pich, A., Manteuffel, R., Hillmer, S., Scholz, G., Schmidt, W., 2001. Fe homeostasis in plant cells: Does nicotianamine play multiple roles in the regulation of cytoplasmic Fe concentration? Planta 213, 967-976.

Pich, A., Scholz, G., 1996. Translocation of copper and other micronutrients in tomato plants (*Lycopersicon esculentum* Mill.): Nicotianamine-stimulated copper transport in the xylem. Journal of Experimental Botany 47, 41-47.

Pimentel, D., Pimentel, M., Guerinot, M.L., 2000. To Improve Nutrition for the World's Population. Science 288, 1966-1967.

Plaza, S., Tearall, K.L., Zhao, F.J., Buchner, P., McGrath, S.P., Hawkesford, M.J., 2007. Expression and functional analysis of metal transporter genes in two contrasting ecotypes of the hyperaccumulator *Thlaspi caerulescens*. Journal of Experimental Botany 58, 1717-1728.

Polle, A., Schutzendubel, A., 2003. Heavy metal signalling in plants: linking cellular and organismic responses, in: Hirt, H., Shinozaki, K. (Eds.), In Topics in Current Genetics. Plant Responses to Abiotic Stress,. Springer Verlag, Berlin, pp 187-216.

Pongrac, P., Zhao, F.-J., Razinger, J., Zrimec, A., Regvar, M., 2009. Physiological responses to Cd and Zn in two Cd/Zn hyperaccumulating Thlaspi species. Environmental and Experimental Botany 66, 479-486.

Prasad, S.M., Singh, J.B., Rai, L.C., Kumar, H.D., 1991. Metal-induced inhibition of photosynthetic electron transport chain of the cyanobacterium *Nostoc muscorum*. FEMS Microbiology Letters 82, 95-100.

Programme), U.U.N.E., 2008. Draft Final Review of Scientific Information on Cadmium.

Ramesh, S.A., Choimes, S., Schachtman, D.P., 2004. Over-expression of an Arabidopsis zinc transporter in *Hordeum vulgare* increases short-term zinc uptake after zinc deprivation and seed zinc content. Plant Molecular Biology 54, 373-385.

Rascio, N., Navari-Izzo, F., 2011. Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? Plant Science 180, 169-181.

Reeves, R., Baker, A., 2000a. Metal accumulating plants, in: Raskin, I., Ensley, B. (Eds.), Phytoremediation of toxic metals: using plants to clean up the environment. John Wiley and sons, New York, pp 193-229.

Reeves, R.D., Baker, A.J.M., 2000b. Metal-accumulating plants, in: Raskin, I., Ensley, B.D. (Eds.), Phytoremediation of toxic metals: Using plants to clean up the environment. John Wiley & Sons, Inc.,, New York, pp 192-229.

Reeves, R.D., Brooks, R.R., 1983. European species of Thlaspi L. (Cruciferae) as indicators of nickel and zinc. Journal of Geochemical Exploration 18, 275-283.

Repetto, M., Ferrarotti, N., Boveris, A., 2010. The involvement of transition metal ions on iron-dependent lipid peroxidation. Archives of Toxicology 84, 255-262.

Richau, K.H., Kozhevnikova, A.D., Seregin, I.V., Vooijs, R., Koevoets, P.L.M., Smith, J.A.C., Ivanov, V.B., Schat, H., 2009. Chelation by histidine inhibits the vacuolar sequestration of nickel in roots of the hyperaccumulator *Thlaspi caerulescens*. New Phytologist 183, 106-116.

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

Robinson, B., Mills, T., Petit, D., Fung, L., Green, S., Clothier, B., 2000a. Natural and induced cadmium-accumulation in poplar and willow: Implications for phytoremediation. Plant and Soil 227, 301-306.

Robinson, B.H., Mills, T.M., Petit, D., Fung, L.E., Green, S.R., Clothier, B.E., 2000b. Natural and induced cadmium-accumulation in poplar and willow: Implications for phytoremediation. Plant and Soil 227, 301-306.

Robinson, N.J., Procter, C.M., Connolly, E.L., Guerinot, M.L., 1999. A ferric-chelate reductase for iron uptake from soils. 397, 694-697.

Rogers, E.E., Eide, D.J., Guerinot, M.L., 2000. Altered selectivity in an Arabidopsis metal transporter. Proceedings of the National Academy of Sciences of the United States of America 97, 12356-12360.

Roosens, N., Verbruggen, N., Meerts, P., Ximénez-Embún, P., Smith, J.A.C., 2003. Natural variation in cadmium tolerance and its relationship to metal hyperaccumulation for seven populations of *Thlaspi caerulescens* from western Europe. Plant, Cell and Environment 26, 1657-1672.

Ruf, S., Karcher, D., Bock, R., 2007. Determining the transgene containment level provided by chloroplast transformation.

Rutherford, A.W., Boussac, A., 2004. Water Photolysis in Biology. Science 303, 1782-1784.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular biology and evolution 4, 406-425.

Salt, D.E., Prince, R.C., Baker, A.J.M., Raskin, I., Pickering, I.J., 1999. Zinc Ligands in the Metal Hyperaccumulator *Thlaspi caerulescens* As Determined Using X-ray Absorption Spectroscopy. Environmental Science & Technology 33, 713-717.

Salt, D.E., Rauser, W.E., 1995. MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiology 107, 1293-1301.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor (NY).

