Paving the way for genetic improvement of zinc accumulation in *Brassica rapa*

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Chapter 1

General Introduction

The importance of micronutrients

Micronutrients are essential trace minerals and vitamins that are needed in a small amount for various physiological functions of organisms. The fourteen essential trace minerals comprise Fe, Co, Cr, Cu, I, Mn, Se, Zn, Mg, Ni, Mo, Si, V and B. Insufficient supply of these mineral elements results in disturbed physiological process in organisms. However, the micromineral deficiency is a prevalent nutritional problem for human due to the unbalanced diet as well as for crops because of the poor availability of these elements in soils. More than 3 billion people worldwide suffer iron deficiency, 1.5 billion have a shortage of iodine, and 250 million pre-school children are deficient in vitamin A (Graham et al., 2001). Although there is no figures on the extent of Zn deficiency because of the lack of a simple clinical screening procedure, specialists in Zn nutrition consider Zn and Fe deficiencies equally important (Graham et al., 2001). Insufficient intake of these three elements together with vitamin A deficiency is of high priority in primary health care. Other micronutrient deficiencies of importance to human health include selenium and boron deficiency, though their roles in human nutrition are only emerging.

Zn deficiency and human health

A search for genes in the human genome encoding for proteins with a Zn-binding domain revealed that 10% of all proteins potentially bind Zn (Andreini et al., 2006), and as a consequence in humans, as in all organisms, Zn is the most abundant transition metal after Fe (Broadley et al., 2007). Zinc (Zn) is an essential component of many enzymes involved in virtually all aspects of metabolism, gene expression and structure stabilization of proteins and nucleic acids (Vallee and Auld, 1990; Broadley et al., 2007). In humans Zn deficiency was first identified in the 1960s in the Middle East (Prasad et al., 1963). According to the estimation of the WHO, the percentage of the population at risk for low Zn intake ranges from 1-13% in Europe and North Amercia to 68-95% in South and Southeast Asia, Africa, and regions in the eastern Mediterranean. Globally, nearly half of the world's population is at risk for inadequate Zn intake (World Health Organization, 1996). Zn in diet comes directly from plants or indirectly, through animal sources. Plant breeding strategies hold great promise for making a significant low-cost and sustainable improvement of the intake of bioavailable

Zn in human populations (Ruel and Bouis, 1998). It is easy to see that ensuring that plants take up adequate amounts of Zn will help to solve a major human health problem. In addition, it can also significantly improve crop productivity.

Zinc deficiency and crop production

Zn available to plants is present in the soil solution or is adsorbed to the roots in a labile form. Many soil factors affect the availability of Zn to plants. These include the total Zn content, pH, organic matter content, calcium carbonate content, redox conditions, microbial activity in the rhizosphere, soil moisture status and concentration of other micronutrients and macro-elements (Alloway, 2004). Zn deficiency is common in a wide range of soil types, including high pH calcareous soils, sandy soils and high phosphorous-containing fertilized soils (Marschner, 1995). In total about 30% of the soils in the world are affected by Zn deficiency (Sillanpaa, 1990). Plant tolerance to micronutrient-deficient soils, termed micronutrient use efficiency, is a genetic trait that characterizes the adaptation of a genotype to micronutrient-deficient soils compared to the average cultivar of the species (Graham, 1984). Within crop species, individual varieties can often vary considerably in their response to Zn deficiency (Hacisalihoglu and Kochian, 2003). Growing Zn-efficient plants on Zn-deficient soils represents a strategy of “tailoring the plant to fit the soil” in contrast with the alternative strategy of “tailoring the soil to fit the plant” (Ruel and Bouis, 1998), and will be a more environmental friendly and sustainable approach.

The metal homeostasis network in plant

Essential metal accumulation is tightly controlled and regulated at the level of the whole organism as well as at the cellular level. A coordinate functioning of uptake, buffering, translocation and storage processes is required to maintain necessary, but non-toxic, metal concentrations in different tissues and cell compartments.

Mobilization

The availability of essential metals is affected by many soil factors. Generally their bioavailability is low due to the low solubility of metals in oxygenated water and strong binding to soil particles. Active acquisition processes are needed. The best described active mobilization of an essential element is for Fe, which is either reduced from Fe^{3+} to Fe^{2+} by ferric-reductases or chelated as Fe^{3+} upon extrusion of phytosiderophores (Marschner, 1995). Also soil microorganisms play a role as they

were found to significantly enhance Zn accumulation in the shoots of the hyperaccumulator *Thlaspi caerulescens* (Whiting et al., 2001). Both acidification of the rhizosphere and exudation of carboxylates are considered as potential ways for plants to enhance metal mobilization (Clemens et al., 2002).

Uptake and sequestration

Following mobilization, a metal ion has to be captured by root cells. Metals are first bound by the cell wall before they are transported across the plasma membrane through active transport systems. Zn is acquired from the soil solution primarily as Zn^{2+}, but also as complexes with organic ligands. Transition metals can be transported symplastically from the root epidermal and cortical cells through the endodermal cells into the root xylem upon which they are transported to other parts of the plant (Lasat et al., 1996). A lack of specificity of uptake and distribution systems can lead to the accumulation of nonessential and toxic metals such as Cd or As, which pose a threat to the plant.

Xylem transport

In general, solutes have to be taken up into the root symplast before they can enter the xylem (Tester and Leigh, 2001). Metal uptake into the xylem involves several steps: sequestration of metals inside root cells, symplastic transport into the stele and release into the xylem. The transport of ions into the xylem is a tightly controlled process mediated by membrane transporters (Clemens et al., 2001). AtHMA4 has been demonstrated as one of the transporters involved in this process (Verret et al., 2005). In addition, chelation of metals with certain ligands, for instance, citrate (Senden et al., 1995), nicotianamine (Pich et al., 1994), histidine (Krämer et al., 1996), appears to be required for xylem transport. Xylem unloading processes are the first steps that allow the controlled distribution and detoxification of metals in shoot, as well as in a possible re-distribution of metals via the phloem.

Unloading, trafficking and storage

Metals reach the apoplast of leaves via the xylem sap, from where they have to be delivered to leaf cells (Marschner, 1995). Transporter mediated uptake and distribution within the leaf occurs via the apoplast or the symplast (Karley et al., 2001). Metal trafficking systems are therefore essential in ensuring delivery of sufficient metal ions to their target proteins within specific compartments of the cells. The excess of essential metals and the non-essential metals are sequestered in leaf cell vacuoles (Vogeli-Lange and Wagner, 1990). Some metal accumulating species prefer to store metals in

epidermal or trichome cells, which thus appear to play a major role in storage and detoxification of metals (Salt et al., 1995; Küpper et al., 1999; Küpper et al., 2000).

Heavy metal transporters in plants

Recent studies revealed key roles of transport systems in plant metal ion homeostasis. The coordinated action of uptake and secretion systems made up by metal transporters enables the proper metal homeostasis. Plant genomes encode different and often large families of metal transporters that vary in their substrates specificities, expression patterns and sub-cellular localization to govern metal translocation throughout the plant. According to the direction of metal movement from the cytoplasm these transporter families can be divided into uptake and efflux proteins (Colangelo and Guerinot, 2006). The uptake proteins have roles either in uptake at the plasma membrane or in remobilization metals from intracellular organelles. They include the ZIP, NRAMP, COPT and YSL gene families. In contrast, the efflux proteins are involved in metal efflux from the cytoplasm either by movement across the plasma membrane or into organelles. They include the P_{1B}-ATPase, ZIF (MFP) and CDF families.

ZIP family transporters

The ZIP (ZRT, *I*RT- like Protein) transporters are involved in the transport of a variety of cations, including Fe, Zn, Mn and Cd, with family members differing in their substrate range and specificity (Guerinot, 2000; Mäser et al., 2001). The ZIP proteins have several common features such as: eight transmembrane domains (TM) with the amino- and carboxyl- terminal ends situated on the outer surface of the plasma membrane; a variable region between TM3 and TM4 rich in histidine residues, which is predicted to be a potential metal binding domain, and a fully conserved histidine that may form part of an intramembranous metal binding site involved in transport (Guerinot, 2000; Mäser et al., 2001).

AtIRT1, the first metal transporter gene identified in plants, was cloned by functional complementation of a Fe-uptake deficient yeast mutant (Eide et al., 1996), and was demonstrated to be the major transporter for high affinity Fe uptake by roots (Connolly et al., 2002; Vert et al., 2002). The protein encoded by *AtIRT1* localizes to the plasma membrane of predominantly the external cell layers of the root. *AtIRT2*, a homologue of *AtIRT1*, has a narrower substrate range and cannot substitute for the loss of *AtIRT1* (Grotz and Guerinot, 2002). Homologues of *AtIRT1* have also been identified in a number of other plant species, such as rice (*OsIRT1*) (Bughio et al., 2002) and tomato

(*LeIRT1* and *LeIRT2*) (Eckhardt et al., 2001). All could functionally complement the Fe-uptake deficient mutant of yeast, and encode for proteins with a similar role as AtIRT1.

ZIP proteins also contribute to Zn homeostasis in plant. *AtZIP1*, *2* and *3* were isolated by their ability to complement a Zn uptake deficient mutant in yeast (Grotz et al., 1998) along with an incomplete *AtZIP4* gene that did not confer Zn uptake activity in yeast (Grotz et al., 1998). However, its homologue, the *TcZNT1* gene from the Zn hyperaccumulator species *Thlaspi caerulescens* was isolated by functional complementation of a yeast Zn uptake deficient mutant (Pence et al., 2000). *TcZNT1* and the closely related *TcZNT2*, is overexpressed in *T. caerulescens* compared with nonhyperaccumulator congener *T. arvense*, suggesting an important role for these proteins in Zn hyperaccumulation (Assunção et al., 2001). *OsZIP4*, a member of the ZIP family in rice, showed a selective transport activity of Zn in yeast complementation experiments and its mRNA accumulated under Zn deficiency in the phloem and apical meristem of roots and shoots (Ishimaru et al., 2005).

NRAMP family transporters

The NRAMP (Natural Resistance Associated Macrophage Proteins) family is a highly conserved family of integral membrane proteins that are involved in metal ion transport in a wide range of organisms, including bacteria, fungi, plants and animals. In plants, this family was first identified in rice where three NRAMPs (OsNRAMP1-3) were reported (Belouchi et al., 1995, 1997). Plant NRAMP family members have been implicated in the transport of several divalent cations, including Fe and Cd. Six members of *NRAMP* have been identified in Arabidopsis based on whole genome sequencing, among which *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* encode proteins that are functionally characterized as vacuolar membrane localized Fe/Cd/Mn transporters (Thomine et al., 2000). Over-expression of *AtNRAMP1* leads to resistance to Fe toxicity (Curie et al., 2000). The *nramp3 nramp4* double mutant was shown to have arrested seed germination under low Fe nutrition although the Fe content in seed is the same as in wild type. This phenotype was fully rescued by high Fe supply (Lanquar et al., 2005). These results indicate that AtNRAMP3 and AtNRAMP4 function redundantly in the mobilization of Fe from the vacuole during early seedling development. An *AtNRAMP4* homologue, *TjNramp4* was cloned from the Ni hyperaccumulator *T. japonicum* and was suggested to contribute to Ni tolerance based on yeast expression studies (Mizuno et al., 2005).

COPT family transporters

The COPT (COPper Transporter) family of high-affinity Cu uptake proteins are found throughout

eukaryotes, including five members in *A. thaliana* named as *COPT1-COPT5* (Sancenon et al., 2003). All members of the COPT family contain three predicted transmembrane segments and most possess an amino-terminal Met and a His-rich putative metal binding domain (Puig and Thiele, 2002). *COPT1* has been identified from *A. thaliana* as a putative Cu influx transporter gene by functional complementation of a yeast mutant (Kampfenkel et al., 1995). *COPT1* showed its highest expression in leaves and low expression in stems and flowers, but the gene was not expressed in roots (Kampfenkel et al., 1995). Sancenon et al. (2003) complemented a yeast high Cu affinity transporter mutant by *COPT1, 2, 3* and *5*, although *COPT1* and *COPT2* were most efficient in complementing the mutant compared to the other family members. The physiological function of COPT1 was further shown by the decreased uptake of Cu in *COPT1* antisense transgenic seedlings, and a dramatically increased root length which was completely reversed by copper addition (Sancenon et al., 2004).

YSL family transporters

The YSL (Yellow Stripe-Like) gene family encodes plant-specific transporters. YSL proteins have been identified based on the sequence similarity to the maize *Yellow Stripe 1* (*YS1*) gene which encodes a transporter responsible for the primary uptake of iron from the soil (Curie et al., 2001; Schaaf et al., 2004). YS1 transports iron complexed by phytosiderophores (PS), while in non-grass species YSL family members transport Fe, and possibly Cu, Ni and Zn, which is chelated by nicotianamine (NA), a metal chelator which is structurally similar to PS and which is found in all higher plants (Didonato et al., 2004). Based on the sequence similarity, eight YSL family members are predicted in the *A. thaliana* genome (Didonato et al., 2004). *AtYSL2* is one of the members which has been studied in detail and is suggested to function in the lateral movement of metals in the vascular system (Didonato et al., 2004). Recently Gendre et al. (2007) identified three *YSL* genes from *T. caerulescens*, where one of them (*TcYSL3*) is a nicotianamine-Fe/Ni influx transporter that is expressed at equal levels in roots, shoots and flowers.

P_{1B}-ATPase family transporters

P-type ATPase transporters are representatives of a super family which uses ATP to pump a variety of charged substrates across biological membranes. The gene family has been divided into five groups according to their substrate specificity. Heavy metal ATPases have been classified as type P_{IB}-ATPases (Axelsen and Palmgen, 2001; Williams and Mills, 2005). They have also been described as CPx-ATPases as they share a conserved intramembraneous

cysteine-proline-cysteine/histidine/serine motif (CPx motif), which is thought to function in heavy metal transduction. The eight P_{1B}-ATPases in *A. thaliana* are designated Heavy Metal transporting P-type ATPase (*HMA1-8*). The first P-type ATPase gene reported in plants was *PAA1* (*AtHMA6*) from *A. thaliana* (Tabata et al., 1997), which was reported to function in the transport of copper to the chloroplast (Shikanai et al., 2005).Four family members (*HMA1-4*) group with the Zn/Cd/Pb/Co divalent cation transporter class of P-type ATPases, whereas *HMA5-HMA8* encode Cu/Ag monovalent cation transporters (Axelsen and Palmgen, 2001; Williams and Mills, 2005). Metal transport studies of *AtHMA2* and *AtHMA4* in yeast (Eren and Arguello, 2004; Mills et al., 2003; Verret et al., 2005) and the *A. thaliana* double mutant (Hussain et al., 2004) provided evidence of their role in xylem loading of Zn for long distance transport to the shoot. By contrast AtHMA3 localized at the vacuolar membrane with a role in the influx of Cd into the vacuolar compartment (Gravot et al., 2004). *TcHMA4* isolated from *T. caerulescens* mediates metal tolerance in yeast via efflux out of cells of a variety of metals (Cd, Zn, Pb, Cu). However, in *T. caerulescens* it may play a role in Zn hyperaccumulation, rather than Zn tolerance, as a transporter involved in xylem loading, based on the tissue-specific and metal-responsive expression of the gene (Papoyan and Kochian, 2004).

CDF (MTP) family transporters

Members of the ubiquitous CDF (Cation Diffusion Facilitator) family of metal transporters, also called MTPs (metal tolerance proteins), contain six transmembrane domains. They act as proton antiporter that efflux heavy metals out of the cytoplasm (Hall and Williams, 2003). The first CDF transporter gene that was identified in *A. thaliana* was designated *ZAT* (zinc transporter *Arabidopsis thaliana*; van der Zaal et al., 1999). Over-expression of *ZAT* led to a significant increase in Zn tolerance and a strongly increased Zn content in roots under exposure to high Zn (van der Zaal et al., 1999). The protein was shown to localizing in the vacuolar membrane (Kobae et al, 2004; Desbrosses-Fonrouge et al., 2005). More recently, AtMTP3 has been identified as a vacuolar membrane transporter and functions in metal homeostasis by mediating Zn exclusion from the shoot under Fe deficiency and Zn oversupply (Arrivault et al., 2006). Therefore the CDF proteins were proposed to be involved in the vesicular/vacuolar sequestration of Zn and have a role in Zn homeostasis and tolerance. Twelve CDF members are predicted to occur in Arabidopsis based on its genome sequence (Delhaize et al., 2003). CDF genes have also been identified from other plant

species, such as *TcZTP1* from *T. caerulescens* (Assunção et al, 2001), *TgMTP1* from *T. goesingense* (Persans et al, 2001) and *ShMTP1* from *Stylosanthes hamata* (Delhaize et al., 2003), which are involved in hyperaccumulation of or tolerance to Zn, Ni and Mn respectively.

ZIF (MFP) family transporters

ZIF (Zinc Induced facilitator) is a novel transporter family identified recently from characterization of Zn sensitive Arabidopsis *zif1* mutants (Hussain et al., 2004; Haydon and Cobbett, 2007). *ZIF1* encodes a Major Facilitator Superfamily (MFS) transporter with low similarity to any previously characterized proteins. ZIF1 localizes to vacuolar membrane and conferred increased Zn tolerance and caused interveinal leaf chlorosis when it was ectopically over-expressed. Although ZIF1 and ZTP1 both contribute to Zn tolerance and localize in the vacuolar membrane, the functions of the proteins encoded by them seem via independent mechanisms, due to the different phenotypes observed in *mtp1* and *zif1* mutants. The ZIF1 protein seems functions in a novel mechanism of Zn sequestration, possibly by transport of a Zn ligand or a Zn ligand complex into vacuoles.

Thlaspi caerulescens

Thlaspi caerulescens J. & C. Presl, a herbaceous annual, biennial or short-lived perennial, has been known for a long time to hyperaccumulate zinc up to 3 % Zn of dry matter in shoots without suffering from toxicity (Brown et al., 1995; Shen et al. 1997). It belongs to Brassicaceae family and shares 88% DNA identity with *A. thaliana* in coding region (Rigola et al., 2006). These features make *T. caerulescens* a very interesting experimental system for studying mechanisms for heavy metal transport and accumulation in plants. Zn hyperaccumulation is a constitutive trait in *T. caerulescens* (Meerts and van Isacker, 1997). In addition to Zn hyperaccumulation, *T. caerulescens* is one of the three known Cd hyperaccumulators besides *A. halleri* and *Sedum alfredii* (Zhao et al., 2002; Xiong et al., 2004). However, unlike Zn accumulation, Cd and Ni accumulation are not constitutive at the species level in *T. caerulescens* (Assunção et al., 2003a).

Physiological mechanism of Zn hyperaccumulation

T. caerulescens consistently exhibits extremely high foliar Zn concentrations, regardless of the soil Zn status in the field. Zn transport is stimulated at multiple sites along the metal absorption and translocation pathway, including Zn influx into both root and leaf cells, and Zn loading into the xylem, contributing to the hyperaccumulation traits (Lasat et al., 2000; Cosio et al., 2004). *T.*

caerulescens is effective in mobilizing Zn from less soluble fractions in the soil (McGrath et al., 1997). However, rhizosphere acidification (McGrath et al., 1997) or the production of root exudates (Zhao et al., 2001) was not the main mechanism for the hyperaccumulation. Similar to other hyperaccumulators, metal is mainly stored in the leaves, while keeping the root metal concentrations at levels comparable to, or even lower than in non-hyperaccumulators (Lasat et al., 1996). The 4.5-fold higher maximum initial velocity for Zn^{2+} influx in *T. caerulescens* roots cells than in *T. arvense* indicated that enhanced absorption into the root is one of the mechanisms involved in Zn hyperaccumulation (Lasat et al., 1996), while apoplast binding seems not to be the reason for the increase (Zhao et al., 2002). Studies of the kinetics of Zn influx showed similar K_m values in roots between *T. caerulescens* and *T. arvense*, while V_{max} was much higher in *T. caerulescens* than in *T. avense*, suggesting a higher expression of functionally very similar transporters in both species (Lasat and Kochian, 2000). Lasat et al. (1998) also found that Zn efflux out of vacuoles of roots cells of *T. caerulescens* was faster compared to *T. arvense*. Time course studies exhibited a 10-fold greater Zn translocation to the shoot as compared with the non-hyperaccumulator *T. arvense* (Lasat et al., 1996), which was correlated with a 5-fold increase of Zn in xylem sap (Lasat et al., 1998). Kochian et al. (2002) hypothesized that the increased transport could result in a stimulated metal influx across the leaf cell plasma membrane and an enhanced storage in the leaf vacuole. Vacuolar compartmentalization or cell wall binding in leaves is supposed to play a major role in hyperaccumulation of heavy metals (Küpper et al., 2004).

Salt et al. (1999) reported that the majority of intracellular Zn in roots of *T. caerulescens* was associated with histidine, whereas in leaves Zn was associated with organic acids. Cd and Zn complexation in *T. caerulescens* are tissue and age dependent, with high concentrations in mature and senescent leaves. Oxygen ligands (e.g. water or organic acid such as citrate or malate) dominated, while in young and mature tissues (leaves, petioles and stems), a higher percentage of Cd was bound by S-ligands (e.g. phytochelatins, metallothioneins, and other Cys-rich peptides) than in senescent tissues. S-ligands are not involved in Zn tolerance in hyperaccumulator plants (Küpper et al., 2004).

Genetics of Zn hyperaccumulation and tolerance

Although Zn hyperaccumulation is a constitutive and species-wide trait in *T. caerulescens*, there is still considerable variation between different populations (Baker et al., 1994; Meerts and van Isacker, 1997; Assunção et al., 2003b). This provides a possibility for the genetic dissection of Zn

hyperaccumulation within *T. caerulescens*. Genetic analysis the F2 and F3 progeny from a cross between a calamine and a non-metallicolous *T. caerulescens* population (Assunção et al., 2003c) or between metallicolous and non-metallicolous populations (Frerot et al., 2005) showed that Zn accumulation and tolerance segregated largely independently, although there was a significantly degree of association between low accumulation and high tolerance. Using an F3 population, Assunção et al. (2006) identified two quantitative trait loci (QTLs) for root Zn accumulation. The phenotype distribution of shoot Zn concentration in the F3 families was more or less continuous, suggesting Zn accumulation is under the control of more than one gene. Zha et al. (2004) reported that F2s from a cross of high (Ganges) and low (Prayon) Cd accumulating ecotypes showed continuous variation in shoot Zn and Cd concentration, with significant transgression for Zn, indicating that Cd and Zn accumulation is governed by multiple genes. QTL mapping was also performed for an F2 population obtained after crossing a *T. caerulescens* low Zn/Cd accumulation accession (La Calamine) and a high accumulation accession (Ganges) (Deniau et al., 2006). Two QTLs were identified respectively for root Zn and Cd concentrations, three QTLs were mapped for shoot Zn concentration and one QTL was detected for shoot Cd concentration. Among these QTLs, co-localization was found for the loci related with the concentration of the two elements, as well as co-localization of loci for concentrations in shoots and roots of each element (Deniau et al., 2006).

Molecular mechanism of Zn hyperaccumulation

Zn accumulation in *T. caerulescens* has been studied at the molecular level. *ZNT1* was the first Zn transporter gene identified in *T. caerulescens*, using functional complementation of a yeast Zn uptake defective mutant (Pence et al., 2000). *ZNT1* and its paralogue *ZNT2* are highly expressed in roots and at a lower level in shoots under normal and elevated as well as Zn deficient conditions, in contrast to their orthologues in *A. thaliana* and *T. arvense*, which are mainly expressed in roots and only under Zn deficiency (Assunção et al., 2001; van de Mortel et al., 2006). This is also observed in *A. halleri* another Zn/Cd hyperaccumulator (Becher et al., 2004). The mechanism causing the higher expression of these genes in hyperaccumulator species is still unknown. The ideas so far hypothesize on either alterations in Zn-responsive elements and element binding transcription factors in the *ZNT1* promoter (Assunção et al., 2001), or on alterations in the Zn receptor and signal transduction (Lasat et al., 2000; Pence et al., 2000).

In addition to high expression of Zn uptake transporters, a much more efficient Zn sequestration

could contribute to the hyperaccumulation trait (Assunção et al., 2003a). *TcZTP1* is the homologue of *ZAT* belonging to CDF family, which is predicted to localize to vacuolar membrane and involved in cation influx into vacuole. *ZTP1* is overexpressed in *T. caerulescens* compared to nonhyperaccumulator *T. arvense*. Together with its predominant expression in the leaves this suggests the contribution of *ZTP1* expression to Zn tolerance.

T. caerulescens shares 88% sequence identity with *A. thaliana* (Rigola et al., 2006), which means that many molecular tools developed for *A. thaliana* can be used to some extend for heterologous purposes in *T. caerulescens*. By using *A. thaliana* microarrays, heterologous comparative transcriptional analysis was performed for roots of *T. caerulescens* and *A. thaliana*, revealing more than 2,200 genes that were significantly differentially expressed (van de Mortel et al., 2006). An additional shoot transcription profile comparison using *A. thaliana* ATH1 arrays revealed 5,000 genes to be differentially expressed between *T. caerulescens* and *T. arvense*, including genes involved in Zn transport and compartmentalization (Hammond et al., 2006).

Brassica rapa

Brassica is a genus in the family Brassicacea and includes a number of crops with wide adaptation under different agroclimatic conditions. The genetic relation of the major *Brassica* species used as crop plants has been well studied and is referred to as U's triangle (U, 1935). These, include the three diploids *B. rapa* (synonym *B. campestris*, genome AA, 2n=20), *B. nigra* (genome BB, 2n=16) and *B. oleracea* (genome CC, 2n=18), and the three amphiploids (allotetraploids) *B. juncea* (AABB, 2n=36), *B. napus* (AACC, 2n=38) and *B. carinata* (BBCC, 2n=34).

Economic importance and health benefits of B. rapa

Brassica vegetables include important and highly diversified cultivar groups grown world wide that belong mainly to the species *B. oleracea* and *B. rapa*. In the western hemisphere, including Europe, *B. oleracea* is most important. However, in Asia, *B. rapa* is the most cultivated species as exemplified by the great importance of Chinese cabbage. In addition to its use as a vegetable crop, *B. rapa* is also used as an oil crop, although it is increasingly replaced as oil seed crop by *B. napus*. As vegetable crop, different edible parts, such like root (turnip), leaves (Chinese cabbage, Pak choi), inflorescence stems and flowers (Caixin) can be used. In China, Chinese cabbage is the most important vegetable with the highest cultivation area and production (Chinese annual data of agriculture, 2003) compared to other cultivar groups. In the recent years, Pak choi is gaining

increasing popularity in Western diets (Rochfort et al., 2006). These *B. rapa* vegetables provide dietary fiber, vitamine C and other possible salubrious factors as anticancer glucosinolates (Fahey and Talalay, 1995) and also a potentially important source of dietary flavonols (Rochfort et al., 2006).

Genetic structure of B. rapa in comparison with its relatives

The haploid genome equivalent of *B. rapa* is about 529 Mb (Johnston et al., 2005). The species is relatively closely related with the reference plant species Arabidopsis (125 Mb haploid genome equivalent), which has been whole genome sequenced. Arabidopsis and Brassica are speciated around 14.5-20.4 million years ago from one common ancestor (Bowers et al., 2003). Comparative genomic analyses and cytogenetic analysis (reviewed by Snowdon, 2007) has revealed co-linear chromosome segments (Schmidt et al., 2001) in the Brassicacea family and conserved linkage arrangements between Arabidopsis and Brassica (Teutonico and Osborn, 1994; Parkin et al., 2005). However these studies also revealed that *Brassica* chromosomes show a complex rearrangement in comparison to the Arabidopsis genome and have undergone extra rounds of polyploidisation with as a consequence that syntenic regions are often triplicated in Brassica, with some gene loss in the individual synthenic regions. A detailed comparative genetic map between the chromosomes of *A. thaliana* and *B. napus* was generated by Parkin et al. (2005) by localization of orthologous sequences from mapped *B. napus* RFLP markers to the corresponding map positions in *A. thaliana*. The relationship between *A. thaliana* and its relatives in the Brassica genus was reviewed by Schranz et al. (2006) who showed that 24 chromosomal blocks could be identified that allowed the reconstruction of basic chromosome structures in these species. These new insights in synteny will allow a direct use of the model species *A. thaliana* for the identification of genes and gene function in related crop plants such as *Brassica* species by navigating between the Arabidopsis sequence and the Brassica genomes (Lim et al 2006; Snowdon, 2007).

Genetic studies in B. rapa

The high degree of neutral DNA polymorphism of most *Brassica* species (Figdore et al., 1988) has facilitated the development of molecular linkage maps, with at least twenty described to date for *B. rapa* (Song et al., 1991; Chyi et al., 1992; Teutonico and Osborn, 1994), *B. oleracea* (Slocum et al., 1990; Kianian and Quiros, 1992; Lan et al., 2000), *B. nigra* (Lagercrantz, 1998), *B. juncea* (Cheung et al., 1997; Pradhan et al., 2003) and *B. napus* (Landry et al., 1991; Uzunova et al., 1995). The two

most recent linkage maps of *B. rapa* were constructed by Kim et al. (2006) using 545 sequence-tagged loci and by Suwabe et al. (2006) who based their map on 113 simple sequence repeat (SSR) markers. Using a common marker set, especially of SSR markers (Suwabe et al., 2002; Lowe et al., 2004), allows comparison of linkage groups for different mapping populations. This led to a common nomenclature (Parkin et al., 2005; Suwabe et al., 2006) for *Brassica* linkage groups that is now generally accepted. Thus the *B. napus* linkage groups N1-N10 representing the A genome correspond to *B. rapa* groups R1-R10, and linkage groups N11-N19 representing the C genome correspond to *B. oleracea* O1-O9. With these linkage maps, QTL analysis have been described for a wide variety of morphological and physiological traits such as seed color, pubescence, erucic acid content (Teutonico and Osborn, 1994; Song *et al*., 1995; Nozaki *et al*., 1997), oleic acid concentration (Tanhuanpaa *et al*., 1996), linolenic acid content (Tanhuanpaa & Schulman, 2002), and clubroot resistance (Suwabe *et al*., 2006).

Engineering metal accumulation in plants

The understanding of metal accumulation in plants at the molecular levels enables the manipulation of mineral content of plants through genetic engineering. This offers an opportunity to address human mineral deficiency by biofortifying foods before harvest and an opportunity to improve crop productivity, which is often limited by plant mineral deficiencies. Heterogeneous expression has been used to test the effect of metal transporter gene over-expression to increase crop Zn efficiency or for increasing the Zn content in seeds (Ramesh et al., 2004). Over-expression of *AtZIP1* in *Hordeum vulgare* increased seed Zn and Fe content and Zn uptake after Zn deprivation (Ramesh et al., 2004). Another exciting example is the transformation of rice with a soybean *ferritin* gene, encoding for the major plant Fe-binding protein, which resulted in a higher Fe and Zn concentration in the endosperm of the grain (Goto et al., 1999; Vasconcelos et al., 2003) and enhanced tolerance to low Fe availability (Takahashi et al., 2001). The expression of this *ferritin* gene in lettuce enhanced Fe content in leaves, growth rate and biomass (Goto et al., 2000). However, an even higher expression level of the same *ferritin* gene driven by a stronger promoter did not increase Fe content in rice seed as much as was expected by the increase of the ferritin protein level (Qu et al., 2005). This indicates the importance of coordination of the enhancement of mineral uptake, transport and storage in genetic engineering for high micronutrient content crops.

Outline of the thesis

In this thesis the potential for genetic improvement of mineral, especially Zn, Fe and Mn, accumulation in *B. rapa* vegetable crops by classical breeding as well as genetic modification approaches was explored. The natural variation of leafy Zn, Fe and Mn concentration in *B. rapa* and tolerance to Zn toxicity and to Zn deficiency stress is described in chapter 2. Chapter 3 describes QTL analysis of the content of 11 minerals in *B. rapa* leaves and of the shoot biomass yield under different zinc nutrition conditions using a doubled haploid population. The cloning and functional analysis of two ZIP transporter genes (*TcZNT5* and *TcZNT6*) of *T. caerulescens* is described in chapter 4, and that of two *NRAMP* transporter genes (*TcNRAMP3* and *TcNRAMP4*) in chapter 5. The main results described in the previous chapters are discussed in chapter 6, with perspective for future research.

Chapter 2

Characterization of natural variation for zinc, iron and manganese accumulation and zinc exposure response in *Brassica rapa* L.

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Abstract

Brassica rapa L. is an important vegetable crop in eastern Asia. The objective of this study was to investigate the genetic variation in leaf Zn, Fe and Mn accumulation, Zn toxicity tolerance and Zn efficiency in *B. rapa*. In total 188 accessions were screened for their Zn-related characteristics in hydroponic culture. In experiment 1, mineral assays on 111 accessions grown under sufficient Zn supply (2 µM $ZnSO_4$) revealed a variation range of 23.2-155.9 µg g^{-1} dry weight (d. wt) for Zn, 60.3-350.1 µg g^{-1} d. wt for Fe and 20.9-53.3 µg g^{-1} d. wt for the Mn concentration in shoot. The investigation of tolerance to excessive Zn (800 µM $ZnSO_4$) on 158 accessions, by using visual toxicity symptom parameters, identified different levels of tolerance in *B. rapa*. In experiment 2, a selected sub-set of accessions from experiment 1 was characterized in more detail for their mineral accumulation and tolerance to excessive Zn supply (100 µM and 300 µM $ZnSO_4$). In this experiment Zn tolerance determined by relative root or shoot dry biomass varied about 2-fold. The same six accessions were also examined for Zn efficiency, determined as relative growth under 0 µM $ZnSO_4$ compared to 2µM $ZnSO_4$. Zn efficiency varied 1.8-fold based on shoot dry biomass and 2.6-fold variation based on root dry biomass. Zn accumulation was strongly correlated with Mn and Fe accumulation both under sufficient and deficient Zn supply. In conclusion, there is substantial variation for Zn accumulation, Zn toxicity tolerance and Zn efficiency in *Brassica rapa* L., which would allow selective breeding for these traits.

Keywords *Brassica rapa* L., mineral accumulation, Zn excess tolerance, Zn efficiency

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Introduction

Zinc (Zn) is an essential micronutrient required by all organisms for its role in many physiological processes as a structural or catalytic component of proteins. Unfortunately Zn deficiency is a widespread problem by affecting humans in case of Zn shortage in food. About 20 % of rural children are at risk of inadequate Zn intake in China (Ma et al., 2007). Zn deficiency is also affecting crops in case of poor Zn availability in soil. In China Zn deficiency is prevalent on calcareous soil in North China and calcareous alluvial soils of the Middle and Lower Yangtse River valley (Liu, 1994). Breeding and growing of crops with high Zn content and Zn efficiency are promising and sustainable approaches to solve the Zn deficiency problems in humans and soil (Cakmak et al., 1996).

Knowledge on genetic variation of Zn accumulation and Zn efficiency is the prerequisite for breeding of Zn content/efficiency-improved crop cultivars. Previous studies on genetic variation of micronutrients were mainly limited to staple food crops, including wheat (Graham et al., 1997), rice (Graham et al., 1999), bean (Beebe et al., 2000) and maize (Banziger et al., 2000). Little is known about micronutrient content in leaves, which is the main edible organ of leafy vegetables (Kopsell et al., 2004). The ability of a genotype to grow and yield well in soils that are too deficient in Zn for a standard cultivar to grow and yield well, is defined as Zn efficiency (Graham et al., 1992). Progress has been made in screening Zn efficient genotypes and understanding the physiological and biochemical mechanisms of Zn efficiency (Reviewed in Hacisalihoglu and Kochian, 2003). However, knowledge on Zn efficiency in vegetables is limited (Hacisalihoglu et al., 2004).

Brassica rapa L. comprises several cultivar types producing edible roots, stems, leaves, buds or flowers as vegetables (Gomez-Campo and Prakash, 1999). Some of these are the most important vegetables in eastern Asia, especially in China, Korea and Japan, both in terms of production and per capita consumption (Opena et al., 1988). As vegetables are one of the main micronutrient sources of the population in China (Ma et al., 2007), we studied *B. rapa* vegetables to collect more information on the extent of genotypic variation for Zn accumulation and Zn efficiency and their potential for genetic improvement of these traits.

While a shortage of Zn is a problem for plant growth, an excess of Zn is even more detrimental. Zn heavy metal pollution is prevalent in China's industrialized areas (Liu et al., 2005; Nan et al., 2000). *B. rapa* is not known to be a metal hyperaccumulator and showed a significant decrease in biomass with increased root and shoot Zn concentration upon exposure to toxic Zn levels (Ebbs and Kochian,

1997; Coolong et al., 2003; He et al., 2004). However, in general only one accession was tested in each case. We therefore intended to determine the natural variation for excess Zn tolerance among *B. rapa* germplasm.

The objective of this study is to characterize the genotypic variation for Zn accumulation and Zn response in *B. rapa*_upon exposure to different Zn concentrations. Understanding the range of genotypic variation in Zn accumulation and response to Zn nutritional stress will provide a genetic basis for micronutrient and Zn stress tolerance breeding of *B. rapa* vegetables and for further genetic studies on Zn accumulation and tolerance to Zn nutritional stress**.**

Materials and Methods

Plant Material

To determine the genetic variation of Zn accumulation and response to Zn stress, a total of 188 *Brassica rapa* accessions belonging to nine cultivar groups (Table 1) were screened. 184 accessions were obtained from the Institute of Vegetables and Flowers of the Chinese Academy of Agricultural Sciences (IVF-CAAS); two were obtained from the Dutch Crop Genetic Resources Centre (CGN) in Wageningen, and the other two were obtained from Dr. T. Osborn (University of Wisconsin, Madison, USA). 111 accessions were used for shoot (aboveground tissue) mineral analysis and 158 lines were screened for their tolerance to Zn excess stress. On the basis of their performance in this large scale screening experiment (experiment 1), 15 accessions were selected for a detailed accumulation and tolerance testing (experiment 2) as described below. In experiment 2, six additional accessions were added, which are the parents of additional doubled haploid (DH) populations that are under development.

Table 1 Overview of *B. rapa* accessions, according to cultivar group used in the described experiments. ZA: Zn accumulation experiment; ZT: Zn tolerance experiment; ZE: Zn efficiency experiment; Exp 1: experiment 1; Exp 2: experiment 2.

