PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF HEAVY METAL TOLERANCE AND TRANSPORT IN THE HYPERACCUMULATOR PLANT SPECIES, *THLASPI CAERULESCENS*

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PHYSIOLOGICAL AND MOLECULAR MECHANISMS OF HEAVY METAL TOLERANCE AND TRANSPORT IN THE HYPERACCUMULATOR PLANT SPECIES, *THLASPI CAERULESCENS*

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Heavy metal pollution of the environment is significant problem throughout the world. One possible avenue for heavy metal decontamination of the environment is phytoremediation, which is a technology based on the remarkable abilities of certain plant species to tolerate and accumulate extremely high concentrations of heavy metals. One of the best known heavy metal hyperaccumulator plant species is *Thlaspi caerulescens*, which is a Zn/Cd-hyperaccumulator that can accumulate and tolerate up to 30,000 ppm Zn and 10,000 ppm Cd in the shoots without exhibiting toxicity symptoms. The research described in this dissertation focuses on identifying gene(s) that may be responsible for the extreme heavy metal accumulation phenotype in *Thlaspi caerulescens*.

In the research conducted here, it was demonstrated that xylem metal loading may play a key role in heavy metal hyperaccumulation. In initial studies, the influence of altered plant metal status on metal (Zn, Cd) accumulation in *Thlaspi caerulescens* showed that increased metal status stimulated subsequent heavy metal (Cd) accumulation in the shoots but not roots, suggesting that growth on high metal levels stimulates metal loading into the xylem. Subsequently, a heavy metal transporting P1B-type ATPase, *TcHMA4*, was cloned from *Thlaspi caerulescens* and shown to mediate cellular heavy metal efflux and tolerance when expressed in yeast. TcHMA4

is expressed primarily in the root vascular tissue and its expression is strongly upregulated upon exposure to high concentrations of heavy metals. These findings indicate that TcHMA4 may be responsible for metal xylem loading, and thus play a key role in the enhanced root to shoot metal translocation that is so important to hyperaccumulation. Furthermore, peptides derived from the C terminus of the TcHMA4 protein that harbor several heavy metal binding domains were shown to confer a significant increase in metal accumulation and tolerance when expressed in transgenic yeast (*Saccharomyces cerevisiae*) and plants (*Arabidopsis thaliana*). These findings indicate that the C terminus peptides have the capacity to serve as heavy metal binding ligands, and may be useful for enhancing the phytoremediation potential of plants via biotechnology.

BIOGRAPHICAL SKETCH

The author of this work, Ashot Papoyan, was born on April 1st 1977 (yes, the April Fools Day) in the capital of a small beautiful country Armenia, near Caucasus. Ashot has always had a great love for nature. At the very young age he started spending his summers with his grandparents in the village up in the mountains, which gave him a chance to study and learn great deal about animals, and plants far away from big cities and civilization. In 1983, at the age of 6, Ashot moved to Peru with his parents and brother, a South American country with amazing natural diversity which was so different from what he was used to see back home. This experience helped him better understand the complexity of natural world and different ecosystems, the contrast between the rainforests of Peru and dry mountains of Armenia. Papoyan family returned back to Armenia in 1986 when Ashot was 9 years old and he continued sharing his time between the city during the school semesters and the village during the summer. Soon his yearly fascination with biology started to pay off, when he participated and won several national Olympiads in biology during his high school years. After graduation from high school Ashot successfully passed the tests and was admitted to Yerevan State University's department of Biology, from which he graduated with honors under the supervision of Professor Emil Gevorgyan. With help and encouragement from his brother Ashot came to Cornell University as a summer student during his senior year at the Yerevan State University. He spent 4 months in the laboratory of Dr. Richard Cerione studying signal transduction mechanisms in the cell. During his stay in Ithaca Ashot came to introduce himself to Dr. Leon Kochian as a fellow Armenian. Little that he knew at the time, that Leon will become his good friend and mentor in the years to come. He returned back to Armenia and finished his bachelor's degree at Yerevan State University, and most importantly got married to his college sweetheart Helen. With support from Dr. Kochian Ashot was accepted to the

department of Plant Biology at Cornell University as a non-degree graduate student in the fall of 1999, and was transferred to a full degree program in the fall of 2000. It took him six years to complete his thesis and write this lines, may be because he also spent time with his friends, went hunting and fishing, bought a house, got a dog, became the President of Armenian Student Association at Cornell, and did many-many more activities that wonderful Ithaca and Cornell offer to newcomers.

I would like to dedicate this work to my beloved parents, Haykaz Papoyan and Hasmik Galstyan, for their unconditional love and support.

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I would like to thank all past and present members of Kochian Lab for their support and friendship during my program. I specifically would like to mention Dr. Miguel Pineros, whom I befriended from the beginning of my arrival to Kochian Lab, for all the guidance I have received during our everyday “coffee breaks”. I would also like to thank Jon Shaff for all the help that he has given to me during my experiments, and for answering my questions ranging from “how to make the particular solution” to “where can I buy stamps from”.

I want to thank my wife Helen for making my life colorful for last 12 years. We met the first day of our freshman year in University, and from that day on she has been my best friend. Her endless love and support helped me get through the difficult times. I would like to thank my parents for all the sacrifices they have made in the name of my education. They have never given up on me, even at the times when it was very difficult not to give up. At last but not least I would like to thank all my friends from Armenian community both from Ithaca and all over the U.S. for the friendship through these years, and for making me feel at home far away from home.

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CHAPTER I

Introduction and Literature Review

Introduction to "heavy metals".

The term "heavy metal" is a difficult term to define, since no single definition is available which clearly identifies an element as a heavy metal. This term was introduced into the scientific literature by the Danish scientist Niels Bjerrum (Bjerrum et al 1936), where he described heavy metals as elements that have a density of 7.0 g cm^{-3} and above. Later the density associated with heavy metals went through several changes and more recently, a metal with a density above 5 g cm^{-3} is sometimes defined as a heavy metal (Hawkes et al 1997). There have also been attempts to define the term "heavy metals" in other ways, for example, as those metals having a larger atomic weight than that of sodium, or elements that are able to form soaps when combined with fatty acids (Duffus et al 2002). However none of the available definitions of heavy metals are universal and, furthermore, there are many exceptions to each particular definition. In addition, the bioavailability of a metal and its effect on the plant and other organisms is also an important factor and needs to be taken in consideration when defining the term "heavy metal". Nevertheless, for the purposes of plant biology in general and for this work in particular, it seems acceptable to define a heavy metal" as an element with a density of more than 5.0 gr cm^{-3}. Divalent metals including zinc, cadmium, lead, and nickel, are considered heavy metals in this study in order to keep the nomenclature simple.

Heavy metals in the environment and heavy metal pollution.

Heavy metal pollution of the environment is an increasingly significant problem in the industrialized world. Heavy metals can enter the environment via several pathways, both natural and anthropogenic. The main sources of heavy metal

pollution are the mining industry, agriculture, and the automotive industry (Sterrett et al 1996). It is interesting to note that even in developed countries the automotive industry is the largest contributor to heavy metal pollution, in particular due to water run off from roads. For example, a major source of Zn contamination is from motor oil, brake emissions, and corrosion of galvanized parts, while metal tire components, fuel burning, and batteries have been a significant source of Cd and Pb contamination, (Falahi-Ardakani et al 1984). However, it should be noted that Pb contamination has been decreasing since the prohibition of leaded gasoline in the US, but many countries in the developing world still continue using leaded gasoline (Michaelowa et al 1998). Heavy metals pose a significant risk to human health, mainly due to the fact that heavy metals tend to bioaccumulate in tissues. Therefore, there can be a significant build up of heavy metals in internal organs such as the liver, kidneys, and lungs. Some of the heavy metals are also micronutrients and therefore are essential, at low levels, for the normal functioning of most of organisms, while other non-micronutrient heavy metals have no known biological function. Both essential and non-essential heavy metals can be extremely toxic at high concentrations.

Zinc

Zinc (Zn) is probably among the most interesting and important heavy metal to study since it plays an important role in many biological processes. However, other toxic heavy metals, such as Cd, can replace Zn in those processes, which in turn can lead to toxicity symptoms. Zinc is an essential micronutrient and is absolutely required for the normal functioning of all organisms. The affinity of Zn for binding with organic molecules is relatively low compared to other divalent heavy metals, such as cadmium and lead (Marschner 1995). This has important consequences with regards to metal toxicity, as the heavy metals with a stronger binding affinity can then often

replace zinc in metalloprotein complexes. More than 300 enzymes have been identified that require Zn for their normal function (Marschner 1995). Alcohol dehydrogenase, carbonic anhydrase, Cu/Zn superoxide dismutase, and alkaline phosphatase are examples of enzymes that require zinc for their normal biological function. The Zn requirement for enzyme function can be divided into three categories – the first group of enzymes require Zn as a structural component, the second group requires Zn for catalytic purposes, and in the third group of enzymes, Zn has a co-catalytic function (in such enzymes zinc has both catalytic and structural functions) (Marschner 1995). In addition, it has been shown that Zn plays an important role for processes associated with DNA replication and gene expression (Coleman et al 1992, Nagano et al 2001). Plants that are grown under sufficient Zn conditions accumulate between 20-100 ppm dry weight Zn in their shoot and roots. Under low Zn conditions a number of Zn deficiency symptoms may occur, including stunted shoot growth with a shortening of internodes and decreased leaf size, as well as leaf chlorosis and necrosis. Zn deficiency is a more common problem in high pH soils, where Zn is less bioavailable (Takkar et al 1993, Welch et al 1991). Conversely, in low pH soils Zn solubility and uptake increases, and when the Zn concentration in the tissues of non-hyperaccumulator plants reaches several hundred ppm, toxicity symptoms occur. Zn toxicity symptoms include inhibited root elongation, reduced photosynthesis, and iron deficiency (Marschner 1995). It is thought that excess Zn may be able to replace similarly sized ions such as Fe and Mg in different biological structures (Marschner 1995).

Cadmium

Cadmium (Cd) is an extremely toxic heavy metal that until recently was believed to not have any biological function. However, a biological function for Cd

has now been demonstrated for marine diatoms, where it is required for the proper function of carbonic anhydrase (Lane et al 2000). In plants, Cd is toxic at relatively low levels. For example, in non- hyperaccumulator plant species, Cd accumulation to concentrations higher than 50 ppm often results in severe toxicity with symptoms including stunted growth, leaf chlorosis, and reduced root growth. As mentioned above, Cd competes with Zn in many biological processes including competition for root Zn uptake and translocation to the shoot. Cadmium is very toxic to humans as well and exposure to high concentrations can cause severe illnesses such as osteoporosis, emphysema, irreversible renal tubular injury, anemia, and cancer (Wiesberg et al 2003). As discussed earlier, Cd can accumulate in the organism, for example, the average half life of cadmium in humans is 15-20 years (Jin et al 1998). Because of its danger to human health, there are strict regulations regarding the levels of Cd allowed in foods, which, in turn, is becoming an issue in agriculture. For example, most of the durum wheat produced in the United States is grown in the Northern plains area (North and South Dakota) that have naturally elevated levels of soil Cd, because the soils are derived from parent materials with elevated cadmium concentration. Consequently, the durum wheat grown in this region of the US (and Canada) often has slightly elevated levels of Cd in the grain. Many countries have reduced the maximum allowable grain Cd concentration, which, in turn, will create strict restrictions on the export of US and Canadian durum wheat (Norvell et al 2000).

Hyperaccumulator plant species and phytoremediation.

A small number of terrestrial plant species not only tolerate high levels of heavy metals in the soil but accumulate them to unusually high levels in their shoots. These fascinating plant species, first coined hyperaccumulators by (Brooks et al 1977), are loosely categorized as plants that can accumulate metals in the shoot from 100- to

1000-fold higher levels than normal, non-accumulator plants (McGrath et al 2002). Hyperaccumulating plant species have been identified for a number of heavy metals, including Ni, Zn and Cd, as well as for the metalloids, Se and As. Probably the best known metal hyperaccumulator is *Thlaspi caerulescens*, a member of the Brassica family that has been the object of interest in the plant biology community for over a century, based on its ability to colonize calamine and serpentine soils that contain naturally elevated levels of heavy metals such as Zn, Cd, Ni and Co. Certain ecotypes of *Thlaspi caerulescens* can accumulate Zn and Cd to extremely high levels in the shoot, with Zn reaching levels as high as 30,000 ppm (Brown et al 1995) and shoot Cd concentrations as high as 10,000 ppm (Lombi et al 2000). By comparison, shoot Zn concentrations in Zn sufficient non-accumulator plants are around 100 ppm, with 30 ppm defined as adequate and 300-500 ppm as toxic (Mengel and Kirkby 1987). The growing awareness of metal hyperaccumulating plants has spurred the considerable recent interest and research activity into phytoremediation as a "green" technology for the clean up of heavy metal contaminated soils. A number of laboratories around the world are studying metal hyperaccumulators such as *Thlaspi caerulescens* as model plant systems to gain a better understanding of the mechanisms of heavy metal hyperaccumulation and tolerance, as well as a potential source of genes for developing high biomass plant species that are better suited for phytoremediation of metal contaminated soils.

Phytoremediation is the use of plants to clean up environmental contamination of surface soils. It is a more cost effective and environmentally appealing approach than other currently available methods for soil detoxification, such as excavation and removal of the polluted soil to a chemical treatment facility or long term storage in special landfills. These methods are very costly ($100-400/cubic meter) for the decontamination of large areas and are usually quite destructive to the environment.

On the other hand, phytoremediation technologies are much less expensive, costing only $15-40/cubic meter and are much less perturbative to the environment (Schnoor et al 1997).

There are a number of different uses for plants under the broad umbrella of phytoremediation depending on the type and concentration of the contaminant as well as the desired end result. These are depicted and summarized in Figure 1.1 Phytoextraction is the use of plants to reduce the total metal concentration in the soil, via transfer of the metal from the soil to the above ground biomass where it can then be harvested, put through a volume reduction procedure (ashing or composting), and then the resulting metal-contaminated shoot ash stored in a special landfill. Phytovolatilization disperses contaminants into the atmosphere by the way of plant transpiration. Phytostabilization involves reducing the bioavailability or mobility of pollutants in the soil, which helps to prevent them from leaching into ground and surface waters. Phytodegradation uses plants and the soil microorganisms associated with roots to degrade organic pollutants in the soil. Phytoremediation can be used to reduce the soil levels of organic contaminants such as trinitrotoluene (TNT) or trichloroethylene (TCE), as well as inorganic contaminants such as heavy metals (Pilon-Smits et al 2005).

As described earlier, hyperaccumulator plant species for specific toxic metals have been identified, including those that hyperaccumulate Ni, Zn and Cd, As and Se. Table 1.1 shows a partial list of hyperaccumulating plant species for different toxic metals as well as the shoot metal concentrations that can be achieved by these plants. Unfortunately, most hyperaccumulator plant species are relatively slow growing and produce small amounts of shoot biomass; thus they are usually not good candidates

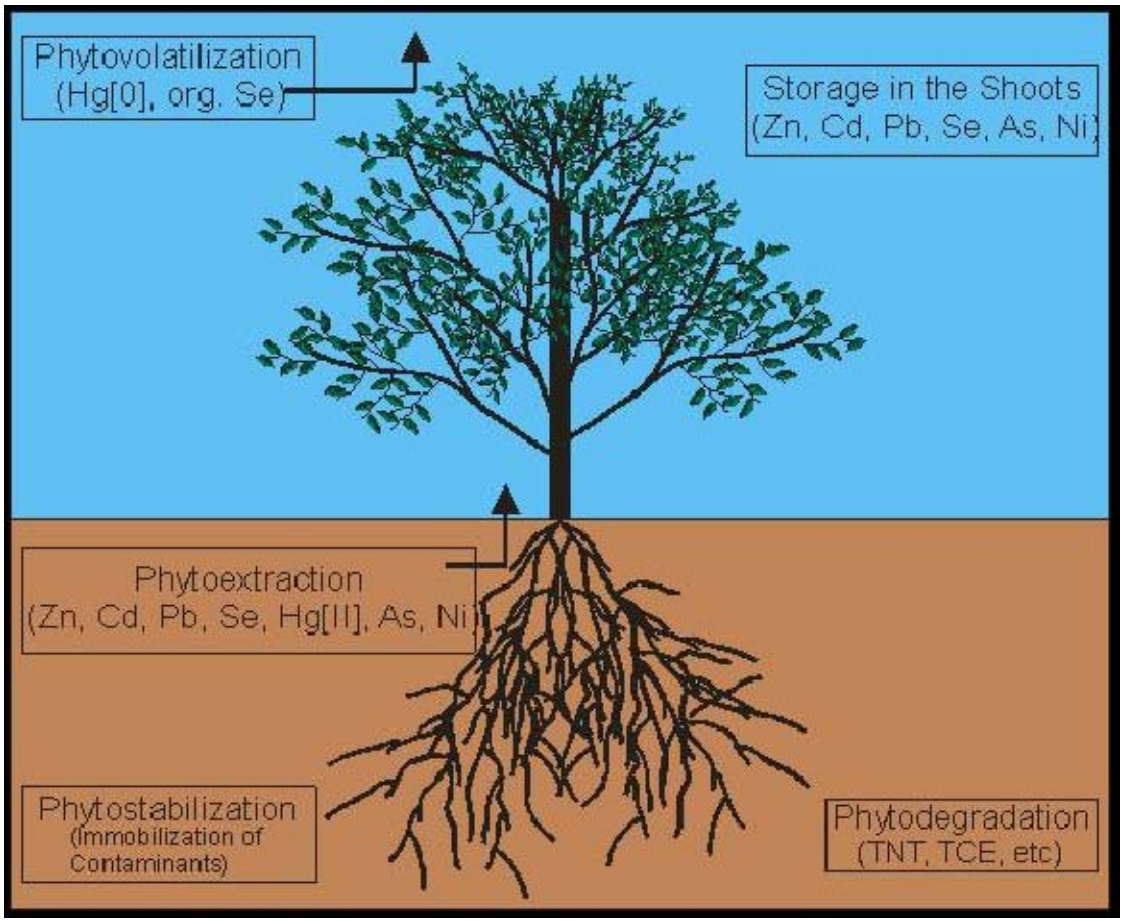

Figure 1.1 Depiction of the various uses of a plant growing on a contaminated soil with regards to environmental remediation.

Heavy metal phytoremediation involves phytoextraction of the toxic metal from the soil and the subsequent transport and storage of the metal in the shoots (for removal). For some toxic metals that form volatile organic forms in the shoot, plants can release these volatile forms into the air (phytovolatilization). Other uses of plants for remediation involve the degradation of organic contaminates in the soil by microbes associated with plant roots, and the use of plants to stabilize and reduce the bioavailability of certain contaminants (phytostabilization).

Table 1.1 List of Selected Naturally-Occurring Metal Hyperaccumulating Plant Species

Element	Plant Species	Metal Accumulation
Selenium (Se)	*Astragalus bisulcatus*	6,500 ppm (Pickering et al 2003)
Arsenic (As)	*Pteris vittata*	7,500 ppm (Ma et al 2001)
Zinc (Zn)	*Arabidopsis halleri*	30,000 ppm (Zhao et al 2000)
Cadmium (Cd)	*Thlaspi caerulescens*	14,000 ppm (Lombi et al 2000)
Nickel (Ni)	*Alyssum lesbiacum*	23,000 ppm (Kupper et al 2001)

for phytoremediation on a commercial scale (Baker et al 2000, Reeves et al 1999). The Zn/Cd hyperaccumulator, *Thlaspi caerulescens*, is an excellent model organism for studying the fundamental physiological and molecular mechanisms underlying heavy metal hyperaccumulation, because of it's small size and relatively easy growth in laboratory conditions. Scientists from a number of laboratories are studying hyperaccumulators such as *Thlaspi caerulescens* in order to identify metal hyperaccumulation genes and the associated mechanisms (Assuncao et al 2003). One of the goals of this research is to build a molecular toolbox that can be used to engineer plants that produce a large shoot biomass and also have the ability to tolerate and accumulate high concentrations of heavy metals in the shoots. In order for a plant to be useful in phytoremediation, it needs to have two distinct features: efficient and effective transport of heavy metals from the roots to the shoots and the ability to detoxify and store the transported heavy metal in the above-ground biomass.

Translocation of heavy metals in non-accumulator and hyperaccumulator plants.

As discussed earlier, it is important for plants to regulate the concentrations of micronutrients that are heavy metals since the micronutrient is essential for plant function but also toxic at high concentrations. This aspect needs to be kept in mind when considering the processes involved in root to shoot metal translocation. There are three fundamental components associated with the movement of metals from the soil to the shoot that must occur in a regulated manner. The first step is the transport of heavy metals into the root from the soil solution. After entering the root epidermal cells, ions move radially through the root symplast and/or apoplast to the root stele where xylem loading occurs. Thus all metal ions (and any other solutes moving from the root to the shoot) must cross the endodermis, where the Casparian strip effectively blocks the further radial apoplastic movement of ions and other solutes. At this cell

layer, the metal ions must cross the plasma membrane and enter the root symplasm to gain access to the stele. This step allows the plant to regulate the movement of ions into the xylem and limit transport of ions to the rest of the plant, if necessary. The next important step is loading of the ions into the xylem and subsequent translocation to the shoots via transpirational pull of water and solutes in the xylem. This step is believed to be mediated by the regulated efflux of metal ions from xylem parenchyma cells into the lumen of non-living xylem vessels (Taiz and Zeiger 1998). Xylem loading is also under tight metabolic control and regulated via xylem parenchyma plasma membrane transporters such H^+-ATPases and ion efflux channels. The last step is movement of metal ions from the xylem sap into the leaf with subsequent transport across the plasma membrane of leaf cells (Fisher et al 2000, Taiz and Zeiger 1998). These three controlled steps are present in both non-accumulator and hyperaccumulator plants and is achieved by metal transporters. In contrast, expression and the activity of these transporters are significantly different between accumulator and non-accumulator plants. Comparative physiological investigations of root Zn^{2+} influx between the hyperaccumulator, *Thlaspi caerulescens*, and the related non-accumulator, *Thlaspi arvense*, have shown root Zn^{2+} uptake is much larger in *Thlaspi caerulescens* (Lasat et al 1996). This increased root Zn uptake is associated with much higher expression of a candidate gene for the root Zn uptake transporter, *ZNT1*, which is a member of the ZIP family of micronutrient transporters, compared with the expression of its homolog in *Thlaspi arvense* (Pence et al 2000). Although *Thlaspi caerulescens* maintained a greater rate of heavy metal transport into the roots, root metal accumulation is much less in hyperaccumulator plants than in non-accumulator plants. In non accumulator plants such as *Thlaspi arvense*, heavy metals are stored and compartmentalized in the roots, which may be a defense mechanism to keep the toxic metals away from the shoots, as the photosynthetic apparatus is one of the most sensitive structures to heavy

metal toxicity in plants (Kupper et al 1998). In contrast, a much larger fraction of the heavy metals that enter the roots of *Thlaspi caerulescens* is actively transported to the shoot (Lasat et al 1998; 2000). Molecular data also support the previous findings that xylem metal loading is increased in hyperaccumulator plants compared to non-accumulator plants. HMA4 is a heavy metal transporter and member of the P-type ATPase family for which evidence has been published in *Arabidopsis* that it is a key player in the xylem loading of heavy metals in higher plants (Hussain et al 2004; Papoyan et al 2004, Verret et al 2004). As shown later in this dissertation, the expression of the HMA4 homolog in *Thlaspi caerulescens* is strongly upregulated by root exposure to high levels of Cd and Zn (Papoyan et al 2004), while the expression of the *Arabidopsis* HMA4 transporter is not upregulated by high metal levels (Mills et al 2003) (a detailed analysis of the *TcHMA4* transporter will be provided in Chapter 3). On the shoot level, non-hyperaccumulator plants employ metal binding ligands such as metallothioneins, phytochelatins, and organic acids to detoxify heavy metals (Cobbett et al 2002). However, it has been shown in *Thlaspi caerulescens* that phytochelatins probably do not play a significant role in heavy metal detoxification (Ebbs et al 2002). Instead, it is suggested that in hyperaccumulator plants, heavy metals are detoxified by sequestering them into the vacuoles (Krämer et al 2000) or storing them in specialized epidermal cells (Küpper et al 1999).