Sanità di Toppi, L., Gabbrielli, R., 1999. Response to cadmium in higher plants. Environmental and Experimental Botany 41, 105-130.

Sarret, G., Harada, E., Choi, Y.-E., Isaure, M.-P., Geoffroy, N., Fakra, S., Marcus, M.A., Birschwilks, M., Clemens, S., Manceau, A., 2006. Trichomes of Tobacco Excrete Zinc as Zinc-Substituted Calcium Carbonate and Other Zinc-Containing Compounds. Plant Physiology 141, 1021-1034.

Sarret, G., Saumitou-Laprade, P., Bert, V., Proux, O., Hazemann, J.-L., Traverse, A., Marcus, M.A., Manceau, A., 2002. Forms of Zinc Accumulated in the Hyperaccumulator *Arabidopsis halleri*. Plant Physiology 130, 1815-1826.

Schat, H., Vooijs, R., Kuiper, E., 1996. Identical major gene loci for heavy metal tolerances that have independently evolved in different local populations and subspecies of *Silene vulgaris*. Evolution 50, 1888-1895.

Schillberg, S., Fischer, R., Emans, N. 2003. Molecular farming of recombinant antibodies in plants. Cellular and Molecular Life Sciences 60, 433-445.

Schmidke, I., Stephan, U.W., 1995. Transport of metal micronutrients in the phloem of castor bean (*Ricinus communis*) seedlings. Physiologia Plantarum 95, 147-153.

Schreiber, L., Hartmann, K., Skrabs, M., Zeier, J., 1999. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. Journal of Experimental Botany 50, 1267-1280.

Schwitzguébel, J.P., van der Lelie, D., Baker, A., Glass, D.J., Vangronsveld, J., 2002. Phytoremediation: European and American trends: Successes, obstacles and needs. Journal of Soils and Sediments 2, 91-99.

Semane, B., Dupae, J., Cuypers, A., Noben, J.-P., Tuomainen, M., Tervahauta, A., Kärenlampi, S., Van Belleghem, F., Smeets, K., Vangronsveld, J., 2010. Leaf proteome responses of *Arabidopsis thaliana* exposed to mild cadmium stress. Journal of Plant Physiology 167, 247-254.

Senden, M.H., Van Der Meer, A.J., Verburg, T.G., Wolterbeek, H.T., 1995. Citric acid in tomato plant roots and its effect on cadmium uptake and distribution. Plant and Soil 171, 333-339.

Shah, K., Russinova, E., Gadella Jr, T.W.J., Willemse, J., De Vries, S.C., 2002. The Arabidopsis kinase-associated protein phosphatase controls internalization of the somatic embryogenesis receptor kinase 1. Genes and Development 16, 1707-1720.

Shahzad, Z., Gosti, F., Frérot, H., Lacombe, E., Roosens, N., Saumitou-Laprade, P., Berthomieu, P., 2010. The five AHMTP1 zinc transporters undergo different evolutionary fates towards adaptive evolution to zinc tolerance in Arabidopsis halleri. PLoS Genetics 6.

Sharma, R.K., Agrawal, M., 2005. Biological effects of heavy metals: An overview. Journal of Environmental Biology 26, 301-313.

Sharma, S.S., Dietz, K.-J., 2009. The relationship between metal toxicity and cellular redox imbalance. Trends in Plant Science 14, 43-50.

Shi, G., Liu, C., Cai, Q., Liu, Q., Hou, C., 2010. Cadmium accumulation and tolerance of two safflower cultivars in relation to photosynthesis and antioxidantive enzymes. Bulletin of Environmental Contamination and Toxicology 85, 256-263.

Shojima, S., Nishizawa, N.-K., Fushiya, S., Nozoe, S., Irifune, T., Mori, S., 1990. Biosynthesis of Phytosiderophores. Plant Physiology 93, 1497-1503.

Sinclair, S.A., Krämer, U., 2012. The zinc homeostasis network of land plants. Biochimica et Biophysica Acta - Molecular Cell Research 1823, 1553-1567.

Singh, J.B., Prasad, S.M., Rai, L.C., 1991. Inhibition of photosynthetic electron transport in Nostoc muscorum by Ni2+ and Ag+. Journal of General and Applied Microbiology 37, 167-174.

Singla-Pareek, S.L., Yadav, S.K., Pareek, A., Reddy, M.K., Sopory, S.K., 2006. Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. Plant Physiology 140, 613-623.

Smeets, K., Cuypers, A., Lambrechts, A., Semane, B., Hoet, P., Van Laere, A., Vangronsveld, J., 2005. Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. Plant Physiology and Biochemistry 43, 437-444.

Solti, Á., Gáspár, L., Mészáros, I., Szigeti, Z., Lévai, L., Sárvári, É., 2008. Impact of iron supply on the kinetics of recovery of photosynthesis in Cd-stressed poplar (Populus glauca). Annals of Botany 102, 771-782.

Song, W.Y., Sohn, E.J., Martinoia, E., Lee, Y.J., Yang, Y.Y., Jasinski, M., Forestier, C., Hwang, I., Lee, Y., 2003. Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nature Biotechnology 21, 914-919.

Sun, Y., Zhou, Q., Xie, X., Liu, R., 2010. Spatial, sources and risk assessment of heavy metal contamination of urban soils in typical regions of Shenyang, China. Journal of Hazardous Materials 174, 455-462.