Cultivar group	Total	No. of accessions				
		ZA		ZT		ZE
		Exp1	Exp2	Exp1	Exp2	
Chinese cabbage (sp. *pekinensis*)	69	45	7	46	1	1
Pak Choi (sp. *chinensis*)	64	39	6	61	2	2

Caixin (sp. *parachinensis*)	23	15	1	23	1	1
Turnip (sp. *rapa*)	11	6	1	10		
Wutacai (sp. *narinosa*)	8	4	1	8	1	1
ZiCaitai (sp. *chinensis* var. *purpurea*)	5	2	1	5		
Mizuna (sp. *nipposinica*)	6	4	2	5	1	1
Oil seed (Yellow Sarson) (sp. *tricolaris*)	1	1	1			
Rapid cycling	1	1	1			
Total	188	117	21	158	6	6

Plant Culture

For experiment 1, three plants for each accession were grown in a greenhouse without climate control in Beijing, China, from mid March till May. The environmental conditions were 20-30°C/10-15°C (day/night temperature), 30,000-40,000 Lux light intensity and 50-60 % relative humidity. Seeds were germinated in vermiculite and watered every three days with half-strength Hoagland's nutrient solution after germination. After 14 days (mineral accumulation experiment) or 7 days (Zn tolerance experiment) seedlings were transferred to hydroponic culture trays each containing three individuals from 24 accessions in 20 L half-strength Hoagland's nutrient solution. The solution was buffered with 2 mM MES (2-morpholinoethanesulphonic acid) at pH 5.5. A concentration of 2 µM $ZnSO_4$ was used as sufficient Zn supply. Nutrient solutions were replaced once a week until harvesting. After 7 days at sufficient Zn, plants for the Zn tolerance experiment were transferred to excess Zn nutrient solution containing 800 µM $ZnSO_4$, and were exposed for 14 days. The solution was refreshed after one week

For experiment 2, plants were grown in a climate-controlled growth cabinet set at 75 % humidity and 22/16 °C (16h/8h) day/night temperature regime. Seeds were germinated in fertilized potting soil watered with tap water. Seedlings were transferred to hydroponic solution after 14 days for the mineral accumulation experiment or after 7 days for the Zn tolerance and Zn efficiency experiments. For the mineral accumulation experiment plants were grown for 14 days in medium with sufficient Zn (2 µM $ZnSO_4$). For the Zn tolerance experiment plants were first grown for 7 days in medium with sufficient Zn (2 µM $ZnSO_4$) before exposure to excess Zn for 14 days. Instead of the very high concentration of 800 µM $ZnSO_4$, plants were transferred to 100 and 300 µM $ZnSO_4$ as excess Zn

concentrations and to 2 µM $ZnSO_4$ as the sufficient Zn control. For the Zn efficiency experiments, one-week-old germinated seedlings were transferred directly to a nutrient solution without $ZnSO_4$ or with 2 µM $ZnSO_4$ as control and grown for 15 days before assessment. For each line three pots were used with one plant per pot for the mineral accumulation experiment and three plants per pot for the Zn efficiency and Zn tolerance experiments. In all these experiments the nutrient solutions were refreshed twice a week.

Mineral Determination

In experiment 1, shoots were harvested from plants with a similar size after 27 to 36 days of growth. For each accession shoots from 2-3 individual plants were combined in one sample. Harvested shoots were washed with de-ionized water and lyophilized. Samples were ground by mortar and pestle before wet-digestion in concentrated HNO_3 : $HClO_4$ (87 : 13, V/V) subsequently at 60 °C for 3 hours, 100 °C for 1 hour, 120 °C for 1 hour and 195 °C for 2.5 hours. The digests were diluted with 5 ml 20% HCl and deionized H_2O to a final volume of 20 ml before analysis by inductively coupled plasma – atomic emission spectrometer (ICP-AES) (Leeman-DRE DR6009, USA) at the IVF-CAAS in Beijing.

In experiment 2, shoots and roots were harvested separately per plant. After oven-drying at 65 °C for 3 days, shoot and root dry biomass were measured. Shoot samples were ground by mortar and pestle before wet-digestion in concentrated HCl : HNO_3 (1 : 4, V/V) at 140 °C for 7 hours. Mineral assays were performed by using a flame Atomic Absorption Spectrometer (AAS) (model 1100, Perkin-Elmer) at the Vrije Universiteit, Amsterdam. Seed mineral content of the 21 accessions used for experiment 2 was determined in samples of about 100 mg ground seeds. Seed mineral determination assays were as described for shoots.

Zn efficiency and tolerance

Shoots and roots were harvested separately and dried at 65 °C for three days to determine their dry biomass. Zn efficiency (ZE) was calculated for shoots and roots based on relative biomass production using the following calculation: *ZE (%) = [dry biomass at 0 µM Zn/ dry biomass at 2 µM Zn] * 100%*.

In the first Zn tolerance (ZT) experiment, a ranked set of five Toxicity Symptom Parameters (TSPs) representing different levels of deterioration of the leaves was used to score plant response after exposure to 800 µM $ZnSO_4$: 1=slight chlorosis of leaves, plant is still growing; 2=chlorosis of leaves;

3=severe chlorosis of leaves, leaves started withering; 4=most of the leaves seared; 5=plant has died. Accessions with mean TSP values below 2 were classified as Zn tolerant and above 4 as Zn sensitive. For the second ZT experiment, ZT was calculated in two different ways. One calculation was made based on dry root or shoot biomass: $ZT_{biomass}$ *(%) = [dry biomass at toxic level Zn/ dry biomass at 2µM Zn] * 100%*. Another calculation was based on root elongation during exposure. For this analysis, roots were dyed with active charcoal before transferring plants to excess Zn medium. Root elongation during exposure (non-stained part of the root) was measured according to Schat and Ten Bookum (1992): ZT_{root} *(%) = [root elongation at toxic level Zn/ root elongation at 2µM Zn] * 100%*.

Statistics

Statistical analyses of metal concentration and root length were conducted using one-way ANOVA followed by the Student-Neuman-Keuls posthoc analysis (SigmaStat, SPSS Science, Chicago, IL, USA). The variation within the mean is presented as the standard error. Significance of correlation was determined using simple linear regression. We considered differences significant at $P \leq 0.05$.

Results

Zn, Fe and Mn accumulation

When examining the shoot Zn concentration for 111 accessions, belonging to seven cultivar groups of *B. rapa*, grown in hydroponic culture with sufficient Zn supply for about four weeks, large variations were found between accessions, ranging from 23.2 to 155.9 µg Zn g^{-1} d. wt (Fig 1A). Accessions with a Zn concentration lower than 50 µg g^{-1} d. wt, between 50-100 µg g^{-1} d. wt and above 100 µg g^{-1} d. wt accounted for respectively 37%, 56% and 7% of the total. The same samples were used to determine the Fe and Mn concentrations. The Fe concentration varied from 60.3 to 350.1 µg g^{-1} d. wt (Fig 1B). Accessions with a Fe concentration lower than 100 µg g^{-1} d. wt, between 100-200 µg g^{-1} d. wt and above 200 µg g^{-1} d. wt accounted respectively for 28%, 71% and 1% of the total. The Mn concentration ranged from 20.9 to 53.3 µg g^{-1} d. wt (Fig 1C). The proportions of accessions with a Mn concentration lower than 30 µg g^{-1} d. wt, between 30-50 µg g^{-1}d. wt and above 50 µg g^{-1}d. wt were respectively 28%, 69% and 3% of the total. There was no significant difference in average Zn or Fe concentration between the different cultivar groups, however the average Mn concentrations in Wutacai and Mizuna accessions were significantly higher compared to those of the other cultivar groups (Table 2). Zn concentration varied most in Wutacai with a variation coefficient

as high as 60%.

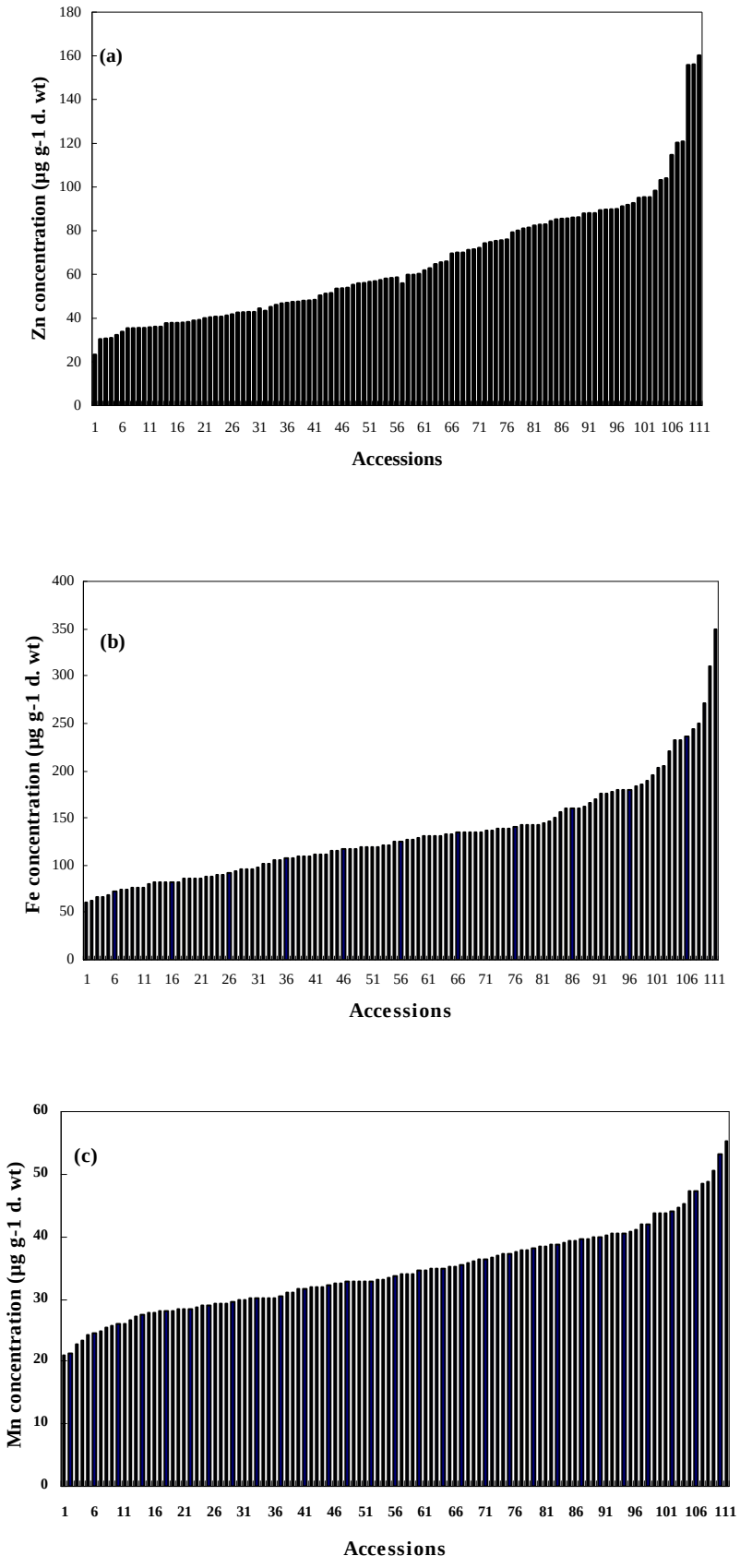


Fig 1 Genotypic variation of shoot Zn (a) , Fe (b) and Mn (c) concentrations of 111 *B. rapa*

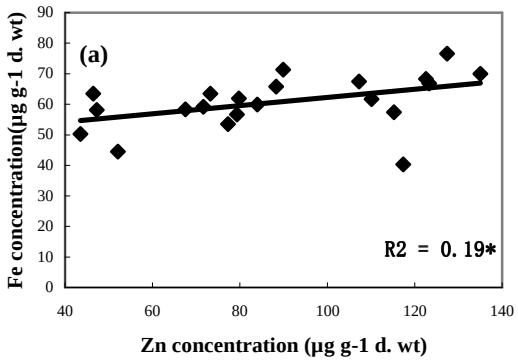
accessions grown in half-strength Hoagland's nutrient solution containing 2 µM Zn for 27-36 days. Data are based on a mixed sample of 2-3 plants per accession. Accessions are ordered according to increasing mineral content.

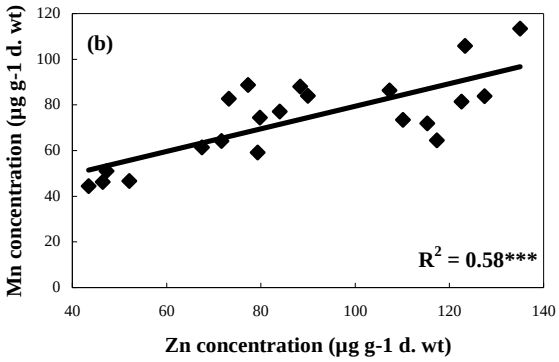
Table 2 Average Zn, Fe and Mn concentrations (µg g^{-1} d. wt.) in shoots of a number of *B. rapa* accessions (No. acc.) according to cultivar groups. All plants were grown in half-strength Hoagland's nutrient solution containing 2 µM $ZnSO_4$. Data are presented as means ± S.E. Only for Mn significant differences ($P \leq 0.001$) were found between the cultivar groups as indicated by different letters. Significance was determined by ANOVA followed by Student Neuman-Keuls posthoc analysis.

Cultivar group	No. acc.	Zn	Fe	Mn
Chinese cabbage(sp. *pekinensis*)	44	60 ± 30	142 ± 50	32 ± 7b
Pak Choi (sp. *chinensis*)	37	69 ± 24	118 ± 46	34 ± 5b
Caixin (sp. *parachinensis*)	15	60 ± 21	125 ± 72	37 ± 5b
Wutacai (sp. *narinosa*)	4	83 ± 50	153 ± 48	45 ± 7a
Turnip (sp. *rapa*)	5	44 ± 10	145 ± 48	39 ± 6b
Mizuna (sp. *nipposinica*)	3	75 ± 10	142 ± 37	44 ± 5a
ZiCaitai (sp. *chinensis* var. *purpurea*)	2	80 ± 15	185 ± 67	34 ± 0b

Five accessions with low Zn concentration (on average 40 µg g^{-1} d. wt), four accessions with moderate Zn concentration (on average 67 µg g^{-1} d. wt) and six accessions with high Zn concentration (on average 135 µg g^{-1} d. wt) were selected for further confirmation in a subsequent experiment, with plants grown under climate-controlled conditions (Experiment 2). Six additional accessions, which are the parents of recently developed DH populations, were also included. In experiment 2, the range of Zn concentration was slightly less (43.5-135.0 µg g^{-1} d. wt) than in experiment 1 (Table 3). The mean Zn concentration was comparable in both experiments. In general, the Fe concentration was lower in experiment 2 compared to experiment 1 (ranging from 40.4-70.6 µg g^{-1} d. wt), whereas it was the reverse for the Mn concentration (ranging from 44.5-113.4 µg g^{-1} d. wt). The data from the two experiments were not significantly correlated (R^2= 0.10 for Zn, 0.06 for

Fe and 0.24 for Mn). When comparing the 21 accessions, the Fe concentrations were not significantly different, but there were significant differences for the Zn and Mn concentrations (Table 3). The Zn, Fe and Mn concentrations of these accessions were positively correlated (Fig 2). The correlation between Zn and Mn (R^2=0.58, P≤0.001) was much higher than that of Zn and Fe (R^2=0.19, P≤ 0.05), however, when excluding the data of outlier accession L144 from the data set, the correlation between Zn and Fe concentrations was significant at P≤0.005 (R^2=0.47). Omitting this accession from the correlation analysis did not affect the significance level for the correlation between Zn and Mn concentrations (R^2=0.67, P≤0.001) or Fe and Mn concentrations (R^2=0.43, P≤0.005). In addition to shoot mineral concentration, the seed weight, seed mineral content and plant biomass were determined for these accessions (Table 4). When comparing the shoot mineral concentration to the dry shoot or root biomass (Table 4), shoot Fe concentration was positively correlated with dry shoot biomass (R^2=0.32, P≤0.05) and root biomass (R^2=0.34, P≤0.05), while neither Zn concentration nor Mn concentration was correlated with biomass. There was no significant correlation between shoot concentration and content per seed for Zn and Fe (Table 4), however, a significant correlation was found for seed Mn content and shoot Mn concentration (R^2=0.25, P≤0.05).





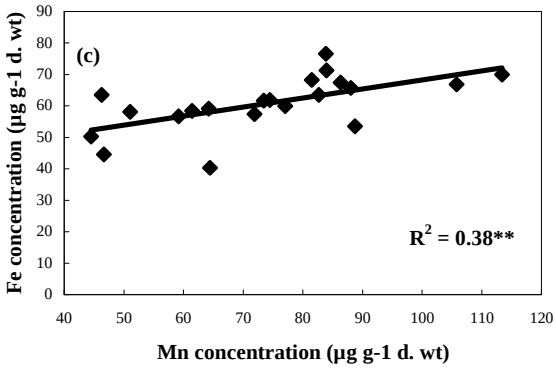


Fig 2 Correlations between shoot Zn, Fe and Mn concentrations of 21 selected accessions grown in half-strength Hoagland's nutrient solution with 2 µM $ZnSO_4$ for 14 days. (a) Correlation between shoot Zn and Fe concentrations. (b) Correlation between shoot Zn and Mn concentrations. (c) Correlation between shoot Fe and Mn concentrations. Correlation between shoot Zn and Fe concentrations was significant at $P \leq 0.005$ (R^2=0.47) when excluding the low Fe outlier L144. *, ** and *** are statistically significant at $P \leq 0.05$, $P \leq 0.005$ and $P \leq 0.001$ levels respectively. Significance was determined by simple linear regression; R^2= squared linear regression coefficient.

Table 3 Comparison of Zn, Fe and Mn concentrations (µg g^{-1} d. wt) in shoots of 21 selected *B. rapa* accessions (Acc.) for experiments 1 and 2. The accessions are arranged according to cultivar groups and in declining order of Zn concentration in experiment 2. Data in experiment 1 are values of mixed samples of 2-3 individual plants; data in experiment 2 are presented as means ± S.E, n=3. All plants were grown in half-strength Hoagland's nutrient solution containing 2 µM $ZnSO_4$. Different letters

indicate significant differences at $P \leq 0.001$. Significance was determined by ANOVA followed by Student Neuman-Keuls posthoc analysis. The squared correlation coefficients (Correl) were determined between data in the two experiments for each mineral by linear regression.

Cultivar	.	Zn		Fe		Mn	
group	Acc	Exp 1	Exp 2	Exp 1	Exp 2	Exp1	Exp 2
Chinese	L107w	104	127 ± 1ab	244	77 ± 6 a	40	84 ± 10 ae
Cabbage	L113w	160	107 ± 7 ae	127	68 ± 6 a	38	86 ± 5 ad
	L64w	57	80 ± 6 ae	90	62 ± 3 a	29	75 ± 2 ce
	L127w	120	79 ± 16 ae	203	57 ± 1 a	28	59 ± 6 ce
	L140w	156	72 ± 3 be	87	59 ± 4 a	40	64 ± 5 ce
	L23w	48	52 ± 3 de	134	45 ± 3 a	37	47 ± 0 de
	L146	n.d.	46 ± 3 e	n.d.	64 ±13 a	n.d.	46 ± 7 de
Pak	L196w	156	135 ± 12 a	151	70 ± 5 a	47	113 ± 2 a
Choi	L86w	64	123 ± 10 ac	168	67 ± 2 a	50	106 ± 4 ab
	L66w	48	123 ± 4 ac	160	68 ± 5 a	38	82 ± 3 ae
	L175	n.d.	115 ± 21 ac	n.d.	57 ± 4 a	n.d.	72 ± 2 be
	L67w	115	110 ± 13 ad	157	62 ± 7 a	34	73 ± 7 be
	L78w	23	90 ± 1 ae	98	71 ± 0 a	23	84 ± 7 ae
Mizuna	L203w	42	68 ± 1 ce	160	58 ± 4 a	39	61 ± 2 ce
	L19	n.d.	44 ± 6 e	n.d.	50 ± 6 a	n.d.	45 ± 4 e
Wutacai	L56w	41	88 ± 2 ae	110	66 ± 3 a	38	88 ± 3 ac
Caixin	L58w	71	73 ± 4 be	117	64 ± 2 a	34	83 ± 7 ae
Zi Caitai	L62w	76	84 ± 5 ae	90	60 ± 6 a	37	77 ± 5 ae
Yellow Sarson	L143	n.d.	77 ± 7 ae	n.d.	54 ± 5 a	n.d.	89 ± 10 ac
Rapid Cycling	L144	n.d.	117 ± 6 ac	n.d.	40 ± 2 a	n.d.	64 ± 10 ce
Turnip	L115	n.d.	47 ± 6 e	n.d.	58 ± 5 a	n.d.	51 ± 3 ce
Correl		0.10		0.06		0.24	
Mean		85	89	102	61	37	74

Table 4 Seed weight (mg per 100 seeds), seed mineral content (ng seed^{-1}) and dry biomass (mg plant^{-1}) after 28 days of growth under sufficient Zn supply of the 21 *B. rapa* accessions used in experiment 2.

Cultivar group	Acc.	Seed weight	Zn	Fe	Mn	Dry shoot biomass	Dry root biomass
Chinese	L107w	428	199	289	153	880 ± 65	55 ± 6
Cabbage	L113w	154	107	145	67	380 ± 68	32 ± 4
	L64w	267	238	309	91	517 ± 42	46 ± 6
	L127w	248	139	153	87	645 ± 47	36 ± 2
	L140w	233	124	214	87	345 ± 44	23 ± 4
	L23w	218	179	227	74	175 ± 54	9 ± 4
	L146	246	245	237	112	754 ± 101	48 ± 4
Pak Choi	L196w	266	176	278	124	474 ± 98	34 ± 7
	L86w	318	222	314	140	422 ± 29	36 ± 4
	L66w	306	189	246	138	601 ± 10	43 ± 1
	L175	199	134	209	83	321 ± 41	22 ± 4
	L67w	179	143	229	102	361 ± 14	21 ± 5
	L78w	287	242	391	173	449 ± 38	34 ± 6
Mizuna	L203w	297	221	332	117	413 ± 117	22 ± 6
	L19	123	73	222	74	606 ± 97	40 ± 9
Wutacai	L56w	203	137	202	96	182 ± 21	11 ± 1
Caixin	L58w	189	85	201	80	465 ± 56	35 ± 6
Zi Caitai	L62w	202	128	173	92	356 ± 70	29 ± 1
Yellow Sarson	L143	448	261	469	187	342 ± 41	40 ± 6
Rapid Cycling	L144	124	94	88	101	57 ± 17	5 ± 1
Turnip	L115	183	141	206	74	434 ± 3	28 ± 2

Zn tolerance

Although *B. rapa* is not known to be particularly tolerant to excess Zn exposure, we assessed the initial set of 158 accessions for their tolerance to 800 µM $ZnSO_4$ exposure for 14 days (Experiment 1). Tolerance was determined using Toxicity Symptom Parameters (TSPs), and tolerant, average and sensitive accessions accounted for respectively 8 %, 45 % and 46 % of the total (Fig 3). Exposure to 800 µM $ZnSO_4$ is rarely encountered by plants in the field. To determine if comparable results could be obtained when exposing plants to less extreme Zn concentrations, two tolerant accessions (L56w and L86w), three average accessions (L58w, L64w and L203w) and one sensitive accession (L66w) were used in experiment 2. In this experiment, plants were exposed to 2 µM $ZnSO_4$ (as normal Zn supply) and 100 and 300 µM $ZnSO_4$ as excess Zn supply. Zn tolerance (ZT) was determined in terms of dry biomass production rather than with TSPs (Table 5). As expected, biomass was reduced when plants were grown at high Zn concentrations and the growth inhibition increased along with the increase in Zn concentration (Table 5). ZT based on shoot dry biomass varied almost 2-fold among accessions at both 100 µM and 300 µM $ZnSO_4$. A comparable range was found for ZT based on root dry biomass. In line with the initial selection based on TSP values, the two tolerant accessions L56w and L86w maintained a high relative shoot growth at 300 µM Zn (94 %), whereas the sensitive accession L66w showed only 50 % relative shoot growth at 300 µM Zn (Table 5). The range of relative growth was different between roots and shoots (Table 5). The 300 µM Zn treatment induced a drastic decrease in root biomass by 65- 82 %, while this induced only a moderate reduction in shoot biomass of between 6 to 50%.

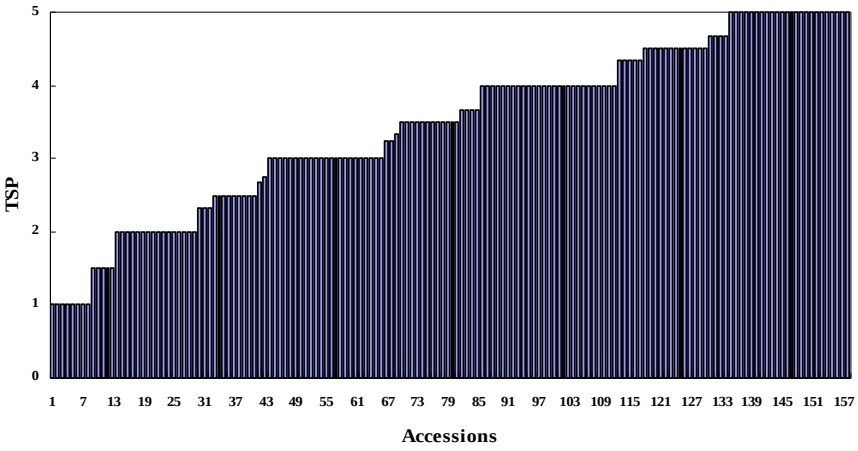


Fig 3 Excess Zn stress response of 158 *B. rapa* accessions grown in half-strength Hoagland's nutrient solution with 800 µM Zn for 14 days. TSP (Toxic Symptom Parameter): 1=slight chlorosis, still growing; 2=chlorosis; 3=severe chlorosis, leaves started withering; 4=most of the leaves seared; 5= dead. Values are means of two or three plants per accession.

Table 5 Average dry shoot and root biomass (mg plant^{-1}) and Zn tolerance (ZT) of shoots and roots of six accessions (Acc.) grown for 14 days in half-strength Hoagland's nutrient solution containing 2 µM, 100 µM or 300 µM $ZnSO_4$. ZT (in %) is calculated as relative biomass compared to 2 µM $ZnSO_4$. Data are presented as means ± S.E., n = 9.

	2 µM		100 µM				300 µM			
Acc.	shoot	root	shoot	ZT	root	ZT	shoot	ZT	root	ZT
L56w	247 ± 49	20 ± 5	257 ± 28	104	15 ± 3	74	231 ± 20	94	7 ± 1	35
L86w	441 ± 58	71 ± 10	354 ± 40	80	46 ± 5	65	417 ± 6	94	18 ± 1	26
L58w	476 ± 62	62 ± 12	341 ± 29	72	37 ± 5	59	312 ± 32	66	13 ± 2	21
L64w	688 ± 77	73 ± 6	491 ± 67	71	48 ± 7	66	429 ± 61	62	14 ± 3	19
L203w	660 ± 44	109 ±11	440 ± 95	67	37 ± 11	34	391 ± 50	59	20 ± 2	18
L66w	516 ± 38	58 ± 6	274 ± 34	53	24 ± 3	41	256 ± 44	50	11 ± 2	19

When determining ZT in terms of root elongation, it became clear that the effect on root biomass was not reflected by an effect on root length (Table 6), as the relative growth calculated for the increase of maximum root length is not consistent with the previously determined ZT based on root dry biomass. Root elongation of accessions L56w and L86w, that showed the highest relative root dry biomass with respectively 35 % and 26 % of the control, was strongly inhibited at 300 µM Zn to respectively 11 % and 9 % of the control. Root elongation of all six accessions was inhibited at 100 µM Zn and this inhibition was enhanced at 300 µM Zn. The difference in root elongation between accessions decreased with increased Zn concentration. When plants were grown at 300 µM Zn there was no significant difference in the mean root elongation among the six accessions.

Table 6 Comparison of root elongation (RE; in mm) and relative root growth (RG; in %) of six accessions (Acc.) grown in half-strength Hoagland's nutrient solution with 100 µM or 300 µM Zn supply for 14 days. RG is expressed as percentage of the growth at 2 µM Zn. Data are presented as means ± S.E, n=9. Values followed by different letters are significantly different at $P \leq 0.05$. Significance is determined by ANOVA followed by Student Neuman-Keuls posthoc analysis.

Acc.	2 µM	100 µM		300 µM	
	RE	RE	RG	RE	RG
L56w	17 ±3 c	13 ± 3 b	77	2 ± 1 a	11
L86w	33 ± 6 ab	17 ± 3 b	52	3 ± 3 a	9
L58w	16 ± 3 c	13 ± 4 b	82	5 ± 2 a	31
L64w	49 ± 8 a	35 ± 4 a	72	4 ± 1 a	8
L203w	45 ± 9 a	19 ± 5 b	42	11 ± 4 a	24
L66w	29 ± 6 ab	12 ± 5 b	41	7 ± 4 a	24

Zn efficiency

In experiment 2, the six accessions (L56w, L58w, L64w, L66w, L86w, L203w) used in ZT experiment were also examined for their Zn efficiency (ZE), i.e. the ability to grow under low Zn supply. Plants began to show typical symptoms of Zn deficiency, such as interveinal chlorosis, a purple stem and reduced growth, already after growing for one week in Zn deficient medium. After two weeks, the difference in phenotype compared to untreated accessions was easily distinguished by eye with the exception of accessions L64w and particularly L56w, which appeared comparatively healthy (Fig 4).

Both root and shoot dry matter production was reduced in all accessions due to Zn deficiency (Table 7), but this reduction was much less in L64w for both shoot (69.8 %) and root (98.0 %) . L64w is therefore considered to be the most zinc efficient accession of the six accessions tested. ZE varied more for shoot biomass (2.6-fold) than for root biomass (1.8-fold). In addition to ZE we also examined the relative root:shoot biomass ratio (RSR) for the six accessions. Zn deficiency enhanced the RSR in most accessions and thus has a stronger effect on shoot biomass production than on root biomass production. However, since the most Zn efficient accession L64w has a very similar RSR as the least Zn efficient accession L86w, there does not seem to be a very strong correlation between ZE and RSR. When comparing ZE based on shoot biomass, root biomass or RSR with seed weight and mineral content per seed (Table 4), no significant correlation was found (data not shown).

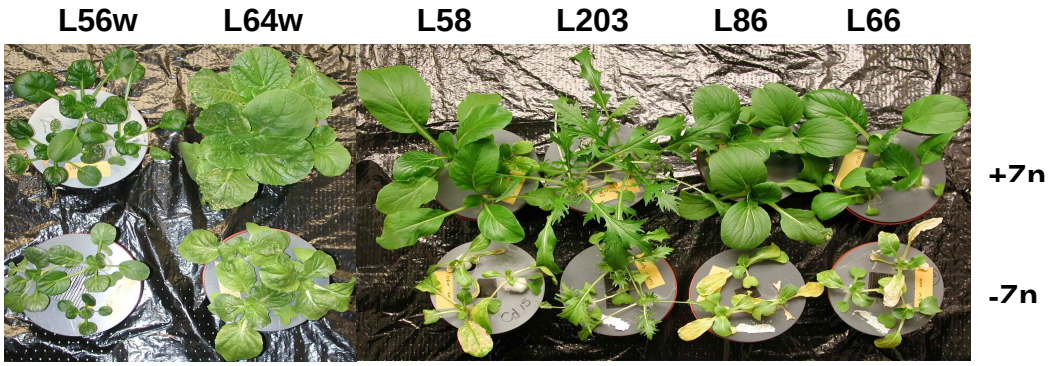


Fig 4 Visible phenotypic response of *B. rapa* accessions to Zn deficiency. Plants were grown in half-strength Hoagland's nutrient solution for 15 days with (2 µM Zn; + Zn) or without $ZnSO_4$

supply (0 µM Zn; - Zn).

Table 7 Average dry biomass (in mg plant^{-1}), Zn efficiency (ZE; in %) of roots and shoots and the relative root:shoot biomass ratio (RSR) of six accessions (Acc.) grown for 15 days in half-strength Hoagland's nutrient solution without (0 µM) or with (2 µM) Zn supply. ZE is determined as the biomass at 0 µM Zn compared to 2 µM Zn (in %). RSR is determined as the root:shoot ratio (Root:Shoot) at 0 µM Zn compared to 2 µM Zn (in %). Data are presented as means ± S.E., n = 3.

	Root			Shoot			Root:Shoot		
Acc.	0 µM	2 µM	ZE	0 µM	2 µM	ZE	0 µM	2 µM	RSR
L64w	44 ± 1	45 ± 6	98.0	379 ± 26	543 ± 32	69.8	0.113	0.083	1.4
L66w	15 ± 2	25 ± 4	60.0	140 ± 12	290 ± 31	48.5	0.107	0.086	1.2
L56w	19 ± 1	29 ± 3	66.0	160 ± 11	352 ± 11	45.4	0.094	0.082	1.2
L203w	20 ± 1	52 ± 4	38.7	276 ± 30	647 ± 49	42.9	0.072	0.080	0.9
L58w	23 ± 4	53 ± 6	43.4	175 ± 40	434 ± 42	42.7	0.131	0.122	1.1
L86w	24 ± 3	48 ± 2	50.6	197 ± 23	529 ± 6	37.2	0.122	0.091	1.3

Shoot Zn concentration did not differ among the six accessions after plants were grown under Zn deficient condition for 15 days with a reduction of 76 %-84 % (Table 8). There was no significant correlation between ZE and the shoot Zn concentration at 0μM or 2 μM Zn (data not shown). In general, both shoot Fe and Mn concentrations were increased under Zn deficient condition when compared to Zn sufficient condition (Table 8). Four out of six accessions showed a significant increase in shoot Mn concentration, while two of these accessions also increased significantly in Fe concentration. There was no correlation between ZE and shoot Mn or Fe concentration of plants grown at 0 or 2 μM Zn (data not shown).

Table 8 Shoot Zn, Fe and Mn concentrations (μg g^{-1} d. wt) of plants grown for 15 days in half-strength Hoagland's nutrient solution without (0 μM) or with (2 μM) Zn supply. Data are presented as means ± S.E., n = 3. * significantly different from 2 μM Zn supply ($P \leq 0.05$). Significance is determined by one-way ANOVA.

Acc	Zn		Fe		Mn	
	0 μM	2 μM	0 μM	2 μM	0 μM	2 μM
L64w	6 ± 0 *	33 ± 1	90 ± 17	45 ± 2	51 ± 2	53 ± 2
L66w	8 ± 1 *	41 ± 2	297 ± 135	43 ± 1	164 ± 30*	43 ± 1
L56w	7 ± 1 *	43 ± 3	131 ± 29 *	45 ± 2	128 ± 18*	65 ± 1
L203w	6 ± 1 *	27 ± 1	59 ± 8	45 ± 2	65 ± 5 *	46 ± 3
L58w	7 ± 1 *	36 ± 2	99 ± 54	52 ± 1	88 ± 16	66 ± 1
L86w	6 ± 0 *	32 ± 1	84 ± 6*	50 ± 4	123 ± 9 *	50 ± 4

Discussion

In total 117 *B. rapa* accessions were screened for Zn, Mn and Fe accumulation characteristics as a general survey for genotypic variation among *B. rapa* vegetables. This survey showed that there is considerable genotypic variation for shoot Zn, Mn and Fe concentration in *B. rapa*. This variation is not limited to one or a few cultivar groups and there is also no clear correlation between mineral concentration and cultivar group. When the selected accessions were re-examined at a second location, different results were obtained for some of these accessions and in general the correlation between locations was lower than expected. This does not reflect errors in sampling or measuring mineral concentrations, but largely illustrates the difficulty associated with studying a trait that is

easily affected by genotype x environment interactions, which is often the case for mineral accumulation. Genetic variation within accessions is another factor that may have caused differences in mineral concentration. Although the accessions had been propagated for some generations in the resource collection they originated from, the occurrence of self-incompatibility, which is common in *B. rapa*, is expected to maintain some genetic variation within each accession.

Previous studies revealed variation ranges of 10-60 µg g^{-1} for Zn and 10-90 µg g^{-1} for Fe in seeds (Beebe et al., 2000; Graham et al., 1999; Graham et al., 1997; Banziger et al., 2000). Kopsell et al. (2004) reported that leaf Zn concentration based on fresh weight ranged from 29.1 to 71.9 mg $kg^{-1}$and Fe concentration ranged from 53.1-114.2 mg kg^{-1} in *B. oleracea* vegetables. In the present study a wider variation range (7-fold) and higher highest concentration were found for Zn and Fe in *B. rapa* shoots. Of course this may reflect a physiological difference in the accumulation process between shoot and seed. Shoot accumulation largely depends on xylem transport, whereas seed accumulation requires additional phloem transport. Also different from screening plants in hydroponic culture, as was done in our study, the investigations on above staple crops were all carried out in soil, which might cause lower mineral availability. Seed weight and mineral content influence plant growth at the early vegetative stage (Rengel and Graham, 1995a) and therefore affect shoot mineral accumulation. In the present study the correlation of Mn concentration in shoot and Mn content in seed supports the previous conclusion, however, there was no correlation between shoot concentration and seed content for Zn and Fe. Thus, the variation for Zn and Fe concentration as observed in this study suggests that there is sufficient genetic variation to dissect the genetic mechanism controlling shoot Zn and Fe accumulation by quantitative trait locus (QTL) analysis and/or to improve Zn and Fe content in *B. rapa* vegetables by breeding.

In addition to growth under sufficient Zn supply we also studied the response to Zn excess and deficiency in *B. rapa*. Both relative shoot and root growth have been suggested as good indices of tolerance to excess Zn in different species (Bert et al., 2000; Escarre et al., 2000; Meerts and Van Isacker, 1997; Schat and Ten Bookum, 1992; Yang et al., 2004). The comparable range in variation of Zn tolerance we observed, which was based on dry shoot and root biomass, suggests that the same holds for *B. rapa*. However, Zn tolerance determined by maximum root length did not correlate with the Zn tolerance determined by dry biomass, although both root elongation and biomass increase were inhibited in a concentration-dependent manner when exposed to toxic Zn concentrations. Our

results thus support the suggestion by Ebbs and Kochian (1997) that the toxic effect of excess Zn on the root development in *Brassica* ssp. has more effect on lateral root elongation than on lateral root density.

To examine the response to deficient Zn exposure, relative shoot growth was reported as a suitable index to determine Zn efficiency (Cakmak et al., 1999; Grewal et al., 1997; Hacisalihoglu et al., 2004; Rengel and Römheld, 2000), although also the relative root:shoot biomass ratio (RSR) has been suggested to be adequate (Rengel and Graham, 1995b). In the present study, both the relative shoot and relative root biomass index were effective in distinguishing differences in Zn efficiency. Accession L64w clearly stood out as the least affected by low Zn supply when compared to the other accessions. The RSR generally increases under Zn deficiency as an initial response to Zn deficiency (Grewal et al., 1997; Khan et al., 1998; Loneragan et al., 1987). Higher RSRs correlating with Zn efficiency have also been reported for *B. napus* and *B. juncea* (Grewal et al., 1997). A comparable result was obtained in the present study, with the exception that, based on biomass production, the relatively Zn inefficient accession L86w had a similarly high RSR as the Zn efficient accession L64w. Considering this, the RSR does not seem to be the optimal Zn efficiency index for *B. rapa*.

While variation for both Zn tolerance and Zn efficiency was observed among the six accessions, there was no significant correlation between these traits. The absence of correlation between Zn efficiency and seed weight, seed mineral content or shoot mineral concentration also indicates that Zn efficiency is genetically independent from these traits. When grown under Zn deficiency, the limited Zn supply resulted in an almost uniform shoot Zn concentration for all accessions that was below 10 µg g^{-1} d. wt. Since a leaf Zn concentration below 10-15 µg g^{-1} d. wt is considered to be the critical Zn deficiency level for normal plant growth (Marschner, 1995), this explains the negative effect of the Zn deficiency treatment on growth of the *B. rapa* accessions.

In addition to an effect on Zn concentration, we found that when plants were grown under Zn deficient conditions, both Fe and especially Mn concentration in shoots increased, comparable to what has been found in wheat (Rengel and Graham, 1996) or Arabidopsis (van de Mortel et al., 2006). Most of the known metal transporters belong to large gene families covering a broad range of metal specificities. Several of the Zn transporters can also transport Fe or Mn (Connolly et al., 2002; Mills et al., 2003; Vert et al., 2001). Decreased shoot Fe and Mn concentration was found for *Brassica* plants grown in high level Zn conditions (Ebbs and Kochian, 1997). This is also in line with

the correlations we observed between shoot Zn, Fe and Mn concentrations when plants were grown under sufficient Zn supply (Fig 2). In both experiments (sufficient and deficient Zn supply), the strongest correlation was found between Zn and Mn, suggesting that Zn and Mn accumulation share more common elements than Fe and Mn or Fe and Zn accumulation.