Channels, Carriers, and Pumps

Plant transporters play an important role in many essential metabolic processes throughout the plant's life. Nutrient acquisition is done via various kinds of transporters that are able to transport both inorganic and organic molecules. Signal transduction is an important process that is often triggered by movement of various ions in and out of the cell. In addition transporters help facilitate

compartmentalization, since different metabolites often are kept in different compartments of the cell and are transported as needed for a wide range of processes. Also, turgor pressure which is critical for plant growth and function is achieved by balancing the internal and external concentrations of inorganic and organic ions (Sander and Bethke 2000).

Plant transporters can be categorized into three main groups: channels, carriers, and pumps. Each has distinctive characteristics in terms of substrate specificity and energy source that is used to mediate the transport of the many diverse solutes across biological membranes. Channels are characterized by their extremely high rate of transport across the membrane that can reach up to 10^8 molecules per second (Sander and Bethke 2000). This large transport rate is presumably achieved because channels can be considered, in the simplest sense, water filled pores through which the ions flow down their electrochemical potential gradient. Carriers, on the other hand, undergo conformational changes in protein structure that facilitate transport across the membrane. They bind to the substrate on one side of the membrane and after the conformational change; the substrate is released on the other side of the membrane. The conformational changes associated with transport reactions in carriers result in a slower rate of carrier-mediated transport, approximately 10^3 molecules per second (Sander and Bethke 2000). Carriers can be further subdivided into passive transporters, which like channels mediate the downhill transport of solutes, and symporters/antiporters. Symporters and antiporters drive the active, thermodynamically uphill transport of solutes via a coupling on the same transporter with the passive flux of H^+, with the transmembrane H^+ gradient generated via the functioning of H^+ ATPases or "pumps". The slowest transporters are the active pumps that have the transport rate on the order of 10^2 molecules per second (Sander and Bethke 2000). These ion pumps directly use the energy of ATP hydrolysis to drive the

active, uphill transport of ions and other solutes. These pumps, or ATPases, describe a large family of transporters that can be further classified into several sub-groups (Sander and Bethke 2000). F-type ATPases are multi-subunit complexes involved in both ATP synthesis coupled to H^+ fluxes as well as transmembrane H^+ transport in mitochondria. V-type ATPases are also multi-subunit protein complexes that are expressed in the plant vacuolar membrane and mediate H^+ fluxes across that membrane. P-type ATPases are single protein transporters, the best known being the plasma membrane H^+- ATPases that is the central player in energizing the plant cell plasma membrane. P-type ATPases can also transport other solutes, including heavy metal ions. The coupling of ATP hydrolysis to transport in P-type ATPases has a unique feature that distinguishes them from all other ATPases. In the E1 state, the solute to be transported binds to the ATPase, which then binds one molecule of ATP. The terminal phosphate of the ATP molecule becomes covalently bound to an aspartyl residue of the transporter. Hydrolysis of the acyl-phosphate bond provides energy for transformation of the solute-bound transporter to the E2 state. In the E2 state, the affinity of the solute to the transporter is much lower compared to the E1 state and the solute is released on the opposite side of the plasma membrane. Additionally, hydrolysis of the phosphate bond returns the transporter to its original E1 state where it is ready for the next cycle. P-type ATPases consume one molecule of ATP per one transported molecule which makes them relatively energy inefficient. On the other hand, due to the specific substrate transporter binding, the selectivity of ATPases is much higher compared to ion channels. All P-type ATPases share common characteristics which include 8-12 membrane spanning domains, a highly conserved sequence at the ATP binding site which is normally located between fourth and fifth transmembrane domains, sensitivity to orthovanadate which is able to effectively

block the E1 to E2 cycle by replacing the phosphate in the ATP-transporter bond (Sander and Bethke 2000).

Heavy Metal ATPases

As discussed above, P-type ATPases can transport a variety of substrates across the plasma membrane. P-type ATPases are present in all life forms and currently approximately 160 of them have been discovered. Remarkably, the largest number of P-type ATPases occur in higher plants. For example, 45 different ATPases are predicted in the *Arabidopsis* genome (Axelsen et al 1998), and 43 are found in rice (Buxter et al 2003). It is not clear why plants would need so many ATPases, many of which have similar or duplicate functions (Axelsen et al 1998). One possible explanation is that since plants are immobile, they need to adjust to be able to rapidly fine-tune to changing environmental conditions such as water and nutrient availability and temperature, and having many highly selective transporters may help plants to more finely adapt to their environment. P-type ATPases are classified into five major branches based on their substrate specificity and not according to their evolutionary relationship. Type I ATPases are pumps that are responsible for transport of different kinds of heavy metals across the plasma membrane (Axelsen et al 2001). Na^+/K^+-ATPases, and H^+/K^+-ATPases are members of the type II ATPase subgroup. Type III ATPases are able to transport protons, Mg^{2+}, and Ca^{2+}. Type IV ATPases are involved in the transport of phospholipids across the plasma membrane. Finally, type V ATPases is a group of pumps that to date, have no known substrate for their transport (although it is thought that type V ATPases may be involved in the transport of anions) (Axelsen et al 1998).

P1B-type Heavy Metal ATPases

Among the type I ATPases in *Arabidopsis* thaliana, a group of eight transporters is subcategorized as Type1B-ATPases. Members of this subgroup are the HMA (Heavy Metal Association) ATPases that are further subdivided, based on the metals they can transport, into HMA1-4 (the Zn/Cd/Co/Pb subgroup) and HMA5-8 (the Cu/Ag subgroup) (Axelsen et al 2001). These HMA transporters are also referred as CPx-type ATPases since they all have a conserved cysteine-proline-cysteine or cysteine-proline-histidine motif that is thought to be involved in translocation of the substrate metal (Solioz et al 1996). In addition, these ATPases also share several other structural characteristics including: 1) they all exist as single subunit transporters; 2) they contain a conserved amino acid motif, D-K-T-G-T, that is the phosphorylation domain; and, 3) they all contain one or two heavy metal binding domains (Cobbatt et al 2003, Williams et al 2005). In recent years significant progress has been made in understanding the structure and biological function of these HMAs using model plant species such as *Arabidopsis thaliana* and *Thlaspi caerulescens*.

Cu/Ag Subgroup of P1B-Type ATPases

Copper is an essential micronutrient that plays an important role in key physiological oxidation reactions, however, it is also very toxic at elevated tissue concentrations. Therefore, it is not surprising that plants have evolved transporters and detoxification mechanisms for copper to avoid its toxic effects. Utilizing the availability of complete *Arabidopsis* genome sequence and similarities between members of HMA family of transporters, researchers have been able to clone and characterize the AtHMA5 transporter. *AtHMA5* is expressed mainly in *Arabidopsis* roots and its expression is strongly and exclusively upregulated by copper (Andres-Colas et al 2006). Interestingly, *Arabidopsis* mutants in which *Athma5* has been

knocked out are hypersensitive to copper but not to other heavy metals such as zinc or cadmium. In addition, these *hma5* mutant plants accumulate more copper in the roots compared to the wild type plants when treated with excess copper. Based on these findings, it has been suggested that AtHMA5 plays an important role in Cu compartmentalization and detoxification. The first plant P1B-type ATPases to be cloned were initially called PAA1 and PAA2, and then later were renamed HMA6 and HMA8, respectively. Both of these transporters play a crucial role in Cu transport to Cu-requiring proteins in the chloroplast. HMA6/PAA1 transports Cu across the plastid envelope to be incorporated into stromal Zn/Cu superoxide dismutase, while HMA8/PAA2 transports Cu into the thylakoid lumen for incorporation into Cu requiring steps in photosynthetic electron transfer (Tabata et al 1997; Shikanai et al 2003; Abdel-Ghany et al 2005). HMA8/PAA2 is only expressed in shoots while HMA6/PAA1 is expressed in both shoots and roots, which further supports the hypothesis that HMA8/PAA2 has an exclusive role in photosynthetically active plastids. AtHMA7, also known as RAN1, was first identified by screening *Arabidopsis* mutants that had altered sensitivity to the plant hormone, ethylene. HMA7/RAN1 has strong sequence similarity to other known Cu ATPases such as the human Welson/Menkes disease and yeast CCC2 proteins. Results from transformation of HMA7/RAN1 into a yeast *ccc2* Cu sensitive mutant strain confirmed its function as a Cu transporter. It is thought that HMA7/RAN1 ATPase is involved in transporting Cu into a post-Golgi endomembrane compartment where it is subsequently incorporated into a membrane-targeted ethylene receptor (Hirayamma et al 1999).

Zn/Cd/Co/Pb Subgroup of P1B-Type ATPases

HMA1 is a P-1B type ATPase that is expressed in the chloroplast envelope. Using a chloroplast envelope-targeted proteomics approach in *Arabidopsis thaliana*,

researchers recently found that AtHMA1 was localized in the chloroplast envelope proteome (Seigneurin-Berny et al 2005). In this study, it was shown that HMA1 is mainly expressed in green tissues, and *hma1* knockout mutants in *Arabidopsis* exhibit decreased chloroplast copper content. In addition, transformation of *Arabidopsis* plants with an HMA1/GFP translational fusion confirmed the predicted localization of the HMA1 protein to the chloroplast. Yeast cells transformed with *AtHMA1* have an increased accumulation of copper. This result was explained by the targeting of HMA1 protein to one of the endomembrane compartments in yeast, and the resulting depletion of copper from the cytoplasm triggering elevated Cu influx into the yeast cell (Seigneurin-Berny et al 2005).

AtHMA2 is another member of this P1B-type ATPase subgroup that has been extensively studied both *in vitro*, based on enzymatic studies with recombinant protein, and *in planta*. It has been shown that the HMA2 transporter functions as a classical ATPase, forming an acid stable phosphorylated intermediate and the ATPase activity is inhibited by vanadate (Eren et al 2004). The HMA2 enzyme was shown to bind Zn^{2+} and Cd^{2+} with a high affinity, suggesting it transports both of these ions. Reports characterizing the *Arabidopsis thaliana hma2* knockout mutant are somewhat conflicting. Eren and coworkers show that when this transporter was knocked out in *Arabidopsis*, the mutant seedlings accumulated more Zn compared to the wild type plant (Eren et al 2004). In a second report by (Hussain et al 2004), it was reported that this *hma2* knockout mutant did not have an apparent phenotype, but a *hma2*/*hma4* double mutant in *Arabidopsis* exhibited increased root Zn accumulation. In the work by Hussain and coworkers, it was also shown that HMA2 is mainly expressed in vascular tissue in both roots and shoots, while protein localization studies with an HMA2-GFP fusion showed that HMA2 is localized to the plasma membrane of plant

cells. The above mentioned findings led investigators to conclude that HMA2 is an efflux transporter and it may be involved in xylem and possibly phloem loading.

AtHMA3 is the most poorly understood transporter among the eight members of HMA subgroup of P-type ATPases in *Arabidopsis thaliana*. In particular, not much physiological information is available on the function of HMA3 in the plant. When transformed into yeast cadmium hypersensitive Δycf1 strain it was shown that *AtHMA3* confers an increased tolerance to high concentrations of cadmium and lead (Gravot et al 2004). Interestingly, when wild type yeast (which are more metal tolerant than the Δycf1 strain) were transformed with *AtHMA3*, there was no significant increase in yeast heavy metal tolerance. Yeast protein localization for a AtHMA3-GFP fusion indicated the transporter in yeast is exclusively expressed in the vacuolar membrane. *In planta*, *AtHMA3* was found to have a constitutively high expression that was not altered in response to high cadmium or zinc. Highest levels of *AtHMA3* expression were found in old rosette leaves, roots, and in cauline leaves. As mentioned above, more work needs to be done to further understand the role of HMA3 in plants, but it may have a possible role in detoxification of heavy metals by sequestering them into the vacuole.

Probably the most studied of the HMAs has been the HMA4 transporter. The *HMA4* gene has been characterized in two model species, the non-accumulator, *Arabidopsis thaliana*, and the hyperaccumulator, *Thlaspi caerulescens*, and comparison of its expression and function in these contrasting plant species may increase our understanding about the mechanistic basis for heavy metal hyperaccumulation. The HMA4 protein shares characteristics common with all other P1B-type ATPases, but in addition it has several interesting features not found in the other ATPases. For example, it has an unusually long C-terminal hydrophilic extension predicted to reside in the cytoplasm which contains several Cys pairs and

eleven consecutive His residues at its 3' end. It has been previously shown that Cys and His residues in proteins may play important roles in heavy metal binding and transport (Gaither et al 2001). Using heterologous expression in *Saccharomyces cerevisiae*, researchers have begun to elucidate the role(s) of different regions of the AtHMA4 protein in metal transport and binding. For example, it was found that transformation of the *AtHMA4* gene into yeast Δycf1 mutants rescues the heavy metal hypersensitive phenotype and results in decreased accumulation of cadmium and zinc in transformed yeast cells (Verret et al 2005). When the 11 His stretch in the C terminal cytoplasmic tail was deleted and this truncated protein was expressed in the yeast Δycf1 strain, Cd hypersensitivity was not complemented, indicating this region is critical for metal transport (Verret et al 2005).

Altering the expression of *AtHMA4* in transgenic *Arabidopsis* seedlings also has yielded significant information about its function. As mentioned earlier, *Athma4* knockouts in *Arabidopsis* had no apparent phenotype when grown on soil, but hma4hma2 double mutant plants showed a Zn deficiency phenotype that could be rescued by Zn supplementation (Hussain et al 2004). This finding suggests that HMA2 and HMA4 may have at least partially redundant functions in plants. Expression of *AtHMA4-GUS* promoter-reporter construct in *Arabidopsis* indicated that *AtHMA4* is expressed in the vascular tissue of the root and shoot, with greater expression in the root, where the protein is expressed in the plasma membrane (Hussain et al 2004). Finally, overexpression of *AtHMA4* under the control of the CAMV35S constitutive promoter increased the accumulation of heavy metals in the shoots and also increased plant tolerance to heavy metals (Verret et al 2004). These findings have led researchers to hypothesize that HMA4 (and also possibly HMA2) are metal transporters involved in the loading of micronutrients and heavy metals into the xylem.

HMA4 is the only P1B-type ATPases that has been characterized in heavy metal hyperaccumulator plants species, as it has been studied in some detail in *Thlaspi caerulescens* (Papoyan et al 2004; Bernard et al 2004). In research detailed later in this thesis, *TcHMA4* was isolated using a functional screening of a *Thlaspi caerulescens* cDNA library in yeast grown on levels of Cd toxic to WT yeast. Expression of both the full TcHMA4 protein and the C terminal region of TcHMA4 in yeast conferred a significant increase in yeast heavy metal tolerance. Moreover, comparison of yeast cells expressing the C-terminus of TcHMA4 and AtHMA4 show that C-termini peptides derived from *Thlaspi caerulescens* HMA4 have much stronger binding capacity for heavy metals compared to the peptides derived from *Arabidopsis thaliana* HMA4 (Bernard et al 2004). In addition, *in planta* expression studies showed that *HMA4* has a much higher constitutive expression in roots of *Thlaspi caerulescens* than in *Arabidopsis thaliana*, and *TcHMA4* expression was also induced by root exposure to high Zn and Cd levels, which was not seen in *Arabidopsis*. A more detailed analysis of *TcHMA4* and its function will be provided in Chapter 3. From the findings characterizing HMA4 in *Thlaspi* and *Arabidopsis*, it was hypothesized that HMA4 may play a key role in heavy metal hyperaccumulation, as the transfer of heavy metals from the root to the shoot is an important component of the hyperaccumulation phenotype.

Heavy Metal Ligands in Plants

Due to the extreme toxicity of heavy metals, plants have evolved various mechanisms of detoxification that include, in addition to transport out of the cytoplasm (the primary site of metal toxicity), the binding of metals by low molecular weight organic cell ligands. The major heavy metal-binding ligands in plants are phytochelatin (PCs), metallothioneins (MTs), and organic acids (Cobbett and

Goldsbrough, 2002). It is important to note that hyperaccumulator and non-accumulator plants may have evolved different mechanisms for heavy metal detoxification, in particular for long term storage. Recent advances in plant molecular biology techniques and the sequencing of the complete *Arabidopsis* genome have allowed researchers to gain a better understanding of the importance and function of heavy metal ligands in plants. Arguably, metallothioneins (MTs) and phytochelatins (PCs) are the best characterized heavy metal chelators in plants. They both are cysteine-rich, small peptides that have a different origin, in that MTs are encoded by genes, while PCs are synthesized enzymatically.

Metallothioneins

Metallothioneins were discovered forty years ago from horse kidney as novel Cd-binding proteins. Originally, it was thought that MTs were present only in the animal kingdom and all MTs discovered from plants were referred to as "metallothionein-like" proteins. MTs are gene-encoded proteins and are products of mRNA translation. They are characterized as cysteine rich, low molecular weight, metal binding proteins that can form mercaptide bonds with various metals. Typically, metallothioneins have two Cys-rich metal binding domains that give them a "dumbbell" conformation. Metallothioneins are divided into four classes (see Cobbett and Goldsbrough, 2002, for detailed discussion on the four MT classes). Class one metallothioneins include MTs that are common in the animal kingdom and contain 20 highly conserved Cys residues that are distributed equally between the two heavy metal binding domains. Class two metallothioneins are found in plants, fungi, and some invertebrates; these MTs also contain two heavy metal binding domains. These domains are separated by approximately 40 amino acids and the signature characteristic of MTII class metallothioneins is that they have a highly conserved N-

terminal domain that starts with a Cys-Cys pair. The spacer region between the two heavy metal binding domains can vary between the different classes of MTs isolated from different species. The third class of metallothioneins is characterized by having only four Cys residues in the N-terminal and six Cys residues in the C-terminal region. In plants, many MTs that are expressed during fruit ripening are class three MTs. The class four MTs are characterized by having three heavy metal binding domains each of which are separated by 10-15 amino acid residues (Cobbet and Goldsbrough, 2002). For a long time it was believed that in plants, metallothionein mRNA was not translated into protein since all the attempts to purify the MT proteins were unsuccessful. Subsequently, it was found that plant MT proteins are extremely unstable in the presence of oxygen (Cobbett and Goldsbrough, 2002). However, transformation of *E.coli* and yeast with plant metallothionein genes conferred increased tolerance to heavy metals (Tomey et al 1991; Zhou et al 1994). Metallothionein genes from the different classes share common but not very strict expression profile. For example, class I metallothioneins are mainly expressed in the roots, while class II metallothioneins have strong expression in the shoots. The class III MTs are very abundant in plants that produce fleshy fruits and expression of class IV metallothioneins is restricted to the developing seed. The biological function of metallothioneins in plants is still not clearly understood, but is believed to be similar to MT function in animals. It is most likely that MTs in plants play an important role in metal homeostasis. There have been several reports showing that metallothioneins play a role in copper homeostasis (Murphy et al 1995; van Hoof et al 2001). The cloning and characterization of expression of two *Arabidopsis* metallothionein genes (MT1a and MT2a) led investigators to suggest that these MTs may be involved in copper tolerance and remobilization in plants (Garcia-Herandez et al 1998). In a subsequent study, researchers used biolistic transformation to express *Arabidopsis*

AtMT1 and *AtMT2* genes in *Vicia faba* guard cells. The transformed cells showed increased tolerance to high concentrations of Cd. In addition, the concentration of reactive oxygen species in the transformed guard cells was lower than in control cells, which may indicate that the increased resistance to high Cd was not due to the direct detoxification of cadmium but rather due to the reduction of Cd-induced generation of reactive oxygen species (Lee et al 2004). Finally, *AtMT1* knockout mutants in *Arabidopsis thaliana* exhibited an increased sensitivity to high concentrations of Cd and increased Cd accumulation in the tissues (Zimeri et al 2005). The recent identification and characterization of metallothioneins genes from *Thlaspi caerulescens* suggests that in *T. caerulescens*, at least certain MTs may also play a role in copper homeostasis (Roosens et al 2005). However, to date, there is no evidence for involvement of MTs in the hyperaccumulation of heavy metals such as Cd, Zn, and Ni in hyperaccumulator plants and it seems likely that in hyperaccumulators, metallothioneins may have similar functions to MTs in non-accumulator plants.

Phytochelatins

Unlike MTs, phytochelatins (PCs) are enzymatically synthesized, cysteine-rich peptides. Originally it was thought that phytochelatins were present exclusively in plants, hence the term "phyto" in the name, but recent findings have shown that PCs are also present in yeast and some invertebrates (Vatamaniuk et al 2002). PCs are peptides with varying numbers of γ-GluCys dipeptides, followed by a terminal Gly: (γ-Glu-Cys)n-Gly, where "n" ranges from 2 to 5. Glutathione (GSH), which has the general structure of (Glu-Cys-Gly), has been shown to be the substrate for phytochelatin biosynthesis. It was not until relatively recently that the enzyme responsible for PCs biosynthesis, PC synthase (*PCS1*) was successfully cloned from *Arabidopsis* and wheat plants (Clemens et al 1999; Ha et al 1999; Vatamaniuk et al

1999). Upon exposure to heavy metals, the concentration of phytochelatins significantly increases in plants. However, the expression level of the phytochelatin synthase gene, *PCS1*, does not change in response to cadmium treatment. It appears that PC synthesis is primarily regulated post-translationally, with heavy metals such as Cd enhancing the activity of the PC synthase enzyme (Vatamaniuk et al 2000). The function of phytochelatins has been elegantly characterized primarily in yeast. Upon exposure to Cd, phytochelatins form a Cd-PC complex in the cytoplasm commonly referred to as a low molecular weight (LMW) complex (Cobbett and Goldsbrough 2002). This LMW PC-Cd complex is, in turn, transported into the vacuole via specific transporters such as the Hmt1 ABC transporter from the yeast, *Saccharomyces pombe*. In the vacuole, the LMW complexes combine with additional Cd^{2+} ions and sulfur, in the form of sulfide ions, to form a high molecular weight PC-Cd complex (Cobbett and Goldsbrough 2002). Phytochelatins are able to form complexes with several heavy metals and metalloids, including Cd, Cu, and As. However, conclusive physiological data supporting a role of PCs in heavy metal detoxification exists only for cadmium and arsenic, and not for other toxic metals. For example, although PC-Cu complexes have been identified in plants, the *Arabidopsis cad1* mutants, which are deficient in PC synthase, do not show any increased sensitivity to copper (Howden et al 1995). More recent studies using transgenic plants overexpressing phytochelatins suggest that this could be a strategy to increase plant heavy metal tolerance, at least for Cd. (Pomponi et al 2006).

To date, there is no strong evidence that PCs play a major role in heavy metal tolerance in hyperaccumulators. Ebbs and colleagues studied levels of PCs in roots and shoots of *Thlaspi caerulescens* and the related non-accumulator, *Thlaspi arvense* in response to different levels of Cd and Zn (Ebbs et al 2002). It was found that the PC levels in these two *Thlaspi* species were not significantly different despite the fact

Thlaspi caerulescens accumulated much more Cd and Zn than did *Thlaspi arvense*. These findings indicate that heavy metal tolerance in hyperaccumulator plants most likely depends on mechanisms that do not utilize phytochelatins.

Heavy Metal Ligands in Hyperaccumulator Plants

As was previously discussed, metallothioneins and phytochelatins may play an important role in heavy metal detoxification in normal, non-accumulator plants. However, it appears that in hyperaccumulator plants some other ligands must be involved in chelation of heavy metals. The conventional rationale is that since hyperaccumulator plants are able to accumulate extremely high concentrations of heavy metals (up to several % of their dry weight), it would be energetically too costly for them to chelate heavy metals with relatively large organic molecules such MTs and PCs. Organic acids may be alternate candidates as heavy metal ligands in hyperaccumulator plants. Using extended x-ray absorption spectroscopy (EXAFS), it was shown that in the Zn/Cd hyperaccumulator, *Arabidopsis halleri*, leaf Zn is predominantly bound to malic and citric acid (Sarret et al 2002). Similar results were found for Zn in leaves of *Thlaspi caerulescens* (Salt et al 1999). It is also important to note that different hyperaccumulator species may employ different chelators for detoxification of specific heavy metals. For example, it was reported that in Ni hyperaccumulator plants such as *Thlaspi goesingense* and *Allysum lesbiacum*, Ni may be associated with histidine (Kramer et al 1996; Ingle et al 2005). There are also tissue and age dependant differences in heavy metal tolerance and storage in hyperaccumulator plants. Kupper and coworkers reported that in *Thlaspi caerulescens*, a higher percentage of Cd was associated with oxygen ligands in mature tissues, while in younger leaves, Cd binding with sulfur ligands dominated (Kupper et al 2004). Another important aspect of heavy metal hyperaccumulation and detoxification is

sequestration of heavy metals into different cell types, which could minimize heavy metal exposure to structures that are quite sensitive to heavy metal toxicity, in particular the photosynthetic apparatus. It has been shown that heavy metals in some hyperaccumulator plants may be sequestered into epidermal cells except the guard cells. This is significant, as in the leaf epidermis, only the guard cells contain chloroplasts (Kupper et al 2001, Psaras et al 2000). A similar "detoxification" strategy may also occur at the root level in *Thlaspi caerulescens* plants, where it was shown that most of the Cd in hairy root cultures of *T. caerulescens* transformed with *Agrobacterum rhizogenes* was in the cell walls (Boominathan et al 2003). It is clear that more work is needed to better understand the mechanisms of extreme metal tolerance and accumulation in heavy metal hyperaccumulator plants. However, with each piece of new information, we are getting one step closer to the ultimate goal of engineering plants that could be used to help clean up heavy metal contamination in soils.