Takahashi, M., Terada, Y., Nakai, I., Nakanishi, H., Yoshimura, E., Mori, S., Nishizawa, N.K., 2003. Role of nicotianamine in the intracellular delivery of metals and plant reproductive development. The Plant cell 15, 1263-1280.

Talke, I.N., Hanikenne, M., Krämer, U., 2006. Zinc-Dependent Global Transcriptional Control, Transcriptional Deregulation, and Higher Gene Copy Number for Genes in Metal Homeostasis of the Hyperaccumulator *Arabidopsis halleri*. Plant Physiology 142, 148-167.

Talukdar, S., 2007. Functional characterization of three zinc transporters in *Thlaspi caerulescens*. Wagenigen University, Netherlands.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., Toh, E.A., 1990. IRA2, a second gene of Saccharomyces cerevisiae that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. Molecular and Cellular Biology 10, 4303-4313.

Tewari, R.K., Kumar, P., Sharma, P.N., 2008. Morphology and physiology of zinc-stressed mulberry plants. Journal of Plant Nutrition and Soil Science 171, 286-294.

Thomas, J.C., Davies, E.C., Malick, F.K., Endreszl, C., Williams, C.R., Abbas, M., Petrella, S., Swisher, K., Perron, M., Edwards, R., Ostenkowski, P., Urbanczyk, N., Wiesend, W.N., Murray, K.S., 2003. Yeast metallothionein in transgenic tobacco promotes copper uptake from contaminated soils. Biotechnology Progress 19, 273-280.

Thomine, S., Wang, R., Ward, J.M., Crawford, N.M., Schroeder, J.I., 2000. Cadmium and iron transport by members of a plant metal transporter family in Arabidopsis with homology to Nramp genes. Proceedings of the National Academy of Sciences of the United States of America 97, 4991-4996.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.

Vamerali, T., Bandiera, M., Mosca, G., 2010. Field crops for phytoremediation of metal-contaminated land. A review. Environmental Chemistry Letters 8, 1-17.

Van De Mortel, J.E., Schat, H., Moerland, P.D., Van Themaat, E.V.L., Van Der Ent, S., Blankestijn, H., Ghandilyan, A., Tsiatsiani, S., Aarts, M.G.M., 2008. Expression differences for genes involved in lignin, glutathione and sulphate metabolism in response to cadmium in *Arabidopsis thaliana* and the related Zn/Cd-hyperaccumulator *Thlaspi caerulescens*. Plant, Cell and Environment 31, 301-324.

Van De Mortel, J.E., Villanueva, L.A., Schat, H., Kwekkeboom, J., Coughlan, S., Moerland, P.D., Van Themaat, E.V.L., Koornneef, M., Aarts, M.G.M., 2006. Large expression differences in genes for iron and zinc homeostasis, stress response, and lignin biosynthesis distinguish roots of *Arabidopsis thaliana* and the related metal hyperaccumulator *Thlaspi caerulescens*. Plant Physiology 142, 1127-1147.

Van der Zaal, B.J., Neuteboom, L.W., Pinas, J.E., Chardonnens, A.N., Schat, H., Verkleij, J.A., Hooykaas, P.J., 1999. Over-expression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiol. 119, 1047-1055.

Van Zaal, B.J.D., Neuteboom, L.W., Pinas, J.E., Chardonnens, A.N., Schat, H., Verkleij, J.A.C., Hooykaas, P.J.J., 1999. Overexpression of a novel Arabidopsis gene related to putative zinc-transporter genes from animals can lead to enhanced zinc resistance and accumulation. Plant Physiology 119, 1047-1055.

Vangronsveld, J., Clijsters, H., 1994. Toxic effects of metals, in: ME, F. (Ed.), Plants and the chemical elements. VCH New York.

Vangronsveld, J., Clijsters, H., 2008. Toxic Effects of Metals, Plants and the Chemical Elements. Wiley-VCH Verlag GmbH, pp 149-177.

Vangronsveld, J., Herzig, R., Weyens, N., Boulet, J., Adriaensen, K., Ruttens, A., Thewys, T., Vassilev, A., Meers, E., Nehnevajova, E., van der Lelie, D., Mench, M., 2009. Phytoremediation of contaminated soils and groundwater: lessons from the field. Environmental Science and Pollution Research 16, 765-794.

Varotto, C., Maiwald, D., Pesaresi, P., Jahns, P., Salamini, F., Leister, D., 2002. The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. Plant Journal 31, 589-599.

Varun, M., D'Souza, R., Pratas, J., Paul, M.S., 2012. Metal contamination of soils and plants associated with the glass industry in North Central India: Prospects of phytoremediation. Environmental Science and Pollution Research 19, 269-281.

Vassilev, A., Schwitzguébel, J.P., Thewys, T., van derLelie, D., Vangronsveld, J., 2004. The use of plants for remediation of metal contaminated soils. Scientific World J 4, 9-34.

Verbruggen, N., Hermans, C., Schat, H., 2009. Molecular mechanisms of metal hyperaccumulation in plants. New Phytologist 181, 759-776.

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

Verret, F., Gravot, A., Auroy, P., Preveral, S., Forestier, C., Vavasseur, A., Richaud, P., 2005. Heavy metal transport by AtHMA4 involves the N-terminal degenerated metal binding domain and the C-terminal His11 stretch. FEBS Letters 579, 1515-1522.