Another observation was that the difference in Zn efficiency based on biomass was not fully reflected in the visual appearance of the accessions. Accessions L64w and L56w seemed to suffer little from deficient Zn supply in terms of plant size and degree of leaf senescence or yellowing of the leaves (Fig 4). However, when scored for biomass production, L56w did not perform better than the other accessions with a comparable ZE value. A similar difference in visible appearance and biomass production was previously found for wheat (Genc and McDonald, 2004). This suggests that the plant response to low zinc can act at different levels and the effect on biomass production is not always easily visible by eye. It also shows that visual selection of Zn efficient plant genotypes by breeders may be misleading with respect to yield.

Based on the screening of a large set of *B. rapa* accessions, we conclude there is substantial genotypic variation for Zn, Fe and Mn accumulation and for tolerance to excessive or deficiency inducing levels of Zn. Relative shoot and root growth calculated on dry biomass yield are suitable indices both for the evaluation of excess Zn tolerance and for Zn efficiency. There is a close relationship between Zn, Mn and Fe accumulation. Our results underline that breeding for improved Zn content, whether or not in combination with enhanced fertilization with Zn, is likely to substantially increase the Zn content of *B. rapa* vegetables and thus offer a desirable Zn supplementation to a vegetarian human diet.

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Chapter 3

Mapping QTLs for mineral accumulation and shoot dry biomass yield under different Zn nutritional conditions in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*)

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Maarten Koornneef, Mark G.M. Aarts, Xiaowu Wang

Abstract

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is one of the most important vegetables in China. Genetic dissection on leaf mineral accumulation and tolerance to Zn stress is important for nutritional breeding of Chinese cabbage. A mapping population with 183 doubled haploid (DH) lines was used to study the genetics of mineral accumulation and the growth response to Zn. The concentration of 11 minerals was determined in leaves for 142 DH lines grown in an open field. In addition shoot dry biomass production (SDB) under Zn normal, deficient and excessive nutritional conditions were investigated in hydroponics experiments. A genetic map was constructed based on 226 AFLPs and 31 SSRs. Seven QTLs, explaining 11.1-18.2 % of Na, Mg, P, Mn, Zn, Sr concentration variance, were identified by multiple-QTL model (MQM) mapping. One common QTL was found affecting SDB under normal, deficient and excessive Zn nutritional condition. An additional QTL was detected for SDB under Zn excess stress only. These results offer insight into the genetic basis of leaf mineral accumulation and plant growth under Zn stress condition in Chinese cabbage, and should be useful for future marker assisted selection.

Keywords *Brassica rapa* L. ssp. *pekinensis*, quantitative trait loci (QTL), minerals, zinc response.

This chapter has been submitted.

Introduction

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) is one of the most important vegetable crops in China with, among vegetable crops, the largest cultivation area and per capita consumption. It is a main source of mineral nutrients for the Chinese population (Ma et al.，2007), and therefore elevating the amount of beneficial minerals in Chinese cabbage can contribute to the reduction of micronutrient malnutrition in China, especially when meat supply is limited. The improvement of mineral content by plant breeding is a recent development that requires knowledge about the genetic variation for this trait and genetic markers linked to relevant genes (reviewed in Ghandilyan et al., 2006). However, genetic studies on mineral content in crops have been limited mainly to seeds, such as for bean (Beebe et al., 2000; Islam et al., 2002), rice (Gregorio et al., 2000), wheat (Monasterio et al., 2000), maize (Banziger and Long, 2000) and also the model species *Arabidopsis thaliana* (Vreugdenhil et al., 2004). QTLs were also identified for mineral concentrations in leaves for P (Bentsink et al., 2003), Cs (Payne et al., 2004) and K (Harada and Leigh, 2006) in *A. thaliana* and for Zn in *Thlaspi caerulescen*s (Assunção et al., 2006; Deniau et al., 2006). These studies have demonstrated the presence of allelic variation affecting mineral content, although the identity of the underlying genes remains unknown. However, very little is known about the genetics of mineral content in leafy vegetables, such as Chinese cabbage.

Zn is an essential micronutrient as it plays both functional and structural roles in enzyme reactions (Vallee and Auld, 1990). However, both Zn deficiency and Zn toxicity occur worldwide, including China, depending on Zn content and availability in the soils (Hacisalihoglu and Kochian, 2003; Liu et al., 2005; Nan et al., 2000). In addition to reduced yield due to Zn deficiency or toxicity, the transfer of excess Zn from soils to plants is an important contribution to human health exposure to Zn and more importantly the Cd that goes along with it (Friberg and Nordberg, 1986). A potential solution is to breed crop cultivars which are more tolerant to high Zn exposure and thus grow relatively well with an acceptable level of Zn accumulation in the leaves.

In *Brassica rapa* vegetables, about 8-, 6- and 2.5-fold genotypic variation was observed for respectively shoot Zn, Fe and Mn concentration, and 2-fold variation for relative shoot or root dry biomass production under Zn deficient or toxic conditions (Wu et al., 2007). Two-fold genotypic variation was reported for Ca, Mg, K, Fe, Zn concentration among *B. oleracea* vegetables (Kopsell et al., 2004) and shoot dry biomass ratio-based Zn efficiency for *B. napus* and *B. juncea* (Grewal et al.,

1997). The observed natural variation for these traits in *Brassica* crops suggests the possibility to analyze these traits genetically.

Mineral accumulation in aboveground organs is a complex trait controlled by a series of processes, including mobilization from the soil, uptake by the roots, translocation and redistribution within the plant, and import and deposition in the organs (Clemens, 2001). This is also the case for plant growth under stressful nutrition conditions because different aspects such as availability, uptake and utilization of nutrient elements can be affected and as a consequence growth, development, and yield of the crop will alter (Gupta, 2005). Quantitative trait locus (QTL) mapping allows the identification of individual chromosomal regions containing genetic factors that contribute to variation in a complex trait (Alonso-Blanco and Koornneef, 2000). Molecular mapping of *B. rapa* was initiated by Song et al. (1991) using RFLP markers and a segregating F_2 population. Several subsequent maps were constructed based on different molecular markers and F_2, Recombinant Inbred Line (RIL) populations (Chyi et al., 1992; Teutonico and Osborn, 1994; Kole et al., 1997; Suwabe et al., 2006; Kim et al., 2006). The recent development of *Brassica* Simple Sequence Repeat (SSR) markers facilitates the anchoring of linkage groups to one common reference map and allows the comparison of map positions in studies involving different populations (Suwabe et al., 2006). Furthermore the syntenic relationship with the related genus *Arabidopsis* is now well established (reviewed by Schranz et al., 2006) and allows the comparison of map positions between *Brassica* and *Arabidopsis*. In *B. rapa* QTL analysis has been described for a wide variety of morphological and physiological traits such as seed colour, pubescence, erucic acid content (Teutonico and Osborn 1994; Song et al., 1995; Nozaki et al., 1997), oleic acid concentration (Tanhuanpaa et al., 1996), linolenic acid content (Tanhuanpaa and Schulman, 2002), and clubroot resistance (Suwabe et al., 2006). As microspore culture can be applied efficiently in *Brassica* species, doubled haploid (DH) populations are presently the preferred mapping populations for QTL studies because of their immortality and homozygosity which allows replicated experiments.

In the present paper we describe the construction of a molecular linkage map containing 226 Amplified Fragment Length Polymorphism (AFLP) markers and 31 SSRs using 183 DH lines derived from a cross between two Chinese cabbage DH lines. Plants from this population were analyzed for the concentration of 11 elements in leaves, including four essential micro-elements (Fe, Zn, Mn and Cu), five macro-elements (Na, Ca, K, P and Mg), and also for two toxic elements (Al

and Sr). QTL analysis was performed for mineral accumulation and for shoot dry biomass (SDB) yield under normal, deficient and excessive Zn conditions.

Materials and methods

Mapping population and DNA isolation

The BrIVFhn mapping population of 183 DH lines was derived from a cross between two DH Chinese cabbage lines. Y177 originated from a winter type Japanese cultivar ‘Jianchun’ and Y195 is derived from a summer type Chinese cultivar ‘Xiayang’. The two cultivars show differences in bolting time, seed colour, trichome density and flower colour. DNA from the parents and the DH plants was isolated from the mature leaves as described by Wang et al. (2005).

AFLP analysis

AFLP markers were analyzed following Vos et al. (1995), using fluorescent labelled *EcoR*I and *Mse*I primers with three selective nucleotides as described by Zhao et al. (2005). AFLPs were scored on the basis of the presence or absence of the band. AFLP markers were named using a code for each *EcoR*I and *Mse*I primer followed by the band number in ascending molecular-weight order (Table 1).

Table 1 AFLP primer combinations used for *B. rapa* ssp. *pekinensis* linkage map construction.

Primer combination	*3' selective nucleotides*		*No. of polymorphic bands*
	*Eco*RI	*Mse*I	
E32M61	AAC	CTG	6
E33M32	AAG	AAC	12
E33M48	AAG	CAC	8
E33M49	AAG	CAG	10
E33M50	AAG	CAT	6
E33M51	AAG	CCA	10
E33M53	AAG	CCG	3
E33M54	AAG	CCT	4
E33M56	AAG	CGA	2
E33M60	AAG	CTC	8
E34M35	AAT	AAT	15
E35M52	ACA	CCC	7
E35M56	ACA	CCT	2
E36M31	ACC	AAA	8
E36M32	ACC	AAC	12
E36M35	ACC	ACA	6
E36M36	ACC	ACC	4
E36M47	ACC	CAA	5
E36M50	ACC	CAT	13
E36M53	ACC	CCA	2
E36M59	ACC	CTA	5
E37M32	ACG	AAC	12
E37M49	ACG	CAG	4
E37M56	ACG	CGA	2
E38M35	ACT	ACA	9
E38M50	ACT	CAT	10
E38M51	ACT	CCA	11
E38M56	ACT	CGA	3
E40M38	AGC	ACT	6
E40M51	AGC	CCA	6
E41M51	AGG	CCA	9
E41M56	AGG	CGA	6

SSR analysis

A total of 202 SSR markers derived from *B. rapa*, *B. nigra*, *B. oleracea* and *B. napus* (Ge et al. 2005;

Kim et al. 2006; Lowe et al. 2004; Suwabe et al. 2002, 2004, 2006; T. Osborn, personal communication; G. King, personal communication; G. Bonnema, personal communication) were screened for their polymorphism between the two parental lines. PCRs were performed in 20 µl 1 X PCR buffer, containing 20 ng DNA template, 1 U Taq DNA polymerase, 0.25 mM of each dNTP, and 40 ng of each primer. Thermocycling was started at 94 °C for 5 min and followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min before holding at 12 °C. The PCR products were separated on 6 % denaturing polyacrylamide gels and visualized by silver staining. The polymorphic SSR markers with clear bands were used to screen 64 DH lines of the mapping population.

Segregation analysis and map construction

The segregation of each marker and linkage analysis were performed using JoinMap version 3.0 (http://www.kyazma.nl/). Segregating data were sorted by order of *Individual genot. fre.* value supplied by Joinmap 3.0. The linkage groups (LGs) were determined using a LOD threshold ≥ 4.0 and a maximum recombination fraction of 0.4. The Kosambi mapping function (Kosambi, 1944) was used to convert recombination frequencies into map distances.

Field trial and phenotypic measurement

The leaf mineral concentration was analyzed for the parents and 142 DH lines growing in an open field of the Henan Academy of Agricultural Sciences in Zhengzhou China in the autumn of 2004. Seeds were first sown in pots (10 cm) with fertilized potting soil. Thirty days after sowing, plants were transplanted to an open field in a randomized two-block design. Soil analysis was carried out according to the method of Agro Services International (ASI) of Orange City, Florida, U.S.A (Table 2). Basal fertilizer (750 kg Di-ammonium phosphate ha^{-1}) was applied to plots at the time of transplanting; 375 kg N ha^{-1} in the form of urea was applied at late rosette stage.

The second fully grown head leaf was harvested from two plants per DH line with each from a different block when plants had been grown for 30 days in open field. Harvested leaves were washed with de-ionized water and lyophilized for three days. Samples were ground by mortar and pestle before being ash-digested at 300 °C for 1 hour and 500 °C for 6 hours. The digests were dissolved in 1.5ml 8 M HNO_3 and doubled de-ionized H_2O to a final volume of 25 ml. Mineral concentration was

analyzed by inductively coupled plasma – atomic emission spectrometer (ICP-AES) (Shimadzu 1000Ⅱ, Japan).

To investigate Shoot Dry Biomass (SDB) yield when plants were grown under Zn nutritional stress condition, the parental lines and randomly selected 140 DH lines were cultivated hydroponically in a greenhouse without climate control in March (experiment 1: Zn deficiency experiment) and April 2006 (experiment 2: Zn toxicity experiment) in Beijing. The environmental conditions were 15-25°C/8-12°C in March and 20-30°C/10-15°C in April (day/night temperature), 30,000-40,000 Lux light intensity and 50-60 % relative humidity. Each experiment included Zn stress treatment (without supplying $ZnSO_4$ to create Zn deficiency or supplying 100 µM $ZnSO_4$ for Zn toxicity) and normal Zn supply (2 µM $ZnSO_4$). Half strength Hoagland's nutrition solution (pH 5.5), buffered with 2 mM MES (2-morpholinoethanesulphonic acid) was used for plant cultivation. In both experiments three replicates were used for each Zn treatment. Seeds were germinated on agar in 1.5-ml eppendorf tubes of which the bottom tips had been being cut off and put directly in hydroponic culture trays. Each tray contained 71 DH lines with one plant per line. The trays were shuffled twice a week to reduce a possible effect of location in the greenhouse. Complete shoots (aboveground tissue) were harvested after 21 days growth and oven-dried at 65 °C for three days for SDB analysis.

Table 2 Soil mineral contents for the open field trial plot. Data are presented as means ± S.D., n=6.

pH		*Element*	*K*	*Ca*	*Mg*	*P*	*Cu*	*Fe*	*Mn*	*Zn*
7.97	±	Content	58.7±10.	1568±18	209±33.	75.3±12.	2.0±0.1	34.3±4.0	12.3±2.	3.2±0.4
0.1		(ppm)	2	5	5	2			8	

Statistical analysis and QTL mapping

For each DH line, the mean values of 11 mineral concentrations were log_{10}-transformed to improve normality of the distribution. Transformed mineral data were used for QTL analysis. For QTL analysis of SDB under normal Zn supply, data from the two experiments were first standardized, and the mean values of the two experiments were used for QTL analysis. Detection of QTL for SDB under deficient and excessive Zn conditions was conducted with untransformed data. The software package MAPQTL version 5.0 was used to identify and locate QTL on the linkage map by using interval mapping (IM) and multiple-QTL model (MQM) mapping methods as described in its

reference manual (http://www.kyazma.nl). A sequential procedure was used for QTL detection. The first step involves finding putative QTLs using IM analysis (Van Ooijen, 1992). The significance thresholds accepting the presence of QTLs were determined by permutation tests (1,000 replications) (Churchill and Doerge, 1994) for each trait at a significance level of $\alpha = 0.05$. For different traits, the genome wide significance threshold varied between 2.9 to 3.1. Therefore QTLs with a LOD score $\geq$ 3.1 were considered to be significant. Markers at these QTLs were taken as cofactors (significant at $P<0.02$ level) to include the effect of the genetic background in MQM analysis (http://www.kyazma.nl). A mapping step size of 1 cM was used for both IM and MQM analyses. The QTL positions were estimated as the position with maximum LOD score on a linkage group. Two-LOD support intervals were established as 95 % confidence intervals (Van Ooijen, 1992) using MQM mapping implemented with MAPQTL 5.0. For the correlation coefficient (r) test between mineral concentrations for 11 elements, a Bonferroni correction to adjust the 0.05 or 0.01 threshold of significance was applied. Broad sense heritability was estimated for each trait by using the following equation: $h^2_b = VG / (VG + VE)$; where VG is the variance between DH lines and VE is the variance within DH lines.

The interaction between the QTLs identified for the same trait was tested by analysis of variance (ANOVA) using the corresponding two markers as fixed factors and the trait as dependent variable at a significance level $P \leq 0.05$. For the interaction between genotype and Zn treatment, ANOVA was tested using corresponding markers and Zn treatment as fixed factor and the SDBs as dependent variable. The calculations were carried out by using the general linear model module of the SPSS version 12.0.1 statistical package (SPSS Inc., Chicago, IL, USA).

Results

Construction of the BrIVFhn linkage map

Out of the 211 AFLP primer combinations tested on the parental lines, 32 that each generated over 8 polymorphic markers were selected for genotyping the BrIVFhn mapping population (Table 1). After removing ambiguous markers, in total 226 polymorphic bands were used for mapping. Among these, 123 bands were provided by parent Y177 and 103 by parent Y195. In addition, a total of 202 SSR markers were screened for polymorphisms between the parental lines. 43 displayed a polymorphism

and out of these, 31 SSRs that could be easily scored using silver stained gels were chosen for genotyping the mapping population.

In total 257 markers (226 AFLPs and 31 SSRs) were assigned to ten linkage groups (LGs) with a LOD score ≥ 4.0 (Fig 1). The total length of the map is 1142 cM, which represents on average one marker for every 4.4 cM. By using the 17 SSRs with known map positions on the common *B. rapa* reference map (Kim et al., 2006; Lowe et al., 2004; Suwabe et al., 2006), 9 LGs could be assigned to their corresponding reference LGs. The remaining LG, which did not contain a SSR marker, we deduced to be R8, also since none of the available R8 SSR markers was polymorphic between the two parents. The individual LGs ranged from 158 cM for R3 with the highest number of markers (40) to 29 cM for R7 with the least markers (10). Only three map-intervals with markers separated more than 20 cM were observed on respectively R1, R5 and R8. The distribution of markers and the marker density make this map a useful framework for quantitative trait loci identification.

Based on a χ^2 test for goodness-of-fit to the expected 1:1 Mendelian segregation ratio, skewed segregation was detected for 165 AFLP loci and 16 SSR loci (70.4 %) at $P<0.05$. For 136 out 181 skewed markers the Y195 allele was in excess. Markers that showed skewedness were clustered in specific regions on LGs R1, R3, R6, R7, R8, R9 and R10, containing clusters of markers with an excess of Y195 alleles, and for LGs R4 and R5 this was the case for Y177 alleles (Fig.1). LG R2 had markers with segregation distortion of alleles from both parents but located in separate regions of the LG.

Trait analysis

Transgression beyond the parental values was observed for nearly all tested elements, including K, Zn and P concentrations for which parental values hardly differed (Table 3, Figure 2). Differences between DH lines were from about 2-fold for P to 6.5-fold for Fe. In most cases the extent of transgressive variation was more to the lower than to higher values. Correlations between mineral concentrations are shown in Table 3. The highest and very significant correlations were found between Ca, Mg and Sr concentrations, which were 0.84 (Ca/Mg), 0.86 (Ca/Sr) and 0.84 (Mg/Sr) respectively (Table 4). In this population, Mn concentration was significantly correlated with all other elements, except for K. In all cases significant correlations were positive except for Na/K.

For the DH lines grown under different Zn conditions, the distribution of the SDB is wide and

there is transgression towards lower as well as higher SDB (Table 3). Parental line Y177 has a considerably higher SDB than Y195 under all three tested Zn conditions. In order to clarify whether the QTL for SDB detected under Zn excess stress condition is same locus involved in tolerance to the Zn deficiency stress, we also investigated SDB under normal Zn condition in both Zn excess and deficiency experiments. On average we found a difference in the SDB of plants growing under normal Zn between experiment 1 and experiment 2, with less biomass for plants in experiment 1, which was carried out earlier in spring. Nevertheless, the correlation of the SDB of DH lines under 2 µM Zn in two experiments was significant ($r = 0.43, P \leq 0.01$).

Broad sense heritability of the shoot mineral concentration was high, ranging from 0.56 for Mg to 0.74 for Mn, respectively (Table 3). The heritability of SDB under different Zn nutritional conditions was lower compared with that of mineral content, with heritabilities varying from 0.30 to 0.60.

Table 3 Variation of leaf mineral concentration and Shoot Dry Biomass (SDB) of the parents (Y177 and Y195) and the DH population (Range, Mean) grown in an open field or, for determination of SDB, under 0, 2 and 100 µM Zn supply.

Trait	*Parents*		*Range*	*Mean*	*Heritability*
Macro mineral content (mg g^{-1} DW)					
K	35.8	34.4	25.3-58.2	36.7	0.70
Na	8.6	14.1	2.4-15.7	7.5	0.70
Ca	17.5	30.3	10.1-26.0	15.2	0.60
Mg	3.4	5.1	2.1- 5.3	3.2	0.56
P	8.8	8.3	5.0- 9.6	7.2	0.64
Micro mineral content (µg g^{-1} DW)					
Al	77.0	171.5	38.9-175.0	88.3	0.60
Cu	6.0	7.75	3.8- 7.9	5.6	0.68
Mn	27.2	30.2	13.9- 34.6	20.0	0.74
Fe	89.0	183.1	46.5-224.2	109.2	0.69
Zn	51.4	53.8	31.1- 72.3	48.2	0.69
Sr	81.8	145.4	44.2-150.2	78.1	0.63

SDB (mg plant^{-1})					
SDB0 [a]	130	70	20.0-165.0	79.1	0.46
SDB2-1[a]	189	84	37.5-254.0	135.9	0.60
SDB2-2[b]	120	85	25.0-176.7	84.1	0.30
SDB100	107	59	21.0-144.3	86.0	0.59

[a] Data in experiment 1; [b] Data in experiment 2.

Table 4 Pearson correlation coefficients of the mineral concentrations in the second head leaf.

Mineral	*Pearson correlation*									
	Na	Ca	Mg	P	Al	Cu	Mn	Fe	Zn	Sr
K	-0.30**	0.02	0.14	0.41**	0.33**	0.33**	0.20	0.36**	0.37**	-0.12
Na		0.38**	0.38**	0.05	0.11	0.17	0.33**	0.13	0.03	0.43**
Ca			0.84**	-0.07	0.56**	0.17	0.63**	0.46**	-0.02	0.86**
Mg				-0.04	0.61**	0.17	0.62**	0.57**	0.02	0.84**
P					-0.05	0.57**	0.31**	0.04	0.64**	-0.18
Al						0.03	0.49**	0.68**	0.02	0.48**
Cu							0.34**	0.22	0.70**	-0.02
Mn								0.44**	0.25*	0.47**
Fe									0.23	0.41**
Zn										-0.20

*significant at P<0.05 level, **significant at P<0.01 level

Detection of QTLs

To identify the genetic loci involved in establishing leaf mineral concentration in Chinese cabbage and shoot dry biomass production under Zn nutritional stress, QTL mapping was performed for the tested 11 minerals and SDBs under different Zn nutritional conditions.

Seven QTLs were mapped on four LGs for Na, Mg, P, Mn, Zn and Sr concentrations, while no

QTL was detected for K, Ca, Al, Fe and Cu concentrations in this population (Fig 1; Table 5). The most significant of these QTLs, localizing on R4, explained 18.2 % of the Zn concentration variance. Another QTL for Zn was detected on R6, explaining 11.3 % of the phenotypic variance. These two QTLs together explained 22.2 % of the Zn concentration variance. When tested by ANOVA, no significant interaction was found between the two QTLs. For the concentration of Na, Mg, P, Mn and Sr, only one QTL was found for each element. QTLs for Mg and Sr co-localized on R8 and explained 11.4 % for Mg and 12.9 % for Sr phenotypic variance. The QTL for Na concentration was detected on R4, accounting for 16.3 % of the phenotypic variance. For Mn concentration, one QTL was found on R5 which explained 15.1 % of the variance. The QTL for P concentration localized on R6, explaining 11.1 % of the phenotypic variance.

QTLs related to SDB were only identified on R3 and R6. The QTL detected on R3 for SDB under Zn excess or deficiency co-localized with the QTL for SDB under normal Zn supply, explaining 15.4 %, 14.6 % and 13.1 % of the phenotypic variance for SDB yield under Zn excess, deficiency and normal conditions respectively. When tested by ANOVA, no significant interaction between genotype and Zn conditions was found for SDB phenotypic variance at this locus. This suggests that this QTL is involved in SDB yield regardless of the Zn supply. A QTL was also found on R6 for SDB under Zn excess condition, accounting for 13.0 % of the phenotypic variance. Although this locus was only detected under Zn excess condition, the interaction between genotype and Zn conditions was not significant. The two loci detected for SDB under excessive Zn supply do not show significant interactions. No QTL specific for SDB at Zn deficiency was detected.

Table 5 Quantitative trait loci (QTL) affecting mineral concentration in leaves and shoot dry biomass (SDB) under different Zn nutrition conditions of a *B. rapa* ssp. *pekinensis* DH population

QTL[a]	*LOD*	*Peak*	*Marker* [b]	*2-LOD interval (cM)*	*Exp (%)* [c]	*Add* [d]
Na4	4.1	81	38M51-9 -- E38M35-7	80-84	16.3	0.15
Mg8	3.1	6	E36M35-6--E33M51-5	0-14	11.4	-0.06
P6	3.5	59	E36M59-5	56-60	11.1	-0.04

Mn5	4.5	42	E33M54-4--E41M56-1	40-43	15.1	-0.06
Zn4	5.7	77	E36M36-6--E36M31-10	31-39	18.2	0.07
Zn6	3.8	95	BC51-E40M51-2	92-96	11.3	-0.05
Sr8	3.2	4	E36M25-6-E33M51-5	0-15	12.9	-0.1
Zn2SDB3	3.8	16	E38M51-5	12-19	13.1	0.73
Zn0SDB3	3.8	16	E38M51-5	14-18	14.6	10.3
Zn100SDB3	3.9	19	E38M51-5-E36M31-8	16-21	15.4	9.9
Zn100SDB6	3.2	65	E41M51-7	62-66	13.0	-9.3

[a] QTL names are indicated as element followed by chromosome number. [b] Peak marker or the marker interval. [c] Explained variance. [d] Additive effect of the Y177 allele.

Discussion

This study reports the construction of a genetic linkage map for a segregating population of 183 *B. rapa* DH lines and the first analysis of genetic control of leaf mineral accumulation in *B. rapa* and shoot dry biomass yield under different Zn nutrition conditions.

Linkage map of B. rapa

The present linkage map includes 226 AFLP markers and 31 SSR markers. These markers were grouped into 10 LGs covering a total map distance of 1142 cM, comparable to the 1005.5 cM of a recently published SSR-based map (Suwabe et al., 2006) and 1287 cM of a sequence-tag-based map (Kim et al., 2006). Nine LGs have been anchored to the corresponding LGs in the common reference map by using published SSR markers. These LGs are comparable with the recently published maps both in length and marker density, except for R7 which is shorter and seems to lack some markers. No available SSR markers previously anchored on R8 were polymorphic between both parents and this LG could therefore not be assigned to a LG in the presented map. As the LG without any SSR is not linked to the shortest LG R7 and it is comparable in length with that of R8 in the reference map, we assume that this LG is R8. Further work will be done to confirm this assumption and improve the present map by adding more anchored SSR markers.

A high percentage of skewed markers (70.4 %) were observed in our study. This is comparable

with a proportion of 64 % AFLP and RFLP markers deviating from a 1:1 ratio as observed by Voorrips et al. (1997) in a *B. oleracea* DH mapping population. Skewed segregation ratios have often been observed in populations of doubled haploid plants for many other plant species (Graner et al., 1991), and are most likely due to selection during microspore culture (Foisset et al., 1996). Segregation of skewed markers may generate errors in linkage analysis, resulting in map stretching or a false order of markers on linkage groups. However, simply deleting the markers which show skewed segregation from the map would result in losing all of the skewed segments for trait QTL analysis and thus we decided to include markers with skewed segregation in the map.

QTLs for mineral accumulation

We analyzed leaf mineral concentration in the mapping population and found transgression for all 11 elements. However no QTL was detected for five of these elements including K, which showed transgressive variation to both sides of the parental values. The failure of detection of QTLs associated with transgressed traits has also been reported for RIL populations of tomato (Saliba-Colombani et al., 2001) and tobacco (Julio et al., 2006). Transgressive segregation that is observed in a population is normally due to the presence of complementary QTL alleles in the two parental lines. However when there are several QTLs and the QTL effects are relatively small they may not be detected.

The trait analysis showed that significant positive correlations existed among the tested minerals. This is also the case for seed mineral content in common bean (Beebe et al., 2000) and Arabidopsis (Vreugdenhil et al., 2004). The correlation between different minerals implicated pleiotropy for genes controlling accumulation of these minerals or close linkage of genes. Therefore consideration must be taken in a breeding program for mineral content improvement, especially when toxic minerals are involved. However we did not find as many as co-localized QTLs as we found significant correlations between concentrations of different minerals. Co-localization of QTL involved in different minerals was reported previously for Arabidopsis seed mineral content (Vreugdenhil et al., 2004), with most of these co-localization of K/Ca and K/Ca/Mn QTLs. Instead we found co-localization of a QTL for Mg and Sr. It is frequently observed that the Ca: Sr: Ba ratio in the shoot is identical to that of the solution to which roots are exposed, and therefore a close correlation had been found between the accumulation of Ca, Sr and Ba by plant species grown in the

same substrate (reviewed by White, 2001). The results in this study further indicate that the accumulations of Ca, Mg and Sr share common mechanisms and are therefore under the same genetic control. It was reported for rice that a QTL affecting K concentrations under saline conditions coincided with *QsHKT8*, a Na transporter (Ren et al., 2005). Although no QTL was detected for K concentration in the present study, the significant negative correlation between K and Na implies that there could be a similar competition of their accumulation in *B. rapa*.

Although the heritabilities for most mineral concentrations were not low, only one or two QTLs with an explained variance per locus varying from 11.1-18.2 % were detected for individual minerals, suggesting that most QTLs had small effects and remained undetected. The observation that no major QTL (explaining more than 30 % of the phenotypic variance) was detected for mineral accumulation indicates that it is under complex genetic control in *B. rapa*.

QTL for SDB

We carried out two independent experiments for plant SDB analysis under Zn deficiency and Zn excess conditions. Relative shoot growth based on dry biomass was previously identified as a suitable parameter for tolerance evaluation of *Brassica* crops to Zn stress (Grewal et al., 1997; Wu et al., 2007). Therefore in the present study we investigated SDB under deficient and excess Zn supply. Considering that SDB represents a complex trait including production-related characters as well as adaptive traits that must be changed in plants in order to adapt to (a)biotic stress (Ronnberg - Wastljung and Gullberg, 1999), we also analyzed SDB under normal Zn supply as a reference to the SDB obtained after Zn deficiency and excess treatments. Only few QTLs were detected for SDB, with low explained variance (≤ 15 %). This is consistent with previous reports that the analyses of plant responses to environmental stress are generally revealing only few major QTL (Koyama et al., 2001), indicating that a large number of genes contribute to the overall phenotype.

A QTL for SDB was mapped on R3 under different Zn nutrition conditions and did not show a significant interaction between the genotype of this locus and the Zn treatment. This suggests that this locus controls plant SDB in general. For the *Zn100SDB6* QTL on R6, which was identified for excessive Zn condition only, the interaction between genotype and Zn conditions is also not significant. As the LOD score of this locus was just above threshold for QTL determination and with a low explained variance, this minor QTL might have escaped QTL detection under normal Zn

condition. The much higher effect of Zn condition (20.9 %) on SDB than that of genotype of this locus (4.5 %), as revealed by ANOVA, indicates that genetic improvement on Zn stress tolerance needs integration of a large number of genes. Inefficient detection of major QTL, specifically related with SDB under Zn stress conditions in the present study is most likely due to the multi-genic inheritance of the trait, which has also been reported for QTL mapping of rice tolerance to Zn deficiency (Wissuwa et al., 2006). More detailed physiological studies that distinguish between sub-processes may allow an improved genetic dissection on tolerance to Zn stress.

Conclusion

In the present study, QTLs were identified for six mineral concentrations in leaves and one QTL for shoot dry biomass yield was specifically detected under Zn excess conditions. These results will contribute to the understanding of mineral accumulation and plant tolerance to Zn nutrition stress in *B. rapa*. These QTLs will be validated in practical marker-assisted selection in *B. rapa* vegetables breeding for high nutritional quality.

Acknowledgements

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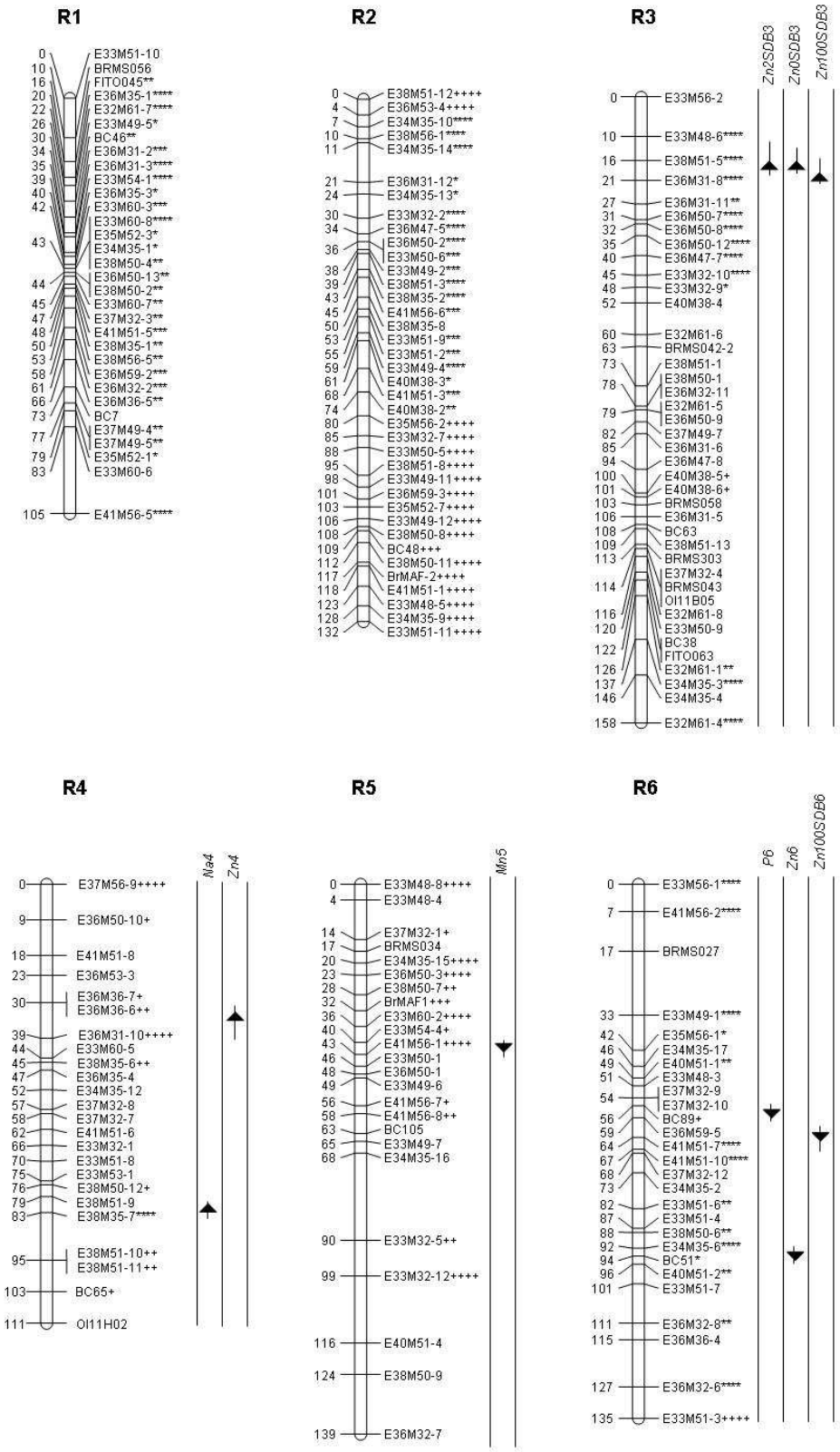
Figure lengends

Fig 1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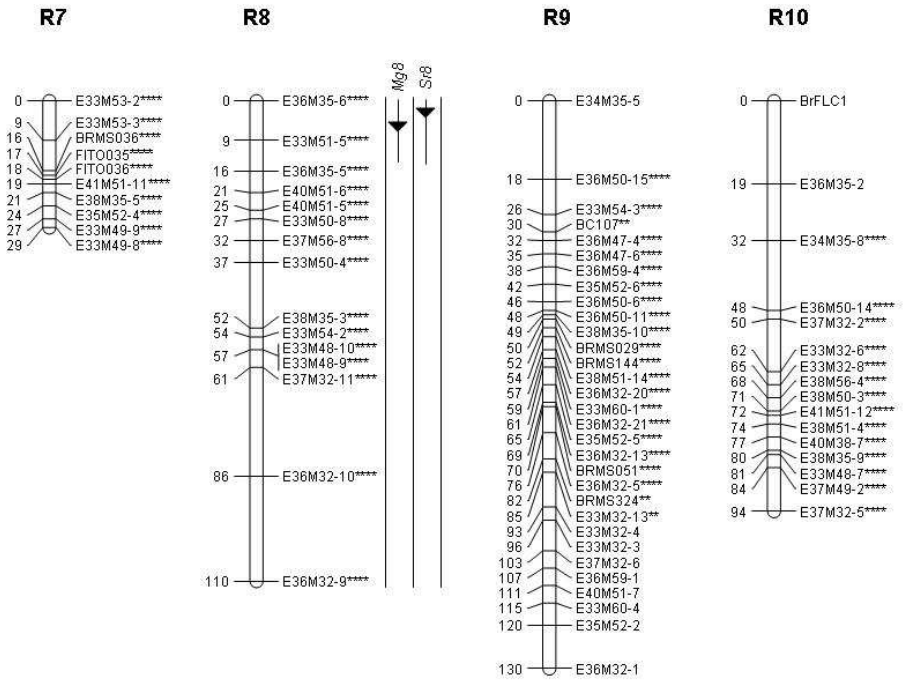
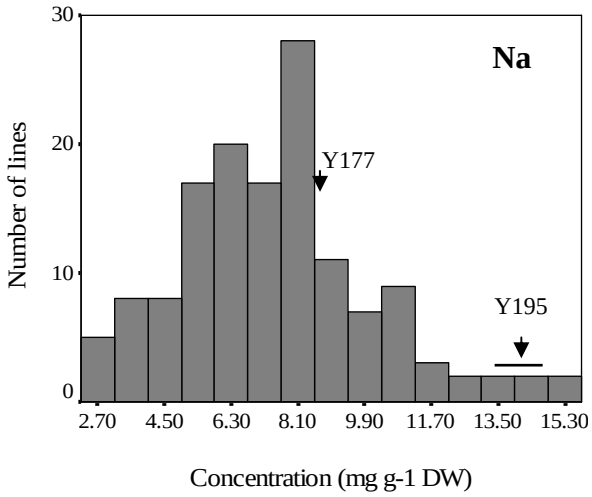
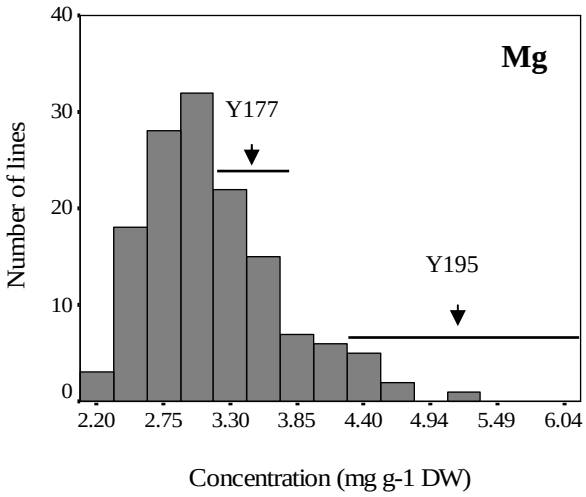
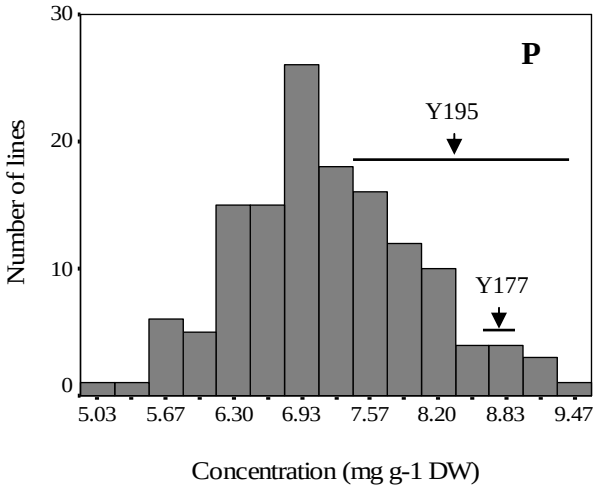


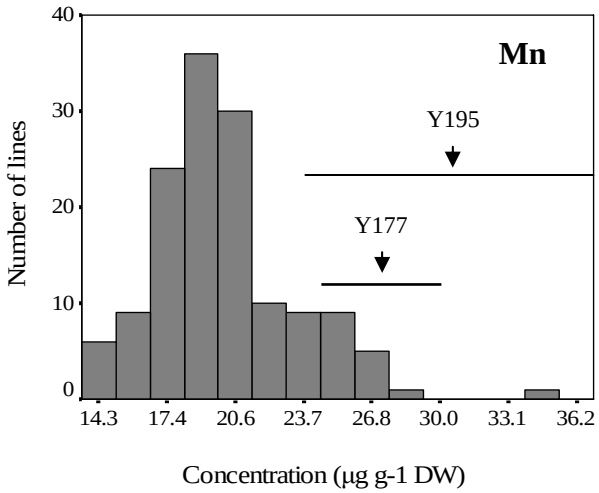
Fig 1 Genetic linkage map based on a population of 183 DH lines of *B. rapa* ssp. *pekinesis*, and positions of QTLs associated with the concentration of six minerals in leaves and with Shoot Dry Biomass (SDB). Marker loci are listed to the right and recombination distances (cM) to the left of each linkage group. Skewed marker loci are indicated with + (skewed to Y177) or * (skewed to Y195), the number of * or + indicates the level of significance with one symbol indicating significance at $P \leq 0.05$; two at $P \leq 0.01$; three at $P \leq 0.005$; and four at $P \leq 0.001$. Locations of QTLs are indicated by bars to the right of the linkage groups. The length of a bar indicates the two-LOD confidence interval. The direction of the arrow head indicates the relative effect of the Y177 allele with upward for increasing and downward for decreasing.