General Goals of the Present Study

As described above the heavy metal tolerance and accumulation in hyperaccumulator plants is a complex mechanism that involves both heavy metal transporters and ligands. Upon exposure to high concentrations of heavy metals in hyperaccumulator plants such as *Thlaspi caerulescens* a complex set of mechanisms appear to be activated that regulate the transport of heavy metals into the roots from the soil, of absorbed heavy metals into the xylem, and storage and detoxification of heavy metals in the leaves. The work detailed here provides a physiological characterization of Zn and Cd accumulation in *Thlaspi caerulescens*. In addition, the cloning and detailed characterization of a heavy metal transporting P1B-type heavy metal ATPase is presented. The possible use of a peptide derived from the heavy metal

transporting ATPase that may function as a heavy metal ligand when transformed into plants is also described in this study.

An interesting phenomenon was observed regarding the effect of high Zn concentrations in the environment on plant Cd accumulation and vise versa. Chapter II shows that *Thlaspi caerulescens* plants that are grown high concentrations of Zn accumulate more Cd in the shoots compared to *Thlaspi caerulescens* plants that are grown on a normal concentration of Zn and the same concentration of Cd. This trend is opposite to what has been observed in non-accumulator plants where a high concentration of Zn reduces the accumulation of Cd in the shoots.

Chapter III details a characterization of the heavy metal ATPase, HMA4, in *Thlaspi caerulescens* plants. This ATPase was found to be expressed mainly in roots and its expression is regulated by exposure to heavy metals. Evidence is provided that HMA4 is a xylem loading heavy metal ATPase and may be an important factor in the metal hyperaccumulation phenotype.

Chapter IV describes TcHMA4 C-terminus derived peptides and their potential use for genetically engineering plants that will have phytoremediation capabilities and/or improved nutritional value. *Arabidopsis thaliana* plants that are transformed with these peptides show increased tolerance to and accumulation of heavy metals in the shoots. In addition, transformed plants show increased accumulation of Zn in the seeds when grown under Zn deficient condition, which may be a beneficial feature since Zn is an important micronutrient.

Finally, Chapter V summarizes and provides an overview of all of the the work in this thesis as well as a description of potential future research.

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CHAPTER II

The effect of Cd^{2+} treatment on micronutrient metal accumulation in *Thlaspi caerulescens*, a heavy metal hyperaccumulator plant species.

Abstract

Thlaspi caerulescens is capable of accumulating and tolerating extremely high concentrations of heavy metals such as Zn and Cd. In this study, experiments were conducted looking at the influence of changes in plant Zn status on the accumulation of Cd and other micronutrient metals. It was found that plants grown under a high Zn regimen (500μM) were able to tolerate a high concentration of Cd (200 μM) much better compared to normal Zn-status plants (grown on 1 μM Zn). Furthermore, Cd concentrations in the shoots of plant s grown on high Zn were significantly higher than shoot Cd levels for normal Zn-grown plants. A positive correlation was also found between shoot Zn accumulation and increasing plant Cd status; such that plants grown on increasing levels of Cd exhibited an increasing shoot Zn accumulation, especially at lower Zn levels. Interestingly, for plants grown on high levels of Zn, the effect of increasing Cd status disappeared, as increasing the concentration of Cd in the growth solution had no effect on shoot Zn accumulation. In contrast, in the roots, the interactions between Zn and Cd accumulation were what might be expected for a non-accumulator species, in that increasing levels of one metal reduced the root accumulation of the other metal. Radiotracer ^{109}Cd root flux experiments indicated that plants grown on high (500 μM) Zn maintained significantly higher root Cd^{2+} influx values, when compared to plants grown on normal (1 μM) Zn. In addition, the effect of plant Zn status on the shoot accumulation of other micronutrients that also are heavy metals (Cu, Ni, and Mn) was also examined. It was found that both Ni and Cu shoot accumulation were also stimulated by high plant Zn status, while Mn accumulation was not affected. Overall, our findings have led us to speculate that

xylem loading may be one of the key sites responsible for altered Zn and Cd accumulation in hyperaccumulator plants.

Introduction

Heavy metal transport, and subsequent sequestration and accumulation are important aspects of plant's ability to tolerate high concentrations of heavy metals. Essential micronutrients such as Zn, Ni, Cu, and Mn play an important role in different aspects of plant's metabolism; however, they also are potentially toxic heavy metals. Additionally, other non-essential toxic heavy metals such as Cd and Pb do not have biological functions, but are able to enter plants on the transport systems in place of essential micronutrients. All of the mentioned heavy metals are divalent cations with similar physicochemical properties, and are therefore able to compete, antagonize and displace each other in various biological processes.

Heavy metal hyperaccumulator plants such as *Thlaspi caerulescens* have evolved mechanisms for extreme heavy metal accumulation and detoxification. There is great interest in understanding the mechanisms underlying the hyperaccumulating phenotype; thus there is a need to understand how metal transport and accumulation processes differ between normal and hyperaccumulator plants, as well as how the different essential and non-essential heavy metals interact and influence each other. "Normal" non-hyperaccumulator plants tend to store absorbed heavy metals in the roots, where as hyperaccumulator plants are capable of transporting most of the accumulated heavy metals to the shoots (Lasat et al 1998). This is consistent with the several fold higher Zn concentrations found in the xylem sap of *Thlaspi caerulescens* when compared to that found in the related non-accumulator *Thlaspi arvense*. It has also been reported that hyperaccumulator plants have stronger influx of heavy metals into the roots compared to that observed in non-accumulator species (Lasat et al

1996). In a study investigating the competitive effect between Zn and Cd transport in *Thlaspi caerulescens*, the authors concluded, that in the leaf, Cd is transported via cellular Zn transporters (Cosio et al 2004). Similar results were obtained when comparing root heavy metal transport in the *Thlaspi caerulescens* ecotypes Prayon and Ganges. In the Prayon ecotype, it was suggested that Zn and Cd are transported via the same transporter, while in Gange which is a better Cd hyperaccumulator, circumstantial evidence for a separate Cd-specific transporter has been presented (Lombi et al 2001). It is known that in non-accumulator plants that Zn and Cd uptake and accumulation are negatively correlated (Hart et al 2002; Wu et al 2003). Using ^{65}Zn and ^{109}Cd radiotracer flux techniques, Hart and colleagues showed there was competition between root Zn and Cd uptake, such that high levels of Zn inhibit Cd uptake and high Cd inhibits Zn uptake in the roots of flax and durum wheat. This correlation can be explained by ionic competition between Zn and Cd for the heavy metal binding site in the transport protein.

The ability of *Thlaspi caerulescens* to accumulate high levels of both Zn and Cd even from nutrient solutions or soils that contain high levels of both metals suggests that the competitive interactions between Cd and Zn accumulation in non-accumulator plants may not exist or may be altered in the hyperaccumulator. For these reasons, the current study was conducted, which examined the effect of low and high plant Zn and Cd status on Zn and Cd accumulation, as well as their effects on the essential micronutrients Cu, Ni and Mn.

Materials and Methods

Plant Growth Conditions

Thlaspi caerulescens (ecotype Prayon) seedlings were grown on a modified Johnson's solution that had a macronutrient composition of 1.2 mM KNO_3, 0.8mM $Ca(NO_3)_2$, 0.2mM $NH_4H_2PO_4$ and 0.2 $MgSO_4$ and a micronutrient composition of 50µM KCl, 12.5µMH_3BO_4, 1µM $MnSO_4$, 1µM$ZnSO_4$, 0.5µM $CuSO_4$, 0.1µM Na_2MoO_4, 0.1µM $NiSO_4$, and 7.5µM Fe-EDDHA (N, N'-ethylenediamine-di(O-hydroxyphenylacetic acid). The solution was buffered at a pH of 5.5 with 1 mM MES (2-[N-morpholino]-ethanesulfonic acid) buffer. *Thlaspi* seeds were placed in a drop of 0.7 % (w/v) low temperature gelling agarose on nylon mesh circles (1 mm mesh openings) which, in turn, were positioned on a coarser mesh support sealed to the bottom of black plastic cups. The cups and seeds were fitted into holes cut into black plastic lids covering 5 liter black plastic pots. Seedlings were grown in a growth chamber at 25/15 °C (light: dark, 16:8 h) under a light intensity of 300 µmol photons $m^{-2}s^{-1}$for two weeks.

Heavy Metal Treatment and ICP Analysis

After germination and growth in Johnson nutrient solution for two weeks, the seedlings were transferred into growth containers containing identical nutrient solution supplemented with specific concentrations of Zn (1 or 500 µM) and/or Cd (1, 5, 20, 40, 60, 80, 100 and 200µM). The treatments continued for two months with the treatment solutions being refreshed weekly. After two months, plants were harvested, the root and shoot tissues separated and oven dried for 10 days at 65 degrees ^oC. Subsequently, dry weights for each sample were obtained and then the samples were digested in concentrated HNO_3 to dryness. Dried samples were then resuspended in

5% HNO_3 and the concentrations of specific micronutrients and heavy metals for each sample was determined using an inductively-coupled, plasma trace analyzer emission spectrometer (Model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA). For the root heavy metal content analysis, the tissue was first transferred into a desorption solution containing 5 mM $CaCl_2$ for 20 minutes, to desorb as much of the heavy metal bound to the root cell wall as possible.

^{109}Cd Radiotracer Experiments

Radiotracer (^{109}Cd) flux methodologies were used to determine Cd^{2+} transport into *Thlaspi caerulescens* roots. Plants were grown on nutrient solution containing either 1 or 500 μM Zn for two months, after which the radiotracer experiments were conducted. Plants were placed for 15 minutes into a non radioactive uptake solution of identical composition to that used for the radiolabeled uptake experiment, with the objective of acclimating the plants and avoiding stress effects. Following this pre-treatment, root radiotracer uptake experiments were initiated by the addition of ^{109}Cd at different Cd concentrations for 20 minutes. To terminate the Cd uptake experiment, the roots were transferred into a desorption solution for 20 min that contained a 10 fold excess of "cold" non-radioactive Cd, to desorb as much of the ^{109}Cd bound to the cell walls as possible. It was previously found that this 20 min desorption regime was optimal for desorbing radioactive Zn or Cd from *T. caerulescens* root cell walls while minimizing efflux of radiotracer transported into the root symplasm. Subsequent to the desorption, roots were excised, blotted dry, weighed, and the radioactivity was counted using a Perkin Elmer "WIZARD 3" 1480 Automatic Gamma Counter.

Results

Effect of changing plant status on *Thlaspi caerulescens* Cd tolerance.

It has been well established that *Thlaspi caerulescens* is a hyperaccumulator plant species that is capable of tolerating extremely high concentrations of toxic heavy metals such as Cd and Zn. In the present study the influence of changing the plant Zn status from the normal status (plants grown on 1 μM Zn) to a high Zn status (plants grown on 500 μM Zn) on the ability to tolerate a high level of Cd (200 μM) in the nutrient solution was examined. Figure 2.1 illustrates how plants grown under the high Zn regime are capable of tolerating a high concentration of Cd more effectively than plants that are grown on normal level on Zn. In this Figure it can be seen that the normal Zn-grown plants exhibited some symptoms of Cd toxicity, including reduced shoot biomass and leaf necrosis, while the high Zn-grown plants were asymptomatic. To test the possibility that the increased tolerance to Cd was a consequence of reduced Cd accumulation shoot and root Cd accumulation in high and low Zn-grown plants was measured. As shown in Figure 2.2, Cd accumulation in the shoots was significantly higher in plants grown under the high Zn regime. This increased Cd accumulation was particularly evident at higher Cd levels in the nutrient solution (> 40 μM). In roots, the pattern was reversed as the higher Zn regime resulted in significantly lower levels of root Cd accumulation (Figure 2.3). It should be noted that because the roots were in direct contact with the nutrient solution, and a significant fraction of the root-associated Cd and Zn is in the cell wall, this effect could primarily be on root cell wall binding of Cd and not root Cd uptake.

Given that high plant Zn status stimulated shoot Cd accumulation, the effect of increasing plant Cd status on plant Zn accumulation was studied. As seen in Figure 2.4A for normal (1 μM) Zn-grown plants, increasing the plant Cd status by growing the plants on Cd levels ranging from 1 to 200 μM yielded a significant, two-fold

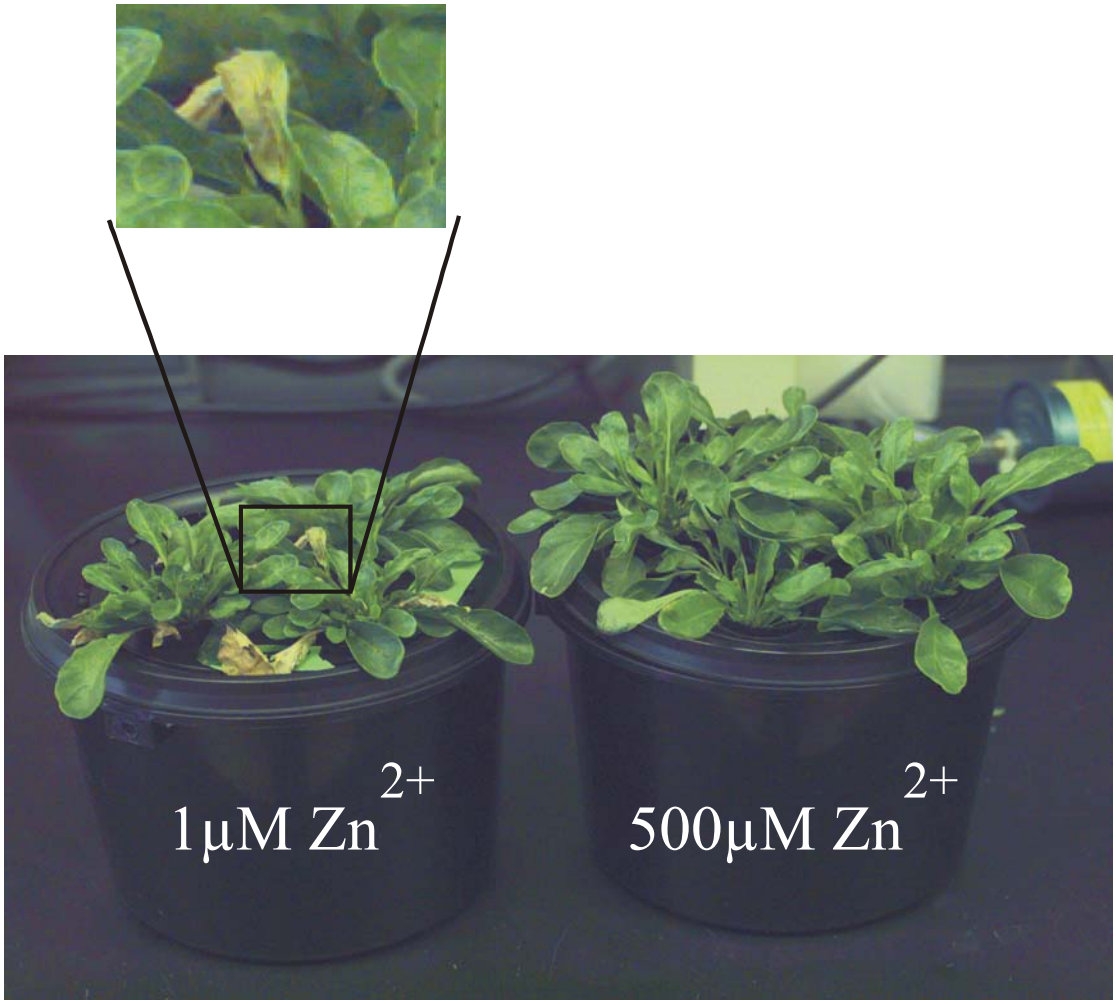


Figure 2.1 *Thlaspi caerulescens* tolerance in different Zn mediums.

Thlaspi caerulescens plants grown on hydroponic nutrient solution containing 1μM or 500μM Zn and 200μM Cd, Zn and Cd were added simultaneously. Magnified section highlights the necrotic symptoms caused by high Cd on the leaves of low Zn grown plants.

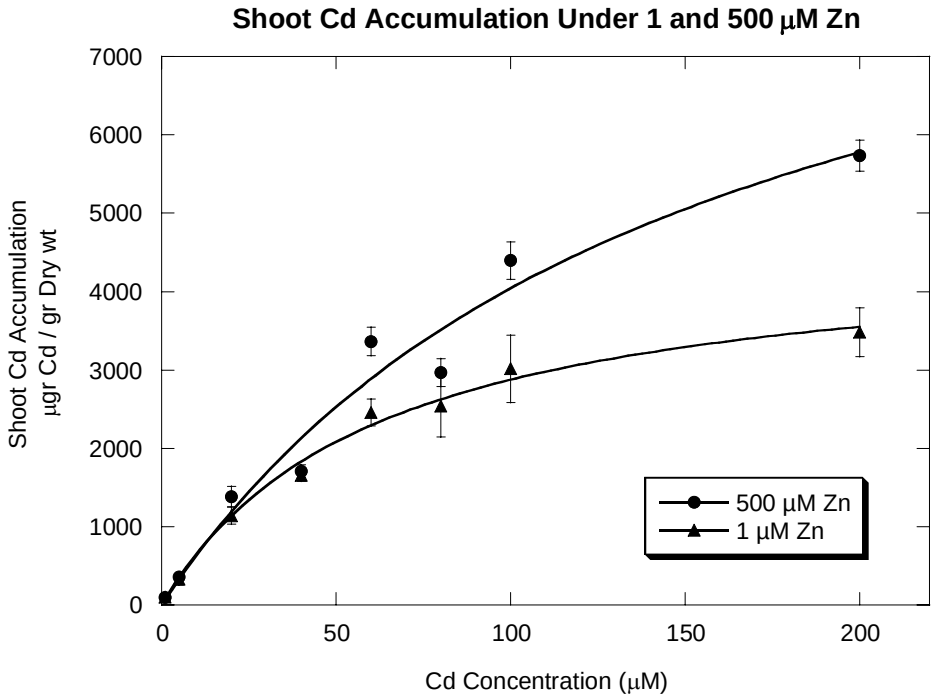


Figure 2.2 Zn-dependent accumulation of cadmium in *Thlaspi caerulescens* shoots.

Triangles represent *Thlaspi caerulescens* plants grown on nutrient solution containing 1μM Zn and circles represent *Thlaspi caerulescens* plants grown on nutrient solution containing 500μM Zn.

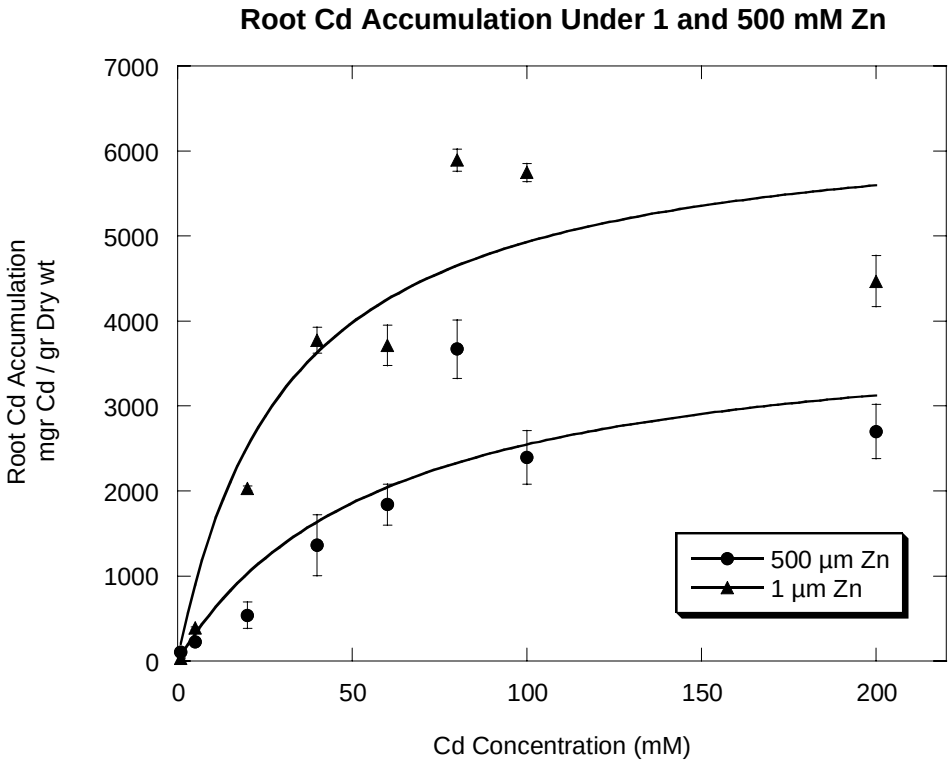


Figure 2.3 Zn dependent accumulation of Cd in the roots of *Thlaspi caerulescens* plants.

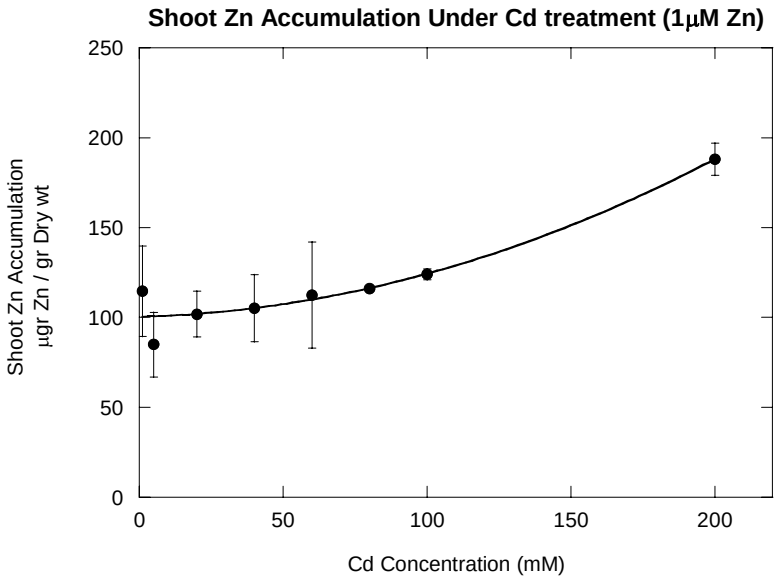
Rectangles represent *Thlaspi caerulescens* plants grown on nutrient solution containing 1µM Zn and circles represent *Thlaspi caerulescens* plants grown on nutrient solution containing 500µM Zn.

Figure 2.4 (A; B) Cadmium dependent accumulation of Zn in the shoots of *Thlaspi caerulescens* plants.