Vert, G., Barberon, M., Zelazny, E., Séguéla, M., Briat, J.F., Curie, C., 2009. Arabidopsis IRT2 cooperates with the high-affinity iron uptake system to maintain iron homeostasis in root epidermal cells. Planta 229, 1171-1179.

Vert, G., Briat, J.-F., Curie, C., 2001. Arabidopsis IRT2 gene encodes a root-periphery iron transporter. The Plant Journal 26, 181-189.

Vert, G., Grotz, N., Dédaldéchamp, F., Gaymard, F., Guerinot, M.L., Briat, J.-F., Curie, C., 2002. IRT1, an Arabidopsis transporter essential for Iron uptake from the soil and for plant growth. The Plant Cell Online 14, 1223-1233.

Vögeli-Lange, R., Wagner, G.J., 1990. Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves: Implication of a transport function for cadmium-binding peptides. Plant Physiology 92, 1086-1093.

Voglar, D., Lestan, D., 2012. Electrochemical treatment of spent solution after EDTA-based soil washing. Water Research 46, 1999-2008.

Vorwieger, A., Gryczka, C., Czihal, A., Douchkov, D., Tiedemann, J., Mock, H.P., Jakoby, M., Weisshaar, B., Saalbach, I., Bäumlein, H., 2007. Iron assimilation and transcription factor controlled synthesis of riboflavin in plants. Planta 226, 147-158.

Wang, H.Y., Klatte, M., Jakoby, M., Bäumlein, H., Weisshaar, B., Bauer, P., 2007. Iron deficiency-mediated stress regulation of four subgroup Ib BHLH genes in *Arabidopsis thaliana*. Planta 226, 897-908.

Waters, B.M., Chu, H.H., DiDonato, R.J., Roberts, L.A., Eisley, R.B., Lahner, B., Salt, D.E., Walker, E.L., 2006. Mutations in Arabidopsis Yellow Stripe-Like1 and Yellow Stripe-Like3 reveal their roles in metal ion homeostasis and loading of metal ions in seeds. Plant Physiology 141, 1446-1458.

Weber, M., Harada, E., Vess, C., Roepenack-Lahaye, E., Clemens, S., 2004. Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine

synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. The Plant journal : for cell and molecular biology 37, 269-281.

Wojas, S., Clemens, S., Hennig, J., Sklodowska, A., Kopera, E., Schat, H., Bal, W., Antosiewicz, D.M., 2008. Overexpression of phytochelatin synthase in tobacco: distinctive effects of AtPCS1 and CePCS genes on plant response to cadmium. Journal of Experimental Botany 59, 2205-2219.

Wong, C.K.E., Cobbett, C.S., 2009. HMA P-type ATPases are the major mechanism for root-to-shoot Cd translocation in *Arabidopsis thaliana*. New Phytologist 181, 71-78.

Wong, M.H., 2003. Ecological restoration of mine degraded soils, with emphasis on metal contaminated soils. Chemosphere 50, 775-780.

Wood, B.W., Reilly, C.C., Nyczepir, A.P., 2004. Mouse-ear of Pecan: a nickel deficiency. HortScience, 1238-1242.

Wu, G., Kang, H., Zhang, X., Shao, H., Chu, L., Ruan, C., 2010. A critical review on the bio-removal of hazardous heavy metals from contaminated soils: Issues, progress, eco-environmental concerns and opportunities. Journal of Hazardous Materials 174, 1-8.

Wu, J., Zhao, F.-J., Ghandilyan, A., Logoteta, B., Guzman, M., Schat, H., Wang, X., Aarts, M., 2009. Identification and functional analysis of two ZIP metal transporters of the hyperaccumulator *Thlaspi caerulescens*. Plant and Soil 325, 79-95.

Xiong, Y.H., Yang, X.E., Ye, Z.Q., He, Z.L., 2004. Characteristics of cadmium uptake and accumulation by two contrasting ecotypes of *Sedum alfredii* Hance. Journal of Environmental Science and Health - Part A Toxic/Hazardous Substances and Environmental Engineering 39, 2925-2940.

Yang, X.E., Long, X.X., Ye, H.B., He, Z.L., Calvert, D.V., Stoffella, P.J., 2004. Cadmium tolerance and hyperaccumulation in a new Zn-hyperaccumulating plant species (*Sedum alfredii* Hance). Plant and Soil 259, 181-189.

Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D., Ling, H.Q., 2008. *FIT* interacts with *AtbHLH38* and *AtbHLH39* in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. Cell research 18, 385-397.

Zhang, B., Georgiev, O., Hagmann, M., Günes, C., Cramer, M., Faller, P., Vasák, M., Schaffner, W., 2003. Activity of metal-responsive transcription factor 1 by toxic heavy metals and H2O2 in vitro is modulated by metallothionein. Molecular and cellular biology 23, 8471-8485.

Zhang, J., 2003. Evolution by gene duplication: an update. Trends in Ecology & Evolution 18, 292-298.

Zhao, F.J., Lombi, E., Breedon, T., McGrath, S.P., 2000. Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant, Cell and Environment 23, 507-514.

SUMMARY

Increasing environmental contamination with metals is a growing risk for the ecological and health associated hazards for the global human community. Phytoremediation is an emerging technology to remediate the metal contaminated soils by using plants. There are numerous natural metal hyperaccumulator plants species found in nature and among them, *Noccaea caerulescens* can accumulate and tolerate very high concentrations of Zn, Cd and Ni. Understanding the molecular mechanisms of metal hypertolerance and hyperaccumulation in this species can help to develop high biomass plant species useful for phytoremediation. It is because natural hyperaccumulators including *N. caerulescens*, exhibit less growth and low biomass production which is not suitable for efficient phytoremediation. This thesis describes the functional analysis of two Zn transporter genes, *NcZNT1* and *NcMTP1*, from *N. caerulescens*. Furthermore, this study explores the applied implications of these genes in the high biomass species *Nicotiana tabacum* useful for phytoremediation purposes.