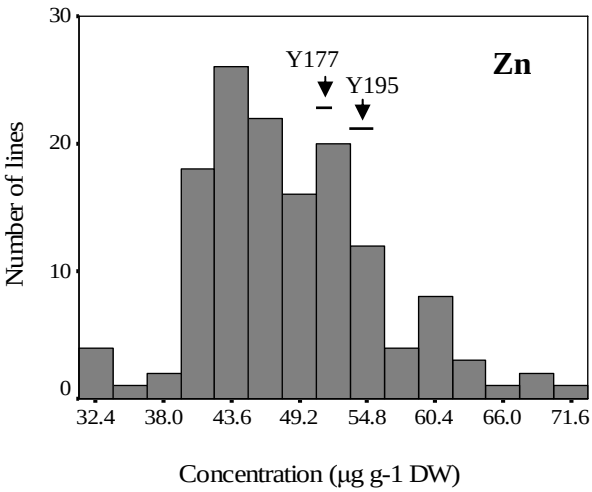
Fig 2 Frequency distributions of non-normalized data for the concentration of six minerals in leaves for which QTLs are identified in the DH population of *B. rapa* ssp. *pekinesis*. The parental values are the mean value of two replicates, indicated with an arrow for each parent. The horizontal bars represent the range of the parental values.

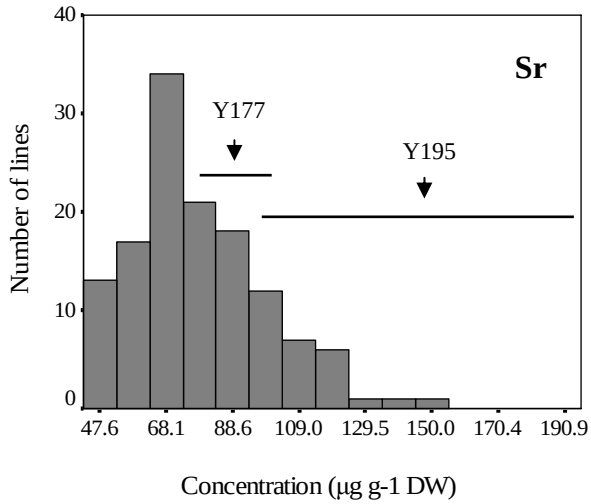

Na
Y177
Y195
Number of lines
0
10
20
30
2.70
4.50
6.30
8.10
9.90
11.70
13.50
15.30
Concentration (mg g-1 DW)


Mg
Y177
Y195
Number of lines
0
10
20
30
40
2.20
2.75
3.30
3.85
4.40
4.94
5.49
6.04
Concentration (mg g-1 DW)


P
Y195
Y177
Number of lines
0
10
20
30
5.03
5.67
6.30
6.93
7.57
8.20
8.83
9.47
Concentration (mg g-1 DW)


Mn
Y195
Y177
Number of lines
0
10
20
30
40
14.3
17.4
20.6
23.7
26.8
30.0
33.1
36.2
Concentration (µg g-1 DW)


Zn
Y177
Y195
Number of lines
0
10
20
30
32.4
38.0
43.6
49.2
54.8
60.4
66.0
71.6
Concentration (µg g-1 DW)


Sr
Y177
Y195
Number of lines
0
10
20
30
40
47.6
68.1
88.6
109.0
129.5
150.0
170.4
190.9
Concentration (µg g-1 DW)

Chapter 4

Identification and functional analysis of two ZIP metal transporters of *Thlaspi caerulescens*

Jian Wu , Xiaowu Wang , Mark G. M. Aarts

Abstract

A cDNA library of *Thlaspi caerulescens* was screened to clone highly expressed putative metal transporters of the ZIP family. Two full-length cDNA clones highly similar respectively to the *Arabidopsis thaliana* genes *ZIP5* and *ZIP6* were isolated and were named *TcZNT5* and *TcZNT6*. Transcript level analysis revealed the constitutively high expression of these two genes in *T. caerulescens* compared to its Arabidopsis orthologues *ZIP5* and *ZIP6*. The expression of *TcZNT5* in roots was higher in the La Calamine (LC) accession of *T. caerulescens* than in the Ganges (GA) accession, regardless of Zn and Cd supply, and was up-regulated in roots by Zn deficiency both in LC and GA. The expression of *TcZNT6* was consistently high in GA roots regardless of Zn or Cd level, but was expressed at a lower level in LC roots and down-regulated upon Cd exposure. Loss of function of the *AtZIP5* gene in Arabidopsis increased its tolerance to Cd, but overexpression of *TcZNT5* in Arabidopsis did not lead to any change in phenotype. In contrast, loss of function of *AtZIP6* in Arabidopsis did not change its phenotype, but overexpression of *TcZNT6* increased the Cd sensitivity of Arabidopsis as measured by reduced root growth. In conclusion, *TcZNT5* and *TcZNT6* expressions respond to changes in Zn or Cd exposure, as do the expressions of *AtZIP5* and *AtZIP6*, but a clear function for these two orthologous gene sets in metal homeostasis could not be established.

Keywords ZIP, *Thlaspi caerulescens*, metal transport

Introdution

The molecular understanding of zinc (Zn) transporters and their role in plant metal homeostasis is important because Zn is essential for all organisms for its key structural and catalytic role in many protein and enzymes. Human Zn deficiency ranks third after Fe and vitamine A deficiency in significance of nutritional diseases (Hambridge, 2000). Food Zn content is very important for human Zn intake, because the supplementation of minerals is often difficult to achieve, especially in developing countries when the diet is based mainly on cereals. Therefore, breeding of crops with increased Zn content has been proposed as a new strategy to reduce Zn deficiency (Graham et al., 1999; Welch and Graham, 1999). Molecular knowledge on Zn accumulation, for which Zn transporters are assumed to be crucial, will therefore have very important biotechnological applications in creating crops with high Zn content (Ramesh et al., 2004).

Using molecular genetics techniques, a wide range of gene families have been identified in plants that are involved in metal transport (see review by Hall and Williams, 2003). Members of the ZRT-IRT-like Protein (ZIP) family were the first metal transporters to be identified in plants (Eide et al., 1996). Fifteen ZIP genes have been identified in Arabidopsis so far, based on whole genome sequencing. Some of them have been characterized and shown to be involved in metal uptake and transport in plants (Mäser et al., 2001). Genes encoding ZIPs have also been identified and characterized from other plant species. In general the ZIPs have the capacity to transport a variety of divalent cations including Zn^{2+}, Fe^{2+}, Mn^{2+} and Cd^{2+} (Guerinot, 2000) and the expression of *ZIP* genes is regulated by environmental metal levels (Grotz et al., 1998). *AtIRT1* and *AtIRT2* have been described to have an essential role in maintenance of iron homeostasis (Vert et al., 2001, Vert et al., 2002; Connolly et al., 2002; Varotto et al., 2002; Henriques et al., 2002). *AtZIP1*, *AtZIP2* and *AtZIP3* confer Zn uptake activity in yeast (Grotz et al., 1998). *GmZIP1*, a homologue of *AtZIP1* in soybean, was identified as a Zn transporter (Moreau et al., 2002). *MtZIP2* was shown to be a Zn transporter localized at the plasma membrane in roots and stems of *Medicago truncatula* with up-regulation in roots by Zn fertilization (Burleigh et al., 2003).

Thlaspi caerulescens, a Zn/Cd/Ni hyperaccumulator, has been used as a model species for dissection of the genetic and molecular mechanism of metal accumulation and homeostasis (Assunção et al., 2003). *TcZNT1* and *TcZNT2*, the proposed *T. caerulescens* orthologues of the Arabidopsis *AtZIP4* and *AtIRT3* genes respectively, are constitutively over-expressed in roots even when exposed to 1 mM

Zn^{2+} (Assunção et al., 2001; Pence et al., 2001). *TcZNT1* was shown to mediate high-affinity Zn uptake and low-affinity Cd uptake (Pence et al., 2000). Homologous genes *TjZNT1* and *TjZNT2* were isolated from the Ni hyperaccumulator *T. japonica* and were shown to be able to transport Zn, Cd and Mn (Mizuno et al., 2005). Two copies of *TcIRT1* were detected in two accessions (Gangs and Prayon) of *T. caerulescens*, with predominant full-length transcript in Ganges and truncated transcript in Prayon (Plaza et al., 2007). The expression of *TcIRT1* was induced by Fe deficiency or by exposure to Cd, while *TcIRT1* showed less effect in mediate Cd sensitivity in yeast than *AtIRT1*. Expression of *TcZNT5* was also compared for the two accessions showing that *TcZNT5* was mainly expressed in roots and the expression level was much higher in Prayon than in Ganges.

Transcript profiling has been used to investigate the molecular mechanism of metal hyperaccumulation (Weber et al., 2004; Becher et al., 2004; Hammond et al., 2006; van de Mortel et al., 2006), assuming that genes involved in metal hyperaccumulation will be higher expressed and differently regulated than those in non-hyperaccumulators. Comparative transcript profiling of the Zn/Cd hyperaccumulator *A. halleri* and *A. thaliana* showed similar expression levels in roots of the two species for *ZIP5* and *ZIP6* (Weber et al., 2004). However, transcription of *ZIP6* in shoots was substantially higher in *A. halleri* than in *A. thaliana* (Becher et al., 2004). Expression of *ZIP5* was upregulated in roots of *A. thaliana* upon Zn deficiency and in *T. caerulescens* this gene was higher expressed compared to Arabidopsis (van de Mortel et al., 2006). These studies indicate that *ZIP5* and *ZIP6* and their orthologues are likely to have a pronounced role as possible Zn/Cd transporters in Zn homeostasis and Zn/Cd hyperaccumulation.

Here we report the cloning of full-length cDNAs of two new ZIP members of *T. caerulenscens* accession La Calamine (LC), which were named *TcZNT5* and *TcZNT6* based on their high similarity to the *A. thaliana* genes *AtZIP5* and *AtZIP6* respectively. Transcription levels of the two *T. caerulescens* genes were compared with their presumed *A. thaliana* orthologues and by comparing the *T. caerulescens* accessions LC and GA, which differ in Cd hyperaccumulation potential. Additional evidence for a functional role in Zn homeostasis was sought in studying T-DNA insertion knock-out mutants of *AtZIP5* and *AtZIP6* and Arabidopsis plants over-expressing *TcZNT5* or *TcZNT6*, for their phenotypic response to growth on media with different Zn and/or Cd supply.

Materials and methods

Library screening

A cDNA library made from roots of *Thlaspi caerulescens*, as described by Assunção et al. (2001) was used for full-length cDNA cloning. Partial cDNA clones RR9nr066 and RR8nr089 in a pAD-GAL4-2.1 vector had been identified as putative orthologues of *AtZIP5* and *AtZIP6* (Rigola et al., 2006). cDNA fragments (approximately 500bp for the *ZIP5* homologue and 650bp for *ZIP6* homologue) cut from the pAD-GAL4-2.1 plasmid by *Eco*RI and *Xho*I were used as probes for cDNA library screening. The probes were labeled with [α-^{32}P]dATP using the Hexalabel^{TM} DNA labeling kit (Fermentas, http://www.fermentas.com/). The isolated cDNA clones were sequenced by ABI PRISM BigDye terminator cycle sequencing technology v2.0, according to the manufacturer's instruction (Applied Biosystems; http://www.appliedbiosystems.com.), using an ABI3700 DNA analyzer. Sequence analysis was performed using the standard BLAST sequence comparison (http://www.ncbi.nlm.nih.gov/BLAST/). Transmembrane domains were defined according to TMHMM (http://www.cbs.dtu.dk/services/TMHMM/. Intracellular localization was predicted using PSORT (www.psort.nibb.ac.jp). Sequence alignment was conducted by MegAlign (DNAstar, Madison WI). Phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al., 2004)

Plant material

Seeds of *A. thaliana* Columbia-0 and *T. caerulenscen*s J. & C. Presl accession LC were germinated on garden peat soil (Jongkind BV, The Netherlands). Three-week-old seedlings were transferred to hydroponics, three plants per pot filled with 1 L modified half-strength Hoagland nutrient solution (van de Mortel et al., 2006). After growth for three weeks on this solution, the *T. caerulenscens* plants were transferred to the same nutrient solution with a deficient (0 μM), sufficient (100 μM), or high (1000 μM) $ZnSO_4$ concentration on which they were grown for 7 additional days. The Arabidopsis plants were transferred to the same nutrient solution with deficient (0 μM), sufficient (2 μM) or high (25 μM) $ZnSO_4$. The nutrient solution was replaced once a week during the first three weeks and thereafter twice a week.

For comparison of transcription levels of *TcZNT5* and *TcZNT6* between *T. caerulescens* accessions LC and GA, plants were grown as described above including two additional cadmium treatments (Zn 100 μM + Cd 1μM and Zn 100 μM + Cd 10 μM).

DNA blot analysis

Genomic DNA was extracted from three *T. caerulescens* accessions: LC, Monte Prinzera (MP), GA and Austria; and four *Thlaspi* species: *T. japonicum, T. praecox, T. minimum* and *T. perfoliatum*, using a modified CTAB method (Fulton et al., 1995). Digestion of genomic DNA, TAE gel electrophoresis, DNA blotting, prehybridization, hybridization and washing were all performed as described by Assunçaõ et al. (2001). Partial cDNA fragments digested from the pAD-GAL4-2.1 vector plasmid containing full-length cDNAs using *Eco*RI and *Xho*I for *TcZNT5* (1032 bp) and *TcZNT6* (1310 bp) were used as probes and labeled as described above.

Semi-quantitative RT-PCR

Leaves or roots of one pot containing three Arabidopsis or three *T. caerulescens* plants per treatment were pooled and homogenized in liquid nitrogen. Total RNA of leaves or roots was extracted with Trizol (Invitrogen) following the manufacturer's instructions. Five micrograms of total RNA was used to synthesize cDNA with MLV reverse transcriptase (Invitrogen) and oligo(dT) as a primer (Invitrogen). The PCR amplification was performed with a 2-µl cDNA aliquot. The *AtZIP5* forward primer was 5'-ATGAGAATCACACAAAACGTCAAGC-3' and the reverse primer was 5'-TGGGATTCACCAGATTCCAC-3'; the *TcZNT5* forward primer was 5'-ACCGGAGCCGAGTTGTG-3' and the reverse primer was 5'-TGGGCCATGATTTGAAGC-3'; the *AtZIP6* forward primer was 5'-GTCACCGGAACAGAGGCAGCAA-3 and the reverse primer was 5'-TTCACCGCAAGTCGTCAGCATCTT-3'; the *TcZNT6* forward primer was 5'-AGAGACGGAGACGCGGCGG-3' and the reverse primer was 5'-CTGATGAAACGAAAGAGTAGCG-3'. Primer pairs for *Tubulin* were used as a control for using similar cDNA quantities for each sample. For *A. thaliana* the forward *Tubulin* primer was 5'-AAGCTTGCTGATAACTGTACTGGT-3' and the reverse primer was 5'GGTTTGGAACTCAGTGACATCA-3'; for *T. caerulescens* the forward primer was 5'-CTACGCACCAGTCATCTCT-3' and the reverse primer was 5'-CGAGATCACCTCCTGGAACA-3'. 25 cycles were performed for *Tubulin* PCR amplification from Arabidopsis and *T. caerulescens* samples. PCR fragments were separated on an ethidium bromide stained 1 % agarose TAE gel.

Construction of expression vectors

The full-length cDNAs of *TcZNT5* and *TcZNT6* were amplified from the pAD-GAL4-2.1 vector plasmids by PCR. Gateway primers were used for PCR, the forward primer was 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGATCGAATTA*GGATCC*TCTGC-3', containing the attB1 sequence (underlined) and a *Bam*HI site (italic) in pAD-GAL4-2.1, and the reverse primer was 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATGGG*CTCGAG*AGTCGAC-3', containing the attB2 sequence (underlined) and an *Xho*I site (italic) in pAD-GAL4-2.1. The PCRs were performed with the proofreading Pfu polymerase (Fermentas, http://www.fermentas.com) at 93 °C for 5 min, followed by 30 cycles of 93 °C for 1 min, 55 °C for 1 min and 68 °C for 3 min, and finished by an extension at 68 °C for 10 min. PCR products were recombined into pDONR207 (Invitrogen, http://www.invitrogen.com) in a 10-µl BP Clonase (Invitrogen) reaction following the manufacturer's instruction. The fragments were transferred from their donor constructs to the binary over-expression vector pGD625 (de Folter et al., 2006), under the control of the double 35S CaMV enhancer, in a 10-µl LR Clonase (Invitrogen) reaction following the manufacturer's instruction. The binary constructs were introduced into *A. tumefaciens* strain AGL0 by electroporation.

Plant transformation

The binary constructs with full-length cDNAs of *TcZNT5* and *TcZNT6* were used to transform *A. thaliana* Columbia-0 (Col) by the standard flower dip method (Clough and Bent, 1998). The primary T1 transformants were selected on 0.5 X MS medium containing kanamycin (50 mg/ml). Kanamycin-resistant plants were transferred to soil, and the T2 seeds resulting from self-fertilization were collected. The T2 seeds were plated on the same selection medium and scored for kanamycin resistance. Transgenic lines that displayed a 3:1 segregation ratio for kanamycin resistance : sensitivity in the T2 generation indicating a single T-DNA insertion locus were selected for further analysis. In the T3 generation, because of silencing of resistance to kanamycin in all the selected lines, we tested 12 T3 progeny plants from ten T2 plants each by PCR using the gene specific primers for *TcZNT5* and *TcZNT6* designed for the semi-quantitative RT-PCR. Genomic DNA was isolated using a modified CTAB method (Fulton et al., 1995). The lines for with all the 12 T3

progenies contained a T-DNA were deemed to be homozygous transgenic lines. T4 seeds from these plants were used for further experiments. The expression levels of *TcZNT5* and *TcZNT6* in the transgenic lines were determined by RT-PCR using cDNA made from T4 plants grown in soil for 30 days.

Selection of homozygous T-DNA insertion plants

SALK_009007 and SALK_116013 Arabidopsis T-DNA insertion lines were obtained from the Nottingham Arabidopsis Stock Centre (NASC). SALK_009007 carries a T-DNA insert approximately 357 bp downstream of the start of the first exon of *AtZIP5* (At1g05300), while SALK_116013 carries a T-DNA insert approximately 363 bp downstream of the start of the first exon of *AtZIP6* (At2g30080) (Alonso et al., 2003). Genomic DNA was isolated from eight plants of SALK_009007 and 16 plants of SALK_116013. Homozygous mutants were identified by PCR using genomic DNA as template and separated combinations of primer LBb1, designed to fit the left border of the T-DNA insert (5'- GCGTGGACCGCTTGCTGCAACT-3') (http://signal.salk.edu), and *AtZIP5* or *AtZIP6* specific primer pairs. The gene specific primer pairs were same as those used in semi-quantitative RT-PCR for *AtZIP5* and *AtZIP6*, only except the reverse primer for *AtZIP6* was 5'-TGCAACCACCAAGACCCAAA-3'. Seeds harvested from the identified homozygous T-DNA insertion plants and homozygous wild-type plants segregating in the respective SALK lines, were used for further phenotype screening.

Metal tolerance screening

Seeds were sterilized and sown on 0.5 X MS medium containing deficient or high Zn (0 μM or 200 μM), deficient Fe (0.5 μM), Cd (50 μM) and Fe deficient (0.5 μM) medium supplemented with Cd (50 μM). 0.5 X MS medium was used as control. For each treatment, five replicates were used for each line with 20 seeds sown in each replicate. After being sown on media, seeds were kept at 4 °C for 4 days in the dark to synchronize germination. Seedlings were grown in a 14 h light, 10 h dark cycle at 20 °C day and 15 °C night temperatures. The phenotypes were studied on 15-day-old plants by measuring root length.

Results

Cloning of full-length cDNA clones encoding *TcZNT5* and *TcZNT6*

A cDNA library prepared from roots of *T. caerulescens* plants from accession La Calamine (LC), grown hydroponically in 10μM Zn (Assunção et al., 2001), was screened to clone the full-length cDNAs for *T. caerulescens* genes orthologous to *AtZIP5* (At1g05300) and *AtZIP6* (At2g30080). Two partial *T. caerulescens* cDNA clones (RR9nr066 and RR8nr089) previously identified to have high identity respectively to *AtZIP5* and *AtZIP6* (Rigola et al., 2006), were used as probes to screen the cDNA library. Three positive clones were obtained for each probe. Nucleotide sequence analysis revealed that one of the three positive clones detected with the RR9nr066 probe had an open reading frame of 1068 bp covering the full predicted *AtZIP5* ORF and encoding a predicted protein of 355 amino acids and 37 KDa. It contains eight transmembrane domains (TMs) (Fig. 1). The sequence has 86% DNA identity and 85% amino acid identity with *AtZIP5* and was provisionally named *TcZNT5-LC*. There is a putative metal binding domain containing a series of repeated histidine residues (HVHAHGHAHG) between TM3 and TM4, and it also has the conserved histidine residue in TM4 found in many members of the ZIP family. According to TAIR (www.arabidopsis.org), both AtZIP5 and the related protein AtZIP3 are targeted to the plasmamembrane. Considering the similarity in sequence also TcZNT5-LC has a cleavable N-terminal signal sequence of 25 residues (PSORT) and will be targeted to the plasma membrane. There are three other full-length cDNA sequences of *TcZNT5* from *T. caerulescens* accessions GA and Prayon (PR) deposited in the NCBI genebank. Remarkable is that the protein predicted to be encoded by *TcZNT5-GA* lacks three amino acids close to the N-terminus. It is not clear if this will interfere with protein localization.

The sequence of the longest of three positive clones identified by the RR8nr089 probe contains an open reading frame of 1023 bp encoding a predicted protein of 340 amino acids, with a molecular weight of 35 KDa. The protein contains eight potential TMs (Fig 2), a cleavable N-terminal signal sequence of 44 residues. This protein is also predicted to be targeted to the plasma membrane, although Swiss-Prot (http://www.ebi.ac.uk/swissprot/) suggests localization to the plastid membrane. The cDNA sequence has 86% DNA identity and 90% amino acid identity with *AtZIP6* and therefore the sequence was provisionally named *TcZNT6-LC*. In contrast to most members of the ZIP family, *TcZNT6-LC* contains only two histidine residues as potential metal binding motifs in the variable region between TM3 and TM4. However, it contains the conserved histidine residue in TM4. The

predicted amino acid sequences of *TcZNT5-LC* and *TcZNT6-LC* were aligned with the other members of the ZIP gene family (Fig 3). Phylogenetic analysis revealed *TcZNT5* is closest to *AtZIP5* as expected based on BLAST analysis, and both are closely related to *AtZIP3*, whereas *TcZNT6* is closest to *AtZIP6* and both are more distantly related to the other ZIP family members. According to the previous studies on ZIP family members in *T. caerulescens* and the nomenclature used before (Pence et al., 2000; Assunção et al., 2001), we propose to maintain the provisional names for these two genes as *TcZNT5* and *TcZNT6*.

DNA blot analysis was performed to try and determine the copy number of the *TcZNT5* and *TcZNT6* genes in the *T. caerulescens* genome and in those of related *Thlaspi* species (Fig 4). We used restriction enzymes *Eco*RI and *Xba*I (the latter only for *TcZNT5*) for genomic DNA digestion, which cut once (*Eco*RI) or not (*Xba*I) in the *TcZNT5* cDNA sequence and not in the *TcZNT6* cDNA sequence. There were always at least two bands for the *Eco*RI and *Xba*I digestions in *T. caerulescens* accessions hybridized with the TcZNT5 probe (Fig 4a)., suggesting there are at least two copies with similarity to the probe. Considering the close similarity of *AtZIP5* and *AtZIP3* (stretches of over 85% DNA similarity) and the much lower band intensity of the second (or third) band, this is likely to be cross-hybridization to a gene with homology to *AtZIP3* (*TcZNT3*) rather than a strong indication of an additional copy of a *TcZNT5*-like gene. Similar cross-hybridization was previously found for *TcZNT1* and *TcZNT2* (Assunção et al., 2001).

As was previously found for *NRAMP* genes, there is much stronger hybridization of the ZNT5 probe to the lanes with *T. japonicum* genomic DNA. As the amount of genomics DNA loaded in each *T. japonicum* lane was comparable to those of the *T. caerulescens* lanes (data not shown) it looks like duplications of the *TjZNT5* gene have occurred in this species. Considering the low number of bands, these duplications are likely to be very similar to each other.

For the *TcZNT6* hybridization, there were accessions with only one hybridizing fragment, suggesting there is only one copy of this gene within *T. caerulescens* (Fig 4b). Since there are also in Arabidopsis no closely related ZIP family members to *AtZIP6*, this supports the cross-hybridization of the *TcZNT5* probe to the putative *TcZNT3*. *TjZNT6* does not seem to be duplicated to the same extent as *TjZNT5* in *T. japonicum*.

Analysis of *TcZNT5* and *TcZNT6* transcript levels

The role of *TcZNT5* and *TcZNT6* in metal accumulation and homeostasis in *T. caerulescens* was firstly evaluated by their expression upon exposure to various metal conditions. Transcript levels of *TcZNT5* and *TcZNT6* in leaves and roots were compared to those of their orthologues *AtZIP5* and *AtZIP6* in the non-hyperaccumulator *A. thaliana* using semi-quantitative RT-PCR on in 4-week-old, hydroponically grown plants. Both *TcZNT5* and *TcZNT6* were more or less constitutively expressed in leaves and roots, whereas expression of *AtZIP5* and *AtZIP6* is clearly different in response to changes in Zn supply (Fig 5). In general, expression of *TcZNT5* and *TcZNT6* is higher than that of *AtZIP5* and *AtZIP6*. Expression of *AtZIP5* was induced under Zn deficiency both in roots and shoots, while expression of *AtZIP6* was induced by high Zn supply. The transcript level of *TcZNT5* was higher in roots than in leaves, while *TcZNT6* was expressed at a similar level in leaves and roots under the tested Zn levels. In Arabidopsis *AtZIP5* is only slightly higher expressed in roots *vs.* leaves. This difference is much more pronounced for *AtZIP6* expression.

As *T. caerulescens* is a Zn/Cd hyperccumulator, we further tested the changes of transcript levels of *TcZNT5* and *TcZNT6* in roots and leaves upon different Zn and Cd status for two *T. caerulescens* accessions, LC and GA. These two accessions are similar in Zn/Cd tolerance, but GA accumulates more Zn and Cd than LC (Assunção et al., 2003b). Semi-quantitative RT-PCR analyses were conducted on the plants grown under deficient (0 μM), sufficient (10 μM and 100 μM) and high Zn (1000 μM) together with sufficient Zn (100 μM) in the presence of low Cd (1 μM) or high Cd (10 μM). As shown before, *TcZNT5* was predominantly expressed in roots in both accessions (Fig 6), but with a higher expression in LC compared to GA. Although much less than what we observed for *AtZIP5*, expression of *TcZNT5* was also slightly up-regulated in roots under Zn deficiency in both accessions. Cd treatment did not alter the expression level of *TcZNT5* in LC roots, whereas in GA the expression is marginally up-regulated at 1 μM Cd (+ 100 μM Zn), but down-regulated at 10 μM Cd (+ 100 μM Zn) compared to 100 μM Zn in the absence of Cd. There was more difference in expression of *TcZNT6* between two accessions, especially in roots. *TcZNT6* was expressed at similar levels in shoot of both accessions, irrespective of the metal supply. In GA this was also the case for root, but in LC the expression in roots was lower than in GA, and down-regulated in response to 10 μM Cd (+100 μM Zn).

Root growth of *zip5* and *zip6* loss of function mutant

Investigating the function of *TcZNT5* and *TcZNT6* in *T. caerulescens* is cumbersome in the absence of a collection of T-DNA insertion mutants like in Arabidopsis. Therefore we analyzed T-DNA insertion mutants of their orthologues in Arabidopsis. Homozygous T-DNA insert plants and wild-type sister (WT) plants were isolated by PCR screening (data not shown). RT-PCR confirmed the expression of the genes in the WT plants. No expression was detected in the mutants (data not shown) indicating that the T-DNA is not spliced from the transcript and the insertion is likely to cause a null allele. When grown in soil, the knock-out mutants showed no visibly different morphological phenotype compared to wild type. When screened for root growth on vertical plates, there was only a statistically significant difference between *zip5* and WT plants when grown on medium containing Cd, with *zip5* plants growing longer roots, suggesting increased Cd tolerance. However this difference disappeared when plants were grown on medium with low Fe supply (0.5 μM) even in the presence of Cd (Fig 9). For the *zip6* mutant plants, no significant differences in root length were found when compared to WT plants, irrespective of the metal exposures, suggesting this gene has no role in conferring metal tolerance to act highly redundant or not.

Transgenic Arabidopsis lines overexpressing *TcZNT6* exhibit increased sensitivity to Cd

T. caerulescens is very tolerant to high Zn and Cd exposure and a strong hyperaccumulator of Zn and, in the case of accession GA, also of Cd. In general, the expression of *TcZNT5* and *TcZNT6* is much higher in *T. caerulescens* than that of the *AtZIP5* and *AtZIP6* orthologues in Arabidopsis, and the *T. caerulescens* expression of these genes is not so much influenced by altered metal supply as it is in Arabidopsis. Thus, it could be that the strong metal accumulation and/or tolerance of *T. caerulescens* is partly caused by a high *TcZNT* expression. To test this further, we transformed *A. thaliana* Col-0 with the *TcZNT5* and *TcZNT6* full-length cDNAs controlled by the strong and constitutive CaMV 35S promoter. Three independent homozygous single-locus transgenic overexpression lines were obtained for each gene. For the *TcZNT5* overexpressor lines, the transgene expression was comparable, as confirmed by RT-PCR. However, for the *TcZNT6* construct, only one of the three lines had a similarly high expression of the transgene as for the *TcZNT5* overexpressors, with two other lines still showing higher expression than expected for the *AtZIP6* gene (Fig 8).

Like for the *zip5* and *zip6* knock-out plants, plants expressing *TcZNT5* and *TcZNT6* did not show obvious morphological alterations when they were grown in soil. These transgenic lines were further

examined for their tolerance to metal stresses. Seedlings were grown for 15 days on vertical plates with different metal levels before the root growth was measured. *TcZNT5* overexpression seems to induce marginally longer roots at low Zn, low Fe and high Cd supply, although these differences were not statistically significant (Fig. 9a). High overexpression of *TcZNT6* resulted in significantly reduced root length compared to the WT plants and plants with modest *TcZNT6* overexpression on medium supplemented with Cd (50 μM), either under sufficient and deficient Fe supply, suggesting a generally increased Cd sensitivity (Fig 9b, c). The exposures to Zn deficiency or excess Zn showed no significant differences in root length (Fig 9b).

Discussion

Screening of a *T. caerulescens* root cDNA library identified full-length cDNA clones of two ZIP family members, *TcZNT5* and *TcZNT6*, the latter of which had not yet been identified in *T. caerulescens* before. The predicted proteins encoded by the two genes share all common features found in ZIP proteins: they have eight putative transmembrane domains (TM); they contain a histidine repeat in the variable region between TM3 and TM4 which has been proposed as the metal binding and/or sensing site (Grossoehme et al., 2006); and they have the conserved histidine residue in TM4 which is predicted to occupy the polar face of the amphipathic helix and has a role in substrate transport through the membrane (Eng et al., 1998; Guerinot, 2000).

The N-termini of the predicted protein sequences of the four identified *TcZNT5* alleles from accessions LC, GA and PR (two alleles) are different from the predicted protein sequence of the Arabidopsis *AtZIP5* gene. All *T. caerulescens* alleles seem to lack one of the codons encoding a phenylalanine residue. However, when comparing the *AtZIP5* predicted protein sequence to that of the related gene *AtZIP3*, it shows that this sequence is also lacking the phenylalanine residue and an adjacent leucine residue, suggesting that the sequence may not be critical in terms of providing proper protein function. Also within the three *T. caerulescens* alleles there are some differences. Most striking is the absence of three codons encoding Lys8-Leu9-Leu10 in the *TcZNT5-GA* predicted protein sequence. In principle this deletion could disturb the subcellular localization of the TcZNT5 protein in GA, but since no other non-sense mutations are present in the sequence, it is likely this allele encodes a functional protein targeted to the same subcellular position as the protein for the other accessions. It will be interesting to determine the subcellular localization experimentally using

a GFP protein fusion.

Both *TcZNT5* and *TcZNT6* are more or less constitutively expressed in *T. caerulenscens,* with both root and shoot expression staying constant over a wide range of Zn supplies (0-1000μM), which differs from the Zn status-dependent regulation of the expression of their orthologues *AtZIP5* and *AtZIP6* in *A. thaliana* (Fig 5). A similarly high and constitutive expression compared to *A. thaliana* was previously reported for the *TcZNT1*, *TcZNT2*, *TgMTP1*, and *AhMTP1* genes in *T. caerulescens* and other metal hyperaccumulator species *T. goesingense* and *A. halleri* (Pence et al., 2000; Assunção et al., 2001; Persans et al., 2001; Dräger et al., 2004). As Zn hyperaccumulation in *T. caerulescens* is a constitutive trait involving strongly enhanced metal uptake and root to shoot translocation (Assunção et al., 2003a), higher cation transport capacity will be needed for taking up metals from soil or for intracellular storage and allocation (Lasat et al. 1996). *T. caerulescens* seems to have dealt with the demand for higher transport capacity in increasing the expression of the available zinc transporter genes.

Both *TcZNT5* and *TcZNT6* are expressed at a higher level in *T. caerulescens* than in *A. thaliana*. The RT-PCR results confirm the root micro-array expression data for *TcZNT5* and *AtZIP5* as determined by van de Mortel et al. (2006). They did not report the higher root expression of *TcZNT6* compared to *AtZIP6* as it probably did not exceed the 5-fold threshold level they used. The higher expression of *TcZNT6* in leaves of *T. caerulescens* was also observed for *AhZIP6* in leaves of the Zn/Cd hyperaccumulator *A. halleri* (Becher et al., 2004). The main difference between Arabidopsis and its hyperaccumulating relatives is a higher accumulation of metals in the shoots. Therefore the stronger expression of *TcZNT6/AhZIP6* in leaves and the increased expression of *AtZIP6* upon increasing Zn conditions suggests these genes play a role in accumulation or storage of Zn, for instance in increased uptake in the mesophyl or epidermal cells of hyperaccumulator species that are considered to be especially enriched with Zn (Ma et al., 2005; Zhao et al., 2000). It will be interesting to study the expression of ZIP6promoter::GUS or ZNT6promoter::GUS fusion constructs to determine the tissue in which this gene is expressed in the relevant species. Alternatively, ZIP6/ZNT6 may not be transporting Zn, but another metal. Enhanced Zn exposure may lead to the induction of deficiency responses for other minerals such as Fe, as was also observed by van de Mortel et al. (2006). To study this further it will be helpful to determine the metal specificity of ZIP6/ZNT6 in heterologous yeast expression studies.

The strongly reduced differential regulation of *TcZNT5* and *TcZNT6* expression in response to different Zn supply compared to Arabidopsis is consistent with the hypothesis that an alteration in the regulation of Zn transport by Zn status plays a role in enhanced Zn uptake and accumulation. In order to further identify the function of both genes in metal homeostasis, we first performed a phylogenetic analysis of the *TcZNT5* and *TcZNT6* genes and determined their copy numbers before the subsequent analysis of Arabidopsis overexpression and mutant analysis. The phylogenetic comparison is in line with previous analysis by Mäser et al. (2001) for Arabidopsis ZIPs, and confirmed the close similarity of *TcZNT5* and *TcZNT6* to Arabidopsis genes *AtZIP5* and *AtZIP6* respectively. It also showed that *AtZIP5* is most similar to *AtZIP3* (Fig 3), probably explaining the cross-hybridization to another ZIP-like gene as we observed upon DNA blot analysis (Fig 4). *AtZIP3* is also induced by Zn deficiency in roots and has been shown to mediate Zn uptake across the plasma membrane (Grotz et al., 1998). Like *TcZNT5* the *T. caerulescens* orthologue of *AtZIP3* (*TcZNT3*) is only marginally induced by Zn deficiency conditions in the roots (van de Mortel et al., 2006). Although the expression of *TcZNT5* is much less up-regulated under Zn deficiency than *AtZIP5* (Fig 5 and Fig 6), the similarities in protein sequence and predominant expression in roots provide further evidence to a presumed function of *TcZNT5* in Zn uptake in roots. Unfortunately the Arabidopsis knock-out *zip5* mutant did not show a clear aberrant phenotype when exposed to different Zn or Fe supplies, but only a mild mutant phenotype of increased root growth under Cd supply. This suggests that the protein has some affinity for Cd uptake but is largely redundant as far as Zn and Fe uptake is concerned, as was previously also found. Inducing expression of iron uptake transporters such as *AtIRT1*, which has a low affinity for Cd transport (Khorshunova et al., 1999), by growing plants on low Fe supply will complement the reduced Cd uptake ability, as was observed when growing the *zip5* plants on Cd medium under low Fe supply (Fig 7). Mineral content analysis of *zip5* mutant plants supplied with Cd should be performed to further confirm this.