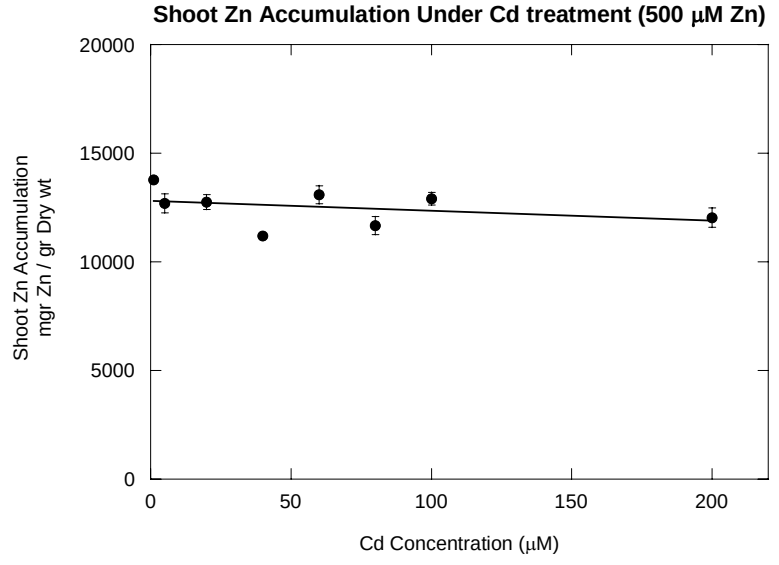
A) Accumulation of Zn in shoots of *Thlaspi caerulescens* plants grown on nutrient solution containing 1μM Zn and a range of Cd concentrations.

B) Accumulation of Zn in shoots of *Thlaspi caerulescens* plants grown on nutrient solution containing 500μM Zn and a range of Cd concentrations.

A


Shoot Zn Accumulation Under Cd treatment (1μM Zn)
Shoot Zn Accumulation
μgr Zn / gr Dry wt
250
200
150
100
50
0
0
50
100
150
200
Cd Concentration (mM)

B


Shoot Zn Accumulation Under Cd treatment (500 μM Zn)
Shoot Zn Accumulation
mgr Zn / gr Dry wt
20000
15000
10000
5000
0
0
50
100
150
200
Cd Concentration (μM)

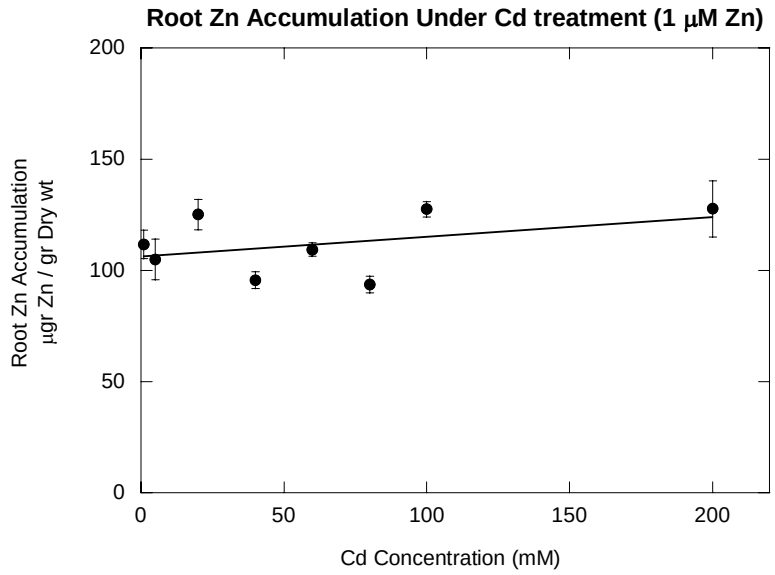
increase in shoot Zn accumulation. However, in shoots of high Zn-grown plants where shoot Zn levels were increased 100- fold compared with normal Zn-grown plants, changes in plant Cd status had no effect on shoot Zn accumulation (Figure 2.4B). With regard to root Zn accumulation, root Zn concentrations for plants grown on high Zn were also 100-fold greater that found in roots from plants grown under normal Zn conditions. However in contrast to the shoot accumulation data, the Zn content in roots from both normal and high Zn treatments remained unchanged regardless of the Cd concentration present in the growth media (Figure 2.5 A, B). The effect of plant Zn and Cd status on the accumulation of the heavy metal micronutrients, Cu, Ni, and Mn was also examined. Figures 2.6 through 2.8 summarize these results. As was seen for the effect of high Zn status on shoot Cd accumulation, high Zn-grown plants also exhibited a significantly higher shoot Cu and Ni accumulation (Figures 2.6A and 2.7A), with Cu and Ni accumulation increased as much as 3-fold. The same inhibitory pattern for high Zn effects on root Cd accumulation were also seen for root Cu and Ni levels, with the presence of 500 μM Zn in the growth solution reducing root Cu and Ni levels by as much as 3-fold (Figures 2.6B and 2.7B). In addition, root Cu accumulation seemed to be influenced by the Cd levels in the growth solution, with Cu accumulation increasing moderately at higher growth solution Cd concentrations.In contrast, increasing Cd levels had a moderate inhibitory effect on shoot Cu accumulation, while having little effect on shoot Ni levels. Unlike plant accumulation of Cu and Ni, shoot Mn accumulation was not stimulated by high plant Zn status, and increasing Cd levels reduced both shoot and root Mn concentrations (Figure 2.8 A,B).

Figure 2.5 (A; B) Cadmium dependent accumulation of Zn in roots of *Thlaspi caerulescens* plants.

A) Accumulation of Zn in roots of *Thlaspi caerulescens* plants grown on nutrient solution containing 1μM Zn and a range of Cd concentrations.

B) Accumulation of Zn in roots of *Thlaspi caerulescens* plants grown on nutrient solution containing 500μM Zn and a range of Cd concentrations.

A


Root Zn Accumulation Under Cd treatment (1 μM Zn)
Root Zn Accumulation
μgr Zn / gr Dry wt
Cd Concentration (mM)
0
50
100
150
200

B

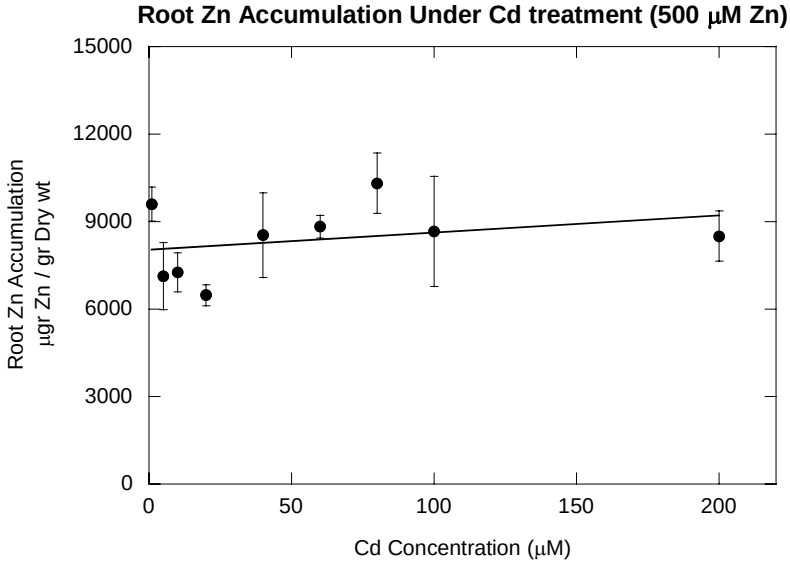
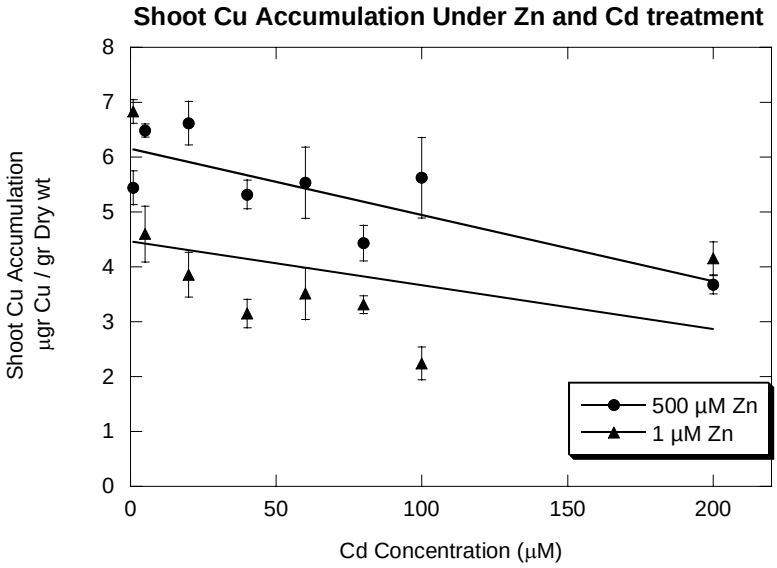

Root Zn Accumulation Under Cd treatment (500 μM Zn)
Root Zn Accumulation
μgr Zn / gr Dry wt
Cd Concentration (μM)
0
3000
6000
9000
12000
15000
50
100
150
200

Figure 2.6 (A; B) Cadmium and zinc dependant uptake of copper in *Thlaspi caerulescens* plants.

A) Cu accumulation in the shoots of *Thlaspi caerulescens* plants, rectangles represent 1μM Zn-grown plants and circles represent 500μM Zn-grown plants. B) Cu accumulation in the roots of *Thlaspi caerulescens* plants grown as described in A.

A


Shoot Cu Accumulation Under Zn and Cd treatment
Shoot Cu Accumulation
µgr Cu / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
1
2
3
4
5
6
7
8
0
50
100
150
200

B

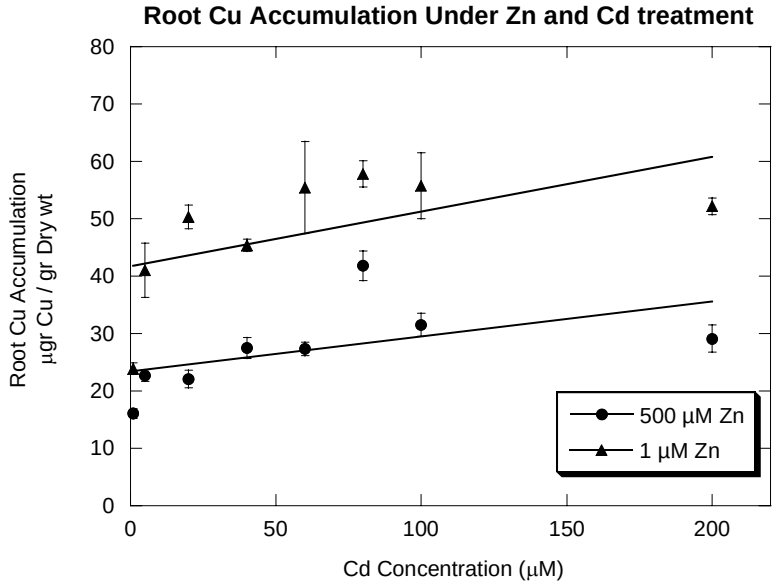
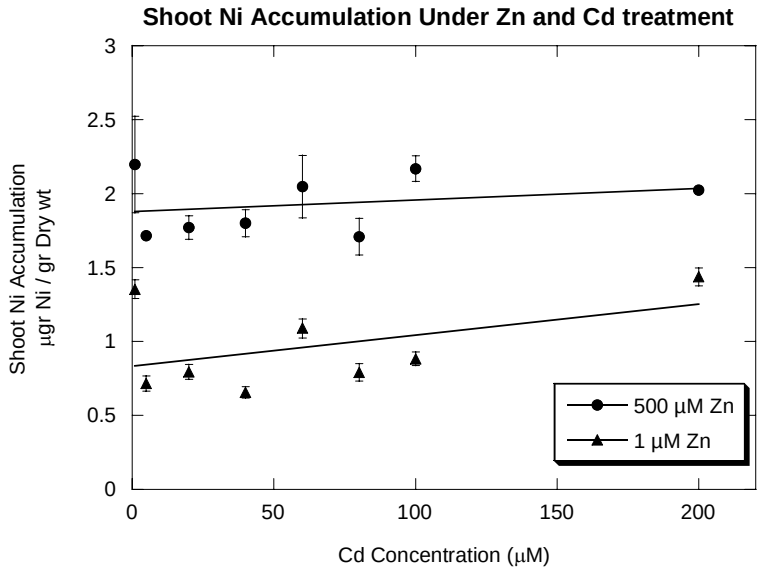

Root Cu Accumulation Under Zn and Cd treatment
Root Cu Accumulation
µgr Cu / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
10
20
30
40
50
60
70
80
0
50
100
150
200

Figure 2.7 (A; B) Cadmium and zinc dependant accumulation of nickel in *Thlaspi caerulescens* plants.

A) Ni accumulation in the shoots *of Thlaspi caerulescens* plants. Rectangles represent 1μM Zn-grown plants and circles represent 500μM Zn-grown plants.

B) Ni accumulation in the roots of *Thlaspi caerulescens* plants grown as described in A.

A


Shoot Ni Accumulation Under Zn and Cd treatment
Shoot Ni Accumulation
µgr Ni / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
0.5
1
1.5
2
2.5
3
0
50
100
150
200

B

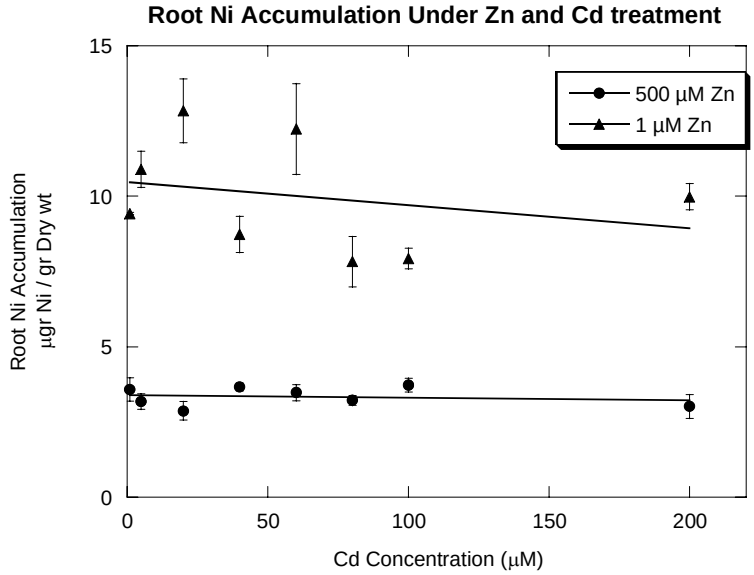
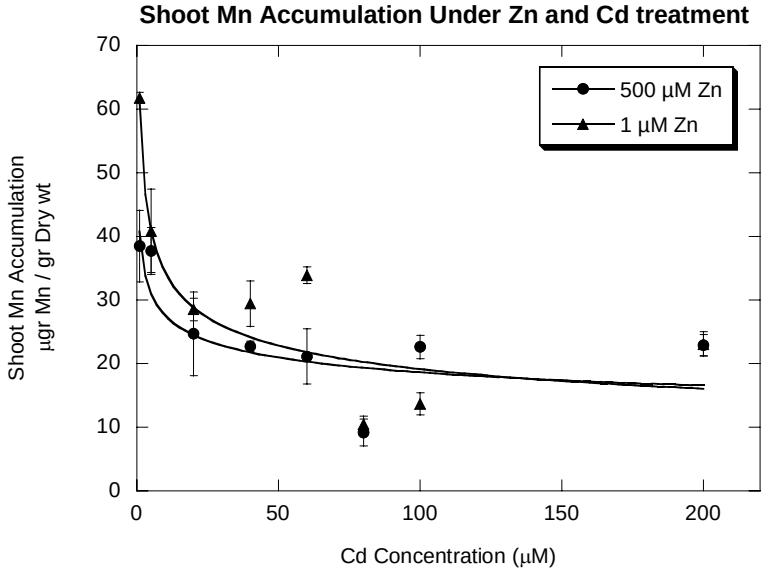

Root Ni Accumulation Under Zn and Cd treatment
Root Ni Accumulation
µgr Ni / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
5
10
15
0
50
100
150
200

Figure 2.8 (A; B) Cadmium and zinc dependant accumulation of Mn in *Thlaspi caerulescens* plants.

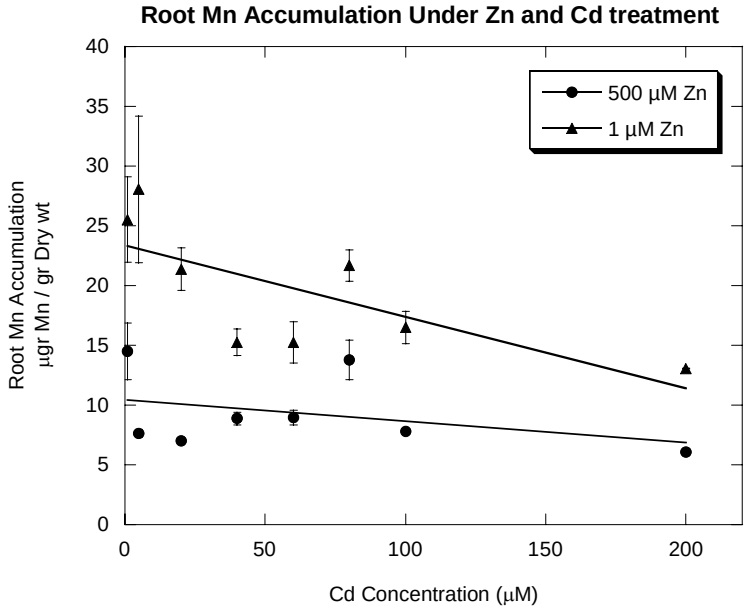
A) Mn accumulation in the shoots of *Thlaspi caerulescens* plants. Rectangles represent 1μM Zn-grown plants and circles represent 500μM Zn-grown plants.

B) Mn accumulation in the roots of *Thlaspi caerulescens* plants grown as described in A.

A


Shoot Mn Accumulation Under Zn and Cd treatment
Shoot Mn Accumulation
µgr Mn / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
10
20
30
40
50
60
70
0
50
100
150
200

B


Root Mn Accumulation Under Zn and Cd treatment
Root Mn Accumulation
µgr Mn / gr Dry wt
Cd Concentration (µM)
500 µM Zn
1 µM Zn
0
5
10
15
20
25
30
35
40
0
50
100
150
200

Measurement of root unidirectional Cd^{2+} influx using ^{109} Cd radiotracer techniques.

Short term ^{109}Cd radiotracer root uptake techniques, which our lab has previously shown to effectively measure unidirectional metal influx into the root symplasm (Lasat et al 1996), were used to investigate the effect of high *versus* low plant Zn status on root Cd uptake. As seen in Figure 2.9, *Thlaspi caerulescens* plants grown on the high Zn regime maintained significantly higher root Cd^{2+} influx rates, particularly at lower Cd concentrations, compared to plants that were grown on the normal Zn concentration (1 μM Zn). The difference in root Cd^{2+} influx was much greater for uptake at the lower levels of Cd (1, 5, and 10 μM; Figure 2.9 A) than for root uptake at higher Cd concentrations (60 - 100μMCd; Figure 2.9B). These findings clearly indicate that the increase in plant heavy metal accumulation with increasing plant Zn status, is due at least in part, to enhanced root metal uptake.

Discussion

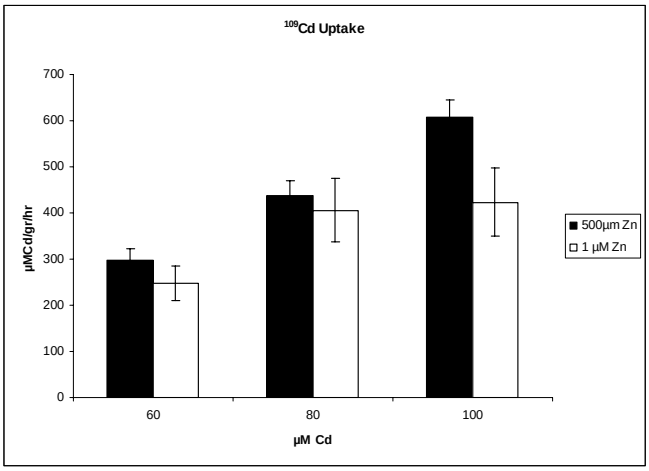
Hyperaccumulating plant species, such as the Zn/Cd/Ni hyperaccumulator, *Thlaspi caerulescens*, have the ability to hyperaccumulate multiple heavy metals at the same time from a complex nutrient or soil solution. In this study, the interactions between Zn and Cd transport and accumulation were studied, in order to better understand the overall hyperaccumulation phenotype. In this study, high and low Zn and Cd grown plants were specifically compared for their ability to tolerate and accumulate these two heavy metals, as well as other heavy metals and micronutrients Cu, Ni, and Mn. In an initial study, it was found that high Zn-grown (500 μM) *Thlaspi caerulescens* plants were considerably more tolerant of high (200 μM Cd) concentrations of Cd compared to the plants that are grown on a "normal" (1μM) level

Figure 2.9 (A; B) Root Cd (^{109}Cd) influx for *Thlaspi* caerulescens plants grown on low (1 µM Zn) and high (500 µM Zn).

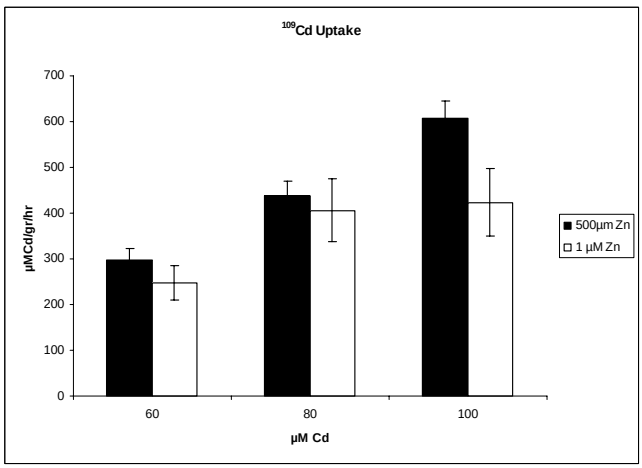
A) Root Cd (^{109}Cd) influx at Cd concentrations of 1, 5, or 10µMCd. Filled bars represent *Thlaspi caerulescens* plants grown on high (500µM) Zn and open bars represent *Thlaspi caerulescens* plants grown on low (1µM) Zn.

B) Root Cd (^{109}Cd) influx at higher Cd concentrations (60, 80, and 100 µM) For plants grown as described in A.

A


109Cd Uptake
700
600
500
400
300
200
100
0
µMCd/gr/hr
60
80
100
µM Cd
500µm Zn
1 µM Zn

B


109Cd Uptake
700
600
500
400
300
200
100
0
µMCd/gr/hr
60
80
100
µM Cd
500µm Zn
1 µM Zn

of Zn (Figure 2.1). On first observation, it was assumed that this phenomenon could be explained by a simple competition between Zn^{2+} and Cd^{2+} ions for entry point for metals into the plant: the active site of metal transporters in the root-cell plasma membrane. However, upon further examination it was found that there is a more complex interaction between Zn and Cd that appears to be occurring at the whole plant level, and involves processes associated with heavy metal translocation from the soil to the shoot. In a non-hyperaccumulator plant ionic competition between Zn and Cd can result in a decreased accumulation of one upon exposure in the root environment with high concentrations of the other metal. It has been shown that in non-accumulators, high Zn treatment decreases the ability to accumulate Cd (Hart et al 2002). Because of its very similar ionic radius and other similar physical characteristics, Cd can replace other divalent metals such as Zn in critical binding sites in proteins and other macromolecules. However, in this investigation of whole plant interactions between Zn and Cd some interesting characteristics that appear to be specific for hyperaccumulator plants were discovered. First, it was found that Zn and Cd accumulation in the shoots of *Thlaspi caerulescens* are positively correlated. *Thlaspi caerulescens* plants grown on high Zn are able to not only tolerate high levels of Cd in the root-bathing media, but also accumulate significantly more Cd in the shoots compared to the plants that are grown on a typically low level of Zn (Figure 2.2). For example, plants grown on 1μM Zn and 100μM Cd accumulated 2800 ppm Cd in the shoots, while plants grown on 500μM Zn and the same concentration of Cd were found to accumulate Cd in the shoots to a concentration of 4200 ppm. On the other hand *Thlaspi caerulescens* roots show the reverse picture in that high Zn-grown plants maintain significantly lower root Cd concentrations that in roots of plants grown on 1 μM Zn. It is likely that this root response, which is also typical of non-accumulator plants, does not involve processes associated with heavy metal transport

and tolerance. Divalent cations that have a high affinity for the fixed negative charges in the cell wall; thus the majority of the divalent metal cations reside in the root apoplast for roots exposed to the metals via nutrient or soil solution (see, for example, Hart et al 1998; Lasat et al 1996).