Functional characterization of the *NcZNT1* gene by expressing it in *Arabidopsis thaliana* and its promoter analysis compared to its homologue *AtZIP4* was performed in order to understand its metal uptake ability, regulation and tissue specific localization. Expression of *pro35S::NcZNT1* in *A. thaliana* improved Zn and Cd tolerance and accumulation of these metals compared to wild-type (WT). There was an upregulation of Fe deficiency responsive genes in *A. thaliana* under excess Zn and Cd. These results, together with the known plasma membrane localization of NcZNT1, illustrate the involvement of *NcZNT1* in Zn and Cd uptake in *N. caerulescens.* Furthermore, Fe transporters can be additionally involved in indirect Zn and Cd uptake in these lines because of their broad metal specificity, including low affinities to Zn and Cd. The differential expression pattern of *proNcZNT1::GUS* and *proAtZIP4::GUS* in *N. caerulescens* demonstrates the presence of additional *cis*- or *trans*-regulatory elements in the latter species, which are likely to control the higher *proNcZNT1* activity in *N. caerulescens*. *proNcZNT1::eGFP* and *proAtZIP4::eGFP* expression in *A. thaliana* and *N. caerulescens* showed the localization of these genes in pericycle, endodermis and/or cortex cells but not in the epidermis. This means that these genes are involved in the loading of Zn

and Cd into the stelar region of the root and thus contributing to long distance transport of metals by enhanced influx in cells associated with xylem loading and ultimately shoot translocation.

NcMTP1 was expressed in Zn and Cd defective mutant yeast and in *A. thaliana* and the expression of *NcMTP1* was knocked down in *N. caerulescens* roots to perform its functional characterization. Transgenic yeast could grow on Zn excess media but had higher sensitivity to excess Cd. Transgenic *A. thaliana* lines showed enhanced Zn and Cd tolerance and accumulation compared with the WT line. Fe deficiency responsive transporters were upregulated under excess Zn and Cd in *A. thaliana* lines. RNAi mediated knockdown of *NcMTP1* enhanced shoot Zn accumulation but reduced Cd accumulation in *N. caerulescens*. The possible vacuolar localization of *NcMTP1* together with these results illustrate the important role of *NcMTP1* in Zn sequestration into vacuoles conferring metal detoxification. The role of *NcMTP1* in Cd sequestration was unexpected as it was previously not found for any *MTP1* orthologue in other species. Furthermore, Fe transporters are again likely to play a role of their own in controlling indirect uptake of Zn and Cd in addition to Fe. Our results also suggest that low sequestration of metals in root vacuoles results in possible higher metals availability for shoot translocation.

In order to enhance the phytoremediation potential of the high biomass species *N. tabacum*, we engineered separate and combined expression of the *NcZNT1* and *NcMTP1* genes in tobacco. Transgenic *N. tabacum* lines were more tolerant to Zn and Cd and exhibited higher accumulation of these metals when grown in a hydroponic system. Furthermore, these lines had less photosynthetic and oxidative damage than the WT lines, illustrating the effectiveness of enhanced metal sequestration. Double transgenic lines had higher Zn and Cd uptake ability than the single transgenic lines. These results demonstrate that it is possible to improve the phytoremediation potential of *N. tabacum* and that the combined expression of *NcZNT1* and *NcMTP1* has an additive effect on metal accumulation.

For the analysis of the improved phytoremediation potential of transgenic *N. tabacum* lines expressing single and double *NcZNT1* and *NcMTP1* constructs, these lines were grown in metal contaminated field soil. Six different

metal contaminated soils were analysed and a suitable toxic soil was used for growing transgenic and WT lines. All transgenic lines had higher chlorophyll a, chlorophyll b, carotenoids and shoot Mg concentrations than the WT line while the *pro35S::NcMTP1* line had the lowest lipid peroxidation of all. The *pro35S::NcMTP1* and *pro35S::NcZNT1 + pro35S::NcMTP1* lines had higher dry biomass and enhanced total Zn and Cd accumulation compared to *pro35S::NcZNT1* and WT lines. The interesting aspect of this study was that our best performing line, *pro35S::NcMTP1*, showed the ability to reduce the Cd pollution level of metal contaminated soil from 2.11 mg kg^{-1} to 1.05 mg kg^{-1} (which lies in the normal range in agricultural soils) in only 14 generations compared to the 28 generations for WT. In contrast to the hydroponically grown plants, the double transformed plants did not perform better than either of the single transformed lines on Zn or Cd containing substrate. It is not clear why that is. However, these results demonstrate that especially the *pro35S::NcMTP1*, but also the *pro35S::NcZNT1 + pro35S::NcMTP1* line, have an improved phytoextraction capacity than WT. *pro35S::NcZNT1* may not be so suitable for phytoremediation in highly polluted soils since it lacks the vacuolar sink for the metals for detoxification and will suffer from metal intoxication. Still, the improved phytoextraction capacity brought about by CaMV 35S mediated expression of two Zn transporters from *N. caerulescens* remains far below the capacity of the natural hyperaccumulators and clearly additional genes will need to be engineered to approach those levels of metal accumulation. Overall our results brought new molecular insights into metal tolerance and hyperaccumulation traits of plants and have proved that genetic engineering can pave the way for the development towards a viable phytoremediation technology.