When comparing the two accessions of *T. caerulescens*, expression of *TcZNT5* in roots is only slightly higher in LC than in GA, also under Cd exposure conditions. Very recently Plaza et al. (2007) reported the lower expression level of *TcZNT5* in GA than in PR. As GA is a much better Cd hyperaccumulator than LC (Assunçao et al., 2003b), *TcZNT5* is unlikely to play a dominant role in Cd uptake in *T. caerulescens* roots. The fact that overexpression of *TcZNT5* did not lead to significantly different root growth upon metal exposure suggests the possible function of *TcZNT5* in

mediating Cd efflux from cytoplasm to the apoplast, which has been proposed by Plaza et al. (2007). Also we cannot rule out that the gene is overexpressed at the transcript level, but not at the protein level, the exact function of *TcZNT5* still needs to be elucidated.

Comparison of the *TcZNT6* expression in GA and LC showed a lower expression in LC compared to GA roots at equal Zn supply and a clear down-regulation in the presence of high Cd. Knowing that GA is a slightly better Zn hyperaccumulator and a much better Cd hyperaccumulator than LC (Assunção et al. 2003b), this support the previously proposed role for this protein in shoot Zn storage and suggests some ability to transport Cd. Additional support for this hypothesis can be found in previous studies revealing that Cd uptake was only significantly suppressed at equimolar concentrations of Zn in accession PR, which like LC is low in accumulating Cd (Lombi et al., 2001b), but not in accession GA. This indicated the presence of a highly selective Cd uptake system in GA roots, presumably caused by high expression of the *TcIRT1* gene in GA and not in PR (Lombi et al., 2001a, 2002; Zhao et al., 2002). Therefore, under the conditions tested (100 µM Zn, 1 and 10 µM Cd), GA will be readily taking up Cd at both Cd exposures, but LC will try to avoid taking up Cd in the roots and the transport to the shoots. Our results suggest that *TcZNT6* may be involved in this process. Based on the high expression of *TcZNT6/AhZIP6* in shoots, the function of the encoded proteins is perhaps not in the initial uptake of Cd from the soil, but rather in its further transport to the xylem for translocation to the shoot and in the uptake into shoot mesophyl and epidermal cells. Further support for this is found in the overexpression phenotypes of 35S::*TcZNT6* Arabidopsis plants, which show increased Cd sensitivity (Fig 9A, C). Analysis of the mineral content of these plants would be needed to verify this. Unfortunately no phenotypic alterations were found in root growth of Arabidopsis *zip6* mutant plants when compared to WT plants growing on vertical agar plates with additional Cd and Zn supply, illustrating once more the redundancy among *ZIP* transporters in Arabidopsis.

In conclusion, *TcZNT5* and *TcZNT6,* two ZIP genes cloned from *T. caerulescens,* likely encode Zn/Cd transporters, similar to the previously cloned *TcZNT1* and *TcZNT2* genes described by Pence et al., (2000) and Assunção et al. (2001). Whereas *TcZNT5* may be involved in the transport of Zn and Cd from cytoplasm to apoplast, *TcZNT6* is more likely to be involved in root to shoot transport and distribution in the shoot. Additional studies are needed to further elucidate the function of these two genes and their exact role in plant mineral homeostasis and metal hyperaccumulation.

Acknowledgement

We thank Andrea Pirondini and Andrea Sacchani for providing us with the seeds of the Monte Prinzera accession, Bettine Aigner for the seeds of Austria accession and seeds of *T. minimum*, Paula Pongrac for the seeds of *T. praecox* and Dr. Takafumi Mizuno for seeds of *T. japonicum*. We thank Judith van de Mortel for her assistance in preparing the *T. caerulescens* cDNA.

Figure legends

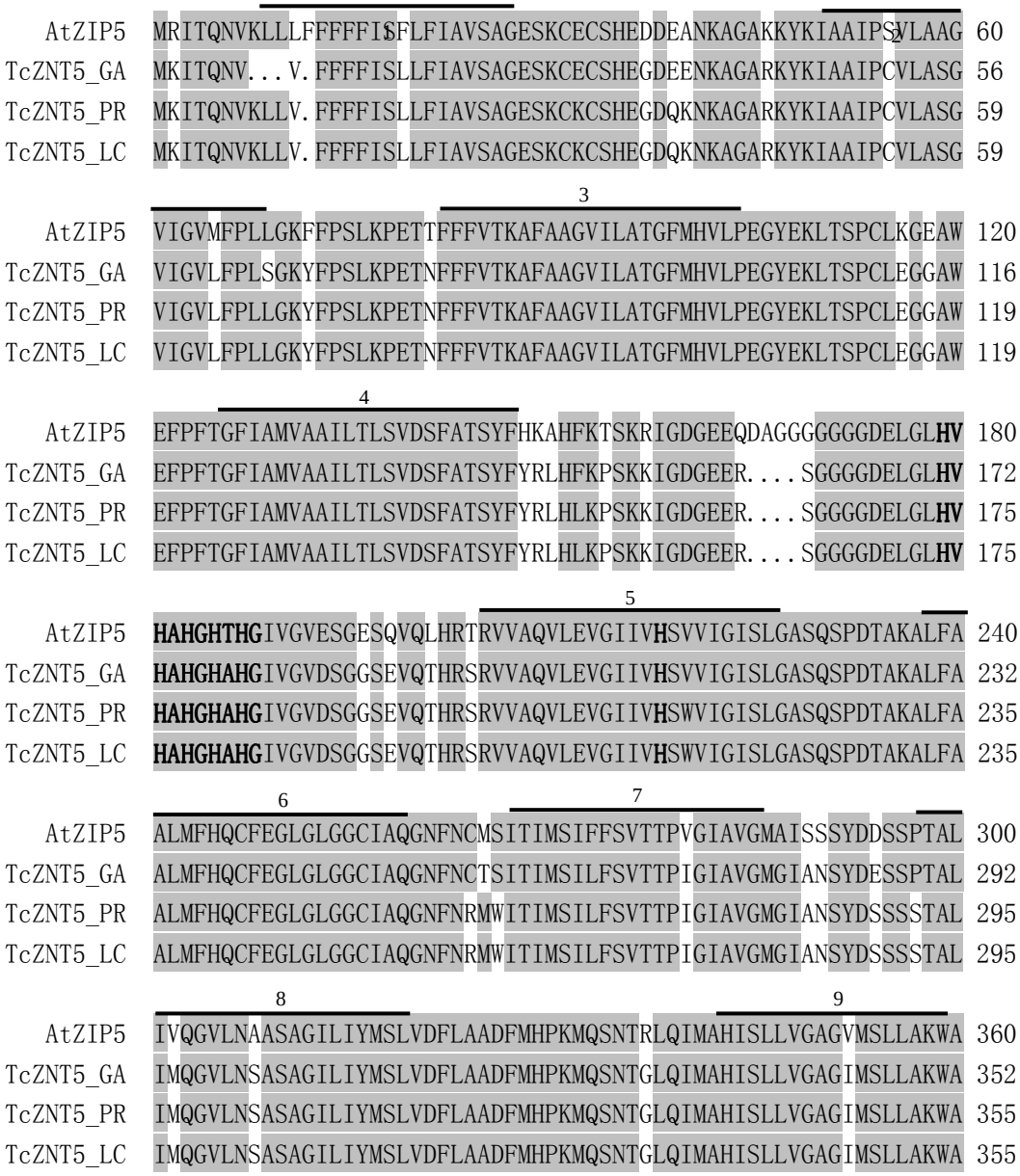


Fig 1. Sequence alignment of predicted protein sequences of *TcZNT5-LC* from *T. caerulenscens* and those of two additional *T. caerulescens* alleles (GeneBank accessions AJ937739 (GA) and AF292029 (PR) and of *AtZIP5* from *A. thaliana* (At1g05300, GeneBank accession AC000098). Sequences are aligned using the ClustalW method. Identical residues of TcZNT5 and AtZIP5 are shaded. The putative transmembrane domains (TM) are numbered and overlined. A conserved stretch of repeated histidine residues between TM3 and TM4 and a conserved histidine residue in TM4 are highlighted in bold.

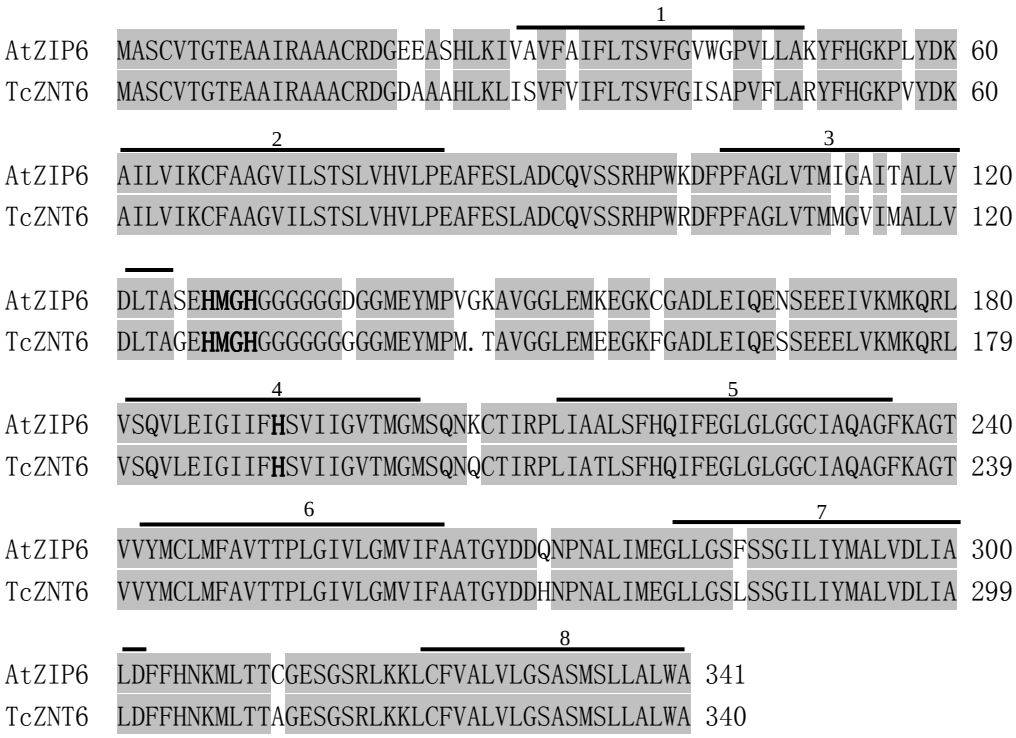
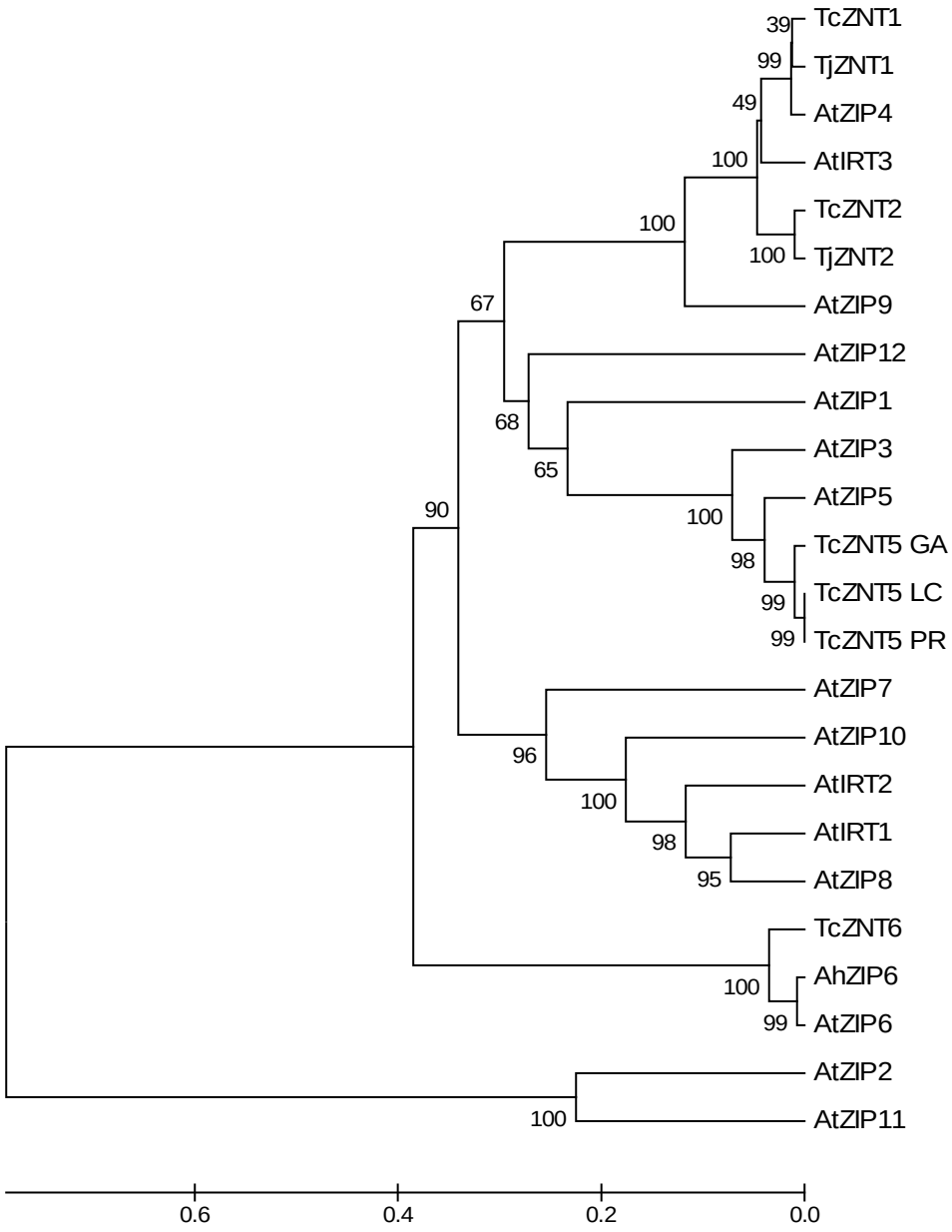


Fig 2. Sequence alignment of predicted protein sequences of *TcZNT6-LC* from *T. caerulenscens* and of *AtZIP6* from Arabidopsis (At2g30080, GeneBank accession AC004680). Sequences are aligned using ClustalW. Identical residues are shaded. The putative transmembrane domains (TM) are numbered and overlined. A conserved stretch of repeated histidine residues between TM3 and TM4, and a conserved histidine residue in TM4 are highlighted in bold.

Fig 3



The bottom bar indicates genetic distance as number of substitutions per unit time.

Fig 3. Phylogenetic comparison of TcZNT5 and TcZNT6 with the predicted protein sequences of 22 ZIP family members from *Arabidopsis thaliana*, *A. halleri*, *Thlaspi caerulescens* and *T. japonicum*. The phylogenetic analysis was conducted using MEGA version 3.1 (Kumar et al., 2004). GenBank accession numbers are: *AtIRT1* (NM_118089), *AtIRT2* (NM_001036593), *AtIRT3* (NM_104776), *AtZIP1* (NM_112111), *AtZIP2* (NM_125344), *AtZIP3* (NM_128786), *AtZIP4* (NM_100972), *AtZIP5* (NM_100409) *AtZIP6* (NM_128563), *AtZIP7* (NM_126440), *AtZIP8* (NM_148089), *AtZIP9* (NM_119456), *AtZIP10* (NM_102864), *AtZIP11* (NM_104468), *AtZIP12* (NM_125609), *AhZIP6*

(AJ580315), *TcZNT1* (AF275751), *TcZNT2* (AF275752), *TcZNT5-PR* (AF292029), *TcZNT5-GA* (AJ937739), *TjZNT1* (AB206397), *TjZNT2* (AB175740).

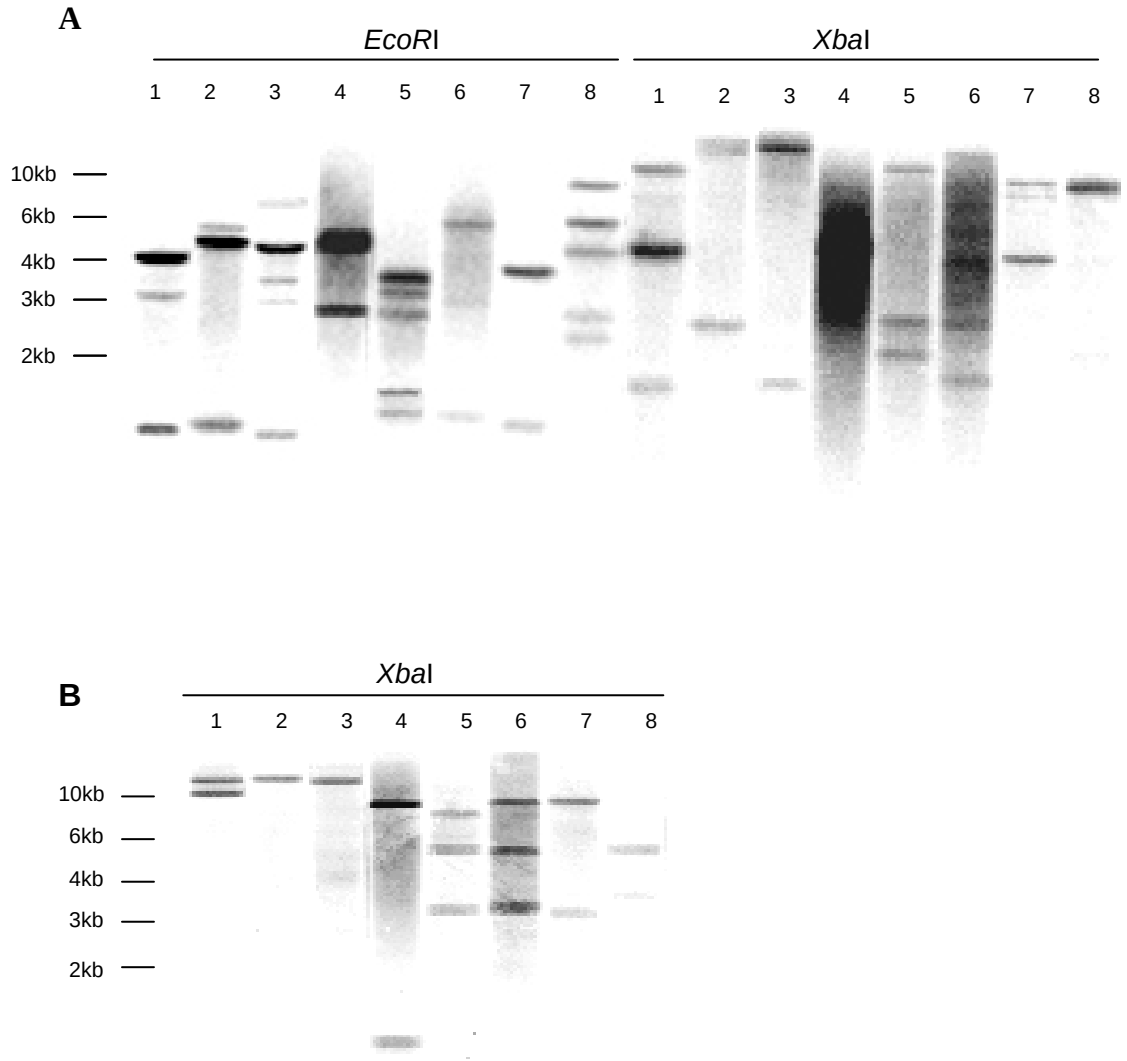


Fig 4 DNA blot analysis of *T. caerulescens* accessions and *Thlaspi* species. Genomic DNA was digested with *Eco*RI or *Xba*I as indicated and the blots were hybridized with a *TcZNT5* (A) or *TcZNT6* (B) specific cDNA probe. The numbers above the lanes designated the different *T. caerulescens* accessions: La Calamine (1), Monte Prinzera (2), Ganges (3)， Austria (7); or *Thlaspi* species: *T. japonica* (4), *T. praecox* (5), *T. minimum* (6) and *T. perfoliatum* (8). Size markers at shown on the left.

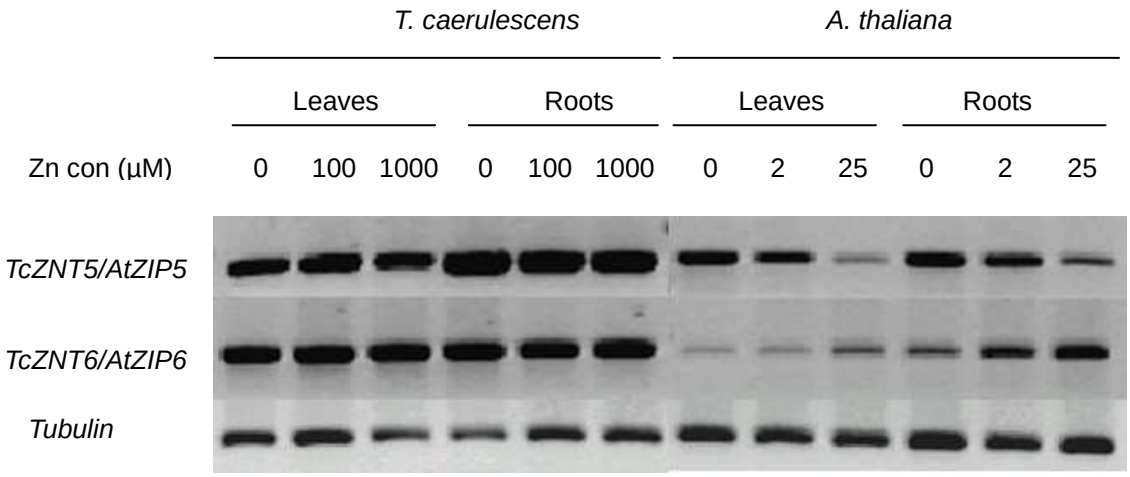


Fig 5. Semi-quantitative RT-PCR analysis of *T. caerulescens TcZNT5* and *TcZNT6* and their *A. thaliana* orthologues *AtZIP5* and *AtZIP6*. Leaves and roots of each species were used, from plants grown at 0, 100 and 1000 µM Zn for *T. caerulescens* or physiologically comparable 0, 2 and 25 µM Zn for *A. thaliana*. RT-PCR was performed using specific primer pairs for each gene. RT-PCR (30 cycles) was used to amplify a 424-bp fragment for *TcZNT5*, a 605-bp fragment for *TcZNT6*, a 593-bp fragment for *AtZIP5* and a 679-bp fragment for *AtZIP6*. *Tubulin* amplification (25 cycles) was used as control for equal use of cDNA.

Fig 6

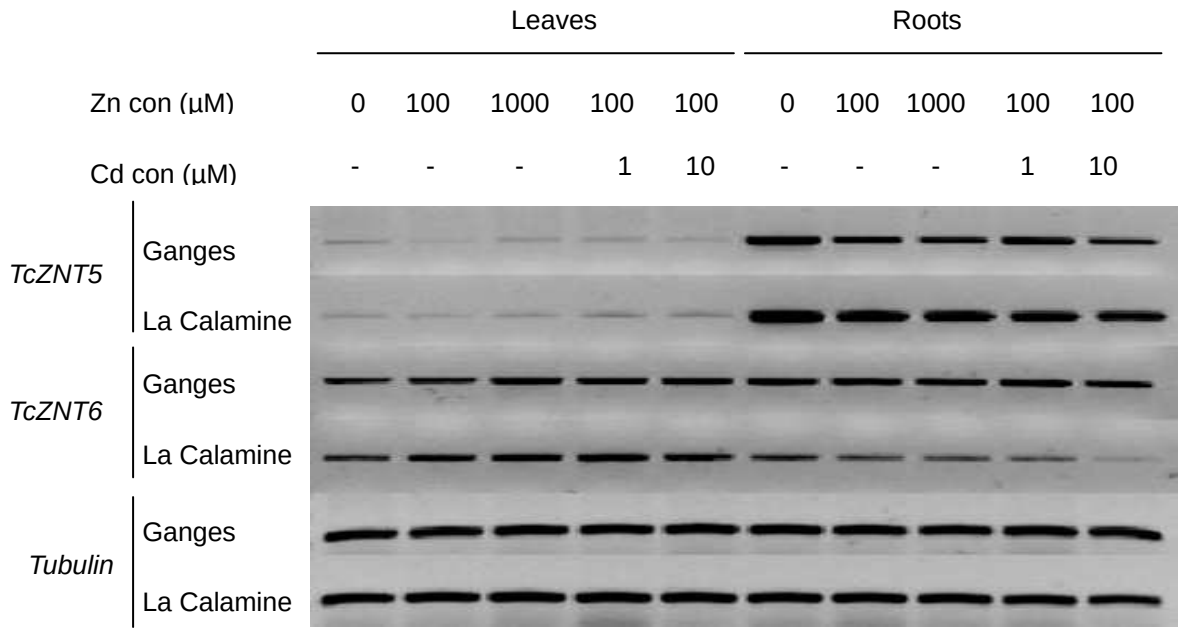
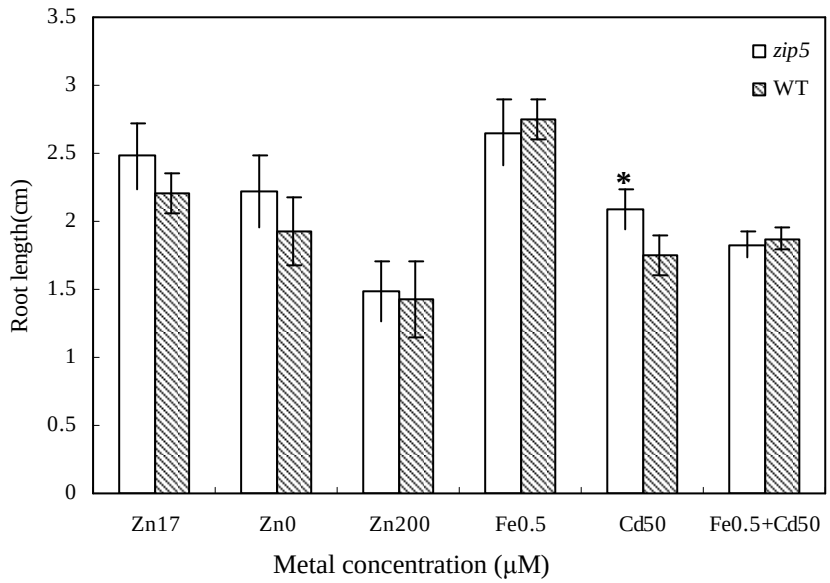


Fig 6 Zn and Cd exposure effects on *TcZNT5* and *TcZNT6* transcript levels in *T. caerulescens* accessions Ganges and La Calamine. Leaves and roots of each accession were used from plants grown at 0, 100 and 1000 µM Zn, or 100 µM Zn supplemented with 1 or 10 µM Cd. RT-PCR was performed (25 cycles) using gene specific primers to amplify a 424-bp fragment from *TcZNT5* and 605-bp fragment from *TcZNT6*. *Tubulin* amplification (25 cycles) was used as a control of equal cDNA use.

A



B

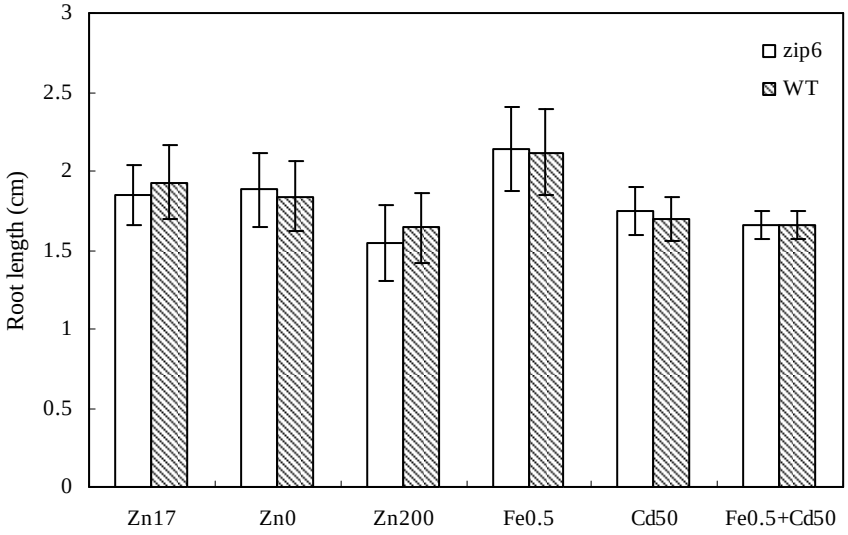


Fig 7 Average root length (± SE) of 15-day-old Arabidopsis seedlings of wild-type (WT) and *zip5* or *zip6* mutant plants germinated and grown on 0.5 x MS medium containing different concentrations of Zn, Fe and Cd. (A): *zip5*; (B) *zip6*, * significantly different at $P<0.05$. Significance is determined by one-way ANOVA. Five replicates with each 12 to 20 seedlings were measured for each treatment.

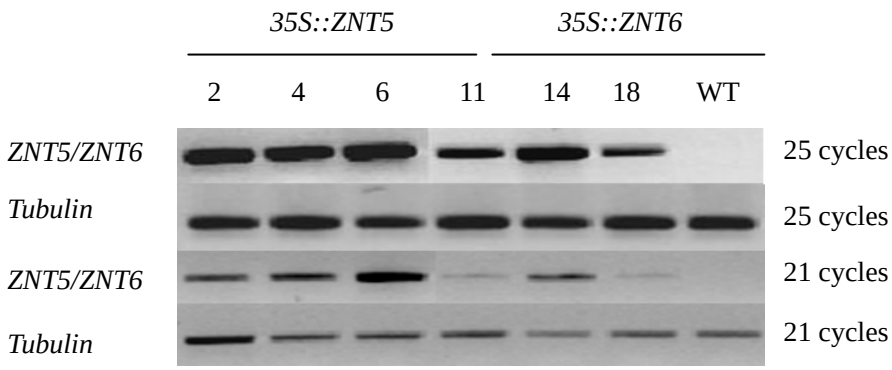
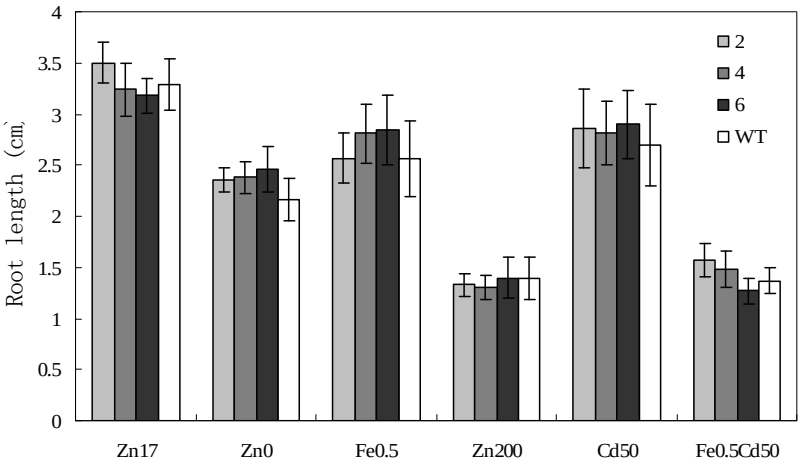
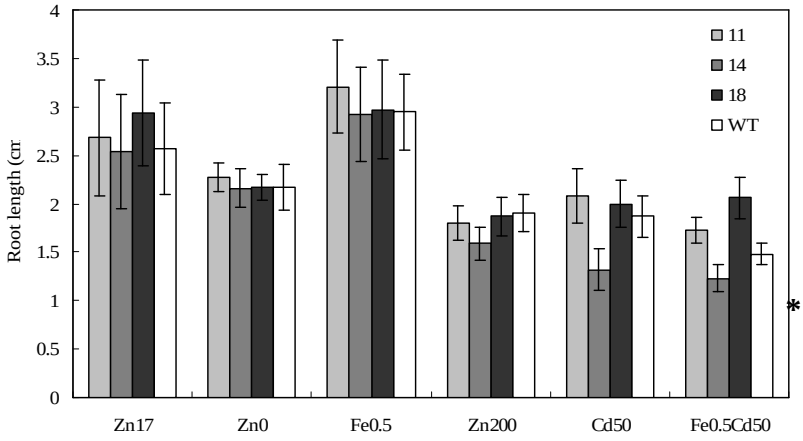


Fig 8 Semi-quantitative RT-PCR analysis of *TcZNT5* or *TcZNT6* expression of wild-type (WT) and homozygous transgenic *A. thaliana* lines transformed with a CaMV 35S::*TcZNT5* (*ZNT5*) or CaMV 35S::*TcZNT6* (*ZNT6*) construct. *Tubulin* amplification was used as a control of equal cDNA use. RT-PCR was performed at 21 and 25 cycles for all the three genes.

A


Root length (cm)
4
3.5
3
2.5
2
1.5
1
0.5
0
2
4
6
WT
Zn17
Zn0
Fe0.5
Zn200
Cd50
Fe0.5Cd50

B


Root length (cm
4
3.5
3
2.5
2
1.5
1
0.5
0
11
14
18
WT
Zn17
Zn0
Fe0.5
Zn200
Cd50
Fe0.5Cd50

*

*

C

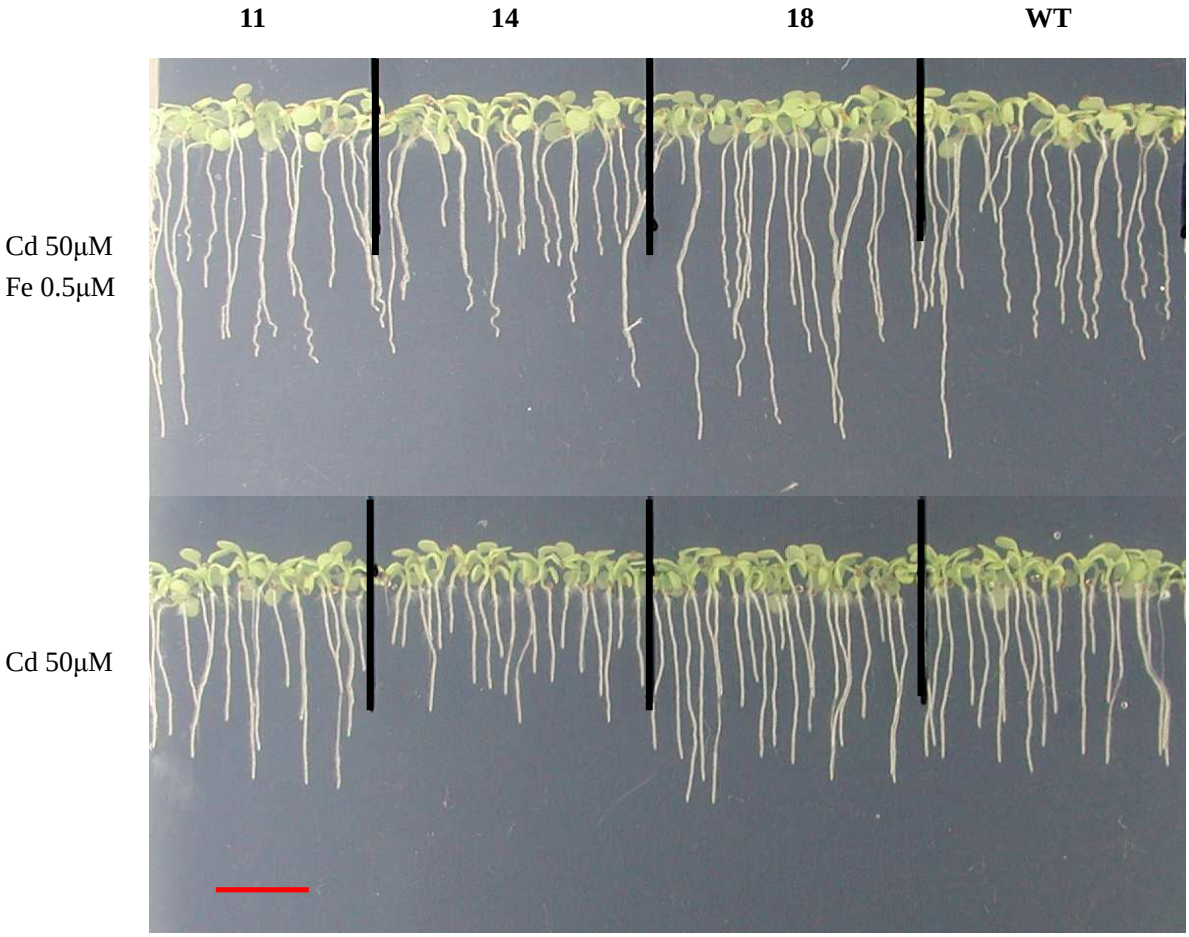


Fig 9 Average root length (± SE) of 15-day-old seedlings grown on 0.5 x MS medium containing different concentrations of Zn, Fe and Cd (indicated in μM). (A): Wild-type (WT) plants and three *35S::TcZNT5* overexpression lines (2, 4, 6); (B) Wild-type (WT) plants and three *35S::TcZNT6* overexpression lines (11, 14, 18), * indicates significantly different at $P<0.001$ as determined by one-way ANOVA; (C): phenotype of transgenic plants overexpressing *TcZNT6* growing on Fe deficient medium (0.5 μM) supplemented with Cd (50 μM) or 0.5 x MS medium containing Cd (50 μM). The bar represents 1 cm. Five replicates, each with 12 to 20 seedlings, were measured for each treatment.

Chapter 5

Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator Thlaspi caerulescens: expression and role in heavy metal tolerance

Ronald J.F.J. Oomen*, Jian Wu*, Françoise Lelievre, Hélène Barbier-Brygoo,

Mark G.M. Aarts, Sébastien Thomine.