The interesting stimulation of shoot Cd and Zn accumulation in response to elevated plant Zn or Cd status appears to be unique for hyperaccumulator plants and it is quit different from normal (non-hyperaccumulator) plants. It is generally believed that since Cd has no known biological function, its entry into the plant is via transport processes that are normally functioning for Zn (Marschner 1995). Therefore, when the concentration of Zn in the environment increases, it increases the ionic competition for transporter proteins between Zn and Cd, which normally leads to decreased plant accumulation of Cd.

Interestingly, this response is broader than just an effect of plant Zn status on Cd accumulation. In addition, it is shown in this study that elevated plant Cd status also increases shoot Zn accumulation. These findings clearly indicate that Zn and Cd transport and accumulation in *Thlaspi caerulescens* are interconnected, while the accumulation of one metal positively affecting the accumulation of the other one in the shoot.

Because there currently is not have enough information concerning the mechanisms underlying this response, one can only speculate about its basis. One possibility is that upon long term exposure to high levels of Zn or Cd, an additional level of metal tolerance is induced, allowing the plants to accumulate even higher levels of heavy metals in the shoot. However, circumstantial evidence argues against this scenario of enhanced metal tolerance. As seen in Figure 2.9, the high Zn-grown plants maintain a much higher root Cd^{2+} influx than do the low Zn plants, at Cd concentrations in the uptake solution that are not toxic to *T. caerulescens* plants. This

finding suggests that there is a more global response to high plant metal status that at least in part stimulates heavy metal entry into the root.

Based on previous physiological analyses of Zn/Cd hyperaccumulation in *T. caerulescens*, it has been suggested that xylem loading is a key transport step involved in moving the majority of the absorbed metal to the shoot (see, for example, Lasat et al 1996, 1998; Papoyan and Kochian, 2004). This is in contrast with non-accumulating plant species that tend to sequester the absorbed heavy metal in the root. Three major transport components are involved in the process of moving metal ions from the soil to the shoots. The first step is metal transport into the root from the soil and several transporters have been identified that may play an important role for this step including ZNT1 (Pence et al 2000). Second is the transport from root to shoot through the xylem and it has been suggested that transporters such as HMA4 may be involved in the process of xylem loading (Hussain et al 2004; Papoyan et al 2004). The third step involves transport of the metal from the xylem to the leaf with subsequent storage in leaf epidermal and mesophyll cells.

It was shown here that there is a stimulation of root Cd influx in response to high Zn which is part of the response to high concentration of heavy metals in the environment. However, it is possible that xylem metal transport may also be involved, and could even be the cause behind the enhanced root Cd influx as most of the absorbed metal would then be efficiently transported from the root to shoot. Since non-accumulator plants do not have a strong metal detoxification mechanism in the shoots, most of the metals are excluded from the shoot via root sequestration, presumably to protect the sensitive photosynthetic apparatus which are extremely sensitive to heavy metals (Kupper et al 1999, 2002). On the other hand, hyperaccumulator plants actively translocate heavy metals into the shoots presumably because they have the mechanisms to tolerate very high metal levels in the shoot.

HMA4 is a heavy metal ATPase that has been shown to be localized to the xylem parenchyma in *Arabidopsis* and *Thlaspi caerulescens* and is presumed to be the micronutrient/heavy metal transporter responsible for loading of these metals into the xylem (Hussain et al 2004; Papoyan et al 2004; Beranrd et al 2004; Verret et al 2004). Interestingly, expression of *HMA4* in *Arabidopsis thaliana* is down-regulated upon exposure to heavy metals (Mills et al 2003), while its expression in *Thlaspi caerulescens* is up-regulated upon exposure to high concentrations of Cd and Zn (Papoyan et al 2004). A study that directly compared levels of expression for the *HMA4* gene between *Arabidopsis* and *Thlaspi caerulescens* showed that *HMA4* has much higher constitutive expression in *Thlaspi caerulescens* compared to *Arabidopsis* (Bernard et al 2004). These qualitative and quantitative differences in expression of this heavy metal transporter that is presumably involved in xylem loading explain the observed Zn and Cd interactions observed. As mentioned above, Cd and Zn transport pathways in *Thlaspi caerulescens* may be the same and transporters such as HMA4 may not be able to significantly distinguish between Zn and Cd ions. Therefore, it is possible that increasing the concentration of either ion up-regulates the expression of xylem loading transporters which in turn increases the accumulation of both metals in the shoot.

The effect of increased plant Cd and Zn status on the accumulation of other divalent heavy metal micronutrients such as Ni, Cu, Mn was examined here and the results suggest that the response of *Thlaspi caerulescens* to elevated metal status is not restricted to Zn and Cd. As seen in Figures 2.6, 2.7, and 2.8, high Zn-grown plants also exhibit a significant increase in shoot Cu and Ni accumulation, while shoot Mn accumulation was not affected. This finding also supports the hypothesis that plants grown on high Zn have more active heavy metal and micronutrient xylem loading. In summary, it was shown in this study that *Thlaspi caerulescens* plants grown on

elevated levels of Zn and/or Cd for extended periods of time exhibit a significantly enhanced ability to accumulate metals in the shoot, including Cd, Zn, Cu and Ni. This stimulated shoot metal accumulation is associated with enhanced root metal influx, and presumably, enhanced xylem transport of these metals from the root to shoot. Based on previous physiological and molecular findings that suggest that xylem loading of metals plays a key role in the hyperaccumulation phenotype, it is possible that this transport step also plays a crucial role in the processes identified here. Also, this enhanced xylem loading triggered by exposure to high heavy metal levels for extended periods may translate into improved heavy metal tolerance, as the metals are more efficiently translocated to the shoots where highly effective metal tolerance mechanisms must be operating.

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CHAPTER III

Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation: Characterization of a novel heavy metal transporting ATPase.

Abstract

Thlaspi caerulescens is a heavy metal hyperaccumulator plant species that is able to accumulate extremely high levels of Zn and Cd in its shoots (up to 30,000 ppm Zn and 10,000 ppm Cd). In this research, *Thlaspi caerulescens* has been studied as a model plant to better understand the mechanisms of heavy metal hyperaccumulation and tolerance, as well as for a potential source of genes for developing high biomass plant species that are better suited for phytoremediation of metal contaminated soils. Here the results of a yeast complementation screen aimed at identifying candidate heavy metal (Cd) tolerance genes in *Thlaspi caerulescens* are reported. A number of *Thlaspi* genes that conferred Cd tolerance in yeast were identified, including possible metal binding ligands from the metallothionein gene family, and a P-type ATPase that is a member of the P_{1B}-subfamily of purported heavy metal-translocating ATPases. A detailed characterization of the *Thlaspi* caerulescens heavy metal ATPase was conducted, in order to investigate its possible role in metal hyperaccumulation. *TcHMA4*, which is a homolog of the *Arabidopsis AtHMA4* gene, was shown to mediate yeast metal tolerance via active efflux of a number of different heavy metals (Cd, Pb, Zn, Cu) out of the cell. However in *Thlaspi caerulescens*, based on differences in tissue-specific and metal-responsive expression of this transporter compared with expression of its homolog in *Arabidopsis thaliana*, we suggest that it is not involved in metal tolerance. Instead, it is proposed that it may play a role in xylem loading of metals and thus could be a key player in the hyperaccumulation phenotype expressed in *Thlaspi caerulescens*.

Introduction

Our laboratory has been studying the physiology and molecular biology of heavy metal hyperaccumulation in *Thlaspi caerulescens* and has previously shown that altered metal ion transport plays an important role in the hyperaccumulation phenotype in this plant species (Lasat et al 1996; 1998). In comparison with related non-accumulating plant species, *Thlaspi caerulescens* mediates a much greater root heavy metal influx, much more rapid and efficient translocation of the absorbed metal from the root to the shoot in the xylem, and effective storage of the absorbed heavy metals in the shoot. One of the distinctive hallmarks of *Thlaspi caerulescens* and other metal hyperaccumulators is their ability to translocate most of the absorbed metal from the root to the shoot. A second hallmark for this hyperaccumulator is its metal tolerance, which is exhibited both in roots and shoots. Mechanisms of metal tolerance can involve both ion transporters that transport the metal out of the cytoplasm (either into an internal compartment or out of the cell), and the synthesis of metal binding ligands that can detoxify the metal in the cytoplasm (Clemens et al 2001).

This study focused on the identification of candidate heavy metal tolerance genes from *Thlaspi caerulescens* via a yeast functional complementation screen. A number of *Thlaspi* genes that conferred Cd tolerance to yeast were identified, including possible metal binding ligands from the metallothionein gene family, and a P-type ATPase that is a member of the P_{1B}-subfamily of purported heavy metal-translocating ATPases (Axelsen and Palmgren, 1998). A detailed characterization of this *Thlaspi* heavy metal ATPase was conducted, in order to investigate its possible role in metal hyperaccumulation. Here evidence is presented that this ATPase facilitates a high degree of heavy metal tolerance in yeast by mediating the active efflux of heavy metals out of the cell. However in *Thlaspi caerulescens*, based on differences in tissue-specific and metal-responsive expression of this transporter

compared with expression of its homolog in *Arabidopsis thaliana*, we suggest that it is not involved in metal tolerance in *Thlaspi*. Instead, it is proposed that it may play a role in xylem loading of metals and thus could be a key player in the hyperaccumulation phenotype expressed in *Thlaspi caerulescens*.

Materials and Methods

Plant Growth Conditions

Thlaspi caerulescens (ecotype Prayon) seedlings were grown on a modified Johnson's solution that had a macronutrient composition of 1.2 mM KNO_3, 0.8mM $Ca(NO_3)_2$, 0.2mM $NH_4H_2PO_4$ and 0.2 $MgSO_4$ and a micronutrient composition of 50µM KCl, 12.5µMH_3BO_4, 1µM $MnSO_4$, 1µM$ZnSO_4$, 0.5µM $CuSO_4$, 0.1µM Na_2MoO_4, 0.1µM $NiSO_4$, and 7.5µM Fe-EDDHA (N, N'-ethylenediamine-di(O-hydroxyphenylacetic acid). The solution was buffered at a pH of 5.5 with 1mM MES (2-[N-morpholino]-ethanesulfonic acid) buffer. *Thlaspi* seeds were placed in a drop of 0.7 % (w/v) low temperature gelling agarose on nylon mesh circles (1 mm mesh openings) which, in turn, were positioned on a coarser mesh support sealed to the bottom of black plastic cups. The cups and seeds were fitted into holes cut into black plastic lids covering 5 liter black plastic pots. Seedlings were grown in a growth chamber at 25/15 °C (light: dark, 16:8 h) under a light intensity of 300 µmol photons $m^{-2}s^{-1}$for two weeks, and then seedlings were transferred into identical growth containers containing modified Johnson's solution supplemented with specific concentrations of Zn (0, 1, 5, or 100µM) or Cd (10 or 100µM). Tissue samples were harvested after 20 days growth in the Zn and Cd supplemented medium.

Yeast Growth Conditions

The wild type yeast strain DY1457 (MATa *ade6 can1 his3 trp1 ura3*) were transformed either with the empty yeast expression vector pFL61 (Minet et al, 1992) (referred to as control cells) or pFL61 containing *Thlaspi caerulescens* cDNA. Transformed yeast strains were grown on synthetic dextrose (SD) minimal medium (Rose et al 1990) supplemented with 0.1% casamino acids, adenine sulfate (20mg/Ml), L-trypthophan (20mg/ml), L-histidine (20mg/ml), l-leucine (30mg/ml). This supplemented media will be referred to as SD media in the manuscript. Solid media consisted of the same SD media supplemented with granulated agar (Difco Laboratories, Sparks, MD) at a concentration of 20g/L. To test the heavy metal tolerance of control and *Thlaspi* transformed yeast, growth was conducted on high Cd plates consisting of SD solid media supplemented with 90 μM $CdCl_2$. Plates were inoculated with an aliquot of liquid media yeast culture and incubated at 30^{o} C for three days before plates were photographed to document the relative Cd tolerance of the different yeast strains.

Functional Complementation Assay for Heavy Metal Tolerance

A cDNA library was constructed with combined $polyA^{+}$ RNA from roots and shoots of *T. caerulescens* seedlings grown on both Zn-deficient and Zn-replete nutrient solution. The cDNA was synthesized using the Superscript Choice System (Gibco-BRL), then ligated using BstXI/EcoRI adapters into the bifunctional yeast/*Escherichia coli* expression plasmid vector pFL61 (Pence et al., 2000). This vector contains a yeast phosphoglycerate kinase promoter and a uracil selection marker. Preliminary experiments determined that the yeast DY1457 wild type stain was unable to grow on solid SD media containing 90μM Cd. Subsequently, wild type yeast was transformed with *T. caerulescens* cDNA and plated on high Cd (90μM)

solid SD media. After growth for three days at 30^{o} C, 35 individual yeast colonies were found to express the ability to grow on the high Cd media. Yeast from each of these colonies was replated on high Cd plates to verify the metal tolerance phenotype, and then DNA was extracted from each of these colonies and the sequence was determined for each sample (Applied Biosystems Automated 3730 DNA Analyzer, Cornell University).

Quantification of Yeast Metal Accumulation

Control (wild type DY1457 yeast strain containing the empty pFL61 vector) and *TcHMA4*-transformed yeast were grown on liquid SD medium. At the mid-log phase of growth, the metal accumulation experimented was initiated by adding either $CdCl_2$ or $PbCl_2$ to a final concentration of 20 or 10 μM, respectively. After 5, 30, and 70 minutes of metal accumulation, aliquots of yeast cells were taken, centrifuged at 10,000 x g for 2 minutes to separate yeast cells from the metal containing media, washed once with 5 mM $CaCl_2$ to desorb Cd^{2+} or Pb^{2+} from the yeast cell walls, and then centrifuged again at 10,000 x g for 2 minutes. The heavy metal content of the yeast cell pellet was analyzed using an inductively-coupled, plasma trace analyzer emission spectrometer (Model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA) 1, 3, and 5 hours of yeast Cd accumulation. All the metal accumulation values are the average $\pm$ the standard error determined from four replicate experiments.

Determination of $^{109}Cd^{2+}$ Influx and Efflux in Yeast

Radiotracer (^{109}Cd) flux methodologies were used to determine Cd^{2+} influx and efflux in control and transformed yeast cells (transformed with either full length or partial *TcHMA4* clones). For the Cd^{2+} influx experiments, the control and *TcHMA4*-

transformed yeast were grown on liquid SD medium until they reached the mid-log phase of growth. At this time, the Cd^{2+} influx experiment was initiated by adding $^{109}CdCl_2$ to a final [Cd] of 10 μM. Yeast was sampled at 30sec (which was an approximation of Cd bound to cell walls), 3, 5, 10 and 15 min. The yeast aliquots for each time point were transferred into 1.5ml plastic microfuge tubes containing a 200 μl silicon oil/dinonyl phthalate pad on top of 2μl of 40% perchloric acid. Tubes were centrifuged at 7,000 x g for one minute to separate the yeast from the radiolabeled uptake solution and the supernatant was removed. The microfuge tube with the remaining cell pellet was placed in a scintillation vial and the amount of ^{109}Cd accumulation determined using a Perkin Elmer "WIZARD 3" 1480 Automatic Gamma Counter. The amount of ^{109}Cd associated with yeast cells after 30 seconds was determined in preliminary experiments to be a measure primarily of Cd associated with the yeast cell wall and this value was subtracted from each time point to obtain a measure of Cd accumulation in the yeast cell symplasm. Preliminary experiments determined that Cd^{2+} accumulation was linear between 30 sec and 15 minutes and these values were used to calculate Cd^{2+} influx values.

For the Cd efflux experiments, the control and *TcHMA4*-transformed yeast cells were grown on liquid SD medium and again, at the mid-log phase of growth, $^{109}CdCl_2$ was added to a final concentration of 10μM. The yeast cells were allowed to accumulate $^{109}Cd^{2+}$ for 90 minutes, centrifuged at 7,000 x g for 2 min, washed briefly in 5mM $CaCl_2$ and then centrifuged again. The washed yeast cells were then transferred to liquid SD medium containing non-radioactive $CdCl_2$ (10 μM) to initiate the Cd efflux, and yeast aliquots were sampled at 30, 60, 90, and 120 minutes. The yeast were treated as described above for the influx experiments to determine the amount of ^{109}Cd appearing in the efflux solution as well as the amount of ^{109}Cd disappearing from the yeast cells. The rate of Cd efflux was calculated from both the

difference in the rate of appearance of ^{109}Cd in the efflux solution at different time points as well as in the difference in the rate of disappearance in ^{109}Cd from yeast cells. All of the Cd influx and efflux values are the average $\pm$ the standard error determined from four replicate experiments.

Comparative Determination of Cd Tolerance for Different Yeast Genotypes

To determine the relative Cd tolerance of control and *TcHMA4*-transformed cells (transformed with either full or partial clones), the different yeast genotypes were grown on regular liquid SD medium until they achieved an optical density (OD) of 1.0. At this point, the cells were harvested and serial dilutions were made to achieve OD's of 0.1, 0.01, and 0.001. SD plates were made containing the appropriate concentration of $CdCl_2$, and a 20µl aliquot for each cell dilution was spotted on to the plates, as described by (Lee et al 2003). The plates were placed in a 30°C incubator for 3 days after which pictures were taken.

Southern and Northern Analysis in *Thlaspi caerulescens*

For southern analysis, genomic DNA was isolated from *Thlaspi caerulescens* plants and digested with the restriction enzymes, BamHI, EcorI, and HindIII. The DNA was run on a 0.8 % agarose gel and transferred onto a nylon membrane (Hybond N^+; Amersham Pharmacia). For Northern analysis, *Thlaspi caerulescens* plants were grown in modified Johnson's nutrient solution that was supplemented with different Zn^{2+} and Cd^{2+} concentrations, as described above Total RNA was isolated from roots and shoots, denaturated, separated with denaturating agarose gel electrophoresis, and transferred to a nylon membrane (Hybond N^+; Amersham Pharmacia). For both southern and Northern analysis, 3'end probes of the *TcHMA4* gene were labeled with [α-^{32}P]dCTP using random hexamer primers and hybridized to the membrane

overnight. 20 μg of RNA was loaded in each lane and equal loading was insured by ethidium bromide staining of ribosomal subunits. Following hybridization at 65^{o}C, the nylon membranes were washed twice for 15 minutes at 65^{o}C in a low stringency wash solution (2x SSC, 0.1% SDS). Each Northern experiment was repeated at least twice.

Production of Anti-TcHMA4 Antibodies

A peptide antibody was generated to the TcHMA4 protein based on a region selected from deduced amino acid sequence of TcHMA4 that is unique to this member of the HMA4 family and has a high degree of probability of reacting with the antibody. The peptide sequence, DKEKAKETKLLLASC, is derived from the 3' cytoplasmic tail of the protein and was provided to Sigma Genosys for antibody production (Sigma Aldrich, Woodland, TX). The peptide was conjugated with KHL (keyhole limpet hemocyanin), the conjugated peptide was injected subcutaneously into two rabbits and after several boost injections, three bleeds were performed on days 49, 56, and 77 after the injection. A 1:1000 dilution of antiserum from the third bleed was successful in detecting a single band of the predicted size for the TcHMA4 protein in plasma membrane vesicles isolated from *Thlaspi caerulescens* roots and was used in subsequent experiments.

Plasma membrane fraction isolation and Western blot analysis.

Plasma membranes were isolated using the aqueous two phase partitioning technique which preferentially partitions plasma membranes into the upper phase and the other membranes (i.e. membranes from Golgi, ER, chloroplast, tonoplast, etc.) into the lower phase (Schaller et all 1995). 100 grams of fresh roots from *Thlaspi caerulescens* plants grown on 1/5 strength Johnson solution supplemented with 10uM Cd were hand homogenized for 10 minutes using fresh blades in a solution containing

330mM sucrose, 1 mM Na-EDTA, 10 mM Tris-KOH, pH 7.5, and 1mM PMSF (added just before use). The homogenate was filtered through 3 layers of cheese cloth. Cellular debris was precipitated using a 20,000xg centrifugation for 5 minutes. Subsequently, the supernatant was centrifuged at 95,000xg for 4 hours to pellet the microsomal membrane fraction (Beckman SW28 rotor). The microsomal pellet was resuspended in phosphate buffer containing 330uM sucrose and 5mM potassium phosphate (pH7.8). The plasma membrane fraction was isolated from the microsomal membranes by placing 2ml of the microsomal fraction on top of a 14 gram aqueous polymer two-phase system which had a final concentration of 6.2% Dextran (w/w), 6.2% PEG (w/w), 330mM sucrose, 5mM potassium phosphate (pH 7.8), and 3mM KCl. Enrichment of plasma membranes in the upper phase was obtained following 3 batch procedures by washing the upper phase with fresh lower phase. Subsequently, the upper and lower phases were centrifuged at 100,000xg for 2 hours. The resulting pellets were resuspended in the same media used for the microsomal membranes to give a final membrane concentration of 200µg protein per milliliter.

Western blot analyses were performed using the two phases enriched in plasma membrane and microsomal membranes. Proteins from both membrane fractions were solubilized in SDS gel loading buffer (50mM TRIS-CL, 100mM DTT, 2% SDS, 0.1% Bromophenol Blue, 20% glycerol pH6.8) and separated on a 12% SDS-page gel in a Mini Protean II Cell (Bio-Rad Laboratories, Hercules, CA) and transferred to a Trans-Blot pure nitrocellulose membrane (0.45 µm, Bio-Rad) using a mini trans-blot transfer cell with transfer buffer containing 39mM glycine, 48mM Tris (pH 8.3), 0.037% SDS and 20% methanol. After the transfer, the membranes were incubated in PBS (Phosphate Buffered Saline)) blocking buffer solution (5% dry milk (Non Fat Dry Milk, BIO-RAD)) at 4^0 C overnight. The next day the primary rabbit TcHMA4 polyclonal antibody solution 1:1000 dilution in blocking buffer was added and

membranes were incubated at room temperature with agitation for 1hr. Membranes were then washed 3 times for 5 min each in PBS, and incubated with HRP (Horse Radish Peroxidaze) labeled secondary goat anti-rabbit IgG for an additional 1hr (1:5000 dilution in blocking buffer). Membranes were washed again with PBS 3 times for 5 min each and incubated with 10 ml of an equal volume mixture of Supersignal West Pico Luminol/Enhancer Solution and Stable Peroxide Solution (Pierce Biotechnologies, Rockford, IL) with agitation for 5 min. The membranes were then drained and exposed on Kodak BioMAX MS film for 10 min.

TcHMA4 expression in oocytes and mammalian cells.

cRNA was prepared using the RNA Capping Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions from ScaI -digested pGEM –4Z plasmid DNA, which contained the TcHMA4 gene coding region between the 3'- and 5' untranslated regions of a Xenopus β-globin gene. Harvesting of stage V to VI *Xenopus laevis* oocytes was performed as described by Golding (1992). Defolliculated oocytes were maintained in ND96 solution (96mM NaCl, 2mM KCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, 5mM HEPES, and 1M NaPyravete) supplemented with 50 $\mu g/ml^{-1}$ Gentamycin overnight prior to injections. *X. laevis* oocytes were injected with 48 nl of water containing 30 ng of cRNA encoding TcHMA4 gene (or 48 µl of water as control), and incubated in ND96 at 18°C for 2 to 4 days. One, three, and five days after the injection, the accumulation studies were performed using ^{109}Cd radiotracer experiments.

Mammalian Hek293 cells were transfected with TcHMA4::GFP DNA in the NT-TOPO GFP vector. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 g/ml penicillin, and 100 g/ml streptomycin. The expression vectors containing GFP or the

TcHMA4::GFP construct were transfected into HEK-293 cells using LipofectAMINE reagent The transfection was performed using LeipofectAMIN method according to manufactures recommendations (Life Technologies).