ACKNOWLEDGEMENTS

Praise and glory be to Allah Almighty, Who is the Creator of the universe and Whose blessings have enabled me to strive in the field of knowledge.

First of all, I am thankful to the Higher Education Commission (HEC) of Pakistan for proving me scholarship to pursue PhD studies, which enabled me to perform my research and helped me to develop my scientific vision and personality. I offer my gratitude to the Netherlands Organization for International Cooperation in Higher Education (NUFFIC) for the continuous management of my scholarship and health insurance. I appreciate Graduate School Experimental plant Sciences (EPS) for suporting my project and offering all the nice courses. I am thankful to the European Cooperation in Science and Technology (COST) "Action FA0905" for providing me an "STSM" grant to perform research and travel grants to attend various scientific conferences.

I express my heartiest gratitude to my supervisor Dr. Mark Aarts for providing me the opportunity to perform research in his lab and for the continuous mentoring during my PhD studies. Mark, your supervision and scientific criticism has enabled me to develop in my field of research. I always enjoyed your hospitality and chit chat during social gatherings. I appreciate the kind supervision of my promoter Prof. Dr. Maarten Koornneef and his critical reading and suggestions for the preparation of this manuscript. Maarten, I loved the way you speak about science in our meetings and enjoyed your accompany during various dinners.

I offer my sincerest gratitude to my former lab colleagues Hedayat, Wessel, Ana Assunção, Artak, Diana and Vanessa for their valuable suggestions and help during my research work. I am thankful to my lab fellows Mohammed, Ana Carolina, Ya-Fen, Charles, Mina, Nihal, Jimeng, Pingping, Yanli, Pádraic, Frank, Aina, Roxanne, Michele, Sam, Ariadne, Guan, Christiane and Valeria for all the help during my experiments, kind and supportive attitude and amusing conversations. My experiments would not have been

possible without the technical support of Corrie. I always liked to talk to you Corrie and you were always very helpful to me. Many thanks to Aafke and Wytske for the management of official formalities regarding my project and for their helpful and kind behaviour towards me.

Many thanks to Prof. Dr. Jaco Vangronsveld, Prof. Dr. Ann Cuypers, Tony Remans, Ann Wijgaerts, Els Keunen and Marijke Jozefczak for their generous assistance in performing experiments in Hasselt University, Belgium.

My beloved friends living in Wageningen will always remain close to my heart. I would love to thank Imran, Masood, Mustafa, Abbas Shah, Abbas Gull, Shafqat, Kashif, Liyakat, Nadeem bhai, Rashid, Noorullah, Mazhar Ali, Mazhar A. Khan, Mazhar I. Zafar, Hamid, Zakir bhai, Shahid bhai, Mubarak, Luqman, Sohail, Ch. Ghulam Ali, A.R. Bhatti, Hashim, Tom and Pierre for their everlasting friendship. I thank all the Pakistani friends and their families i.e. Imtiaz, Sajid bhai, Akmal, Abid, Farrakh bhai, Nazir Khan, Nazir Tahir, Sabaz, Sultan, Uncle Matteen, Munawwar bhai, Uncle Amin, Jamil bhai and Ali bhai for arranging all the nice gatherings and conversations. I appreciate all my friends outside Wageningen i.e. Hasnain bhai, Farooq, Mazhar Iqbal, Imran Bhatti, Aamir Aslam, Aamir Bhai, Tariq Bhai, Waseem Bhai, Shabaz, Muzammil, Yahya, Nadeem and Majid for all the affection and hospitality which they bestowed upon me. I express my special and heartiest thanks to my everlasting friends Khurshid, Tayyab and Fraz for their sincerity, support and best wishes. I would like to say thanks to my friends living in Pakistan i.e. Shajar bhai, Azhar Islam, Imran, Ali, Kashif, Zeeshan bhai and Musafir bhai for their kind support and encouragement from long distance.

Finally, I express my sincere gratitude, wholeheartedly, to my parents, wife and sisters for their love, support and patience. They are the endless source of affection, prayers and strength for my life. May Allah Almighty bless them.

Zeshan Hassan

CURRICULUM VITAE

Zeshan Hassan was born in Layyah, Pakistan on October 23, 1982. He acquired primary and higher secondary education at Layyah Public School and Govt. Degree College, Layyah respectively. In 2001, he joined the University of Agriculture Faisalabad (UAF) and completed his B.Sc. (HONS.) Agriculture in 2005 with major Plant Beeding and Genetics. During B.Sc., he performed an internship at Central Cotton Research Institute (CCRI) in Multan, where he worked on maintenance and development of Cotton genetic stock and designed layout of various field experiments. After bachelor studies, he completed M. Phil Biotechnology degree at National Institute for Biotechnology and Genetic Engineering (NIBGE), Faisalabad in 2008. In his M.Phil thesis, he studied the "Development and Transformation of different Gene Constructs for Insect Resistance in Model Plant" Tobacco. In late 2007, he joined Wageningen University, The Netherlands after being awarded an overseas "MS leading to PhD" scholarship provided by the Higher Education Commission of Pakistan (HEC). He started his first year by undertaking M.Sc. courses and completed his M.Sc. thesis in the Laboratory of Genetics in early 2009 entitled "Functional Analysis of Zn Transporter Genes *TcZTP1* and *TcZNT1* of *Thlaspi caerulescens* for Metal Hyperaccumation Purposes" under the supervision of Dr. Mark Aarts. He continued his M.Sc. research topic and worked as a PhD student from 2009 till 2013, under the supervision of Dr. Mark Aarts and Prof. Dr. Maarten Koornneef in the Laboratory of Genetics, Wageningen University.