Abstract

The ability to tolerate and accumulate heavy metals by metal hyperaccumulating plant species is likely the result of an adapted metal homeostasis. NRAMP metal transporters play an important role in Fe mobilization in *Arabidopsis thaliana* and were found to be highly expressed in metal hyperaccumulating plants such as *Thlaspi caerulescens* and *Arabidopsis halleri*. Here, we identify and characterize the *T. caerulescens* orthologues of AtNRAMP3 and AtNRAMP4. Both *TcNRAMP3* and *4* are expressed at a higher level than their *A. thaliana* orthologues. Additionally, expression analysis in two *T. caerulescens* accessions with contrasting Cd accumulation shows that difference in Cd accumulation ability is associated with differences in the regulation of NRAMP expression. In contrast, our study did not reveal functional differences between the *T. caerulescens* and *A. thaliana* NRAMPs: they transport the same metals when heterologously expressed in yeast and are all four localized at the vacuolar membrane. Here, we show that inactivation of both AtNRAMP3 and AtNRAMP4 results in strong Cd and Zn hypersensitivity in *A. thaliana*. *nramp3nramp4* mutant hypersensitivity to Cd and Zn is readily rescued by TcNRAMP3 expression. Nevertheless, neither

nramp3nramp4 plants overexpressing TcNRAMP3, nor wild type plants overexpressing TcNRAMP4 show enhanced Zn or Cd tolerance compared to wild type *A. thaliana*. Our data thus indicate that enhanced expression of TcNRAMP3 and 4, with functional properties similar to AtNRAMP3 and 4, may be important to maintain metal homeostasis in *T. caerulescens* exposed to high levels of Zn or Cd.

Key Words NRAMP, *T. caerulescens*, hyperaccumulator, Zn, Cd, metal transport

* Both authors contributed equally to this work

Introduction

Among the plant species adapted to environments containing high levels of transition metals, some species called hyperaccumulators have developed the exceptional ability to accumulate metals to levels 10 to 100 times higher than other species. Many Ni hyperaccumulators have been identified (> 300 species; Baker et al., 2000) while only about 15 species display Zn hyperaccumulation (Baker et al., 1992; Brooks, 1994). These Zn hyperaccumulators were mostly identified on Zn, Cd and/or Pb contaminated industrial or mining sites, even though for some species like *Thlaspi caerulescens* and *Arabidopsis halleri*, non-metallicolous accessions have also been reported (Meerts and Van Isacker, 1997; Bert et al., 2000; Schat et al., 2000). Cd hyperaccumulation is even more rare and only identified in some *T. caerulescens* and *A. halleri* accessions and in *Thlaspi praecox* (Brown et al., 1995; Kupper et al., 2000; Lombi et al., 2000; Vogel-Mikus et al., 2006). Hyperaccumulation likely results from an adapted metal homeostasis rendering these plants tolerant to heavy metals and allowing their enhanced accumulation. Such an adapted metal homeostasis requires functional and/or transcriptional modification of metal transporters, metal chelators and other proteins to maintain a balance between plant metal uptake, distribution and storage. Initial studies performed in *Arabidopsis thaliana* led to the identification of several genes encoding mostly metal transporters and chelators (reviewed by Clemens, 2001; Cobbet and Goldsbrough, 2002; Mäser et al., 2001). Elevated transcript levels for some of these genes in *A. halleri* and *T. caerulescens* (Bert et al., 2000; Assunção et al., 2003b), provided first hints about their involvement in metal tolerance and accumulation. Further global transcriptomic studies, comparing hyperaccumulating species with *A. thaliana* by using *A. thaliana* microarrays resulted in the identification of several candidate genes putatively involved in metal tolerance and hyperaccumulation (Becher et al., 2004; Van de Mortel et al., 2006; Talke et al., 2006; Weber et al., 2004). Several genes identified in these studies had previously been reported as transporters or chelators of Zn and Cd and are thus very likely to play an important role in the tolerance to and accumulation of Zn and/or Cd in these plants. The identification of elevated expression levels of genes that are associated with homeostasis of other metals than Zn and Cd suggests that these may play a role in adapting the general metal homeostasis in the plant to the altered levels of Zn and Cd in plant tissues (Talke et al., 2006).

Among other genes, transcriptomic studies identified NRAMP as one of the gene families that is highly expressed in hyperaccumulating plants and differently regulated by variations in the Zn

concentrations around plants. Weber et al. (2004) showed that *NRAMP3* displays constitutively high expression in *A. halleri* compared to *A. thaliana* and van de Mortel et al. (2006) showed that, in *T. caerulescens*, *NRAMP3* expression is generally higher than in *A. thaliana* but is also induced by Zn deficiency and excess, which is not found in *A. thaliana*. Mouse Nramp1 (natural resistance associated macrophage protein), the first NRAMP family member, was discovered as a gene responsible for resistance to infection by myco-bacteria. Since then, homologues have been identified in many organisms from bacteria to man. They have been shown to play important roles in metal ion homeostasis, especially iron uptake and recycling in mammals and Mn uptake in yeast and bacteria. Most NRAMPs, however, are able to transport multiple metal ions such as Mn, Zn, Cu, Fe, Cd, Ni and Co (reviewed in Nevo and Nelson, 2006). NRAMPs have been identified in many plant species including Arabidopsis, rice, tomato, soybean, and *Thlaspi japonicum* (Curie et al., 2000; Lanquar et al., 2005; Belouchi et al., 1997; Bereczky et al., 2003; Kaiser et al., 2003 and Mizuno et al., 2005). The first studies on NRAMP functions in plants already indicated a putative role in Fe homeostasis based on their Fe-transport activity in yeast, their regulation by environmental Fe levels and the observation of an enhanced tolerance to Fe of NRAMP1 overexpressing plants (Curie et al., 2000; Thomine et al., 2000 and 2003). Furthermore, the analysis of the *A. thaliana nramp3nramp4* double mutant demonstrated the importance of these proteins in the mobilization of vacuolar Fe during germination (Lanquar et al., 2005). The available data thus clearly point to roles of NRAMP1, NRAMP3 and NRAMP4 in Fe homeostasis *in planta*. On the other hand, expression of AtNRAMP1, AtNRAMP3 and AtNRAMP4 in yeast showed that these proteins are able to transport Cd and that AtNRAMP4 is able to transport Zn. In addition, over-expression of AtNRAMP3 or AtNRAMP4 in *A. thaliana* leads to a slight increase in Cd sensitivity without any modification of Cd content (Thomine et al., 2000, Lanquar et al., 2004).

In this work, we further investigated a possible role of NRAMPs in metal tolerance and hyperaccumulation focusing on the hyperaccumulator *T. caerulescens*. *TcNRAMP3* and *TcNRAMP4* cDNAs (Rigola et al., 2006), highly similar to *AtNRAMP3* and *AtNRAMP4* respectively, were isolated from *T. caerulescens* La Calamine. We show that these two *NRAMP* genes are differently expressed between *A. thaliana* and *T. caerulescens*, but also between *T. caerulescens* accessions when treated with specific Zn and Cd concentrations. We compared the metal transport abilities of TcNRAMPs with their *A. thaliana* homologues by heterologous expression in yeast. We show that

TcNRAMP3 and 4, like their AtNRAMP homologues, are localized on the vacuolar membrane. To provide indication about a possible function of NRAMP3 and NRAMP4 in metal accumulation or tolerance, we analyzed *A. thaliana nramp3nramp4* mutant sensitivity to Cd and Zn. Further, *TcNRAMPs* were expressed in *A. thaliana* using wild-type plants as well as the *nramp3nramp4* double mutant background.

Materials and methods

Library screening

cDNA libraries made from roots of *Thlaspi caerulescens* J. & C. Presl (accession La-Calamine) as described in Rigola et al. (2006) were used for full-length cDNA cloning. The partial cDNA clone RR23nr019 had been identified as an *AtNRAMP3* homologue (Rigola et al. 2006). A 1-kb cDNA fragment cut from the pAD-GAL4-2.1 vector plasmid using *EcoR*I and *Xho*I was used as probe in the cDNA library screening. The probe was labeled with [α-^{32}P]dATP using the Hexalabel^{TM} DNA labeling kit (Fermentas). Eight positive clones were obtained. The three longest cDNA clones were sequenced by ABI PRISM BigDye terminator cycle sequencing technology v2.0, according to the manufacturer's instruction (Applied Biosystems; http://www.appliedbiosystems.com.), using an ABI3700 DNA analyzer. Sequence analysis was performed using standard BLAST method (http://www.ncbi.nlm.nih.gov/BLAST/) and revealed that one of the three positive clones, containing a 1539-bp open reading frame, was homologous to *AtNRAMP3* and one, with a 1494-bp open reading frame, was homologous to *AtNRAMP4* and that both open reading frames were full-length.

Southern blot analysis

Genomic DNA was extracted from four *T. caerulescens* accessions: La Calamine, Monte Prinzera, Ganges and Austria, and four *Thlaspi* species: *T. japonicum*, *T. praecox*, *T. minimum* and *T. perfoliatum* using the CTAB method. Digestion of gDNA, TAE gel electrophoresis, Southern blot, prehybridization, hybridization and washing was performed as described in Assunçaõ et al. (2001). cDNA fragments from digested full-length cDNA clones using *Xho*I for *TcNRAMP3* (462bp) and *Sal*I for *TcNRAMP4* (248bp) were used as probes and labeled as described above.

Plant material for RT-PCR analysis

Arabidopsis thaliana Columbia-0 and *Thlaspi caerulescen*s accession La Calamine seeds were germinated on garden peat soil (Jongkind BV). Three-week-old seedlings were transferred to hydroponics, three plants per pot filled with 1L modified half-strength Hoagland nutrient solution (van de Mortel et al., 2006). After three weeks *T. caerulescens* plants were transferred to the same nutrient solution with a deficient (0 µM), sufficient (100 µM), or excess (1000 µM) $ZnSO_4$ concentration for 7 days. *A. thaliana* plants were transferred to the same nutrient solution with deficient (0 µM), sufficient (2 µM) or excessive (25 µM) $ZnSO_4$. Nutrient solution was replaced once a week during the first three weeks and thereafter twice a week. For comparison of transcription levels of *TcNRAMP3* and *TcNRAMP4* between *T. caerulescens* accessions La Calamine and Ganges, plants were grown as described above, including two additional cadmium treatments ($ZnSO_4$ 100 µM + $CdSO_4$ 1 µM and $ZnSO_4$ 100 µM + $CdSO_4$ 10 µM).

Semi-quantitative RT-PCR

Leaves or roots of one pot containing three Arabidopsis or three *T. caerulescens* plants per treatment were pooled and homogenized in liquid nitrogen. Total RNA of leaves or roots was extracted with Trizol (Invitrogen) following the manufacturer's instructions. 5 µg of total RNA was used to synthesize cDNA with MLV reverse transcriptase (Invitrogen) and oligo(dT) as a primer (Invitrogen). The PCR amplification was performed with a cDNA aliquot (2µl) using the following primers; AtNRAMP3F 5'-CCTCTTTGGGCTGGTGTTG-3' and AtNRAMP3R 5'-ACACACCGCCTCCATATTTC-3'; TcNRAMP3F 5'-TCTCTGGGCTGGTGTCAT-3' and TcNRAMP3R 5'-AGCCGCCTCCATACTTG-3'; AtNRAMP4F 5'-TTTGGGCTGGTGTTGTTATCACT-3 and AtNRAMP4R 5'-TCCGCTATCTCTGTCCCGTAAAAC-3'; TcNRAMP4F 5'-TCTGGGCTGGAGTTGTAATCACC-3' and TcNRAMP4R 5'-TCCGCTATGTCCGTCCCGTAAAAG-3'. Primer pairs for *Tubulin* or *Actin* were used as a control for similar cDNA quantity between the samples. For *A. thaliana*: TubulinF 5'-AAGC TTGCTGATAACTGTACTGGT-3' and TubulinR 5'-GGTTTGGAACTCAGTGACATCA-3', ActinF 5'-GGTAACATTGTGCTCAGTGGTGG-3' and ActinR 5'-AACGACCTTAATCTTCATGCTGC-3'; for *T. caerulenscens* TubulinF 5'-CTACGCACCAGTCATCTCT-3', TubulinR 5'-CGAGATCACCTCCTGGAACA-3'. 25 cycles were performed for *Tubulin* amplification from *A. thaliana* and *T. caerulescens* samples, and 20 cycles for *Actin* from *A. thaliana*. Twenty microliter from the fifty microliter reaction was separated on an ethidium bromide stained 1 % agarose gel.

Construction of expression vectors

TcNRAMP3 and *TcNRAMP4* were amplified from cDNA clones by PCR using Gateway primers. Forward primer was 5'-GGGGACAAGTTTGTACAAAAAAGCAGGCT GATCGAATTA*GGATCC*TCTGC-3', containing the attB1 sequence (underlined) and a BamHI site (italic), and the reverse primer was 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAATGGG*CTCGAG*AGTCGAC3', containing the attB2 sequence (underlined) and an *XhoI* site (italic). PCRs were performed with the Pfu polymerase (Fermentas) at 93 °C for 5 min, followed by 30 cycles 93 °C for 1 min, 55 °C for 1 min and 68 °C for 3 min, and finished by an extension at 68 °C for 10 min. PCR products were recombined into pDONOR207 (Invitrogen) in a 10-µl BP Clonase (Invitrogen) reaction following the manufacturer's instruction. A similar procedure was used for *AtNRAMP3* and *AtNRAMP4* from the plasmids pGEM-T-easy AtNRAMP3 and pGEM-T easy AtNRAMP4 (Thomine et al., 2000) using the primers *AtNRAMP3* Forward 5'-GGAGATAGAACCATGCCACAACTCGAGAACAACG-3' and Reverse 5'-CAAGAAAGCTGGGTCTCAATGACTAGACTCCGCTTTG-3' and *AtNRAMP4* Forward 5'-GGAGATAGAACCATGTCGGAGACTGATAGAGAGC and Reverse 5'-CAAGAAAGCTGGGTCTCACTCATCATCCCTCTGTGG-3' followed by nested PCR with universal Gateway extension primers attB1 5'-GGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATG-3' and attB2 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTC-3'. For plant transformation, the *TcNRAMP* fragments were transferred from their donor constructs to the binary over-expression vector pGD625™ (De Folter et al., 2006), containing the double 35S CaMV enhancer, in a 10-µl LR Clonase (Invitrogen) reaction following the manufacturer's instruction. The binary constructs were introduced into the *A. tumefaciens* strain AGL0 by electroporation. For the generation of yeast expression vectors a similar LR reaction was performed using the *AtNRAMP* and *TcNRAMP* fragments to generate pDR195gtw constructs (pDR195gtw constructed by Andeol Falcon de Longevialle).

Yeast experiments

The yeast strains used in this study were DEY1453 (*fet3fet4*, Eide et al., 1996), ZHY3 (*zrt1zrt2*, Zhao and Eide, 1996) and *smf1* (Supek et al., 1996; Thomine et al., 2000). Yeast cells were grown (at

30ºC) on yeast extract/peptone/dextrose (supplemented with 0.2 mM $FeCl_3$, 0.1 mM $ZnSO_4$ or 0.1 mM $MnCl_2$ for the three strains respectively) before transformation and on synthetic dextrose-URA (with the same metal additions) after transformation. Yeast cells were transformed according to standard procedures (Invitrogen). *fet3fet4* and *smf1* complementation was tested by drop spotting assays, spotting diluted cultures of individual transformants on synthetic dextrose-URA agar plates with 80 µM BPS and 30 µM $FeCl_3$ or 200 µM $FeCl_3$ for *fet3fet4* and plates with 10 mM EGTA with or without 0.1 mM $MnCl_2$ for *smf1*. *zrt1zrt2* complementation was tested by measuring the OD_{600} in 5-ml cultures of so-called Low Zinc Medium (Zhao and Eide, 1996b) with 10 µM $ZnSO_4$ inoculated with 50 µl synthetic dextrose-URA pre-culture at OD_{600}=1. Cd sensitivity was tested by measuring the OD_{600} of *fet3fet4* transformants grown in 5-ml cultures of synthetic dextrose-URA (pH 5.5) with or without 10 µM $CdCl_2$ after inoculation with 150 µl synthetic dextrose-URA pre-culture at OD_{600}=1.

GFP fusion and intracellular localization

To construct a cDNA encoding a translational fusion between TcNRAMP3 or TcNRAMP4 and the green fluorescent protein, RSsm-GFP (Davis and Vierstra, 1998), the *TcNRAMP* fragments were transferred from their donor constructs, by a 10-µl LR Clonase (Invitrogen) reaction following the manufacturer's instruction, into the plant transient expression vector CD3-327 (http://aims.cps.msu.edu/aims/menu/search2.html) adapted to Gateway cloning by L. Gissot. This generated TcNRAMP3 or TcNRAMP4 fusion proteins with the green fluorescent protein as an additional carboxy terminal domain, under the control of the 35S CaMV promoter. The AtNRAMP4::GFP fusion construct or the empty vector CD3-327 were transiently expressed in Arabidopsis protoplasts through polyethylene glycol mediated transformation. The walls of Arabidopsis suspension cells were digested in Gamborg's B5 medium supplemented with 0.17 M glucose, 0.17 M mannitol, 1% cellulase and 0.2% macerozyme. Protoplasts were purified by floatation in Gamborg's B5 medium supplemented with 0.28M sucrose. For transformation, 0.2 million cells were mixed with 5 mg of plasmid DNA in a solution containing PEG 6000 25%, mannitol 0.45 M, $Ca(NO_3)_2$ pH 9 and incubated in the dark for 20 min. Then, the PEG was washed twice with 0.275 M $Ca(NO_3)_2$ and the protoplasts were resuspended in Gamborg's B5 medium supplemented with 0.17 M glucose, 0.17 M mannitol in which they were maintained until

microscopic observation. After 24 to 48 hrs, fluorescent cells were imaged by confocal microscopy (Leica TCS SP2) with excitation at 488 nm and the fluorescence emission signal recovery between 495 and 535 nm for GFP fusion proteins and excitation at 543 nm and the fluorescence emission signal recovery between 578 and 707 nm for DsRed2 (Thomine et al., 2003).

Plant transformation

The constructs with full-length cDNAs of *TcNRAMP3* and *TcNRAMP4* were used to transform *A. thaliana* Columbia-0 or *A. thaliana* accession WS *nramp3nramp4* by the flower dip method (Clough, 2005). For Columbia-0 transformants the primary T1 transformants were selected on MS/2 medium containing kanamycin (50mg/ml). Kanamycin-resistant plants were transferred to soil, and the T2 seeds resulting from self-fertilization were collected. The T2 seeds were plated on the same selection medium and scored for kanamycin resistance. Transgenic lines that displayed 3:1 segregation ratio for resistance to sensitivity in the T2 generation were selected for further analysis. In T3 generation, because of silencing of resistance to kanamycin in all the selected lines, we tested T3 progeny from ten T2 plants, 12 T3 progenies for each T2 plants, by PCR using gene specific primers of *TcNRAMP3* and *TcNRAMP4* used in semi-quantitative RT-PCR. Genomic DNA was isolated using CTAB method. The lines with all the tested 12 T3 progenies amplified with correct product were deemed as homozygous transgenic lines. T4 seeds from these plants were used for further experiments. *A. thaliana* WS-*nramp3nramp4* transformants were selected in a similar procedure but using complementation of the *nramp3nramp4* phenotype on low Fe medium as a criterion. Seeds were sown on plates containing ABIS medium with adjusted Fe concentration and 50 µg/ml Kanamycin (2.5 mM H_3PO_4, 5 mM KNO_3, 2mM $MgSO_4$, 1mM $Ca(NO_3)_2$, Murashige and Skoog microelements, 1% sucrose, 0.7% agar, 1mM MES adjusted with KOH to pH 6.1 and 0.3 µM FeHBED).

The expression levels of transgenic lines were checked by RT-PCR using the total RNA isolated from; (Columbia-0 lines) T4 plants grown in soil for 30 days, or (WS-*nramp3nramp4* lines) T4 plants grown *in vitro* on ABIS medium with 50 µM FeHBED, using the RT-PCR procedure as described above.

Growth assays of transformed plants

Arabidopsis (T3 or T4) seedlings were grown as previously described by Lanquar *et al*. (2005) using

the same media with varying levels of FeHBED, $ZnSO_4$ and $CdCl_2$ as indicated in the figure legends.

Results

T. caerulescens NRAMP3 and NRAMP4 show strong similarity to their *A. thaliana* homologues

The EST collection of *T. caerulescens* accession La Calamine (Rigola et al. 2006) contained one *AtNRAMP3* homologous partial cDNA (RR23nr019). This partial cDNA was used to re-screen the *T. caerulescens* cDNA library (Assunção et al. 2001) and resulted in the cloning of two *NRAMP* homologues. As shown in figure 1, these *TcNRAMPs* show a 96.5% and 95.1% similarity to *AtNRAMP3* and *AtNRAMP4* which resulted in their annotation as *TcNRAMP3* and *TcNRAMP4*, respectively. As these two *NRAMPs* are the closest relatives of *AtNRAMP3* and *AtNRAMP4* isolated so far, we consider them the *T. caerulescens* orthologues. The 2nd closest relative is *TjNRAMP4* from *Thlaspi japonicum*, a Ni-hyperaccumulating species related to *T. caerulescens*.

We performed genomic DNA blot analysis to determine whether NRAMP3 and NRAMP4 are present as single or multi-copy genes in several *T. caerulescens* accessions and other *Thlaspi* species (Figure 2). For digestion of the genomic DNA we used *XbaI* and *EcoRI*, which do not cut in the isolated *T. caerulescens TcNRAMP3* and *TcNRAMP4* cDNAs. In most digests we identified single bands hybridizing with the *TcNRAMP* probes indicating that *NRAMP3* (Figure 2a) and *NRAMP4* (Figure 2b) are present as single copy genes in *Thlaspi* (as in *A. thaliana*). The presence of more than one band for *T. caerulescens* Monte Prinzera (*NRAMP4*), *T. praecox* (*NRAMP3* + *NRAMP4*) and *T. perfoliatum* (*NRAMP3*) is most likely the result of the presence of an *XbaI* (*NRAMP3*) or *EcoRI* (*NRAMP4*) restriction site in the genomic sequences due to DNA polymorphisms. This is in contrast to the result for *T. japonicum* which shows a complex hybridization pattern of multiple bands for *NRAMP3* as well as *NRAMP4* indicating that multiple *NRAMP* copies are present in this *Thlaspi* species.

NRAMP3 and *NRAMP4* are differently expressed between *A. thaliana* and *T. caerulescens*, but also between *T. caerulescens* accessions La Calamine and Ganges

Based on the available Arabidopsis cDNA sequences and newly identified *NRAMP3* and *NRAMP4 T.*

caerulescens cDNA sequences, we compared their expression levels in roots and shoots of *A. thaliana* and *T. caerulescens* La Calamine exposed to deficient, sufficient and excess Zn in hydroponic solutions (Figure 3a). Note that physiologically equivalent sufficient and excess Zn concentrations, resulting in Zn sufficiency and Zn excess symptoms, respectively, are different for the two species: 2 (sufficient) and 25 µM (excess) $ZnSO_4$ and 100 (sufficient), and 1000 µM (excess) $ZnSO_4$ were used for *A. thaliana* and *T. caerulescens*, respectively (Van de Mortel et al., 2006). Independent of Zn status, *TcNRAMP3* shows a much higher overall expression level than *AtNRAMP3*. In *A. thaliana*, *NRAMP3* and *NRAMP4* expression in roots is not modified by varying Zn supply while in leaves both *AtNRAMPs* are up-regulated by Zn deficiency as well as Zn excess. This is in contrast to *TcNRAMP3* and *TcNRAMP4* expression, which does not appear to be altered upon alterations in Zn concentration. It must be noted, however, that when lowering the number of amplification cycles (Figure 3b) a small up-regulation of *TcNRAMP4* (La Calamine) is detectable in deficient Zn (leaves and roots) and excess Zn (leaves) conditions. Taking into account the genomic DNA blots, which show that *NRAMP3* and *NRAMP4* are single copy-genes in *T. caerulescens*, accession La Calamine, we conclude that the differences in expression pattern with *A. thaliana* result from a different regulation of these genes in the two species.

We then compared *NRAMP* expression levels between the *T. caerulescens* accessions La Calamine and Ganges. These accessions show equal levels of Zn tolerance and accumulation, but the Ganges accession displays stronger Cd tolerance and accumulation (Assunção et al. 2003a, Deniau et al. 2006). For both accessions root and leaf material was analysed from plants grown hydroponically at deficient and excess Zn, and at sufficient Zn with or without the addition of Cd (Figure 3b). Note that lower PCR cycle numbers than for the interspecific comparison were used to reveal differences between accessions that strongly express *NRAMP3* and *NRAMP4*. Both *TcNRAMP3* as well as *TcNRAMP4* expression patterns differ between the two *T. caerulescens* accessions. *TcNRAMP3* shows generally higher expression in La Calamine compared to Ganges. However, upon Cd addition, *TcNRAMP3* and *TcNRAMP4* expression in roots remains constant or increases in Ganges while it is obviously reduced in La Calamine. In contrast, in leaves *TcNRAMP4* expression is enhanced in both accessions by an excess of Cd or Zn. It thus appears that the differences in Cd and Zn accumulation and tolerance between *T. caerulescens* accessions La Calamine and Ganges are paralleled by marked differences in the regulation of *TcNRAMP3* and *TcNRAMP4* expression.

T. caerulescens NRAMP3 and NRAMP4 show similar metal transport specificities to their *A. thaliana* homologues when expressed in yeast

To test for functional differences between NRAMP3 and NRAMP4 from *A. thaliana* and *T. caerulescens*, we expressed them in yeast metal uptake deficient mutants allowing determination of their metal transport specificity (Pinner, 1997, Thomine et al., 2000; Lanquar et al., 2004, Curie et al., 2000). We tested Fe, Mn, Zn and Cd transport activity of the *T. caerulescens* NRAMP3 and NRAMP4 proteins and compared it to their *A. thaliana* orthologues (Figure 4). In a first experiment, we investigated the ability of TcNRAMP3 and TcNRAMP4 to transport Fe and Mn by testing for the complementation of *fet3fet4* and *smf1* yeast mutant phenotypes. The *fet3fet4* strain is defective in its low- and high-affinity Fe uptake systems and is unable to grow on a low Fe medium (Dix et al., 1994). The *smf1* strain is disrupted in *SMF1*, a yeast *NRAMP* gene essential for high affinity Mn uptake. *smf1* is unable to grow on synthetic media containing high concentrations of the divalent cation chelator EGTA (Supek et al., 1996) and this growth defect is specifically rescued by Mn supplementation (Figure 4B). We have expressed *AtNRAMP3*, *AtNRAMP4*, *TcNRAMP3*, *TcNRAMP4* and *IRT1*, a metal transporter with a broad substrate range (Korshunova et al., 1999) in *fet3fet4* and checked their ability to grow on high and low Fe medium (Figure 4a). All four *NRAMPs* as well as the *IRT1* control complement *fet3fet4* without any marked difference. Expression of *TcNRAMPs*, *AtNRAMPs* and *IRT1* also rescued growth of the *smf1* strain on synthetic medium with 10 mM EGTA with no major difference between the different transporters (Figure 4b). These data indicate that, like their *A. thaliana* orthologues, TcNRAMPs are able to transport Fe and Mn.

Since *T. caerulescens* and *A. thaliana* differ greatly in their tolerance and accumulation of Zn and Cd, we have used a more quantitative experimental set-up to assess Zn and Cd transport capabilities of the NRAMP3 and 4 proteins from these two species. The *zrt1zrt2* double mutant yeast strain is deficient in high (*ZRT1*) and low (*ZRT2*) affinity Zn transport systems (Zhao and Eide, 1996a, b). This strain cannot grow on synthetic media without the addition of extra Zn. The *zrt1zrt2* strain was transformed with the four *NRAMPs* or an empty vector (control), and grown in Low Zinc Medium (Zhao and Eide, 1996b) supplemented with 10 µM $ZnSO_4$. Only *AtNRAMP4* and particularly *TcNRAMP4* were able to significantly rescue *zrt1zrt2* growth (Figure 4c). In contrast, *zrt1zrt2* strains

expressing *AtNRAMP3* and *TcNRAMP3* do not grow significantly better than *zrt1zrt2* empty vector control strain. When these yeast strains were grown in the same medium supplemented with 100 µM $ZnSO_4$ they showed equal growth (data not shown).

The Cd transport activity of the NRAMPs was tested by using their general ability to enhance Cd sensitivity in yeast, revealed by a reduced growth on Cd containing medium (Thomine et al., 2000). The four *NRAMPs*, *IRT1* a strong Cd transporter (Guerinot, 2000) and an empty vector were introduced in the *fet3fet4* yeast strain and twin cultures, with or without 10 µM $CdCl_2$, were inoculated from pre-cultures of individual transformants. Compared to the empty-vector control, expression of all four *NRAMPs* lead to a significant increase in Cd sensitivity. However, none of them increased yeast Cd sensitivity as much as *IRT1* (figure 4d). The differences become more obvious when growing the culture for a longer time (18h compared to 12h). This result indicates that all four NRAMPs are able to transport Cd. Nevertheless, as for Zn transport (figure 4c), we find a difference between NRAMP3 and NRAMP4 proteins: expression of *NRAMP4* leads to significantly higher yeast Cd sensitivity compared to expression of *NRAMP3*, but no significant difference between *AtNRAMP4* and *TcNRAMP4* is observed.

TcNRAMP3 and TcNRAMP4 proteins are targeted to the vacuolar membrane

To test whether the TcNRAMP3 and TcNRAMP4 intracellular localization is similar to that of AtNRAMP3 and AtNRAMP4, we expressed TcNRAMP transporters fused to GFP in protoplasts made from an Arabidopsis cell suspension (two independent experiments). Free DsRed2 was co-transformed in the same cells (one experiment). GFP and DsRed2 fluorescence were imaged by confocal microscopy. Figure 5 shows that while DsRed2 fluorescence was homogenous in the cytosol and nucleus, GFP fluorescence consistently lined the vacuolar membrane when GFP was fused to the carboxy-terminal end of either TcNRAMP3 or TcNRAMP4. These results indicate that, like their *A. thaliana* counterparts, the NRAMP3 and NRAMP4 from *T. caerulescens* reside on the vacuolar membrane.

***A. thaliana nramp3nramp4* double mutant seedlings are hypersensitive to Zn and Cd**

The yeast assays did not indicate strong differences between the metal transport capacities of

AtNRAMP3, AtNRAMP4 and their *T. caerulescens* orthologues. Nevertheless, our expression study and previous transcriptome data in other metal hyperaccumulating plant species such as *A. halleri* (Becher et al., 2004; Weber et al., 2004; Talke et al., 2006) do suggest a function of NRAMPs in metal tolerance or accumulation. Therefore we tested a possible role of NRAMP3 and NRAMP4 in Zn and Cd tolerance in *A. thaliana*. The *A. thaliana nramp3nramp4* double mutant (Lanquar et al., 2005) shows a reduced seedling development under Fe-deficient conditions, which can be rescued completely by the addition of sufficient Fe. In contrast, supplementation with Zn or Mn results in an inhibition of early plant development, which is more pronounced for the *nramp3nramp4* mutant than for wild-type plants. To further investigate this effect, we sowed *nramp3nramp4* and wild-type seeds on normal growth medium supplemented with 50 µM of FeHBED (which rescues the Fe-deficiency phenotype of the double mutant) and subsequently tested the effect of supplying different concentrations of Cd or Zn (Figure 6). Addition of Zn and Cd inhibited plant growth in a concentration dependent manner in *A. thaliana* accession WS (Figure 6a and c). Root length measurements revealed that the *nramp3nramp4* is strongly hypersensitive to both Cd and Zn. In contrast, single *nramp3* or *nramp4* mutants display little difference in Cd sensitivity in comparison with wild-type seedlings (Thomine et al., 2000, Lanquar et al., unpublished). This effect is not due to *nramp3nramp4* hypersensitivity to Fe deficiency that would be prevented by addition of 50 µM FeHBED in the medium (Figure 6b and c). The complementation of *nramp3nramp4* with *AtNRAMP3* or *AtNRAMP4* both restored its Fe-deficiency phenotype (Lanquar et al., 2005) as well as its sensitivity to Zn and Cd (data not shown). These results indicate that AtNRAMP3 and AtNRAMP4 function redundantly in Zn and Cd tolerance in *A. thaliana*.

TcNRAMP3 expression complements *nramp3nramp4* Zn and Cd hypersensitivities

The RT-PCR analysis (figure 3) showed a particularly high expression of *TcNRAMP3* compared to *AtNRAMP3*. Therefore we decided to study the effect of introducing the *TcNRAMP3* cDNA (driven by the 35S promoter) in the *nramp3nramp4* double mutant on its sensitivity to Zn and Cd. Three homozygous single insertion lines were selected (hereafter referred to as *nr3nr4*_3-1, 3-2 and 3-7) and used for further analysis. Semi-quantitative RT-PCR analysis showed a strong expression of *TcNRAMP3* in all three lines with little variation in expression level (Figure 7a). We also found that expression levels correlated well with TcNRAMP3 protein levels, identified by Western blots using

an AtNRAMP3 specific antibody (data not shown).

The three transformants as well as un-transformed *nramp3nramp4* and wild type were sown on ABIS medium supplemented with 0.3 µM FeHBED and grown for 11 days. Under these conditions the *nramp3nramp4* mutant is unable to develop, in contrast to the wild-type control plants, which grow normally and have a root length of about 80 mm. *TcNRAMP3* expression fully complements the *nramp3nramp4* mutant growth defect on low Fe in all three lines to wild-type-like phenotypes (Figure 7b).

To examine if TcNRAMP3 also rescues plant tolerance to Zn and Cd, the *nramp3nramp4 TcNRAMP3* expressing lines were grown on Zn and Cd containing medium. Figure 7c shows the root length of plants grown on normal (30 µM) and excess (100 and 300 µM) Zn containing medium. As shown in figure 6 the presence of excess Zn inhibits growth of the *nramp3nramp4* mutant stronger than that of the wild-type control. The expression of TcNRAMP3 complements this growth inhibition phenotype. However, strong constitutive expression of TcNRAMP3 driven by the 35S CaMV promoter did not lead to an increase of Zn tolerance above the wild type level (Figure 7c). The analysis of these lines on Cd containing media gave comparable results (Figure 7d). *TcNRAMP3* expression in *nramp3nramp4* rescues its hypersensitivity to Cd but does not improve its tolerance above that of wild-type plants. These results indicate that NRAMPs are certainly important for *A. thaliana* tolerance to Zn and Cd but that TcNRAMP3 is functionally equivalent to AtNRAMP3 and does not lead to higher Zn and Cd tolerance levels.

TcNRAMP4 expression in a wild-type *A. thaliana* Col background does not affect Zn or Cd tolerance

Replacement of *AtNRAMP3* and *4* by *TcNRAMP3* in *A. thaliana* did not result in enhanced levels of Zn or Cd tolerance. Based on the ability of TcNRAMP4 to transport Zn and Cd in yeast, we tested the effect of over-expressing *TcNRAMP4* driven by the 35S CaMV promoter in wild-type *A. thaliana* (Columbia) on Zn and Cd tolerance. Homozygous transformed lines with a single insertion locus were selected and expression levels of the introduced *TcNRAMP4* gene were studied by semi-quantitative RT-PCR analysis. Figure 8a shows the presence of *TcNRAMP4* transcript at various levels in all three selected lines (Further referred to as Col_4-1, 4-2 and 4-7). These three lines were used for analysing their growth on media containing increasing concentrations of Zn or Cd. Figure

8b shows that Zn excess (100 and 300 µM) reduces plant development of *TcNRAMP4* expressing lines to a similar degree as in wild-type plants. When we analysed the tolerance to Cd of these plants we found comparable results (Figure 8c). These experiments indicate that increased expression of Tc*NRAMP4* in wild type background does not result in elevated levels of Zn or Cd tolerance.

Disscusion

In this study, we report the identification and functional characterization of the homologues of *A. thaliana NRAMP3* and *NRAMP4* in the metal hyperaccumulating plant *T. caerulescens*. *NRAMP3* and *NRAMP4* are single copy genes in *T. caerulescens*. In comparison to *A. thaliana NRAMP3* and *NRAMP4*, *TcNRAMP3* and, to a lesser extent, *TcNRAMP4* show enhanced expression levels. By analysing different *T. caerulescens* accessions, we find that contrasting Cd accumulation and tolerance features are paralleled by different patterns of *TcNRAMP* regulation. Expression of *TcNRAMP3* and *TcNRAMP4* in several yeast mutants impaired in metal transport shows functional properties similar to their *A. thaliana* counterparts. To investigate the possible functional significance of the strong *NRAMP3* and *NRAMP4* expression in *T. caerulescens*, we analyze their role in metal tolerance in *A. thaliana*. In agreement with a function of NRAMP3 and NRAMP4 in metal tolerance, we show that Arabidopsis *nramp3nramp4* double knockout mutants are hypersensitive to Cd and Zn excess. Expression of *TcNRAMP3* in *nramp3nramp4* does rescue these phenotypes even though expression of *TcNRAMP4* in wild-type *A. thaliana* does not alter Cd or Zn sensitivity.

NRAMP expression is enhanced in *T. caerulescens*

We show here that *TcNRAMP3* and to a lesser extent *TcNRAMP4* are upregulated in comparison with *AtNRAMP3* and *AtNRAMP4*. These results are in agreement with transcriptome analysis of *T. caerulescens* (van de Mortel et al., 2006). Interestingly, previous cross-species microarray experiments had shown that *NRAMP3* expression is also enhanced in another hyperaccumulating plant, *Arabidopis halleri* (Becher et al., 2004; Weber et al., 2004; Filatov et al., 2006; Talke et al., 2006). The upregulation of other metal homeostasis genes in *T. caerulescens* has also been shown for *ZNT1*, *ZNT2*, *ZTP1*, *HMA4* and *MT3* (Assunçaõ et al., 2001; Bernard et al., 2004; Roosens et al.,

2004). A previous study of the metal tolerance protein MTP1 in *A. halleri* has shown a relation between its expression levels and the number of gene copies (Dräger et al., 2004). Their data suggest duplications of an ancestral *MTP1* gene resulting in multiple copies, some of which play a particular role in Zn detoxification. In contrast, our DNA blot experiments show that *TcNRAMP3* and *TcNRAMP4* are single copy genes. The stronger expression of *TcNRAMP3* and *4* is thus not associated with gene duplication.

TcNRAMP3 and _TcNRAMP4_ are differently expressed between _T. caerulescens_ accessions with different Cd and Zn hyperaccumulation capabilities

TcNRAMP3 and *TcNRAMP4* are differently expressed in the *T. caerulescens* accessions La Calamine and Ganges. The *T. caerulescens* Ganges accession is more tolerant and is a stronger accumulator of Cd. This stronger accumulation and tolerance of Cd by Ganges is correlated with enhanced *NRAMP3* and *NRAMP4* expression levels in roots when Cd is supplied to the medium. In contrast, the less tolerant accession La Calamine shows a decrease in *NRAMP* expression in roots upon Cd addition. These results are in agreement with a function of NRAMP proteins in roots associated with higher Cd tolerance and/or accumulation. Previously, Roosens et al. (2004) already showed such a correlation between elevated expression of *TcMT3* and the Cd tolerance/accumulation of specific *T. caerulescens* accessions. Furthermore, *TcIRT1* was shown to be specifically induced upon Fe deficiency only in Ganges and not in weaker Cd accumulating *T. caerulescens* accessions like Prayon (Lombi et al., 2002).

Since genomic DNA blots show single copies of both *NRAMP3* and *NRAMP4* in the two *T. caerulescens* accessions La Calamine and Ganges, our results suggest that the *T. caerulescens* specific - and their accession specific - expression patterns are most likely due to promoter differences with the *A. thaliana* homologous genes or between *T. caerulescens* accessions. Of course it may also be due to upstream changes in metal perception or stress signalling as previously suggested by Assunçaõ et al. (2003b).