Results

Complementation Screening of Candidate Metal Tolerance Genes from *T. caerulescens*

Preliminary studies determined that when 90 μM Cd was included in solid SD media (modified synthetic dextrose media; see Materials and Methods), both the wild type DY1457 yeast strain as well as DY1457 transformed with the empty pFL61 vector were unable to grow. Thus, this Cd level was used to select for Cd tolerant transformants Figure 3.1. Yeast were transformed with a *T. caerulescens* cDNA library constructed in the yeast expression vector, pFL61, and 35 yeast colonies were identified that were able to grow on this restrictive level of Cd. Yeast from each colony was grown up and replated on the same high Cd media to verify the Cd tolerant phenotype. Subsequently, the plasmids bearing the *T. caerulescens* cDNA inserts were isolated, and the *Thlaspi* cDNAs were subjected to restriction digest analysis and DNA sequencing. Based on this analysis, nine unique *Thlaspi* genes were identified that conferred Cd tolerance to wild type yeast. As depicted in Table 3.1, which lists the identity of each of these putative metal tolerance genes, three had a close resemblance to metallothionein genes previously identified in *Arabidopsis thaliana* and *Brassica*

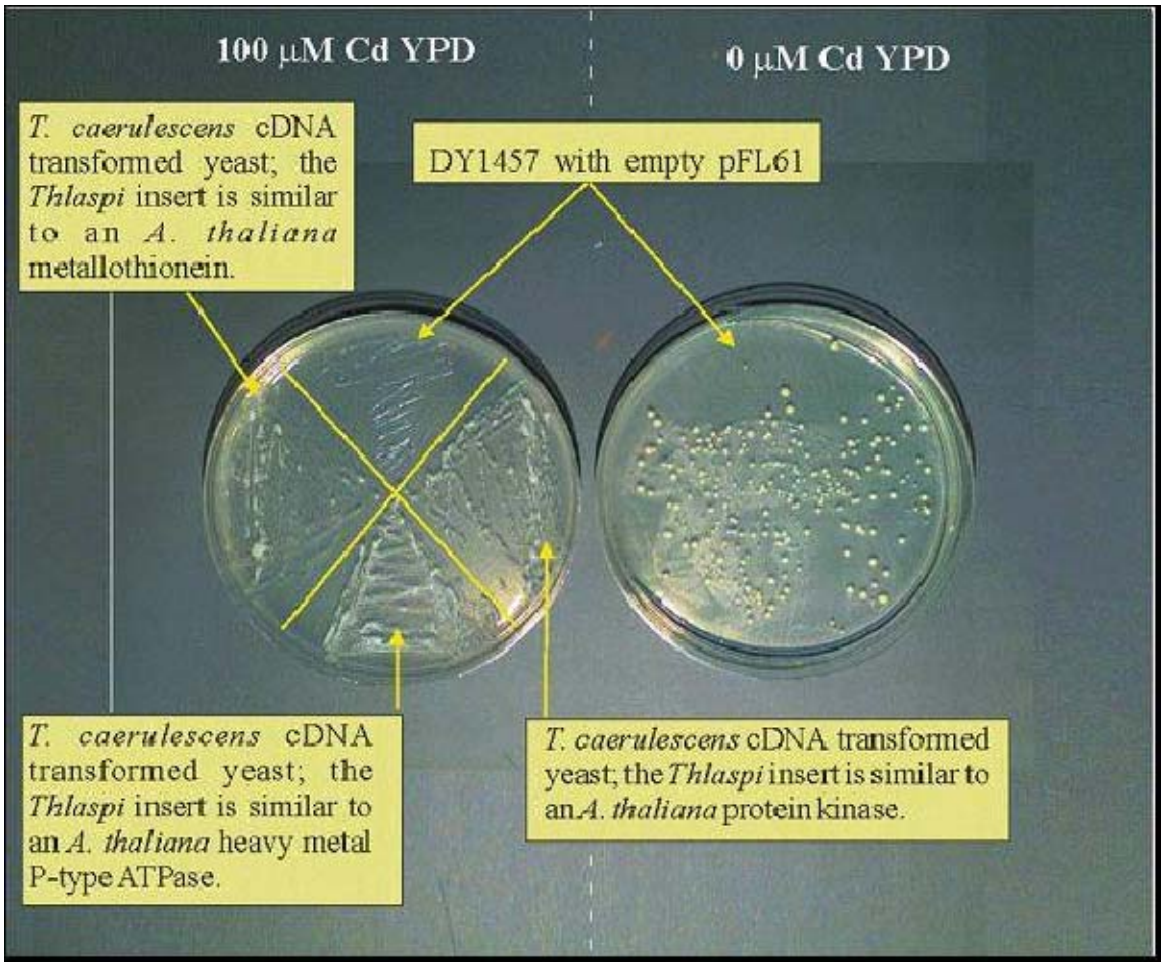

Figure 3.1 Functional screen of yeast for *Thlaspi caerulescens* cDNA's that confer Cd tolerance.

Yeast (DY1457) cells harboring the empty pFL61 yeast expression vector grow normally on medium containing 0μM Cd (right side). On medium containing 100μM Cd, yeast harboring three different *T. caerulescens* cDNAs that confer the ability to grow on high Cd are shown (left side).

Table 3.1 *Thlaspi* caerulescens genes that confer Cd tolerance in yeast.

All the accession numbers provided are GenBank accession numbers.

Clone	Closest Match and Function	Accession #	GenBank Hit/% Identity
1	*Brassica juncea* metallothionein -like protein	AY486002	Y10849/82%
2	*Arabidopsis thaliana* metallothionein	AY486003	L15389/78%
3	*Arabidopsis thaliana* integral membrane putative protein	AY486006	NM_118925/83%
4	*Sinapis alba* subunit of oxygen evolving system of photosystemII	AY486007	Y07498/85%
5	*Arabidopsis thaliana* light regulated kinase	AY486008	Z12120/ 93%
6	*Arabidopsis thaliana* chloroplast inner membrane putative protein	AY486009	NM_116206/ 79%
7	***Arabidopsis thaliana* putative heavy metal P-type ATPase**	**AY486001**	**AF412407/71%**

juncea. Five of the other genes either had an unknown function based on DNA and protein sequence comparisons, or possibly play roles in diverse processes such as photosynthesis, protein synthesis, or signal transduction. The final gene on the list in Table 3.1 was represented by two different length copies of a partial sequence of a gene (423 and 1152 bp in length) that had a strong similarity to the *Arabidopsis thaliana* heavy metal transporting ATPase, *AtHMA4*. RACE-PCR techniques using the GeneRacer kit (InvitrogenLife Technologies, Carlsbad, CA) were employed to clone the full length *TcHMA4* cDNA from *Thlaspi caerulescens*. This approach targets only the 5' end-capped mRNA, and thus allows for the cloning of only the full length cDNA. Because of the possible importance of heavy metal ATPases to metal hyperaccumulation and tolerance in *Thalspi caerulescens*, our subsequent research focused on a detailed characterization of *TcHMA4*.

Both nucleotide and amino acid sequence comparisons between *TcHMA4* and similar sequences from other organisms indicated that it is a member of the P-type ATPase superfamily, and more specifically, in the P_{1B} subfamily of ATPases that are purported to transport heavy metals. As mentioned above, the *Arabidopsis* P-type ATPase that it is most similar to *TcHMA4* is *AtHMA4*, which was recently shown to be associated with in Cd export and tolerance (Mills et al 2003). The deduced amino acid sequence for TcHMA4 and its alignment with AtHMA4 are shown in Figure 3.2. The full-length *TcHMA4* open reading frame is 3561 bp in length and encodes a polypeptide of 1186 amino acids. TcHMA4 is 71% identical to AtHMA4, and contains many of the same predicted motifs found in AtHMA4 and other heavy metal ATPases, including eight predicted membrane spanning domains, and a large C terminal tail that harbors a number of putative heavy metal binding domains (see Figure 3.1). These include a histidine repeat consisting of nine histidine residues, a number of cysteine pairs, and multiple single histidine residues.

Figure 3.2 Sequence alignment of *TcHMA4* from *Thlaspi caerulescens* and the *Arabidopsis* homolog, *AtHMA4*.

Deduced amino acid sequences for *TcHMA4* and *AtHMA4* (accession number 064474) are shown aligned using the Clustal W method. Asterisks indicate identical residues. A number of motifs common to P_{1B}-type ATPases are indicated, including the E1-E2 ATPase phosphorylation site (shaded in dark gray), the highly conserved CPX motif (boxed), and a putative N-terminal heavy metal binding site (underlined). Also, the numerous histidine and cysteine residues in the C terminus are also highlighted.

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Athma4 MALQ----NKEEEKKKVKKLQKSYFDVLGICCTSEVPIIENILKSLDGVKEYSVIVPSRT 56
Tchma4 MALQKEIKNKEEDKKTKKKWQKSYFDVLGICCTSEIPLIENILKSLDGVKEYTVIVPSRT 60
**** **** ** ** *************** * ************** *******

Athma4 VIVVHDSLLISPFQIAKALNEARLEANVRVNGETSFKNKWPSPFAVVSGLLLLLSFLKFV 116
Tchma4 VIVVHDSLLIPPFQIAKALNQARLEANVKVNGETSFKNKWPSPFAVVSGIFLLPSFLKFV 120
********** ******** ******* ******************** ** ******

Athma4 YSPLRWLAVAAVAAGIYPILAKAFASIKRPRIDINILVIITVIATLAMQDFMEAAAVVFL 176
Tchma4 YPPLRWLAVVGVAAGIYPILAKAVASIRRLRVDINILIIITVAATLAMQDYMEAAAVVFL 180
* ******* ************ *** * * ***** **** ******* *********

Athma4 FTISDWLETRASYKATSVMQSLMSLAPQKAIIAETGEEVEVDEVKVDTVVAVKAGETIPI 236
Tchma4 FTTADWLETRASYKANSVMQSLMSLAPQKAVIAETGEEVEVDEVQLNTIIAVKAGETIPI 240
** *********** ************** ************* * **********

Athma4 DGIVVDGNCEVDEKTLTGEAFPVPKQRDSTVWAGTINLNGYICVKTTSLAGDCVVAKMAK 296
Tchma4 DGIVVDGNCEVDEKTLTGEAFPVPKQRDSTVLAGTMNLNGYISVNTTALASDCVVAKMAK 300
****************************** *** ****** * ** ** *********

Athma4 LVEEAQSSKTKSQRLIDKCSQYYTPAIILVSACVAIVPVIMKVHNLKHWFHLALVVLVSG 356
Tchma4 LVEEAQGSKTKSQRLIDKCSQYYTPAIIIISAGFAIVPAIMKVHNLNHWFHLALVVLVSA 360
****** ******************** ** **** ******* ************

Athma4 CPCGLILSTPVATFCALTKAATSGLLIKSADYLDTLSKIKIVAFDKTGTITRGEFIVIDF 416
Tchma4 CPCGLILSTPVATFCALTKAATSGLLIS-AGHLDTLSKIKIAAFDKTGTITRGEFIVIEF 419
*************************** * ********* **************** *

Athma4 KSLSRDINLRSLLYWVSSVESKSSHPMAATIVDYAKSVSVEPRPEEVEDYQNFPGEGIYG 476
Tchma4 KSLSRDISLRSLLYWVSSVESKSSHPMAATIVDYAKSVSVEPRSEEVEDYQNFPGEGIYG 479
******* ********************************** ****************

Athma4 KIDGNDIFIGNKKIASRAGCSTVPEIEVDTKGGKTVGYVYVGERLAGFFNLSDACRSGVS 536
Tchma4 KIDGNNVYIGNKRIASRAGCSTVPEIEVDTKKGKTVGYVYVGERLAGVFNLSDACRSGVA 539
***** **** ****************** *************** ***********

Athma4 QAMAELKSLGIKTAMLTGDNQAAAMHAQEQLGNVLDVVHGDLLPEDKSRIIQEFKKEGPT 596
Tchma4 QAMKGLKDLGIKTAMLTGDNQDSAMQAQEQLGNALDVVHGELLPEDKSKIIQEFKKEGPT 599
*** ** ************* ** ******* ****** ******* ***********

Athma4 AMVGDGVNDAPALATADIGISMGISGSALATQTGNIILMSNDIRRIPQAVKLARRARRKV 656
Tchma4 CMVGDGVNDAPALANADIGISMGISGSALATQTGHIILMSNDIRRIPQAIKLARRAQRKV 659
************* ****************** ************** ****** ***

Athma4 VENVCLSIILKAGILALAFAGHPLIWAAVLVDVGTCLLVIFNSMLLLREKKKIGNKKCYR 716
Tchma4 LQNVIISITLKVGILVLAFAGHPLIWAAVLTDVGTCLIVILNSMLLLREKDKSKIKKCYR 719
** ** ** *** ************** ****** ** ********* * *****

Athma4 ASTSKLNGRKLEGDDDYVVDLEAGLLTKSGNGQCKSSCCGDKKNQENVVMMKPSSKTSSD 776
Tchma4 --------KKLEGVDDQGLDLEAGLLSKS---QCNSGCCGDQKSQEKVMLMRPASKTSSD 768
**** ** ******* ** ** * **** * ** * * * ******

Athma4 HSHPGCCGDKKEEKVKPLVKDGCCSEKTRKSEGDMVSLSSCKKSSHVKHDLKMKGGSGCC 836
Tchma4 HLHSGCCGEKKQESVK-LVKDSCCGEKSRKPEGDMASLSSCKKS---NNDLKMKGGSSCC 824
* * **** ** * ** **** ** ** ** **** ******** ******** **

Athma4 ASKNEKGKE-VVAKSCCEKPKQQVESVGDCKSGHCEKKKQAEDIVVPVQIIGHALTHVEI 895
Tchma4 ASKNEKLKEAVVAKSCCED-KEKTEGNVEMQILNLERGSQKK----------------- 865
****** ** ******** * * * *

Athma4 ELQTKETCKTSCCDSKEKVKETGLLLSSENTPYLEKGVLIKDEGNCKSGSENMGTVKQSC 955
Tchma4 ---VGETCKSSCCGDKEKAKETRLLLASEDPSYLEK-------EERQTTEANIVTVKQSC 915
**** *** *** *** *** ** **** * ******

Athma4 HEKGCSDEKQ--TGEITLASE---EETDDQDCSSGCCVN-EGTVKQSFDEKKHSVLVEKE 1009
Tchma4 HEKASLDIETGVTCDLKLVCCGNIEVGEQSDLEKGMKLKGEGQCKSDCCGDEIPLASEED 975
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Athma4 GLDMETGFC-------------------CDAKLVCCGNTEGEVKEQCR----LEIKKEE 1045
Tchma4 SVDCSSGCCGNKEELTQICHEKTCLDIVSCDSKLVCCGETEVEVREQCDLKKGLQIKNEG 1035
* * * ** ****** ** ** *** * ** *

Athma4 HCKSGCCGEEIQTGEITLVSE--EETESTNCSTGCC---------------VDKEEVTQ 1087
Tchma4 QCKSVRCGDEKKTEEITEETDNLKSESGDDCKSPCCGTGLKQEGSSSLVNVVVESGESGS 1095
*** ** * * *** * ** * *

Athma4 TCHEKPASLVVSG---LEVKKDEHCESSHRAVKVETCCKVKIP--EACASKCRDRAKR-H 1141
Tchma4 SCCSKEGEIVKVSSQSCCASPSDVVLSDLEVKKLEICCKAKKTPEEVRGSKCKETEKRHH 1155
* * * * * *** * * *** ** *

Athma4 SGKSCCRSYAKELCSHRHHHHHHHHHHHVSA 1172
Tchma4 VGKSCCRSYAKEYCSHRHHHHHHHHHVGAA- 1185
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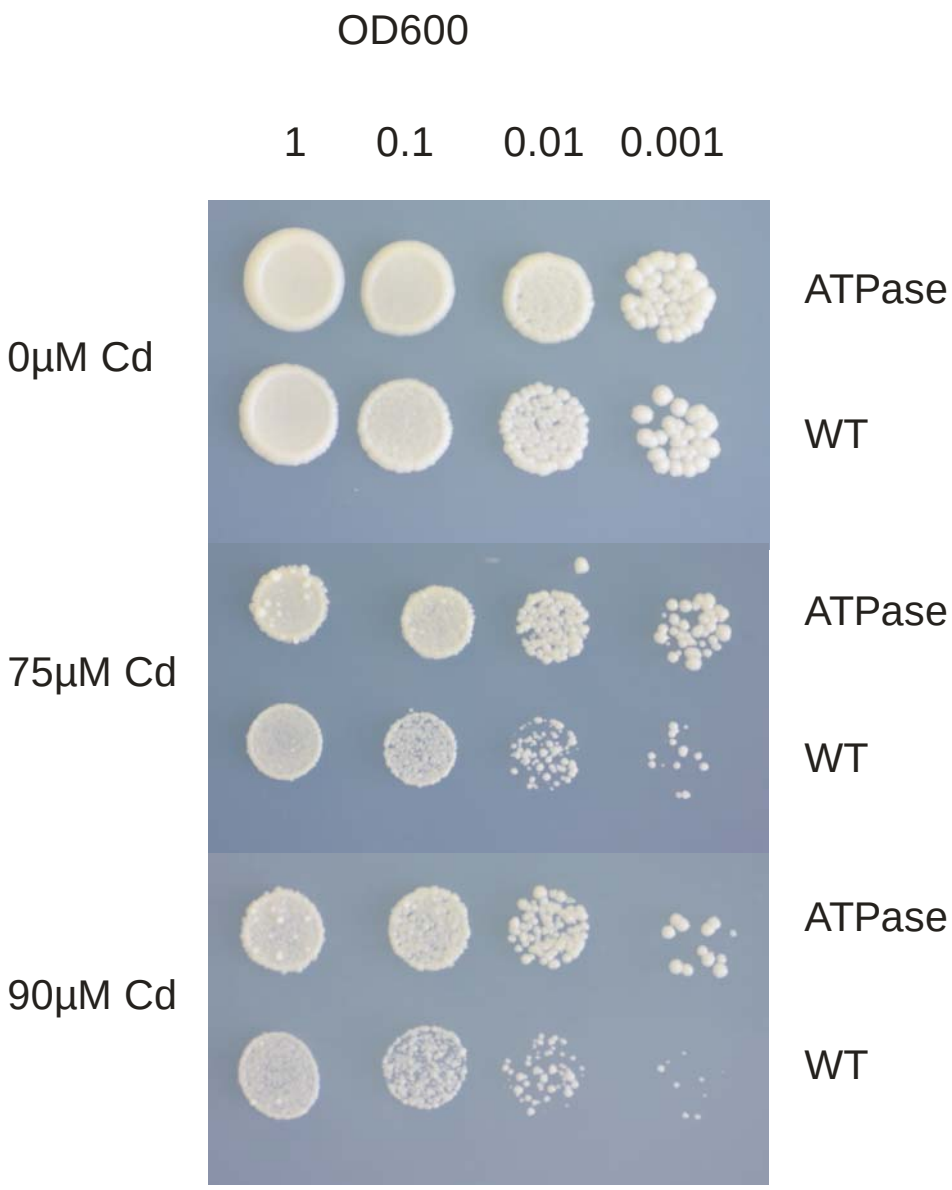


Figure 3.3 Cd tolerance test for WT (transformed with empty pFL61 vector) and *TcHMA4*-transformed yeast cells.

Yeast cells were grown to an OD_{600} of 1.0, serially diluted to an OD_{600} of 0.1, 0.01, and 0.001, and then 20 µL drops spotted on SD plates containing 0, 75, and 90µM $CdCl_2$.

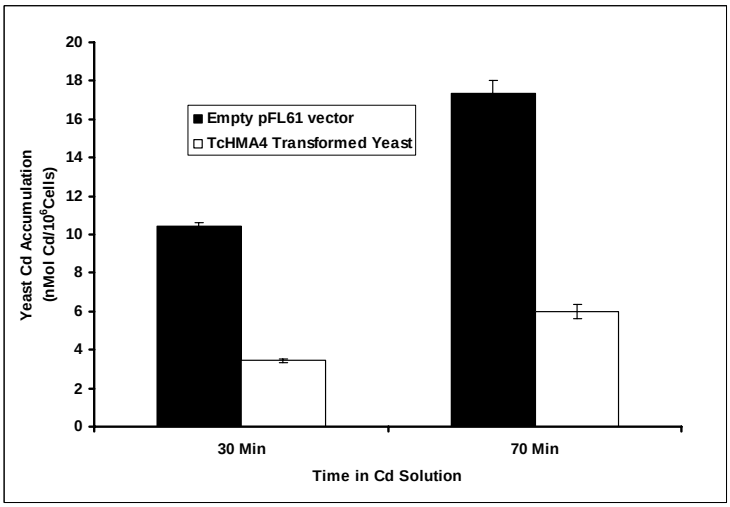
Expression of *TcHMA4* in Yeast Confers Heavy Metal Tolerance

When wild type yeast is transformed with the full-length *TcHMA4*, a strong Cd tolerance is conferred. As shown in Figure 3.3, growth of both the control yeast (wild type expressing pFL61) and the wild type strain (not shown) are strongly inhibited by inclusion of 75 and 90 μM Cd in solid SD media, while expression of *TcHMA4* in yeast allows vigorous growth on both levels of Cd.

Heavy Metal Accumulation in Control and *TcHMA4* Transformed Yeast.

A plant transporter can confer metal tolerance when expressed in yeast either via mediating metal exclusion due to efflux across the plasma membrane, or by sequestering the metal in an endomembrane compartment. The first scenario should result in reduced metal accumulation in yeast, while the second should cause enhanced metal accumulation. Thus, metal accumulation experiments were conducted with control and *TcHMA4*-transformed yeast; these experiments involved incubating both yeast genotypes in liquid SD media or the same media supplemented with either 20 μM $CdCl_2$ or 10 μM $PbCl_2$, and harvesting yeast cells after 30 and 70 min of metal accumulation. As shown in Figure 3.4, expression of *TcHMA4* in yeast resulted in a large decrease in both Cd (Figure 3.4A) and Pb (Figure 3.4B) accumulation. After both 30 and 70 minutes of metal accumulation, *TcHMA4*-transformed cells accumulated approximately 70% less Cd and 50% less Pb. In another accumulation study, the *TcHMA4*-transformed cells also accumulated less Zn and Cu than control cells (data not shown). These findings suggest that TcHMA4 is operating at the yeast plasma membrane to pump metals out of the cell and has the ability to transport a number of different essential micronutrients and heavy metals.

A



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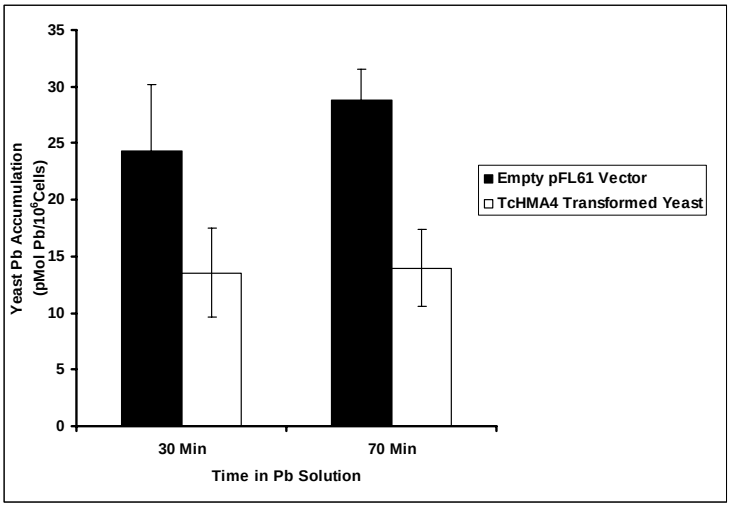


Figure 3.4 Cd and Pb accumulation by WT (wild type yeast transformed with the empty pFL61 vector) and *TcHMA4*-transformed yeast cells.

A) Yeast Cd accumulation for two time periods (30 and 70 min) in liquid SD media supplemented with 20µM $CdCl_2$. B) Yeast Pb accumulation for two time periods (30 and 70 min) in liquid SD media supplemented with 10µM $PbCl_2$. The error bars represent the mean of 4 replicate measurements $\pm$ the standard error of the mean.