PUBLICATIONS

Hassan, Z., Aarts M.G.M., 2011. Opportunities and feasibilities for biotechnological improvement of Zn, Cd or Ni tolerance and accumulation in plants. Journal of Environmental and Experimental Botany 72, 53–63.

Hassan, Z., Lin, Y-F., Talukdar, S., Hong, L., Arulandhu, A., Schat, H., Aarts, M.G.M., 2012. Functional analysis of *NcZNT1* gene and its promoter, a stele-specific zinc transporter of the metal hyperaccumulator *Noccaea caerulescens* confers enhanced zinc and cadmium tolerance and accumulation to *Arabidopsis thaliana*. (submitted)

Hassan, Z., Ochoa, V., Kawachi, M., Fernandes, C.N., Schat, H., Krämer, U., Aarts, M.G.M., 2012. Expressing a vacuolar metal transporter NcMTP1 of *Noccaea caerulescens* enhances Zn and Cd tolerance and accumulation in *Arabidopsis thaliana*. (submitted)

Hassan, Z., Cuypers, A., Schat, H., Vangronsveld, J., Aarts, M.G.M., 2012. Separate and combined expression of NcZNT1 and NcMTP1 metal transporters improves the Zn and Cd phytoremediation potential of *Nicotiana tabacum*. (In preparation)

Iqbal, M., Nawaz, I., **Hassan, Z**., Hakvoort H.W.J, Bliek, Aarts, M.G.M., Schat, H., 2012. Expression of *HMA4* cDNAs under *HMA4* promoters from the zinc hyperaccumulator *Noccaea caerulescens* does not reverse the zinc-deficiency phenotype of the *Arabidopsis thaliana hma2hma4* double mutant. (submitted)

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: **Zeshan Hassan**
Date: **28 January 2013**
Group: **Genetics, Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Functional analysis of zinc transporter genes *ZTP1* and *ZNT1* of *Thlaspi caerulescens* for metal hyperaccumulation purposes	Jan 12, 2009
► **Writing or rewriting a project proposal**	
Functional analysis of zinc hyperaccumulation related genes of *Noccaea* (*Thlaspi*) *caerulescens* for phytoremediation purposes	Jul 2009
► **Writing a review or book chapter**	
Opportunities and feasibilities for biotechnological improvement of Zn, Cd or Ni tolerance and accumulation in plants, Environm. and Exp. Botany 72 (2011) 53-63	Jan 30, 2010
► **MSc courses**	
Plant, Cell and Tissue Culture (PPH-30306), Wageningen University, The Netherlands	Mar-Apr 2008
Gene Technology (MOB-20306), Wageningen University, The Netherlands	May-Jun 2008
► **Laboratory use of isotopes**	
Safe handling of radioactive isotopes, level 5B, Wageningen University, The Netherlands	May 26-28, 2009
Subtotal Start-up Phase	*21,0 credits**

2) Scientific Exposure	*date*
► **EPS PhD Student Days**	
EPS PhD student day, Leiden, The Netherlands	Feb 26, 2009
EPS PhD student day, Utrecht, The Netherlands	Jul 01, 2010
EPS PhD student day, Wageningen, The Netherlands	May 20, 2011
► **EPS Theme Symposia**	
EPS theme 4 'Genome Plasticity', Wageningen University, The Netherlands	Dec 12, 2008
EPS theme 2 'Interactions between Plants and Biotic Agents', Utrecht University, The Netherlands	Jan 22, 2009
EPS theme 1 'Developmental Biology of Plants', Leiden University, The Netherlands	Jan 30, 2009
EPS theme 3 'Metabolism and Adaptation', University of Amsterdam, The Netherlands	Feb 18, 2009
EPS theme 4 'Genome Plasticity', Radboud University, The Netherlands	Dec 01, 2009
EPS theme 1 'Developmental Biology of Plants', Wageningen University, The Netherlands	Jan 28, 2010
EPS theme 3 'Metabolism and Adaptation', Wageningen University, The Netherlands	Feb, 10, 2011
EPS theme 1 'Developmental Biology of Plants', Wageningen University, The Netherlands	Jan 19, 2012
EPS theme 3 'Metabolism and Adaptation', Utrech University, The Netherlands	Apr 26, 2012
► **NWO Lunteren days and other National Platforms**	
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 07-08, 2008
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 06-07, 2009
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 19-20, 2010
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 04-05, 2011
ALW meeting 'Experimental Plant Sciences', Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Seminars (Prof. Dr. Zhenbi Yang; Prof. Dr. Hong-Qing Ling; Prof. Jian-Kang Zhu; Prof. Dr. Sjef Smeekens)	2008
Symposium: RNAomics: Rediscovering RNA and its Multiple Functions, Radboud University, NL	Aug 07, 2008
Symposium: Plant Roots: From Genes to Ecosystems, Radboud University, The Netherlands	Oct 23, 2008
Symposium: The Schilperoort Lectures: Success Stories of Entrepreneurial Scientists, Wageningen UR	Nov 05, 2008
Seminar: Science From an Editor's View, by Dr. Pamela J. Hines, Wageningen UR	Nov 06, 2008
Symposium: New Opportunities for Conservation Genetics with Genome Wide Information, Wageningen	Dec 08, 2008
Mini Symposium: Bibliometrics at Wageningen UR, Wageningen University, The Netherlands	Nov 27, 2008
Seminar: Dr. Hiro Nonogaki	Sep 17, 2009
EPS Symposium: Ecology and Experimental Plant Sciences, Wageningen University, NL	Sep 22, 2009
Farewell Symposium: Dr. Pim Zabel "Art Meets Science", Wageningen University, The Netherlands	Oct 16, 2009
Symposium: National EcoGenomics Day, Amsterdam, The Netherlands	Apr 21, 2010