Functional differences between NRAMP3 and NRAMP4 metal transport abilities

Our results show that *T. caerulescens* NRAMPs have, overall, the same transport abilities as their *A. thaliana* orthologues when expressed in yeast and the same intracellular localization in plant cells. Finally, the complementation of the *nramp3nramp4* Arabidopsis mutant by TcNRAMP3 also

indicates functional similarity between AtNRAMP3, AtNRAMP4 and TcNRAMP3. It thus appears unlikely that the *T. caerulescens* NRAMPs have developed a specific function different from *A. thaliana*, which would play a specific role in metal tolerance or accumulation. Our results however confirm a difference in Zn transport ability: NRAMP4 transports Zn while NRAMP3 does not. A different ability to transport Zn between AtNRAMP3 and AtNRAMP4 was reported before by Lanquar et al. (2004). Since NRAMP3 and NRAMP4 are closely related, the functional comparison of their sequences could help defining the specific amino acid residues that are involved in Zn transport in NRAMP proteins. A difference in metal selectivity within a metal transporter family is not unusual. It has also been described in the ZIP family: *A. thaliana* IRT1 transports Mn and Cd while IRT2 does not (Korshunova et al., 1999; Vert et al., 2001) and rice ZIP3 transports Cd while ZIP2 does not (Ramesh et al., 2003). In addition, *TcNRAMP4* expression rescues the growth of *zrt1zrt2* significantly more efficiently than *AtNRAMP4*. Quantitative assays for Cd transport ability suggest that, apart from the difference in Zn transport, NRAMP3 is also a weaker Cd transporter than NRAMP4 both in *A. thaliana* and *T. caerulescens*. Hence, inability to transport Zn may be associated with decreased ability to transport Cd. In contrast, AtNRAMP4 and TcNRAMP4 abilities to transport Cd, estimated from the induced increase in Cd sensitivity, are not significantly different. This indicates a modest but specific increase in Zn transport ability in TcNRAMP4 compared with AtNRAMP4.

The conserved metal transport properties between TcNRAMP3 and AtNRAMP3 on the one hand and, AtNRAMP4 and TcNRAMP4 on the other hand, contrast strongly with the highly divergent function reported for TjNRAMP4 by Mizuno et al. (2005). They reported that TjNRAMP4 from the Ni hyperaccumulator *T. japonicum* specifically enhances Ni sensitivity and Ni accumulation when expressed in yeast. TjNRAMP4 is however unable to complement yeast strains deficient in Fe, Mn and Zn uptake, in contrast to AtNRAMP4 (Thomine et al., 2000; Lanquar et al., 2004). We did not find a similar enhanced Ni sensitivity when expressing the *A. thaliana* and *T. caerulescens NRAMP3* and *NRAMP4* in yeast (data not shown). Interestingly, our DNA blot experiments show the presence of multiple *NRAMP3* and *NRAMP4* gene copies in the Ni hyperaccumulator *T. japonicum.* This may be an example of gene amplification similar to what has been described for several *A. halleri* metal homeostasis genes (Talke et al., 2006). In this case it is possible that specialization in Ni transport in the particular cDNA reported by Mizuno et al. (2005) resulted in the loss of its other metal transport

abilities.

A. thaliana nramp3nramp4 mutant is hypersensitive to Cd and Zn

The *nramp3nramp4* double mutant shows strong hypersensitivity to Cd and Zn compared to wild type. This phenotype is comparable to the strongest Cd hypersensitive mutants reported so far such as *cad1* and *cad2* (Howden et al., 1995a and 1995b). We previously reported that 35S-*AtNRAMP3* and 35S-*AtNRAMP4* over-expressing plants also showed an enhanced Cd sensitivity (Thomine et al., 2000; Lanquar et al., 2004). However, this phenotype was much weaker than the one we describe here for the *nramp3nramp4* mutant and differs by two major points: its dependence on iron nutrition and its specificity. While *nramp3nramp4* hypersensitivity to Cd and Zn is observed even under high Fe nutrition, 35S-AtNRAMP4 plant hypersensitivity to Cd is apparent only under low Fe nutrition and is abolished at elevated Fe nutrition (Lanquar et al., 2004). In addition, 35S-AtNRAMP4 plant hypersensitivity to metal is specific to Cd *vs* Zn (Lanquar et al., 2004), while *nramp3nramp4* display hypersensitivity to both Cd and Zn.

These different features of metal hypersensitivity observed in *nramp3nramp4* double mutants or triggered by *AtNRAMP3* or *AtNRAMP4* over-expression suggest that they originate from different mechanisms. Cadmium hypersensitivity induced by *AtNRAMP3* or *AtNRAMP4* over-expression was proposed to be due to increased Cd remobilization from the vacuole (Thomine et al., 2003). In contrast, *nramp3nramp4* pleiotropic metal hypersensitivity may be the result of a globally unbalanced metal homeostasis.

TcNRAMP3 expression restores Cd and Zn tolerance in *nramp3nramp4*

In this work, we show that Cd and Zn sensitivity of *nramp3nramp4* can be successfully restored by the expression of *TcNRAMP3* (Figure 7). This result further supports the idea that NRAMPs play an important role in Cd and Zn tolerance, and is in agreement with our finding that TcNRAMP3 shares the same metal transport selectivity and the same intracellular membrane localization with AtNRAMP3.

It is noteworthy that, in yeast, *TcNRAMP3* expression does not complement the *zrt1zrt2* double mutant phenotype (Figure 4) likely because TcNRAMP3 cannot mediate sufficient Zn transport into yeast cells. Although there may be differences in protein function between yeast and plants, the

restoration of Zn tolerance in the *nramp3nramp4* double mutant by *TcNRAMP3* expression is likely not the effect of a re-introduced Zn transport function. An alternative explanation is that in *nramp3nramp4* knockout plants the general metal homeostasis is seriously disturbed, rendering the plants more sensitive to external supply of high metal concentrations. Therefore, we propose that *nramp3nramp4* hypersensitivity to Zn and Cd is related to a general defect in mobilization of essential metals from the vacuole, which would be required for tolerance to metals.

Does elevated *NRAMP* expression contribute to metal tolerance in hyperaccumulating plants?

The Zn and Cd sensitivity of the *nramp3nramp4* double mutant and its rescue by TcNRAMP3 clearly support a role in metal tolerance for NRAMP proteins. Nevertheless, our yeast expression data do not indicate any strong functional differences between *A. thaliana* and *T. caerulescens* NRAMPs. Our experiments could not reveal any specific functional feature of TcNRAMPs. In line with the data from yeast expression, *TcNRAMP3* expression in *nramp3nramp4* restores Cd and Zn tolerance to a level comparable with wild-type Arabidopsis but does not result in highly tolerant plants comparable to *T. caerulescens*. Furthermore, *TcNRAMP4* over-expression in wild-type *A. thaliana* does not alter its Cd or Zn sensitivity under the conditions tested. In addition, QTL analysis for Zn and Cd accumulation, using a cross between the *T. caerulescens* accessions La Calamine and Ganges, did not indicate a role for *TcNRAMP3* or *4* in metal accumulation, although a role in metal tolerance still cannot be excluded (Deniau et al., 2006). The difference in *TcNRAMP* expression between La Calamine and Ganges under Cd exposure is therefore probably not directly related to Zn or Cd transport, but to maintaining general metal homeostasis, possibly through Fe mobilization from the vacuole.

Enhanced expression of NRAMP3 and NRAMP4 is consistently observed in metal hyperaccumulating plants compared to non-accumulating plants. Nevertheless, over expression of NRAMPs alone is not sufficient to enhance metal tolerance in A. thaliana. Enhanced metal tolerance and accumulation would most likely require elevated expression of a whole set of genes. Increased levels of NRAMPs may be needed to maintain intracellular Fe/Mn/Zn homeostasis in metal hyperaccumulating plants when they are exposed to high levels of Zn, Cd or Ni. To test this

hypothesis, future studies should aim at generating stable T. caerulescens RNAi lines to down regulate NRAMP expression in this hyperaccumulating plant.

Acknowledgements

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Fig 1

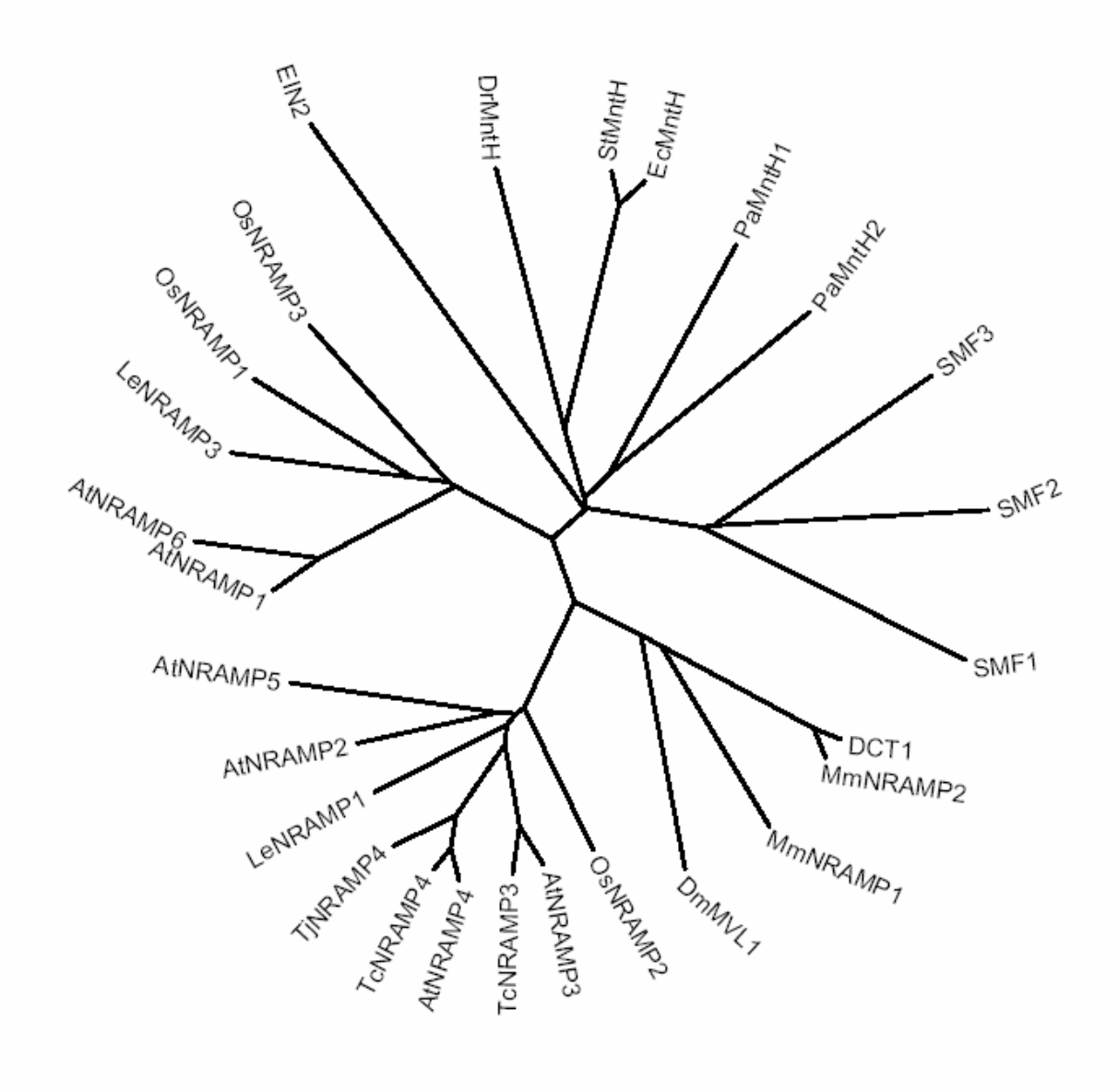

Fig 1 Phylogenetic tree of the *NRAMP* gene family showing the close relatedness of the *NRAMPs* from *A. thaliana* and *T. caerulescens*. The following sequences were used: *A. thaliana*; AtNRAMP1 (AF165125), AtNRAMP2 (AF141204), AtNRAMP3 (AF202539), AtNRAMP4 (AF202540), AtNRAMP5 (At4g18790), AtNRAMP6 (At1g15960), EIN-NRAMP (BAB08388, N-ter only), *T. caerulescens*; TcNRAMP3 (EF639294), TcNRAMP4 (EF639295), *T. japonicum*; TjNRAMP4 (AB115423), rice; OsNRAMP1 (L41217), OsNRAMP2 (L81152), OsNRAMP3 (460767), tomato; LeNRAMP1 (AY196092), LeNRAMP3 (AY196091), yeast; SMF1 (U15929), SMF2 (U00062), SMF3 (XP455861), mouse; MmNRAMP1 (L13732), MmNRAMP2 (L33415), rat; DCT1 (AF008439), Drosophila; DmMVL1 (U23948); bacteria; PaMntH1 (AF161319), PaMntH2 (AF161320), StMntH (Af161317), EcMntH (AF161318), DrMntH (AE002012). The tree was drawn

using PHYLIP drawtree (Felsenstein 1993) at http://bioweb.pasteur.fr/seqanal/interfaces/drawtree-simple.html after comparison of the deduced protein sequences with clustalw at http://www.ebi.ac.uk/clustalw/.

Fig 2

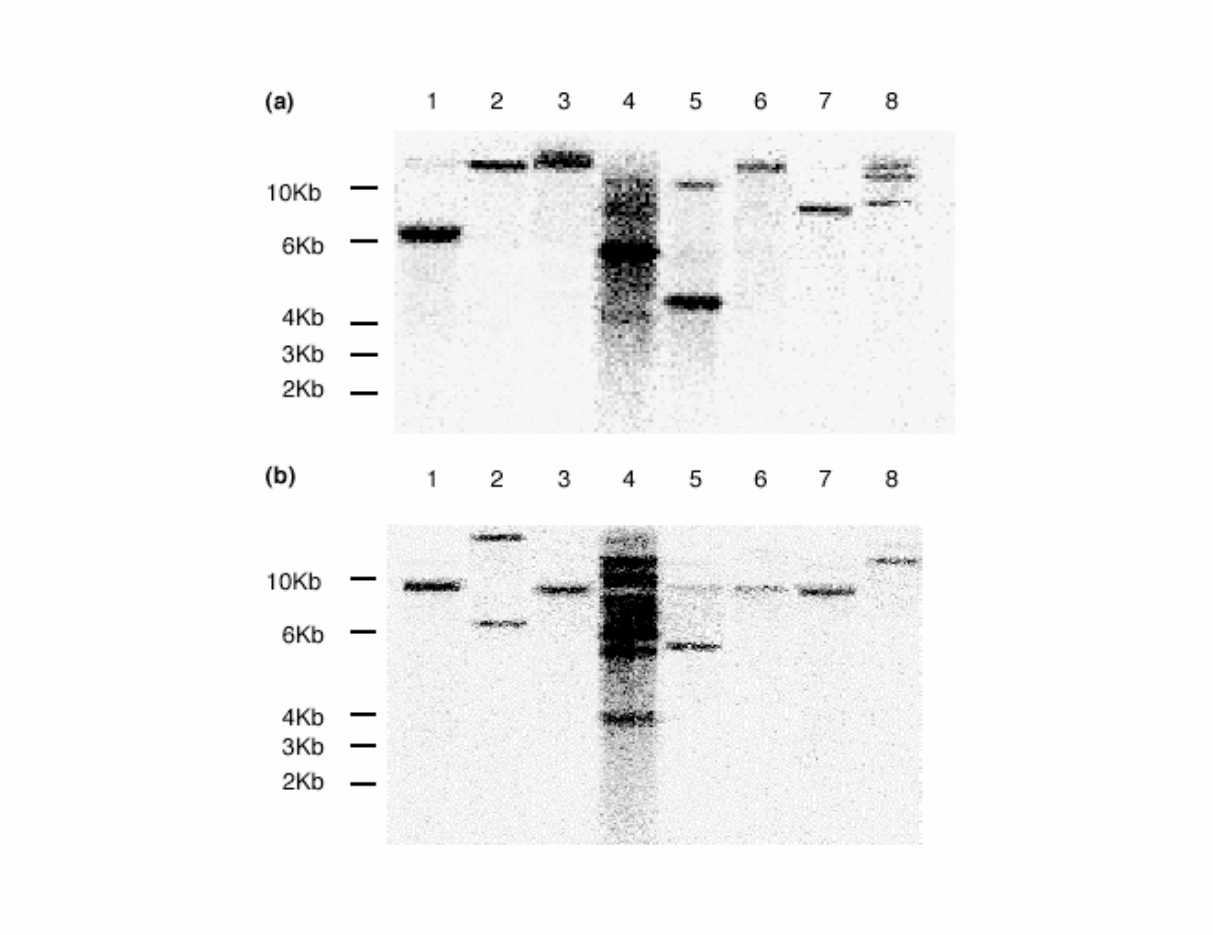

Fig 2 DNA blot analysis of several *T. caerulescens* accessions and *Thlaspi* species.

Genomic DNA was digested with *XbaI* (a) and *EcoRI* (b), and blots were hybridized with a *TcNRAMP3* (a) or *TcNRAMP4* (b) cDNA probe. The numbers above the lanes designate the different *Thlaspi caerulescens* accessions; La Calamine (1), Monte Prinzera (2), Ganges (3), Austria (7), or *Thlaspi* species; *T. japonicum* (4), *T. praecox* (5), *T. minimum* (6), *T. perfoliatum* (8).

Fig 3

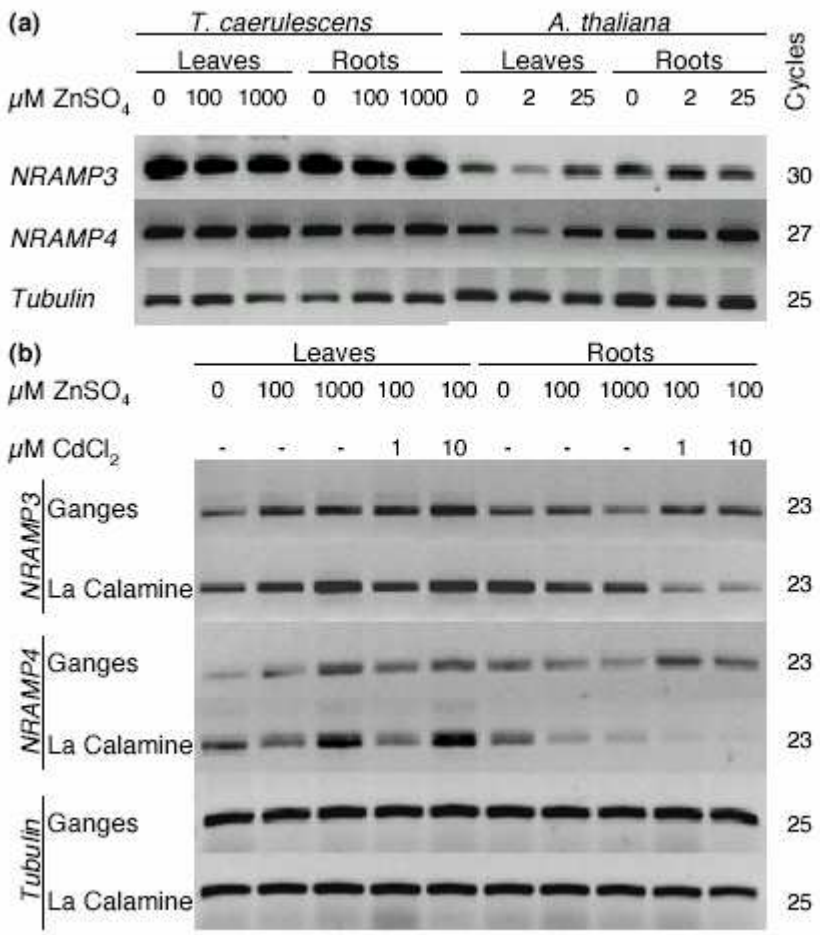

Fig 3 Expression analysis of *NRAMP3* and *NRAMP4* expression levels in *A. thaliana* and *T. caerulescens* and their regulation by Zn and Cd.

(a). Semi-quantitative RT-PCR analysis of *NRAMP3*, *NRAMP4* and *Tubulin* (control) expression in leaves and roots of *T. caerulescens* (La Calamine) and *A. thaliana*. Plant seeds were sown on soil, transferred to hydroponic culture and subsequently transferred to nutrient solutions with deficient (0 µM), sufficient (100 or 2 µM), or excess (1000 or 25 µM) $ZnSO_4$ concentrations for 7 days before separation of roots and leaves followed by RNA extraction. PCR reactions of 30 (*NRAMP3*), 27 (*NRAMP4*) and 25 (*tubulin*) cycles were performed before migration and EtBr visualization in a 1% agarose gel.

(b) Semi-quantitative RT-PCR analysis on leaf and root material from *T. caerulescens* accessions La Calamine and Ganges grown on hydroponic cultures at deficient (0 µM), sufficient (100 µM), or excess (1000 µM) $ZnSO_4$ concentrations and on 100 µM $ZnSO_4$ with the addition of 1 or 10 µM

$CdCl_2$. PCR reactions of 23 cycles were used for *NRAMP3* and *NRAMP4*, while 25 cycles were used for the *Tubulin* control.

Fig 4

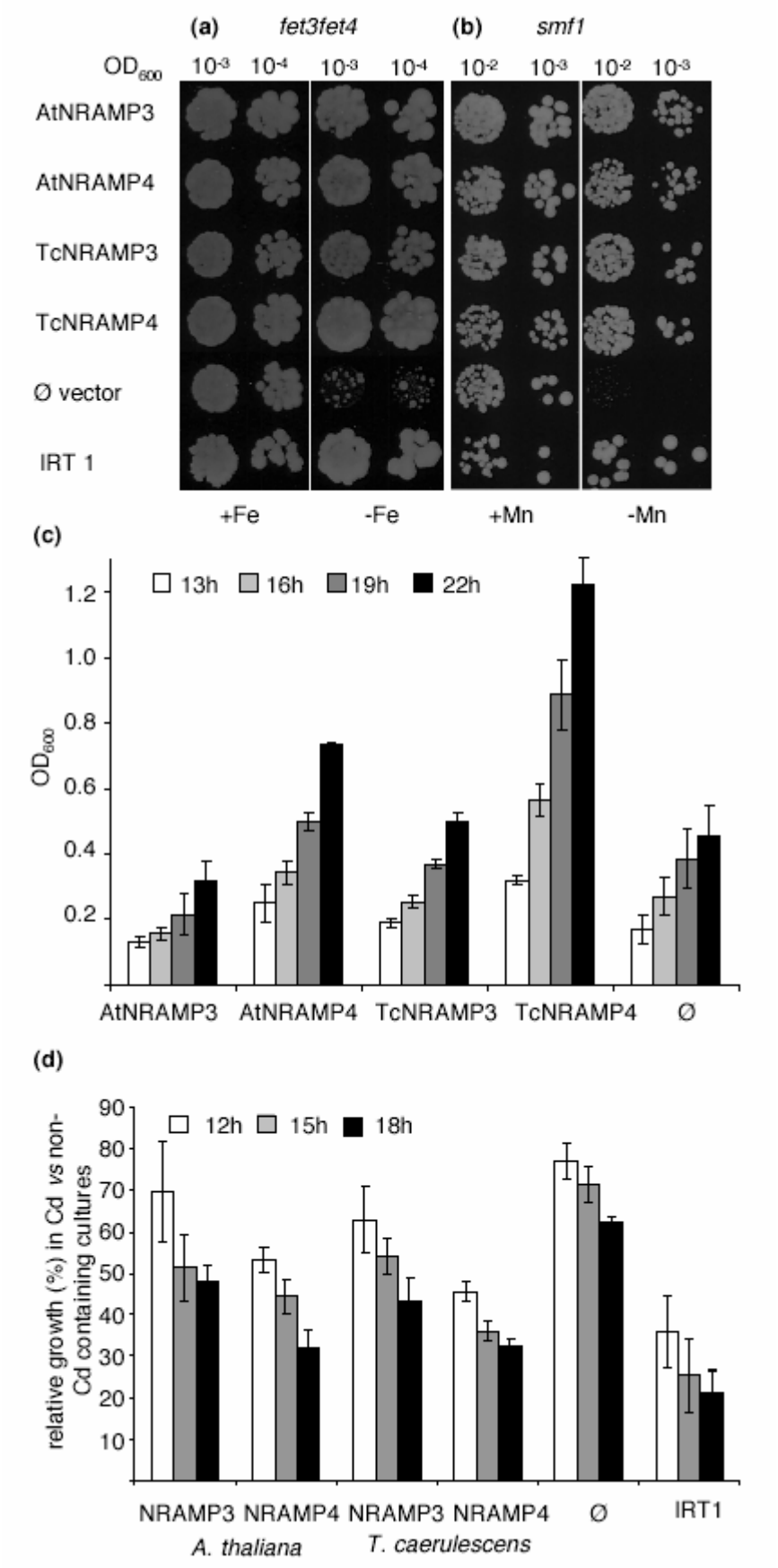
(a) fet3fet4
(b) smf1
OD600
10-3 10-4 10-3 10-4
10-2 10-3 10-2 10-3
AtNRAMP3
AtNRAMP4
TcNRAMP3
TcNRAMP4
Ø vector
IRT 1
+Fe
-Fe
+Mn
-Mn
(c)
13h 16h 19h 22h
OD600
1.2
1.0
0.8
0.6
0.4
0.2
AtNRAMP3 AtNRAMP4 TcNRAMP3 TcNRAMP4 Ø
(d)
12h 15h 18h
relative growth (%) in Cd vs non-Cd containing cultures
90
80
70
60
50
40
30
20
10
NRAMP3 NRAMP4 NRAMP3 NRAMP4 Ø IRT1
A. thaliana
T. caerulescens

Fig 4 In yeast, TcNRAMP3 as well as TcNRAMP4 transport Fe, Mn, and Cd, like AtNRAMP3 and AtNRAMP4, but only the NRAMP4 proteins are able to transport Zn. AtNRAMP3, AtNRAMP4, TcNRAMP3, TcNRAMP4 and IRT1 (control) were expressed in the *fet3fet4*, *smf1*, *zrt1zrt2* yeast mutants (deficient in Fe, Mn and Zn uptake respectively) and subsequently studied for their ability to rescue the mutant phenotype.

(a) Growth of transformed *fet3fet4* yeast cells on synthetic dextrose-URA (pH 5.5) supplemented with 80µM BPS and 200 µM $FeCl_3$ (+Fe, left) or 30 µM $FeCl_3$ (-Fe, right). Pre-cultured yeasts were diluted to $OD_{600}= 10^{-3}$ (left) or 10^{-4} (right) and 10 µl of each sample was spotted and incubated at 30°C for 4 days. (b) Growth of transformed *smf1* yeast cells on synthetic dextrose-URA (pH 6) supplemented with 10 mM EGTA and 0.1 mM $MnCl_2$ (+Mn, left) or no added Mn (-Mn, right). Yeast pre-cultures were diluted to $OD_{600}= 10^{-2}$ (left) or 10^{-3} (right) and 10 µl of each sample was spotted and incubated at 30°C for 4 days. (c) Growth of transformed *zrt1zrt2* yeast cells in low zinc medium (pH 6) supplemented with 10 µM $ZnSO_4$. OD_{600} was measured 13, 16, 19 and 22h after inoculation of 5 ml cultures (at 30°C) with 50 µl of a yeast pre-culture (at $OD_{600} = 1$). Mann-Whitney tests indicate that AtNRAMP4 and TcNRAMP4 significantly rescue *zrt1zrt2* growth ($p<0,001$) while AtNRAMP3 and TcNRAMP3 do not. (d) The ability of the NRAMP proteins to transport Cd was tested by their effect to enhance Cd sensitivity of transformed *fet3fet4* yeast cells. 5-ml cultures of synthetic dextrose-URA (pH 5.5) with or without 10 µM $CdCl_2$ were inoculated with 150 ul pre-culture ($OD_{600} = 1$) and grown at 30°C. The OD_{600} was measured after 12, 15 and 18h and the growth in the Cd containing culture was expressed as a percentage of the growth in its control culture without Cd. Mann-Whitney tests indicate that AtNRAMP3 and 4, and TcNRAMP3 and 4 significantly increase yeast Cd sensitivity compared to control ($p<0,001$) and that AtNRAMP4 and TcNRAMP4 increase Cd sensitivity significantly more than AtNRAMP3 and TcNRAMP3 ($p<0,001$). Figure c and d show representative results of one of four experiments that gave qualitatively similar results. Bars show the mean of duplicate cultures and the arrow bars represent the SD.

Fig 5

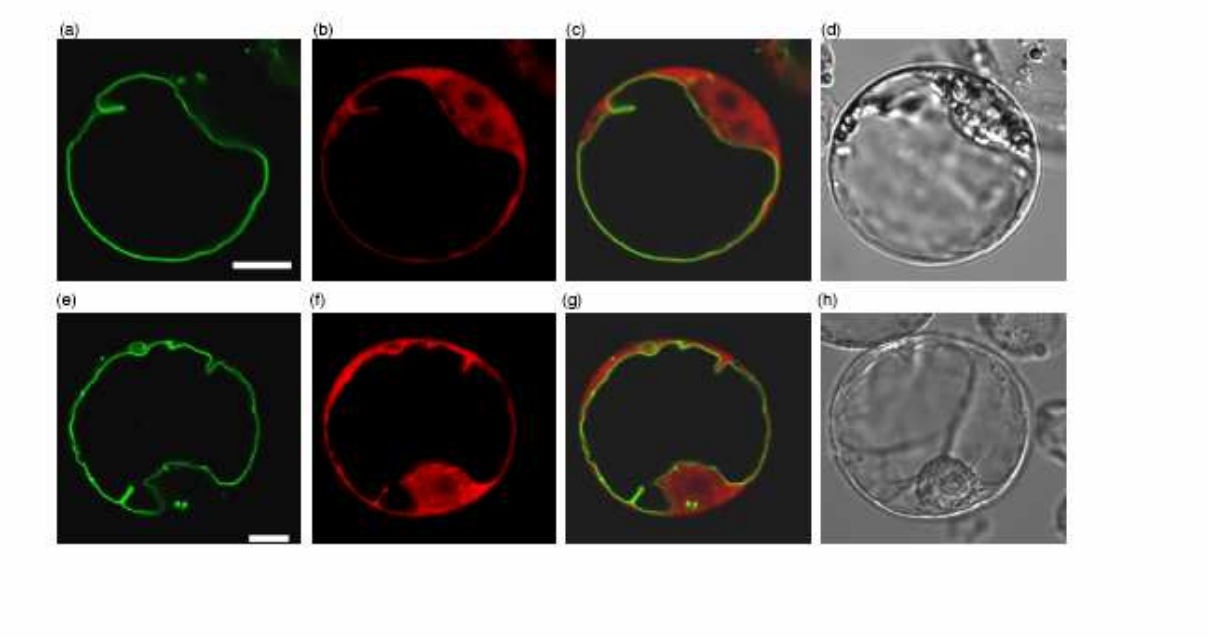

Fig 5 TcNRAMP3::GFP and TcNRAMP4::GFP fusion proteins are targeted to the vacuolar membrane. *Arabidopsis* protoplasts transformed with TcNRAMP3::GFP (a-d) or TcNRAMP4::GFP (e-h) and DsRed2. Confocal cross sections show GFP fluorescence (a, e, excitation 488 nm, emission measured between 495 and 535 nm) DsRed2 fluorescence (b, f, excitation 543 nm, emission measured between 578 nm and 707 nm) and overlay of NRAMP- and free DsRed2 fluorescence (c, g). d, h transmission images of the corresponding protoplasts. GFP fluorescence delimitates the vacuole lumen (black) and the cytosol (red) indicating vacuolar membrane localization. Scale bar 10 μm.

Fig 6

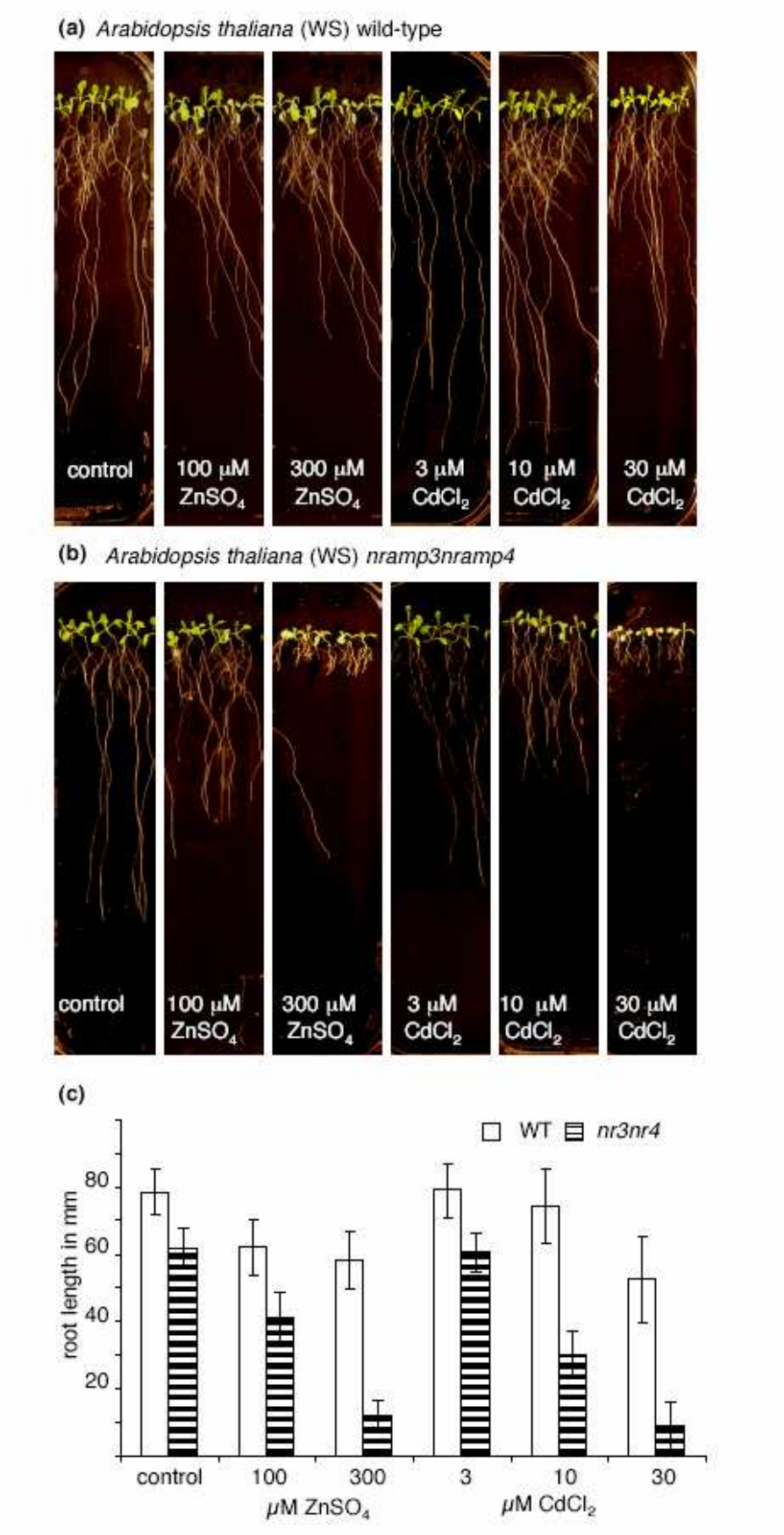
(a) Arabidopsis thaliana (WS) wild-type
control
100 µM ZnSO4
300 µM ZnSO4
3 µM CdCl2
10 µM CdCl2
30 µM CdCl2
(b) Arabidopsis thaliana (WS) nramp3nramp4
control
100 µM ZnSO4
300 µM ZnSO4
3 µM CdCl2
10 µM CdCl2
30 µM CdCl2
(c)
WT
nr3nr4
root length in mm
80
60
40
20
control
100
300
3
10
30
µM ZnSO4
µM CdCl2

Fig 6 The *nramp3nramp4* double mutant shows an enhanced sensitivity for Zn and Cd compared to its *A. thaliana* (WS) wild-type background. Root growth hypersensitivity to Zn and Cd. *A. thaliana* wild-type (a) and *nramp3nramp4* double mutant (b) seedlings were grown vertically for 11 days on ABIS medium containing 50 µM FeHBED or on the same medium supplemented with 3, 10 or 30 µM $CdCl_2$ or 100 or 300 µM $ZnSO_4$ (instead of the regular 30 µM $ZnSO_4$). Pictures represent the result of a representative experiment out of four. (c) Average root length of seedlings, grown in the same conditions as in figure a and b, was determined by measuring ~70 plants per genotype. Arrow bars represent the SD.

Fig 7

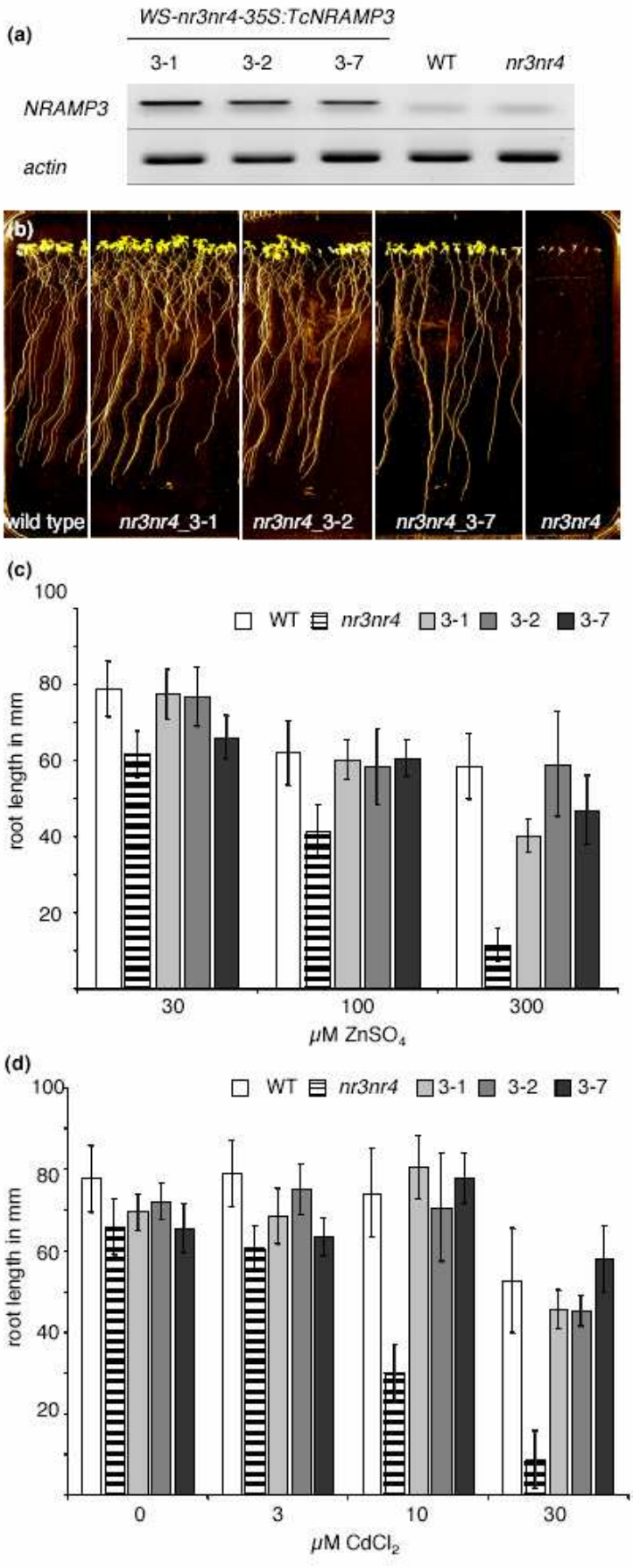
(a)
WS-nr3nr4-35S:TcNRAMP3
3-1
3-2
3-7
WT
nr3nr4
NRAMP3
actin
(b)
wild type
nr3nr4_3-1
nr3nr4_3-2
nr3nr4_3-7
nr3nr4
(c)
WT
nr3nr4
3-1
3-2
3-7
root length in mm
100
80
60
40
20
30
100
300
µM ZnSO4
(d)
WT
nr3nr4
3-1
3-2
3-7
root length in mm
100
80
60
40
20
0
3
10
30
µM CdCl2

Fig 7 Expression of *TcNRAMP3* in the *nramp3nramp4* double mutant complements its phenotypes on Fe-deficiency and Zn and Cd-excess.