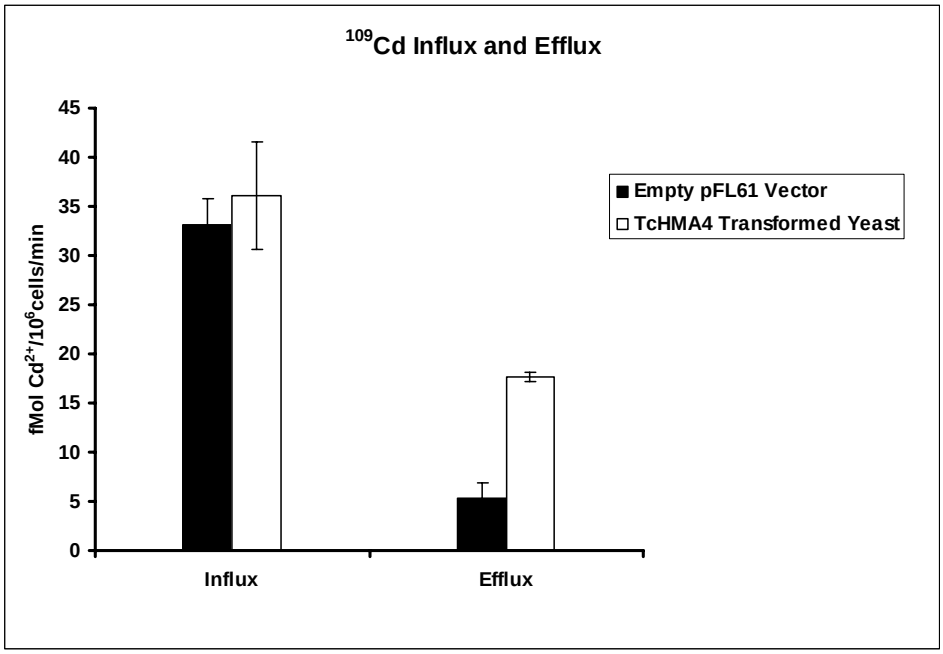


Figure 3.5 Radiotracer Cd influx and efflux

^{109}Cd influx and efflux in wild type yeast cells (transformed with the empty pFL61 vector) and *TcHMA4*-transformed yeast. The error bars represent the mean of 4 replicate measurements $\pm$ the standard error of the mean.

Quantitation of ^{109}Cd Efflux and Influx

In order to more conclusively determine if TcHMA4 is functioning to pump metals across the yeast plasma membrane and out of the cell, ^{109}Cd flux techniques were used to quantify Cd influx and efflux in control and *TcHMA4*-transformed yeast. As depicted in Figure 3.5, short-term ^{109}Cd uptake experiments showed that there were no differences in Cd influx in control *versus TcHMA4*-transformed cells. However, when both yeast genotypes were loaded with ^{109}Cd and allowed to efflux radiotracer into identical, unlabeled solution, the *TcHMA4*-transformed cells maintained a 3.5-fold higher rate of Cd efflux than did control cells not expressing the *Thlaspi* ATPase. This result verified that TcHMA4 is a metal efflux transporter operating at the plasma membrane. It is interesting to note than in these cells, the rates of Cd influx were two to six-fold larger than Cd efflux, indicating that a net Cd uptake was occurring, which presumably is associated with rapidly growing and dividing cells in the mid-log phase of growth.

Tissue-Specific Expression of *TcHMA4* in *Thlaspi caerulescens*

The expression of *TcHMA4* in different plant tissues and in response to changes in plant mineral nutrient and heavy metal status were investigated via Northern analysis in *Thlaspi caerulescens* seedlings. As depicted in Figure 3.6, *TcHMA4* is expressed strongly in roots, and it was also found almost no expression in above-ground plant tissues and organs (data not shown). This finding indicates that TcHMA4 is primarily a root-associated metal transporter. With regards to the relationship of *TcHMA4* expression and plant Zn status, both Zn deficiency as well as exposure of plants to high Zn levels induced a significant increase in *TcHMA4* transcript abundance (Figure 3.6). Furthermore, when plants were challenged with high levels of Cd in the nutrient solution, this also induced a strong increase in

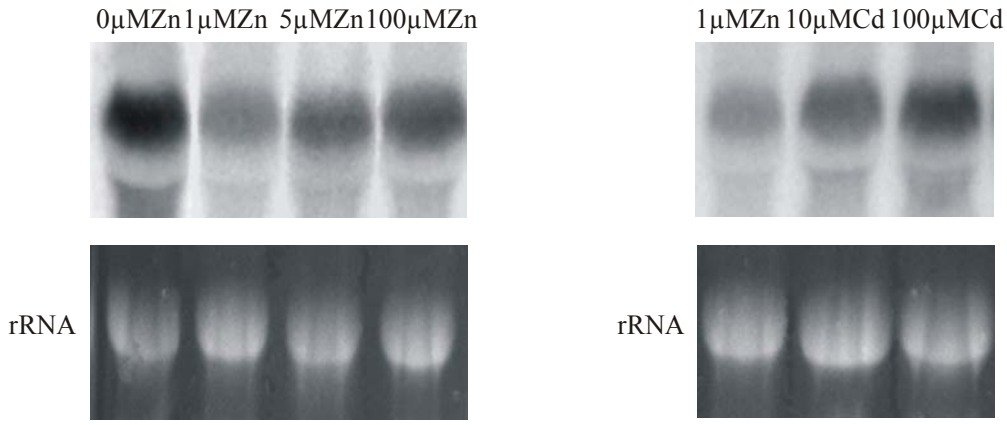


Figure 3.6 Northern analysis

Northern analysis of *TcHMA4* in roots of *Thlaspi caerulescens* plants grown under Zn deficient, sufficient, and high-Zn conditions, or under high Cd conditions. Seedlings were grown on hydroponic media containing 0, 1, 5, 10, and 100µM $ZnCl_2$, or 10 and 100µM $CdCl_2$. The 25S ribosomal bands are shown as loading controls. Each specific Northern was repeated at least twice, with the same results.

TcHMA4 expression (Figure 3.6). This response to increasing plant Cd status in *Thlaspi caerulescens* is quite different than what has been reported for its homolog in *Arabidopsis thaliana*, where it has been shown that root expression of *AtHMA4* is down-regulated by plant exposure to Cd (Mills et al 2003)

Southern analysis of TcHMA4

Southern analysis was conducted to determine if *TcHMA4* is a single copy gene in the *Thlaspi caerulescens* genome. After digestion of genomic DNA from *Thalspi caerulescens* plants with BamHI, HindIII, and EcorI, the blots were hybridized with labeled DNA for the *TcHMA4* gene. As shown in (Figure 3.7A), *TcHMA4* exists as a single copy gene in the *Thlaspi caerulescens* genome, as predicted by the presence of two major bands for the internal cutters, HindIII and BamHI, and one band for the external cutter, EcoRI.

Membrane localization of TcHMA4 protein.

Western blot analysis was conducted with a peptide antibody designed for the 3'-end of the TcHMA4 protein to determine the membrane localization of TcHMA4 in *Thlaspi caerulescens* cells. Aqueous polymer two phase partitioning techniques were used to isolate a purified plasma membrane fraction from the endomembrane fraction. Using standard Western blot protocols, both membrane fractions were tested against the TcHMA4 derived antibody. As seen in (Figure 3.7B), the *Thlaspi caerulescens* HMA4 protein is localized to the plasma membrane, which is in accordance with our hypothesis that TcHMA4 operates as a metal efflux pump that may be involved in xylem loading.

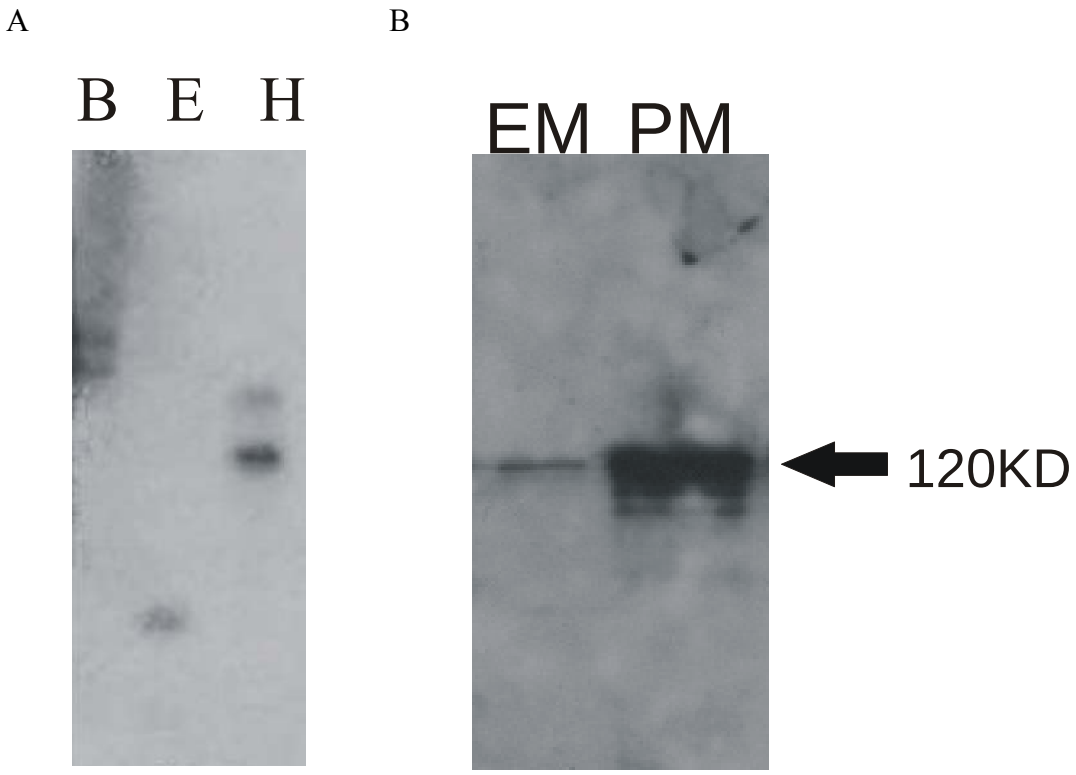


Figure 3.7 Southern and Western blots for TcHMA4 gene and protein.

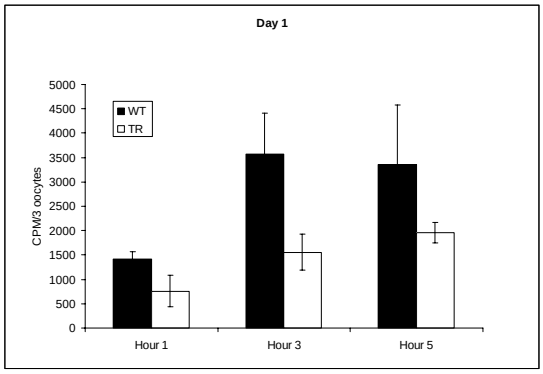
A)For Southern analysis of *TcHMA4* gene, genomic DNA from *T. caerulescens* was digested with the restriction enzymes, B-BamHI, E-EcoRI, and H-HindIII and hybridized against a TcHMA4 probe derived from the C terminus. B) Western blot analysis of TcHMA4 protein in the endomembrane-enriched membrane fraction (EM) from the lower phase, and the plasma membrane-enriched fraction (PM) from the upper phase of the aqueous 2-phase partitioning of membranes isolated from *Thlaspi caerulescens* roots.

TcHMA4 expression in two heterologous systems: Xenopus oocyte and mammalian HEK cells

TcHMA4 was heterologously expressed in *Xenopus laevis* oocytes with a future possible goal of characterizing TcHMA4 transport properties based on electrophysiological techniques (two-microelectrode voltage clamp analysis). Our results show that 24 hrs after injecting oocytes with *TcHMA4* cRNA they accumulated between 30 - 50% less ^{109}Cd compared to the control oocytes (injected with distilled water; Figure 3.8), suggesting that in oocytes TcHMA4 is functioning as a Cd efflux transporter as was seen previously in yeast. However, when oocytes were held for longer periods (2 and 5 days after cRNA injection), the pattern of ^{109}Cd accumulation was reversed. Five days after cRNA injection, the *TcHMA4* expressing oocytes accumulated between 15 and 30% more ^{109}Cd than did control oocytes. It is possible that either continued expression of TcHMA4 protein is somewhat toxic to oocytes. Alternatively, one could speculate that the TcHMA4 protein is not stable in the oocyte plasma membrane and upon degradation, releases Cd-binding peptide ligands into the cytoplasm.

Because of the variation in ^{109}Cd accumulation in TcHMA4-expressing oocytes, we also expressed the *TcHMA4* gene in mammalian HEK cells (human embryonic kidney cells). The HEK cells were transfected with a chimeric C-termini TcHMA4::GFP translational construct. As depicted in (Figure 3.9), the GFP fluorescence from TcHMA4-GFP was more localized to the HEK cell periphery compared with cells expressing soluble GFP, which is consistent with localization of

A



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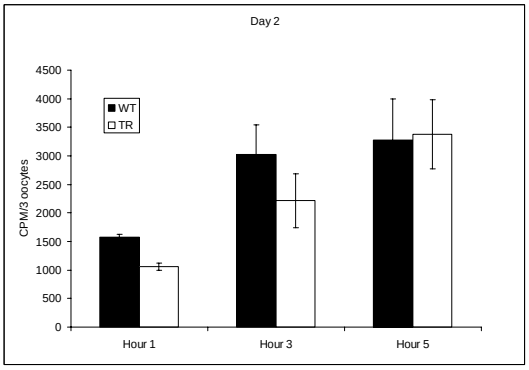

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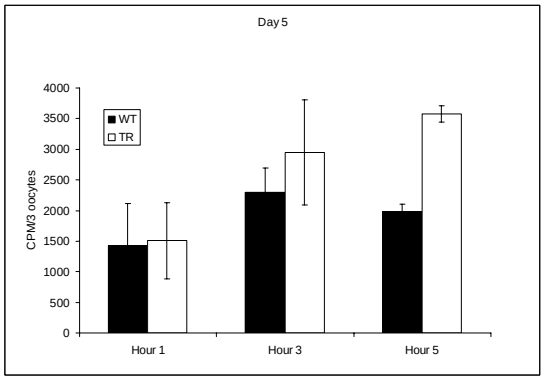
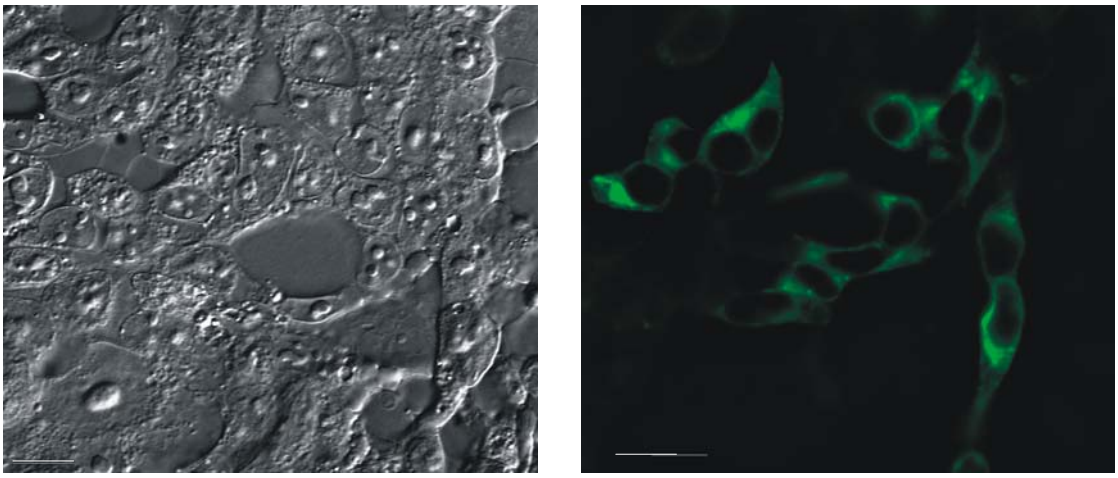


Figure 3.8. Radiotracer ^{109}Cd transport analysis of oocytes expressing TcHMA4. Radioactive ^{109}Cd accumulation in Xenopus oocytes expressing either the *TcHMA4* cRNA (TR) or injected with water (WT). ^{109}Cd accumulation assays were started 24 hrs (A), 2 days (B), and 5 days (C) after injection and ^{109}Cd accumulation measured for 1, 3, and 5 hrs.

A



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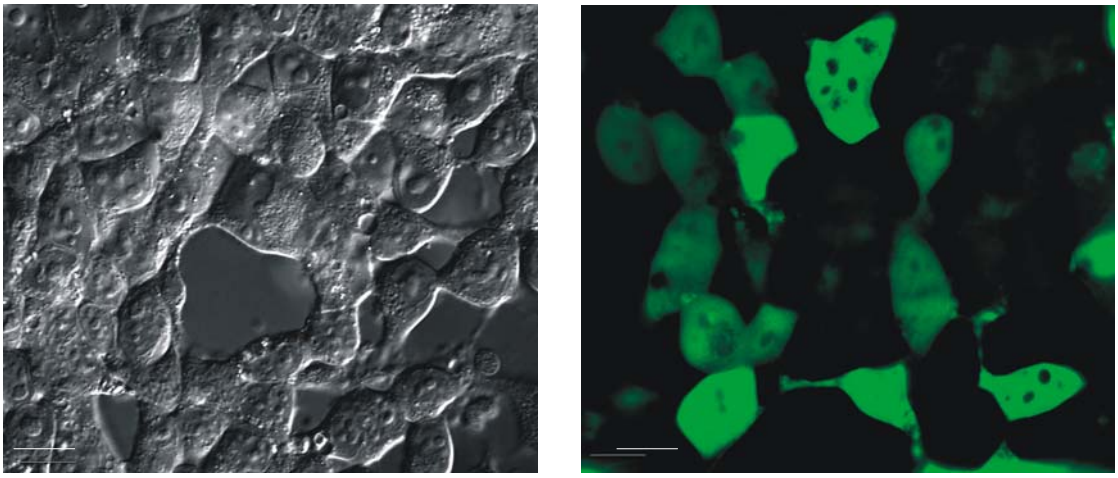

Figure 3.9. Transient expression of HMA4::GFP protein in mammalian HEK 293 cells.

A) HEK293 cells transiently transfected with TcHMA4 observed under bright field microscopy (left) and on the right, under fluorescence microscopy. B) Transient expression of cytoplasmic GFP in transfected HEK293 cells observed under light microscopy (left), and fluorescence microscopy (right).

the TcHMA4 protein to HEK cell plasma membrane. This heterologous expression in mammalian cells may provide future opportunities to conduct an electrophysiological analysis of TcHMA4 transport properties.

Discussion

In this study a number of genes were identified that may be involved in the mechanisms of metal tolerance and hyperaccumulation in the heavy metal hyperaccumulating plant species, *Thlaspi caerulescens* (Table 1). Some of the genes, such as those encoding metallothioneins and heavy metal ATPases, have previously been suggested to play a role in metal tolerance (see, for example, Clemens et al., 2001; Cobbett and Goldsbrough, 2002). However, for most of the other genes in Table 1, it is more difficult to ascribe a function in metal tolerance mechanisms based on sequence similarity to other genes and proteins. In should be noted that because the yeast complementation screen used in this study will identify genes that can confer tolerance at the single cell level, it is possible that these genes participate in other functions in *Thlaspi caerulescens*. This is particularly true if more complex metal tolerance mechanisms based on the operation and interplay of multiple cell types, tissues, and/or organs are operating in higher plants.

TcHMA4 Confers Metal Tolerance in Yeast via Metal Efflux Out of the Cell

From the yeast complementation screen, a P_{1B}-type heavy metal ATPase was identified that is 71% identical on a protein level to the *Arabidopsis thaliana* heavy metal ATPase, AtHMA4 (Mills et al., 2003). The P_{1B} subfamily of ATPases is believed to be involved in the transport of heavy metals such as Zn, Cu, Co, Cd, Pb and Ag that are either essential micronutrients or non-essential toxic metals, although

only a few members of this subfamily have been well characterized. Genome sequencing efforts as well as isolation and analysis of individual members of this sub-group from different organisms indicate these heavy metal ATPases are expressed in a wide range of organisms, ranging from the archaea and bacteria, to eukaryotic organisms including *Arabidopsis thaliana* (see, for example, Rensing et al 1997; Axelsen and Palmgren, 1998, 2001; Tong et al 2002; Mills et al 2003).

This is the first report characterizing a heavy metal ATPase, *TcHMA4*, from a metal hyperaccumulating plant species. *TcHMA4* shares many of the conserved motifs found in other P_{1B}-type ATPases, including eight putative membrane spanning domains, and conserved CPX (amino acids 357-359) and HP (amino acids 441-442) motifs. Also, *TcHMA4* shares certain unique structural features with its *Arabidopsis* homolog, in that both *AtHMA4* and *TcHMA4* have a long polar C terminus region that is predicted to reside in the cytoplasm and contains numerous metal-binding amino acids, including a long (9 amino acid) histidine stretch at the very end of the C terminus, thirteen cysteine pairs, and a large number of single histidine residues (Figure 3.2). As discussed, later, this region of the TcHMA4 protein quite likely participates in the binding of a number of heavy metals, although the involvement of this metal-binding region in transport function is still not clear.

As shown in (Figure 3.3), expression of *TcHMA4* in wild type yeast conferred a significant increase in heavy metal (Cd) tolerance. Metal tolerance at the single cell level can be achieved in a number of different ways. These include: a) metal exclusion, where the metal is either actively pumped out of the cell, or its entry is restricted; b) true tolerance, where the synthesis of metal binding ligands bind and detoxify the metal in the cytoplasm; and c) internal sequestration, where an endomembrane-localized metal transporter sequesters the metal in an internal cellular compartment (e.g., the vacuole). Yeast accumulation experiments indicated that

expression of *TcHMA4* in yeast resulted in metal exclusion, and that this exclusion occurred rapidly, within minutes. Furthermore, TcHMA4 can mediate the exclusion of a number of heavy metals and micronutrients, including Cd and Pb (Figure 3.4) as well as Zn and Cu (data not shown). This TcHMA4-mediated metal exclusion mechanism was investigated in more detail using ^{109}Cd radiotracer flux techniques and these experiments clearly showed that TcHMA4 was functioning to pump metals out of the cell (Figure 3.5). Thus, it is quite likely that in yeast and plants TcHMA4 operates as a plasma membrane-localized metal ATPase to pump Cd and possibly other heavy metals and micronutrients across the plasma membrane and out of the cell.

Heterologous expression of the *TcHMA4* gene in oocytes and mammalian cells

Xenopus leavis oocytes have many advantages for expression of transporters from other organisms, as the oocytes are large cells (dia = 1 mm) and thus are amenable to electrophysiological examination via 2 microelectrode voltage clamp methods. In addition the plasma membranes of oocytes are relatively quiet with respect to transport activity; hence the background activity of endogenous transporters does not significantly interfere with the activity of heterologously expressed transporters. Therefore, TcHMA4 was expressed in oocytes with the hopes of further characterizing its transport properties via electrophysiological analysis. To begin to assess the efficacy of expressing this protein in oocytes, ^{109}Cd radiotracer accumulation was first used to determine if tcthe TcHMA4 protein was functional in oocytes It was found that for the first 24 hrs after injection of the *TcHMA4* cRNA, it appears that the transporter is functioning as a Cd efflux transporter, as was found previously in yeast. That is, as depicted in (Figure 3.8), the oocytes expressing TcHMA4 are accumulating significantly less ^{109}Cd than the water-injected control

oocytes. However, after longer time periods after injection of the *TcHMA4* cRNA, it was observed that the oocytes injected with *TcHMA4* cRNA accumulated more ^{109}Cd than the control oocytes. It is likely that for the longer incubation periods, the TcHMA4 protein is not stable in the oocyte plasma membrane. That is, protein degradation may be occurring, and it is possible that this releases histidine and cysteine-rich peptides from the 3' cytoplasmic tail of the HMA4 protein that are shown in Chapter 4 can serve as metal binding ligands and actually increase ^{109}Cd accumulation when these peptides are expressed in yeast. Circumstantial evidence in support of this hypothesis comes from our attempts to detect the full TcHMA4 protein in oocytes using our anti-TcHMA4 antibody. Based on Western blot analysis of oocytes expressing TcHMA4, numerous peptides were detected whose size where considerably smaller than the full TcHMA4 protein, and these peptides were not detected in water-injected oocytes (data not shown). Hence it was not felt that oocytes would be a useful heterologous system for future investigation of TcHMA4 transport properties. Therefore, it was decided to express the protein in an alternative heterologous system, mammalian HEK cells. As seen in Figure (3.9), transfection of HEK cells with a cDNA expressing a TcHMA4-GFP translation fusion yielded GFP fluorescence microscopic images consistent with TcHMA4 being expressed in the HEK cell plasma membrane. Thus, an electrophysiological examination using the patch clamp technique of HEK cells expressing *TcHMA4* is planned in the future.