	Conference: Technology Transfer in the Plant Sciences: Enhancing national and international benefits, Wageningen, The Netherlands	May 20-21, 2010
	Seminars (Dr. John Yoder; Dr. David Baulcombe; Dr. Robert Kraus)	2010
	Wageningen UR Sequencing Seminar, Wageningen University, The Netherlands	Dec 07, 2011
	Seminar: Prof. Dr. Steffen Abel	Mar 20, 2011
	Seminar Series Plant Physiology-Genetics, Wageningen University, The Netherlands	2008-2012
►	**Seminar plus**	
►	**International symposia and congresses**	
	Genetics of Plant Mineral Nutrition, Hannover, Germany	Sep 30-Oct 02, 2010
	International heavy metals meeting, Wageningen, The Netherlands	Oct 26, 2010
	3rd Joint PhD Retreat, Orsay, France	Jul 05-08, 2011
	COST-Action FA 095 Meeting, Venice, Italy	Nov 24-26. 2011
	ASPB "Plant Biology 2012" meeting, Austin, USA	Jul 20-24, 2012
	4th Joint PhD Retreat, Norwich, UK	Aug 15-17, 2012
	9th International Conference on Phytotechnologies, Hasselt, Belgium	Sep 11-14, 2012
►	**Presentations**	
	EPS theme 3 meeting: Metabolism and Adaptation, Amsterdam, The Netherlands (Oral)	Feb 18, 2009
	Uptake, Sequestration and Detoxification-An Integrated Approach, Szeged, Hungary (Poster)	Apr 15-17, 2009
	Genetics of Plant Mineral Nutrition, Hannover, Germany (Poster)	Sep 30-Oct 02, 2010
	International heavy metals meeting, Wageningen, The Netherlands (Oral)	Oct 26, 2010
	ALW meeting: Experimental Plant Sciences, Lunteren, The Netherlands (Oral)	Apr 05, 2011
	3rd Joint PhD Retreat, Orsay, France (Oral)	Jul 05-08, 2011
	COST-Action FA 095 meeting, Venice, Italy (Oral)	Nov 23-26. 2011
	ASPB "Plant Biology 2012" meeting, Austin, USA (Oral)	Jul 20-24, 2012
	4th Joint PhD Retreat, Norwich, UK (Poster)	Aug 15-17, 2012
	9th International Conference on Phytotechnologies, Hasselt, Belgium (Poster)	Sep 11-14, 2012
►	**IAB interview**	Feb 18, 2011
►	**Excursions**	
	Field excursion for sample collection, La Calamine, Belgium	Mar 24, 2009
	Field excursion for sample collection, Lommel, Belgium	Apr 19, 2012
	Subtotal Scientific Exposure	*29,3 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	PhD work shop: Natural Variation, Wageningen University, The Netherlands	Aug 26-29, 2008
	PhD course: RNAi & the World of Small RNA Molecules, Wageningen University, The Netherlands	Apr 14-16, 2010
►	**Journal club**	
	Literature discussion: Plant Genetics group, Wageningen University, The Netherlands	2009-2012
►	**Individual research training**	
	Atomic Absorption Spectophotometer, Vrije University, Amsterdam, The Netherlands	Feb 03-05, 2009
	Imaging Microscopy, PCB, Wageningen University, The Netherlands	Aug 12, 2011
	Analysis of Plant Redox State, Hasselt University, Belgium	Nov 07-18, 2011
	Subtotal In-Depth Studies	*8,1 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Workshop: Uptake, Sequestration and Detoxification-An Integrated Approach, Szeged, Hungary	Apr 16-17,2009
	Course: Project and Time Management, Wageningen, The Netherlands	Nov 3, 17, Dec 15, 2009
	WGS Workshop: Scientific Publishing, Wageningen, The Netherlands	Nov 19, 2009
	PE&RC Day: Selling Science, Wageningen, The Netherlands	Oct 28, 2010
	EPS Career Event: ExPectationS Day, Wageningen, The Netherlands	Nov 19, 2010
	Workshop: Digital Art, Austin, USA	Jul 21, 2012
	Workshop: Case Study Teaching-Engaging Students in Plant Biology, Austin, USA	Jul 22, 2012
	Minisymposia: Education and Outreach, Austin, USA	Jul 23, 2012
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*3,3 credits**

TOTAL NUMBER OF CREDIT POINTS*	**61.7**

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

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

This project was financed by the Higher Education Commission of Pakistan (HEC) and the Graduate School Experimental Plant Sciences (EPS), Wageningen University, The Netherlands.