(a) Semi-quantitative RT-PCR of *TcNRAMP3* and Actin (RNA concentration control) expression in the three *nramp3nramp4 35S::TcNRAMP3* lines, a wild-type and *nramp3nramp4* control. PCR reactions of 22 (TcNRAMP3) and 20 (Actin) cycles were performed and visualised as before. (b) Seeds of the three *nramp3nramp4 35S::TcNRAMP3* lines were sawn on Fe-deficient medium (ABIS with 0.3 µM FeHBED), together with wild-type WS as positive control and *nramp3nramp4* as negative control and grown vertically for 11 days. (c) Root length of the same genotypes grown vertically for 11 days on ABIS containing 50 µM FeHBED and 30 (normal), 100 or 300 µM $ZnSO_4$. Values are the average of measurements on 10-12 roots and bars represent SD. (d) Root length of the same genotypes grown vertically for 11 days on ABIS containing 50 µM FeHBED and 0, 3, 10 or 30 µM $CdCl_2$. Values are the average of measurements on 10-12 roots and bars represent SD.

Fig 8

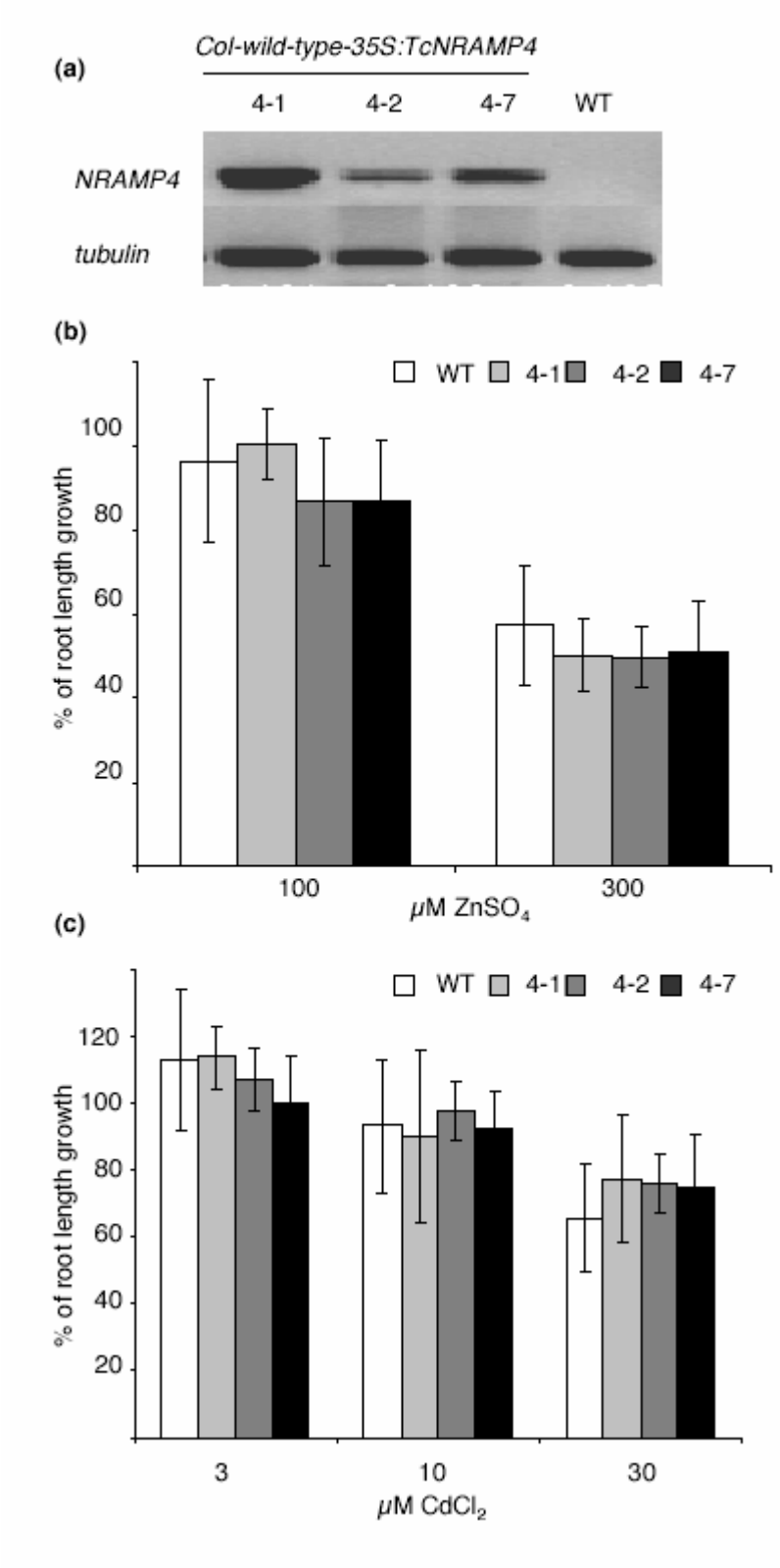

Fig 8 35S::TcNRAMP4 expression in *A. thaliana* (Columbia) results in unaltered tolerance to Cd or Zn excess. (a) Semi-quantitative RT-PCR analysis of *TcNRAMP4* and *Tubulin* (as control) expression in the *35S::TcNRAMP4* expressing lines and a Col wild-type control. (b) Seedlings of the wild type

and *TcRNAMP4* expressing lines were grown vertically on regular ABIS medium containing 30 (normal), 100 or 300 µM $ZnSO_4$. Root growth was measured for 10-12 plants per genotype per condition. The graph shows the percentage of root growth on 100 and 300 µM of $ZnSO_4$, with the root length on 30 µM $ZnSO_4$ set at 100%. Bars represent SD. (c) Root growth in seedlings of wild type and *TcNRAMP4* expressing lines on medium containing 3, 10, 30 µM $CdCl_2$ expressed as a percentage of the growth per genotype on no Cd containing medium. For each genotype the root length of 10-12 plants was measured and bars represent SD.

Chapter 6

General discussion

The content of essential micronutrient minerals in crops is very important for reduction of micronutrient malnutrition in the human diet. Mineral accumulation in plant is a rather complex physiological trait under complex genetic control and greatly influenced by the environment, e.g. the mineral content and bioavailability of the soil. Low contents will be easier leading to mineral deficiencies and high contents will lead to mineral toxicities in plants. Considering the complex genetic control of mineral homeostasis in plants, there is likely to be substantial genetic variation for this control, which means that the improvement of mineral accumulation and tolerance to low or excess mineral stress can be achieved by classical breeding or genetic engineering. The studies reported in this thesis mainly aimed at exploring genetic potentials for the improvement of Zn accumulation in *B. rapa* vegetable crops for these three aspects. To this aim, we performed natural variation screening and quantitative trait analysis for mineral content and Zn stress tolerance in *B. rapa* and cloning of Zn transporter genes from hyperaccumulator *Thlaspi caerulescens*.

Genetics of Zn accumulation in *B. rapa*

The genetic variation of micronutrient content in crops is one of the most powerful tools for achieving a nutrient balance in a given diet on a large scale (Graham et al., 2001). The natural variation was initially investigated in a large germplasm collection with accessions from nine cultivar groups covering most of the geographic distribution of *B. rapa* vegetables in China. With the screening, we showed that there is markedly genotypic variation for leaf Zn, Fe and Mn concentration and tolerance to Zn nutrition stress in *B. rapa* (Chapter 2). The natural variation in metal accumulation and tolerance to Zn stress provides genetic material for breeding programs and also the opportunity for further dissection of the genetics of these traits. We showed that there was no correlation between Zn concentration under normal Zn condition and tolerance to high or low Zn stress, which was in constant with the absence of co-localization of quantitative trait loci (QTL) for these traits in the genetic analysis of a *B. rapa* ssp. *pekinesis* DH population (Chapter 3). This fits with observations made for the hyperaccumulator plant species *T. caerulescens* and *A. halleri* that Zn

accumulation and tolerance are under independent genetic control (Macnair and Smirnoff, 1999; Assunção et al., 2003a; Filatov et al., 2006; Willems et al., 2007).

The QTL analysis of the *B. rapa* DH population was used to further dissect the genetics of mineral accumulation in *B. rapa* (Chapter 3). Seven QTLs related to Na, Mg, P, Mn, Zn, Sr concentrations in leaves were identified, while no QTL was found for K, Ca, Al, Fe and Cu. This together with the fact that no major QTL (with an explained variance exceeding 30% of the genetic variance) was detected in the present study underlines the complex genetic control involving many loci with low contribution to mineral accumulation in *B. rapa*. Furthermore this indicates that the genetic improvement of these traits by classical breeding will be very complicated requiring combination of many genes contributing to the traits. Taking into account the strong effect of environment on the expression of traits as was also observed for Arabidopsis (A. Ghandilyan and M.G.M. Aarts, unpublished data), this may well run into practical limitations prohibiting breeding progress altogether.

However, part of our inability to discover QTLs for some mineral accumulations may well be caused by the nature of the mapping population that was used. We used a DH population in our study, which has the advantage over F2 or F3 populations to allow repeated phenotypic testing of genotypes due to their genetic homozygosity. However, the two parental lines are both of the “Chinese cabbage”-type, which means they are fairly closely related and therefore the low range of variation within the population does not improve the efficiency in QTL detection in this study. Furthermore there was a large amount of markers (70%) showed skewed segregation which is also believed to reduce the power of QTL detection and to affect the estimate of QTL effects, because it reduces the effective size of the progeny by reducing the size of one genotypic class (Bradshaw et al., 1998).

One of the possible advantages of QTL analysis in *B. rapa* over species from other families is that it is related to the general plant reference species *Arabidopsis thaliana* (Arabidopsis) of which the full genome has been sequenced. With increasing knowledge on the synteny between this crop and Arabidopsis it may allow the recognition of similar loci on the bases of map position. The recent advances in comparative genomics in the Brassicaceae family are paving the way for a unified comparative genomic framework (Schranz et al., 2006). However, at the moment there is not yet sufficient genome synteny information available to compare the *B. rapa* QTLs detected in this thesis with previous reports on mineral accumulation and tolerance in Arabidopsis (Vreugdenhil *et al*., 2004;

Harada and Leigh, 2006; Reymond et al., 2006) and *Thlaspi caerulescens* (Assunção et al. 2006; Deniau et al. 2006). With the development of the comparative mapping in Brassicaceae species, the updated framework will accelerate the transfer of information from model species like Arabidopsis and *T. caerulescens* to *Brassica* crops and the integration of the knowledge from all these species.

Cloning and functional analysis of metal transporters

Metal transporters play a key role in metal accumulation and homeostasis in plants (Nelson, 1999). Comparing metal hyperaccumulator and non-accumulator plant species offers a good approach for dissection of metal accumulation and homeostasis both at the physiological/biochemical and the molecular level (Assunção et al, 2003b). Therefore full-length cDNAs of metal transporters were cloned from the Zn/Cd/Mn hyperaccumulator *T. caerulescens* to explore the possibility of genetic engineering for micronutrient accumulation and tolerance to excess soil mineral stress in crops (Chapter 4 and 5). It has been proposed that the hyperaccumulation is more likely due to the altered regulation of metal homeostasis genes rather than involving genes with new functions (Pence et al., 2000). This hypothesis is largely based on the fact that some metal transporters genes identified from the hyperaccumulators *T. caerulescens* or *A. halleri* are constitutively higher expressed compared with their orthologues in related non-accumulator species (Pence et al., 2000; Assunção et al., 2001; Dräger et al., 2004). However, recent analysis also showed that the metal specificity of a transporter can be altered in *T. caerulescens* compared to Arabidopsis (Talukdar S, 2007). In Chapter 4 and Chapter 5 we showed that two *ZIP* genes (*TcZNT5* and *TcZNT6*) and two *NRAMP* genes (*NRAMP3* and *NRAMP4*) are much higher expressed in *T. caerulescens* than their respective orthologues in *A. thaliana* regardless of the external Zn or Cd supply. This strongly suggests a function of the proteins encoded by these genes in metal hyperaccumulation or tolerance in *T. caerulescens*. DNA blot analysis revealed that the different expression levels are unlikely to be caused by a much higher copy number of these genes in the hyperaccumulator. The differential regulation of metal transporter genes in the hyperaccumulator species indicates that the enhanced expression of these genes by genetic engineering is a feasible option for improvement of metal content in crops.

To further characterize the function of these genes in metal homeostasis and to try to mimic the high expression of these genes in the metal hyperaccumulator, we overexpressed cDNAs for the identified metal transporters in Arabidopsis. Unfortunately, no phenotypic change in root growth under

stressful Fe, Zn or Cd conditions was observed for the overexpression lines of the two *NRAMP* genes and *TcZNT5*. This suggests that enhancing the expression level of single transporter genes will have little effect on improving the total metal accumulation of the plant. Most likely the general network of metal homeostasis is sufficiently robust to withstand relatively minor rearrangements of the homeostasis machinery. As the network of metal homeostasis in plant is tightly controlled at the organismal as well as the cellular level (Clemens, 2001), only enhancing the expression of genes at rate limiting steps will potentially generate obvious phenotypes.

In contrast to the absence of a phenotypic change for the over-expression of *TcNRAMP3, TcNRAMP4* and *TCZNT5*, the over-expression of *TcZNT6* increased the sensitivity to Cd as shown by a significantly reduced root length when compared to wild type (Chapter 4). The reports so far on plant ZIP proteins showed they function at the plasma membrane to influx cations to cytoplasm (reviewed in Hall and Williams, 2003). Nevertheless, the ZRT3 protein in yeast was proposed to function in mobilization of stored Zn from the vacuole (MacDiarmid et al., 2000). Although such vacuolar ZIPs have not been reported for plants yet, we cannot exclude that ZIP6/ZNT6 has a vacuolar localization. We can hardly propose the exact function and location of ZNT5 and ZNT6 from the result in this thesis, however, the ongoing analysis of mineral accumulation in the over expression lines of *TcZNT5* and *TcZNT6* will be helpful in clarifying the function of these two proteins in metal homeostasis. The sub-cellular targeting of TcZNT5 and TcZNT6 is also very important for declaring their functions in metal transport.

Molecular strategy for improvement of mineral accumulation and tolerance

The achievements in genetic engineering of new crop plants hold the promise of dramatic improvement in the nutritional balance (Graham et al., 2001), while a major potential problem for genetic improvement of plants for essential micronutrients is that the lack of metal specificity of uptake and distribution systems could lead to the accumulation of nonessential and even toxic elements (Clemens et al., 2002). The genes encoding transporters specific for one or more essential mineral elements are the most favourable candidates for either genetic engineering or marker assisted breeding. Unfortunately Fe transporters often transport Cd as well. However, there are exceptions, like the vacuolar membrane transporter VIT1 which is an iron transporter without Cd transport capacity (Kim et al., 2006). Also the plasma membrane transporter IRT1 of Arabidopsis can be

engineered to lose its Cd transporting ability (Rogers et al., 2000), therefore there are good prospects of biotechnological applications of metal specific essential mineral transporters in crops.

As the complete genome sequencing of *Brassica rapa* is on its way (Multinational Brassica Genome Project, http://www.brassica.info) the sequence information of *B. rapa* will soon facilitate the identification of metal transporters based on the available molecular genetic knowledge from Arabidopsis and *T. caerulescens*. This will be a very important step towards improving the mineral content in *B. rapa* crops either by development of gene specific markers for the marker assisted selection or by genetic engineering, both important components of molecular breeding strategies. The integration of information obtained from genomic approaches with genetic-based breeding will accelerate the success for metal accumulation and tolerance to external mineral stress.

However, experience of others (Sreenivasulu et al., 2007) and ourselves in overexpressing single genes taught us that in most cases constitutive overexpression of stress-tolerance genes is likely to cause unwanted effects if any. Therefore the improvement of mineral content for crops by genetic engineering may require a much more coordinated change of different processes of the whole metal homeostasis network, including uptake, buffering, translocation and storage. This will require the alteration of expression of more genes and only in certain tissues or at certain developmental stages of in response to certain environmental stimuli rather than constitutively in the whole plant. Therefore, it is highly desirable to obtain relevant organ-specific and stress-responsive specific promoters. Such promoters could also be used to prevent gene silencing when gene pyramiding is sought as a feasible strategy to obtain higher tolerance levels. To this end, a better understanding of the mechanisms underlying functional diversity of the metal transporters is certainly required.

More knowledge on the regulation of specific genes is another prerequisite for genetic engineering. The accumulation of most minerals in plant is tightly controlled because they are hazardous when present in too high or too low cytoplasmis concentration because they resemble toxic elements, such as Cd. IRT1, the major Fe transporter in plants, is controlled at the level of transcription and protein accumulation (Connolly et al., 2002). In the case of the post-translational regulation, the over-expression of certain transporter genes will not generate phenotypic changes because the protein degraded soon after the translation. Therefore revealing the complex regulatory networks controlling mineral accumulation mechanisms is certainly required for genetic engineering of mineral accumulation improvement, which means that there is still a long way ahead to realize this aim.

In summary, the research in this thesis revealed the existence of genetic variation on mineral accumulation and tolerance to Zn stress in *B. rapa*, and led to the identification of QTLs related with mineral accumulation and Zn stress tolerance. This provides clues for a genetic approach to improve these traits in *B. rapa* vegetables. Several candidate genes for genetic engineering of tolerance to external metal stress were also analyzed in this thesis, although more studies need to be done, such as the subcellular location of these proteins, the leafy mineral concentration in the over-expression lines and the phenotypic change by combining overexpression or preferentially temporary, spatially or environmentally controlled expression of several metal transporter genes. The development of the *B. rapa* sequencing program and the increasing knowledge of molecular basis of metal homeostasis network in plants will provide a more concert basis for molecular breeding of improved mineral content in *B. rapa* vegetables.

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Summary

Paving the way for genetic improvement for Zn accumulation in *Brassica rapa*

Brassica rapa L. comprises several vegetable crops, some of which are among the most important vegetables in China, serving as one of the main resources of mineral nutrition for Chinese people. However, the knowledge on the genetics of micronutrient accumulation, including Zn, Fe and Mn, is lacking in *B. rapa*. By exploring the genetic potential for the improvement of Zn accumulation in *B. rapa* vegetables I expect to contribute to the reduction of micronutrient malnutrition in China.

To characterize the natural variation of Zn accumulation and Zn response upon exposure to different Zn concentrations, I screened a large collection of germplasm representing nine cultivar groups covering the geographic distribution of *B. rapa* vegetables in China (Chapter 2). The result revealed that there was marked variation in accumulation of Zn (23.2-159.9 µg g^{-1}), Fe (60.3-350.1 µg g^{-1}) and Mn (20.9-53.3 µg g^{-1}) in *B. rapa*. Zn accumulation correlated with Fe or Mn accumulation both under normal and deficient Zn supply. No significant correlation was detected for the accumulation of these three elements with cultivar groups. A two-fold variation was found for dry-biomass based tolerance to Zn deficiency or excessive Zn. The wide natural variation provides a base for the genetic dissection by quantitative trait locus (QTL) analysis or for developing breeding programs for improved mineral content.

QTL analysis is a powerful tool in dissection of complex genetic traits. A doubled haploid (DH) population developed from two Chinese cabbage (*B. rapa*. ssp. *pekinesis*) varieties was used for QTL analysis of the accumulation of 11 minerals in leaves and for tolerance to deficient or toxic Zn supplies (Chapter 3). The trait analysis showed significantly positive correlations between the leaf concentrations of the tested minerals, indicating that this must be taken into consideration in breeding programs, especially when toxic minerals (Cd, As) are involved. Seven QTLs were detected for Na, Mg, P, Mn, Zn and Sr leaf concentrations, with an explained variance ranging from 11.1 % to 18.2 % . The tolerance to Zn stress was evaluated by dry shoot biomass of plants grown under different Zn supplies. One common QTL was found affecting Shoot Dry Biomass (SDB) under normal,

deficient and excessive Zn supply conditions. An additional QTL was detected for SDB only under Zn excess stress, with an explained variance of 13.0 %. The fact that no major QTL was detected indicates the complexity of the genetic control of the traits, probably involving many loci, each with a limited contribution to mineral accumulation in *B. rapa*. Furthermore this indicates that the genetic improvement of these traits by classical breeding will be very complicated, as it will require a combination of favourable alleles at many of the genes contributing to the traits. Screening additional populations for larger effect QTLs may provide an alternative.

Metal transporters play important roles in maintaining metal homeostasis in plants and are involved in processes of metal uptake, internal transport and storage. *Thlaspi caerulescens* J&C Presl. is a Zn/Cd/Ni hyperaccumulator, which has been used as a model for plant metal homeostasis research. It has been proposed that the hyperaccumulation is most likely due to the different regulation of the metal homeostasis genes rather than involving genes with novel functions (Van de Mortel et al., 2006). I cloned four metal transporter full-length cDNAs by screening *T. caerulescens* cDNA libraries, encoding two ZIP transporters (*TcZNT5* and *TcZNT6*, in Chapter 4) and two so-called NRAMP transporters (*TcNRAMP3* and *TcNRAMP4*, in Chapter 5). These four genes showed strongly increased expression in *T. caerulescens*, as compared to the non-accumulator *Arabidopsis thaliana* in a wide range of Zn supplies or in the presence of Cd, indicated that these genes were involved in metal hyperaccumulation or tolerance in *T. caerulescens*. Expression analysis in two *T. caerulescens* accessions with contrasting Cd accumulation (La Calamine and Ganges) further revealed that their difference in Cd accumulation ability was associated with differences in the regulation of the expression of ZNTs and NRAMPs .

Loss of function of the *AtZIP5* gene in Arabidopsis increased its tolerance to Cd as measured by reduced root growth, but overexpression of the *T. caerulescens* orthologue *TcZNT5* in Arabidopsis did not lead to any change in phenotype. In contrast, loss of function of *AtZIP6* in Arabidopsis did not change its phenotype, but overexpression of the *T. caerulescens* orthologue *TcZNT6* in Arabidopsis increased Cd sensitivity.

The Arabidopsis double mutant *nramp3 nramp4* showed hypersensitivity to Cd and toxic Zn, in addition to its previously reported hypersensitivity to Fe deficiency. Our study did not reveal functional differences between the *T. caerulescens* and *A. thaliana* NRAMPs, which transport the same metals when heterologously expressed in yeast and all four are localized at the vacuolar

membrane in plants. Furthermore the phenotype caused by the inactivation of the *AtNRAMP3* and *AtNRAMP4* genes in Arabidopsis can be rescued by expression of *TcNRAMP3*. Nevertheless, neither *nramp3 nramp4* plants expressing *TcNRAMP3*, nor wild-type plants expressing *TcNRAMP4* at high levels show enhanced Zn or Cd tolerance compared to wild type Arabidopsis.

The data presented in this thesis indicate that enhanced Zn accumulation, Zn tolerance or Cd tolerance probably requires elevated and controlled expression of a set of genes, rather than only single metal transporter genes.

Samenvatting

Op weg naar genetische verbetering van Zn accumulatie in *Brassica rapa*

Brassica rapa L. omvat verschillende groentegewassen, waaronder enkele van de meest belangrijke groentes in China. Deze dienen als een belangrijke bron van mineralen in het dieet van een groot deel van de Chinese bevolking. De genetische kennis van de accumulatie van micronutriënten, zoals zink (Zn), ijzer (Fe) en mangaan (Mn), in *B. rapa* is echter zeer spaarzaam. Door het genetische potentieel voor de verbetering van Zn accumulatie in *B. rapa* groentes te onderzoeken verwacht ik een nuttige bijdrage te kunnen leveren aan het verminderen van micronutriënt ondervoeding in China.

Om de natuurlijke variatie van Zn accumulatie en de respons op blootstelling aan verschillende Zn concentraties te karakteriseren, heb ik een grote collectie van *B. rapa* groenteaccessies onderzocht. Deze collectie omvatte vertegenwoordigers van alle negen cultivargroepen die de volledige geografische distributie van *B. rapa* in China bestrijken (Hoofdstuk 2). Uit dit onderzoek bleek dat er een duidelijke variatie aanwezig is in *B. rapa* voor de accumulatie van Zn (23.2-159.9 µg g^{-1}), Fe (60.3-350.1 µg g^{-1}) en Mn (20.9-53.3 µg g^{-1}). Accumulatie van Zn correleert met die van Fe en Mn, zowel onder normale Zn gift als onder Zn tekort (ondermaat Zn). Er werd geen significante correlatie gevonden voor de accumulatie van deze drie elementen met de verschillende cultivargroepen. Twee-voudige variatie werd gevonden voor tolerantie voor ondermaat of overmaat Zn op basis van de hoeveelheid droge stof. De gevonden brede natuurlijke variatie voor Zn accumulatie en tolerantie biedt de basis voor genetische ontrafeling van deze eigenschappen middels de genetisch analyse van loci voor quantitative eigenschappen (QTL) of voor het ontwikkelen van veredelingsprogramma's voor verbetering van mineraalgehaltes.

QTL analyse is een efficiënte methode om complexe genetische eigenschappen te ontrafelen. Voor de QTL analyses van de accumulatie van 11 mineralen in blad en voor de QTL analyse van de tolerantie voor ondermaat of overmaat Zn gift is gebruik gemaakt van een verdubbelde haploid (DH) populatie afgeleid van een kruising tussen twee Chinese koolvariëteiten (*B. rapa* ssp. *pekinensis*; Hoofdstuk 3). De analyse van de accumulaties liet significant positieve correlaties zien tussen de bladconcentraties van de geteste mineralen. Dit is nuttige informatie voor het ontwikkelen van veredelingsprogramma's,

vooral in verband met het voorkomen van ongewenste co-accumulatie van toxische mineralen (Cd, As). Zeven QTLs zijn gevonden voor Na, Mg, P, Mn, Zn en Sr-accumulatie in blad, met een verklaarde variantie van 11.1-18.2 %. De tolerantie voor Zn stress (te veel of te weinig Zn) is bepaald aan de hand van de droge stof opbrengst van planten gegroeid onder verschillende Zn giften. Er is één gemeenschappelijke QTL gevonden voor droge stof in scheut (SDB) onder normale, ondermaat en overmaat Zn en één additionele QTL uitsluitend voor SDB onder Zn overmaat met een verklaarde variantie van 13.0 %. Er is geen majeure QTL gevonden, hetgeen waarschijnlijk veroorzaakt wordt door de genetische complexiteit van mineraalaccumulatie, waar veel genetische loci bij betrokken lijken te zijn, elk met een relatief klein effect op de mineraalaccumulatie in *B. rapa*. Tevens geeft dit aan dat verbetering van mineraalaccumulatie door klassieke veredeling zeer moeilijk zal zijn, aangezien een moeilijk te verkrijgen combinatie van gunstige allelen op verschillende loci nodig zal zijn. Het onderzoeken van splitsende populaties, gebaseerd op andere ouderlijnen, voor QTLs met een groter effect kan hiervoor een uitkomst bieden.

Metaaltransporteiwitten betrokken bij metaalopname, intern metaaltransport en metaalopslag spelen een belangrijke rol bij het handhaven van metaalhomeostase in een plant. *Thlaspi caerulescens* J&C Presl. is een Zn/Cd/Ni hyperaccumulatorsoort, die veel gebruikt wordt als model voor metaalhomeostase onderzoek in planten. Eerder is gesuggereerd dat de hyperaccumulatie in deze soort waarschijnlijk veroorzaakt wordt door differentiële regulatie van algemeen voorkomende metaalhomeostasegenen en niet zozeer door de aanwezigheid van geheel nieuwe genen specifiek voor de metaalhyperaccumulator (Van de Mortel et al., 2006). Ik heb vier volledige metaaltransporter cDNAs gekloneerd na het doorzoeken van een *T. caerulescens* cDNA bank. Deze coderen voor twee ZIP transportereiwitten (*TcZNT5* en *TcZNT6*; Hoofdstuk 4) en twee NRAMP transportereiwitten (*TcNRAMP3* en *TcNRAMP4*; Hoofdstuk 5). Deze vier genen laten een sterk verhoogde expressie zien in *T. caerulescens* vergeleken met dezelfde genen in de niet-accumulator *Arabidopsis thaliana* (Arabidopsis). Deze verhoogde expressie wordt nauwelijks beïnvloed door de concentratie Zn in het groeimedium of door de aanwezigheid van Cd, dit in tegenstelling tot de expressie van Arabidopsis genen, hetgeen een sterke aanwijzing is dat deze genen betrokken zijn bij Zn hyperaccumulatie of hypertolerantie in *T. caerulescens*. Vergelijkende analyse van de expressie van deze genen in twee *T. caerulescens* accessies (La Calamine en Ganges) met een verschillende Cd- accumulatie liet verder zien dat dit verschil in Cd accumulatie sterk geassocieerd is met verschillen in de regulatie van

expressie van deze *ZNT* en *NRAMP* genen.

Uitschakeling van het *AtZIP5* gen in Arabidopsis verhoogde de tolerantie voor Cd, zoals bepaald aan de hand van wortelgroei, maar overexpressie van de *T. caerulescens* ortholoog *TcZNT5* in Arabidopsis leidde helaas niet tot een verandering in fenotype. Dit in tegenstelling tot de overexpressie van het *T. caerulescens TcZNT6* gen dat zorgde voor een verhoogde gevoeligheid voor Cd in Arabidopsis, terwijl uitschakeling van het orthologe *AtZIP6* gen in Arabidopsis niet tot een verandering in fenotype leidde.

De Arabidopsis *nramp3nramp4* dubbelmutant vertoont een overgevoeligheid voor overmaat Cd of Zn, bovenop de eerder aangetoonde overgevoeligheid voor een tekort aan Fe. Ons onderzoek bracht geen functionele verschillen tussen de *T. caerulescens* en Arabidopsis NRAMP transporters aan het licht. De orthologe eiwitten transporteren dezelfde mineralen als ze tot heterologe expressie gebracht worden in gist. Alle vier de eiwitten bevinden zich normaliter in de vacuolaire membraan in een plantencel. Uitschakeling van de Arabidopsis *AtNRAMP3* en *AtNRAMP4* genen kan gecompenseerd worden door expressie van het *TcNRAMP3* gen. Echter sterke expressie van *TcNRAMP3* in Arabidopsis *nramp3nramp4* dubbelmutanten, noch sterke expressie van *TcNRAMP4* in wildtype planten, leidde tot een verhoogde tolerantie voor Zn of Cd overmaat in vergelijking met ongetransformeerde wildtype controleplanten.

De resultaten van de experimenten die in dit proefschrift beschreven worden geven aan dat het verhogen van Zn accumulatie of tolerantie voor Zn tekort of Zn/Cd overmaat waarschijnlijk alleen bereikt kan worden door de verhoogde en gecontroleerde expressie van een set genen in plaats van een enkel metaaltransporter gen.

中文摘要

武剑

白菜类蔬菜由多个栽培种群组成，包括中国最重要的蔬菜作物，也是中国人口重要的矿质营养来源之一。但是目前对白菜类作物的矿质元素积累，包括 Zn 和 Fe，遗传机理的了解还非常有限。研究白菜类蔬菜矿质元素积累的遗传控制和改良潜力，将对降低中国人口中存在的微量元素不良的状况起到促进作用。

首先，我们对白菜类蔬菜的 Zn 积累和 Zn 胁迫条件下反应的自然变异进行了大规模的种质资源筛选。这些材料由 9 个栽培种群组成，代表了中国不同地理分布的白菜类蔬菜（第二章）。结果表明，白菜类蔬菜存在显著 Zn、Fe、Mn 积累的自然变异，其中 Zn 为 23.2-159.9 $\mu g\ g^{-1}$，Fe 为 60.3-350.1 $\mu g\ g^{-1}$，Mn 为 20.9-53.3 $\mu g\ g^{-1}$。在正常供 Zn 水平和缺 Zn 条件下，叶片的 Zn 积累与 Fe 和 Mn 的积累均相关。Zn、Fe、Mn 的积累与栽培种群均无关。以地上部干物重为参数对白菜类蔬菜对高锌胁迫和缺锌胁迫的耐性进行比较，结果显示白菜类蔬菜中存在大约 2 倍的对锌胁迫耐性的自然变异。这些自然变异为白菜类蔬菜的锌积累的数量性状位点(QTL)分析和常规育种改良白菜类蔬菜的锌积累提供了基础。

QTL 分析是解析复杂性状遗传的有力工具。研究中我们利用大白菜的双单倍体（DH）群体对 11 种矿质元素在叶片中的积累和高锌和缺锌条件下的耐性进行了 QTL 分析（第三章）。性状分析表明叶片中 11 种矿质元素的含量存在显著正相关，这说明在进行白菜类蔬菜的矿质元素含量改良育种时，必须考虑到不同矿质元素积累的协同性，特别是当涉及到有毒元素的时候。研究中共检测到 7 个控制叶片 Na、Mg、P、Mn、Zn 和 Sr 含量的 QTL，贡献率为 18.2 %－11.1 %。对锌胁迫耐性的评价以在不同锌处理条件下植株地上部的干物重为参数，检测到 1 个在正常、高锌和缺锌胁迫下都出现的 QTL。研究中我们没有检测到主效 QTL（贡献率>30%），说明这些性状受到复杂的遗传控制，有可能涉及到许多位点，而每个位点对于白菜类蔬菜的叶片矿质元素积累的效益值不大。此外，这也说明利用常规育种对这些性状进行改良的过程会非常复杂，需要将控制这些性状的许多位点进行组合。本研究中使用的 DH 群体是来自 2 个大白菜 DH 系杂交的 F1，因此遗传背景相对狭窄，这也限制了 QTL 检测的能力，对其他遗传背景更广泛的群体进行分析，例如来自不同栽培种群的材料构建的群体，有可能检测到具有更高效应值的位点。

金属转运蛋白在维持植物体金属平衡方面起着重要作用，它们在金属的吸收，运输和贮存过程中具有重要作用。*Thlaspi caerulescens* 是锌/铁/锰的超富集植物，是研究金属积累和平衡机理的模式植物。金属超富集的遗传机理被认为是对基因表达的调控的差异，而不是具有新功能的基因参与。我们通过对 *T. caerulescens* cDNA 文库进行筛选，克隆了四个金属转运蛋白的全长 cDNA，分别编码 2 个 ZIP 转运蛋白（ZNT5 和 ZNT6）和 2 个 NRAMP(NRAMP3 和 NRAMP4)转运蛋白。研究结果表明在不同的 Zn 供应水平和 Cd 处理条件下这四个基因在 *T. caerulescens* 中的表达大大高于在非富集植物 *Arabidopsis thaliana* 的表达。这说明这些基因可能参与到金属超富集和耐性中。对 *T. caerulescens* 具有不同 Cd 积累特性的两个生态型(La Calamine 和 Ganges)的这 4 个基因的表达进行比较，进一步说明 Cd 积累能力上的差异与这 2 个 ZNT 和 2 个 NRAMP 基因的表达调控差异相关。

由于没有现成可以利用的 *T. caerulescens* 的基因敲除突变体，我们对 *A. thaliana* 这 4 个基因的同源基因的突变体进行了分析。以根长为指标，*TcZNT5* 的同源基因 *AtZIP5* 的敲除提高了 Arabidopsis 对 Cd 处理的耐性，但在 Arabidopsis 中过表达 *TcZNT5* 没有显著的表型改变。相反，*TcZNT6* 的同源基因 *AtZIP6* 的基因敲除植株与野生型相比没有表型差异，但过表达 *TcZNT6* 的 Arabidopsis 植株对镉处理的敏感性提高。

Arabidopsis 的 *nramp3 nramp4* 双突变体已被报道对缺铁处理的敏感性增强，我们的研究结果表明 *nramp3 nramp4* 双突变体对镉和高锌处理也具有超强敏感性。我们的研究没有表明 NARMP3 和 NRAMP4 在超富集植物 *T. caerulescens* 和非富集植物 Arabidopsis 之间功能上的差异：它们在酵母中表达时具有相同的金属转运活性，并且亚细胞定位都位于液泡膜，Arabidopsis *nramp3 nramp4* 的植株表型能够被 *TcNRAMP3* 的表达所恢复。但是无论是在 *nramp3 nramp4* 背景中过表达 *TcNRAMP3*，还是在野生型背景中表达 *TcNRAMP4* 都不能提高 Arabidopsis 植株对高 Zn 处理和 Cd 处理的耐性。我们的数据表明提高植物的锌积累和对高锌胁迫或镉胁迫的耐性不是改变单个基因的表达能够实现的，需要提高或控制在金属吸收、运输和贮存等一系列环节中的基因的协同表达。

Curriculum vitae

Jian Wu was born in Henani (China) on the 25th of April 1974. She started her studies in Handan Agricultural College where she continued to work after graduation as a fellow of the horticulture department. In September 1999, she started to study in Nanjing Agricultural University where she obtained her M.Sc. degree in 2002 presenting a thesis entitled “High-efficiency Regeneration System of Radish”. Thereafter, she went to Institute of vegetables and Flowers-Chinese Academy of Agricultural Sciences (IVF-CAAS) to continue her study and research as a PhD student. In the same year he got the chance to join the “Joint PhD training program between WUR and CAAS” and became a sandwich PhD student under the supervision of Mark Aarts and Maarteen Koornneef at WU and Xiaowu Wang and Rifei Sun at IVF-CAAS.

Publication

1. **Wu J,** Schat H, Sun RF, Koornneef M, Wang XW, Aarts MGM. (2007) Characterization of natural variation for zinc, iron and manganese accumulation and zinc exposure response in *Brassica rapa* L. Plant and Soil 291: 167-180
2. **Wu J**, Zhao JJ, Song XF, Li Y, Li XN, Sun RF, Koornneef M, Aarts MGM, Wang XW. Mapping QTLs for mineral accumulation and shoot dry biomass yield under different Zn nutritional conditions in Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) Submitted
3. Oomen Ronald JFJ, **Wu J**, Lelievre F, Barbier-Brygoo H, Aarts MGM, Thomine S. Functional characterization of NRAMP3 and NRAMP4 from the metal hyperaccumulator *Thalspi caerulescens*: expression and role in heavy metal tolerance. Submitted
4. **Wu J**, Wang XW, Aarts MGM. Identification and functional analysis of two ZIP metal transporters of *Thalspi caerulescens*. To be submitted
5. Zhang Xiao-wei, **Wu Jian**, Zhao Jianjun, Song Xiaofei, Li ying, Zhang Yanguo, Xu Donghui, Sun Ri-fei, Yuan yuxing, Xie Conghua, Wang Xiao-wu. 2006. Identification of QTLs Influencing Bolting in *Brassica rapa* ssp. *pekinensis* (syn. *Brassica campestris* ssp. *pekinensis*). Agricultural Sciences in China 2006 5(4): 265-271
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