Possible Function of *TcHMA4* in Heavy Metal Hyperaccumulation

Heavy metal hyperaccumulation in *Thlaspi caerulescens* is associated with several traits, including: 1) the ability to tolerate high levels of metals both in the soil and within the plant; 2) an enhanced ability to absorb metals from the soil; 3) the ability to efficiently and rapidly translocate the absorbed metals from the root to the

shoot; and 4) the ability to store very high levels of the metals in leaf epidermal cells (Lasat et al 1996, 1998; Küpper et al 1999). One of the most distinctive hallmarks that differentiate metal hyperaccumulator plants from non-accumulators is their ability to translocated most of the absorbed metal to from the root to the shoot. Non-accumulator plants tend to sequester heavy metals in the roots, while hyperaccumulators, and especially *Thlaspi caerulescens*, rapidly translocate the bulk of the heavy metal to the shoot for storage in the leaf epidermis (Lasat et al 1996; 1998).

When one examines the metal transport and gene expression data for *TcHMA4* presented here in comparison with recently published expression data for *AtHMA4* in *Arabidopsis*, the findings lead us to speculate about an intriguing scenario regarding the role of TcHMA4 in metal hyperaccumulation in *Thlaspi*. In *Arabidopsis*, it has been reported that the homolog of *TcHMA4* is expressed throughout the plant, with stronger expression in roots than in other plant organs and tissues (Mills et al 2003). It was also reported that *AtHMA4* expression was down-regulated by plant exposure to high levels of Cd. However, as seen in Figure (3.6), *TcHMA4* is expressed strongly and almost exclusively in the *Thlaspi* root and its expression is strongly up-regulated by seedling exposure to high levels of Cd and Zn, as well as by Zn deficiency. Furthermore, Birnbaum et al recently published a gene expression map for 22,000 genes in the *Arabidopsis* root. From the data contained in the root gene expression database from (Birnbaum et al 2003), it is seen that in the *Arabidopsis* root, *AtHMA4* is expressed almost solely in the stele of the mature root. Given the root-specific localization of *TcHMA4* expression, it is reasonable to assume that *TcHMA4* follows a similar cell-specific expression pattern in *Thlaspi* roots. The *Thlaspi* and *Arabidopsis HMA4* expression data, along with our findings that are consistent with TcHHA4 operating at the root-cell plasma membrane to pump heavy metals and micronutrients

out of root cells, lead us to speculate that TcHMA4 does not directly play a role in heavy metal tolerance in *Thlaspi caerulescens*. Instead, it is proposed that it functions in metal xylem loading by mediating heavy metal and micronutrient efflux from xylem parenchyma into xylem vessels, and thus plays a key role in the mechanisms underlying metal hyperaccumulation in *Thlaspi caerulescens*. Further support for this role for TcHMA4 comes from evidence presented here localizing TcHMA4 to the root cell plasma membrane based on Western analysis of TcHMA4 with plasma membrane vesicles derived from *Thlaspi caerulescens* roots (Figure 3.7). The role of TcHMA4 in metal loading into the xylem is also consistent with the observed up-regulation of expression of this transporter by heavy metal exposure. It is interesting to also note that this transporter is also induced by Zn deficiency. Thus *TcHMA4* may be involved not only in heavy metal hyperaccumulation but also in *Thlaspi* Zn nutrition; that is, under Zn deficiency expression of this transporter is increased in an attempt to maintain shoot Zn status for reproduction and the completion of the plant's life cycle.

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Chapter IV

The Role of Peptides from the C Terminus of TcHMA4 in Altering Heavy Metal Homeostasis

Abstract

The ability to detoxify heavy metals is one of the important features that heavy metal hyperaccumulator plants such as *Thlaspi caerulescens* possess. There are several possible mechanisms of heavy metal detoxification in higher plants such as complexation with organic ligands, sequestration in the vacuole, and binding to cell walls. During a functional screen of a *Thlaspi caerulescens* cDNA library in yeast to identify genes involved in heavy metal tolerance described in Chapter 3, several yeast colonies were identified that were tolerant of high levels of Cd. Upon further investigation we found that these yeast clones were harboring two different length partial sequences that encoded peptides from the C terminus of the *TcHMA4* gene. In this chapter we present evidence showing that the C terminus of the TcHMA4 protein, which contains numerous histidine and cysteine repeats, appear to participate in heavy metal binding. When partial peptides from the C terminal domain of TcHMA4 were expressed in yeast, they conferred high level of Cd tolerance and Cd hyperaccumulation. In addition, we show that when these peptides are expressed in transgenic *Arabidopsis thaliana* plants, a measurable increase in heavy metal tolerance was seen. These transgenic *Arabidopsis* plants maintained more shoot biomass and increased root length when grown on high concentrations of Cd compared to wild type plants. Also it was found that the transgenic *Arabidopsis* plants accumulate more heavy metals in the shoots and seeds than the wild type plants. The possibilities for enhancing the metal tolerance and phytoremediation potential of higher plants via expression of these metal-binding peptides in higher biomass, non-hyperaccumulator plants are discussed.

Introduction

Thlaspi caerulescens, a member of the Brassicaceae family, is a well studied Zn/Cd hyperaccumulator plant species. Certain ecotypes of *Thlaspi caerulescens* have been shown to accumulate and tolerate up to 40,000 ppm Zn in their shoots (normal foliar [Zn] for hydroponically grown plants is around 100-200 ppm, while 30 ppm is considered adequate) (Chaney et et al 1993; Brown et al 1995a,b). This species also accumulates Cd in the shoot to levels as high as 10,000 ppm (typical shoot Cd levels are around 1-100 ppm). Certain ecotypes of *Thlaspi caerulescens* have been reported to accumulate high levels of other metals, including Ni and Co. The unique physiology of heavy metal transport and tolerance in *Thlaspi caerulescens* makes it a very interesting experimental system for basic research aimed at elucidating mechanisms of heavy metal hyperaccumulation in plants.

Given the important role that heavy metals play in human nutrition, health, and well being, the ability to alter plant heavy metal content in a controlled manner can be expected to have a broad and significant impact on agriculture, particularly at the interface between agriculture and human health.

Contamination of soils with heavy metals is a worldwide problem both for human health and agriculture. Cleanup of hazardous wastes by current engineering-based technologies has been estimated to cost at least $400 billion in the U.S. alone. Recently, there has been considerable interest in the use of terrestrial plants as an alternative, “green technology” for the phytoremediation of surface soils contaminated with toxic heavy metals. A major factor behind the recent interest in the phytoremediation of metal polluted soils has been the growing awareness by the scientific community of the existence of a number of metal hyperaccumulating plant species. Over 200 terrestrial species have been reported to be endemic to metalliferous

soils and can tolerate and accumulate high levels of heavy metals such as Zn, Cd, Cu, and Ni in their shoots. The existence of these interesting metal hyperaccumulator species suggests that the genetic potential exists for phytoremediation to be successful. Most of these hyperaccumulator species, however, are small and slow growing, and because they produce limited shoot biomass their potential for large-scale decontamination of polluted soils is limited. Transferring the genes expressing the hyperaccumulating phenotype to higher shoot biomass-producing plants has been suggested as an avenue for enhancing the potential of phytoremediation as a viable commercial technology

Two main groups of heavy metal binding ligands have been identified in plants that play an important role in heavy metal detoxification, particularly in non-hyperaccumulator plants. Metallothioneins (MT) and phytochelatins (PC) have been shown to be involved in heavy metal detoxification in higher plants. Metallothioneins are low molecular weight proteins, characterized by cysteine-rich heavy metal binding sites (Kagi et al 1993). Unlike MTs, phytochelatins are enzymatically synthesized molecules that have a general structure of (γ-GluCys)n-Gly where n can vary from 2-5. Glutathione is considered to be the main substrate for phytochelatin synthesis (Cobbett et al 2000). Since these two classes of small peptides have been shown play a role in heavy metal detoxification in non-hyperaccumulator plants via binding of the metals to amino acids such as cysteine and histidine in the peptides (Cobbett et al 2002), we investigated the possibility that cysteine and histidine-rich peptide domains in the C terminus of TcHMA4 from *Thlaspi caerulescens* might be used to alter heavy metal accumulation and tolerance in a non-accumulator plant, in this case, *Arabidopsis thaliana*.

In this chapter the identification of partial sequences from the *Thlaspi caerulescens HMA4* gene are described as well as the use of peptides derived from

these sequences to confer metal tolerance and accumulation in both yeast as a model, heterologous system, and also plants. The potential to use these peptides in genetically modified plants and possibly other organisms to facilitate the ability to accumulate high concentrations of heavy metals without exhibiting toxicity symptoms is discussed. This feature possibly could be used to develop transgenic plants better suited for the phytoremediation of surface soils contaminated with heavy metals.

Materials and Methods

Identification of TcHMA4 partial sequence clones.

During our initial screen of a *Thlaspi caerulescens* cDNA library expressed in the yeast, *Saccharomyces* cerevisiae, we identified Cd tolerant yeast colonies that were transformed with partial sequences of the *TcHMA4* gene. This cDNA library was constructed with combined polyA$^+$ RNA from roots and shoots of *T. caerulescens* seedlings grown on both Zn-deficient and Zn-replete nutrient solution. The cDNA was synthesized using the Superscript Choice System (Gibco-BRL), then ligated using BstXI/EcoRI adapters into the bifunctional yeast/*Escherichia coli* expression plasmid, vector pFL61 (Pence et al 2000). This vector contains a yeast phosphoglycerate kinase promoter and a uracil selection marker. Preliminary experiments determined that the yeast DY1457 wild type stain was unable to grow on solid SD media containing 90μM Cd. Subsequently, wild type yeast was transformed with *T. caerulescens* cDNA and plated on 90μM Cd in solid SD media. After growth for three days at 30^o C, 35 individual yeast colonies were observed that were able to grow on the high Cd media. Yeast from each of these colonies was replated on high Cd plates to verify the metal tolerance phenotype, and then DNA was extracted from each of these colonies and the sequence was determined for each sample (Applied Biosystems Automated 3730 DNA Analyzer, Cornell University).

Characterization of yeast harboring partial *TcHMA4* clones.

The Cd tolerance and Cd accumulation of yeast expressing the *TcHMA4* partial sequences transformed yeast Cd tolerance level was examined in more detail via growth on liquid culture. To determine the relative Cd tolerance of control and *TcHMA4*-transformed cells (transformed with either full length or partial clones), the different yeast genotypes were grown on regular liquid SD medium until they achieved an optical density (OD) of 1.0. At this point, the cells were harvested and serial dilutions were made to achieve OD's of 0.1, 0.01, and 0.001. SD plates were made containing the appropriate concentration of $CdCl_2$, and a 20µl aliquot for each cell dilution was spotted on to the plates, as described by (Lee et al 2003). The plates were placed in a 30°C incubator for 3 days after which pictures were taken. To assay metal accumulation, control (wild type DY1457 yeast strain containing the empty pFL61 vector) and *TcHMA4*-transformed yeast were grown on liquid SD medium. At the mid-log phase of growth, the metal accumulation experiment was initiated by adding either $CdCl_2$ or $PbCl_2$ to a final concentration of 20 or 10 µM, respectively. After 1, 3, and 5 hours of metal accumulation, aliquots of yeast cells were taken, centrifuged at 10,000 x g for 2 minutes to separate yeast cells from the metal containing media, washed once with 5 mM $CaCl_2$ to desorb Cd^{2+} from the yeast cell walls, and then centrifuged again at 10,000 x g for 2 minutes. The heavy metal content of the yeast cell pellet was analyzed using an inductively-coupled, plasma trace analyzer emission spectrometer (Model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA).

Transformation of *Arabidopsis thaliana* plants.

Arabidopsis thaliana ecotype Colombia seedlings were transformed using the Agrobacterium floral dip transformation method. Both of the partial *TcHMA4* sequences were inserted into the EcoRI site of the pBARII vector, which in turn was transformed into Agrobacterium cells. Using the standard Agrobacterium floral dipping transformation protocols described by (Clough et al 1998), these vectors were transformed into *Arabidopsis* plants. At the stage of plant development when most of the secondary bolts where about 10cm long, the aerial parts were dipped for a few seconds in 200ml of solution containing resuspended Agrobacterium cells harboring the *TcHMA4* sequences in the pBARII vector. Plants were grown to completion, the seeds were harvested and germinated, and the T_0 seedlings screened for transformants via resistance to the herbicide, BASTA, which contains glufosinate ammonium as an active ingredient. Seeds were collected from the surviving plants and were used for future experiments.

Selection of lines that had high transgene expression and were homozygous for transgene expression.

After transformation with the partial *TcHMA4* sequences, transgenic T_0 seedlings were screened for high transgene expression. Northern analysis for the partial *TcHMA4* sequences was conducted with mRNA isolated from T_0 seedlings. . From this screen 5 lines that had the highest expression of the *TcHMA4* partial sequence were selected for future study. Subsequently, these plants were allowed to self, and progeny from each line were tested for BASTA resistance. The lines in which all the seeds were resistant to the herbicide were deemed to be homozygous. Two lines each for the transformation of the “short” and “long” partial *TcHMA4* sequences that

had high expression and were homozygous for the transgene were used for subsequent studies.

Plant growth conditions for characterization of transgenic lines.

Transformed and wild type *Arabidopsis thaliana* plants were grown hydroponically using 1/5 strength Johnson nutrient solution. The nutrient solution consisted of a macronutrient composition of 1.2 mM KNO_3, 0.8mM $Ca(NO_3)_2$, 0.2mM $NH_4H_2PO_4$ and 0.2 $MgSO_4$ and a micronutrient composition of 50µM KCl, 12.5µMH_3BO_4, 1µM $MnSO_4$, 1µM$ZnSO_4$, 0.5µM $CuSO_4$, 0.1µM Na_2MoO_4, 0.1µM $NiSO_4$, and 7.5µM Fe-EDDHA (N, N'-ethylenediamine-di(O-hydroxyphenylacetic acid). The solution was buffered at a pH of 5.5 with 1mM MES (2-[N-morpholino]-ethanesulfonic acid) buffer. Prior to germination, seeds were placed in 0.1% agarose solution and placed in the cold room for four days for stratification. Subsequently, seeds were placed on a 250 μm^2 plastic mesh that was just in contact with the nutrient solution. Seedlings were grown in a growth chamber at 25/15 °C (light: dark, 16:8 h respectively) under a light intensity of 300 µmol photons $m^{-2}s^{-1}$. Seedlings were grown for 2 weeks in 1/5 strength Johnson solution, after which the appropriate heavy metal treatment was imposed (5, 10, 25 and 30 µM Cd). In another experiment investigating the response of transgenic seedlings to Zn deficient conditions, after the first 2 weeks of growth on 1/5 strength Johnson solution, the solution was replaced with the same nutrient solution that lacked Zn and also contained 5µM EDDHA to complex any contaminating Zn.

Determination of shoot biomass, root length and heavy metal content in *Arabidopsis thaliana* plants.

Transgenic and wild type *Arabidopsis* plants were grown hydroponically as described above and roots and shoots were harvested after 20 days of growth on the specific metal treatment and root and shoot tissues were collected separately. Root lengths were determined and then the shoot and root tissues were oven dried at 65^{o}C for 10 days. Dry weights were determined and then tissues were digested in concentrated HNO_3 and dried. Dried samples were resuspended in 5% HNO_3 and the heavy metal content of each sample was analyzed using an inductively-coupled, plasma trace analyzer emission spectrometer (Model ICAP 61E, Thermo-Jarrell Ash, Waltham, MA).

Results

Analysis of Partial *TcHMA4* Clones

As described in Chapter 3 regarding the yeast complementation screen for *Thlaspi* Cd tolerance genes, two different partial *TcHMA4* clones conferred a significant degree of Cd tolerance when expressed in yeast. These two clones encode peptides predicted to be 384 and 141 amino acids in length that encompass most (for the 384 aa peptide) or a portion (for the 141 aa peptide) of the large C terminal cytoplasmic tail of TcHMA4 that harbors a number of putative heavy metal binding domains. These domains include a number of cysteine pairs, a his-9 repeat, and numerous single histidine residues (Figure 4.1). As neither of these peptides contain any predicted membrane spanning domains, it is clear that they are not functioning as metal transporters. Thus, the possibility that they confer heavy metal tolerance in yeast by acting as metal-binding peptide ligands in the yeast cytoplasm was investigated. As shown in Figure 4.2, both the 141 and 341 amino acid peptides conferred a very high

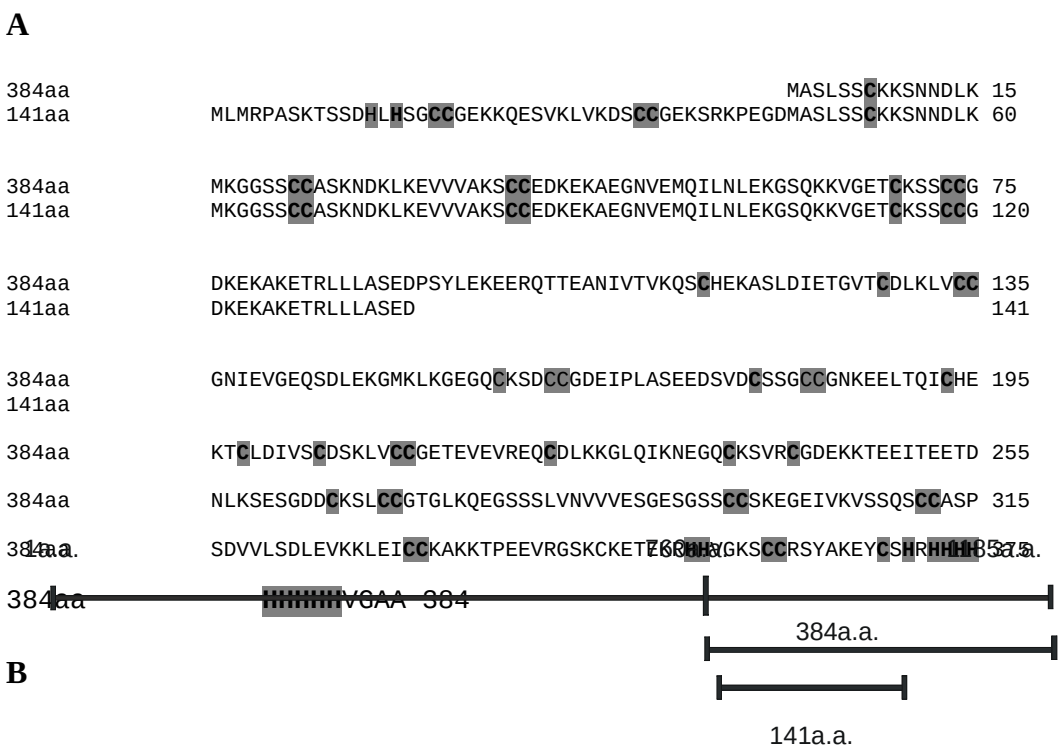


Figure 4.1 Amino acid sequence alignment and schematic representation of two partial peptides.

A) Amino acid sequence alignment of the 384 and 141 amino acid partial peptides from the C terminus of TcHMA4 that were identified from the initial yeast complementation screen. The conserved histidine stretch, numerous cysteine repeats, and single histidine residues in the C terminal region of the peptide are indicated in light gray.

B) Schematic representation of location of two partial peptides sequences relative to each other and to the full length TcHMA4 protein.

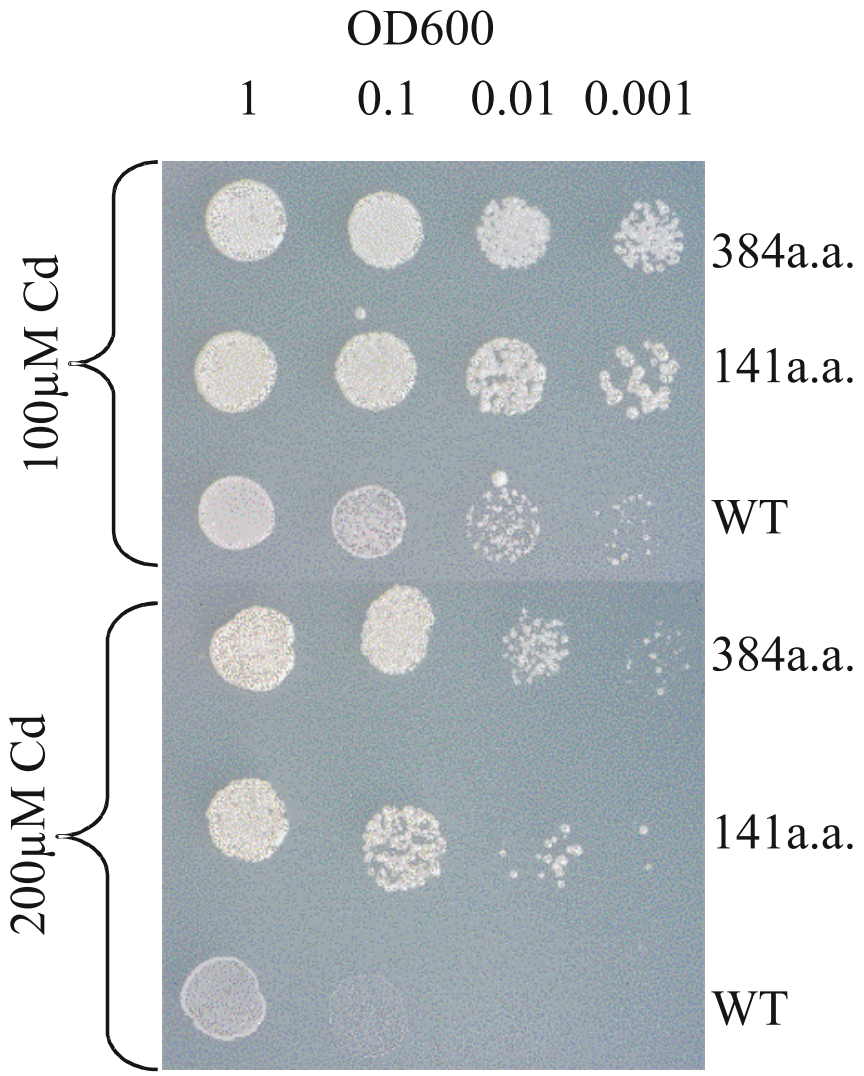


Figure 4.2 Cd tolerance test for yeast expressing the partial TcHMA4 peptides. Yeast were transformed either with the empty pFL61 vector (WT) or with the 384 or 141 amino acid peptides. Metal tolerance was assayed by visualizing growth on SD plates containing 100 and 200 μM $CdCl_2$

degree of Cd tolerance, resulting in significant yeast growth even in solid media supplemented with 200 μM $CdCl_2$. Thus these peptides confer a considerably higher degree of tolerance than does the full TcHMA4 protein, as yeast expressing the full TcHMA4 protein would not grow on media containing Cd at concentrations higher than 120 μM. It is interesting to note that the longer TcHMA4 peptide confers a greater degree of Cd tolerance than does the shorter, 141 aa peptide (see Figure 4.2). The longer peptide contains both the his-9 repeat as well as the numerous cysteine pairs and single histidine residues, while the shorter peptide lacks the his-9 repeat. Thus it might be expected that the longer peptide, which covers most of the C terminus cytoplasmic tail with its numerous potentially metal-binding amino acids, might have a greater capacity to bind metals and afford a greater degree of metal detoxification in the cytoplasm. It can predicted for a heavy metal tolerance mechanism based on the production of a metal-binding ligand, that this would confer a greater degree of cellular metal accumulation compared with wild type yeast cells. As shown in Figure 3, this is exactly what was found. In yeast cells transformed with the partial clones, yeast Cd accumulation after 1 hr was four to six-fold greater than control yeast expressing the empty pFL61 vector. Furthermore, Cd accumulation in yeast cells expressing the longer peptide was always greater than in yeast expressing the shorter peptide, at all three time points where Cd accumulation was measured (Figure 3). Again, this correlates nicely with the differing degree of Cd tolerance conferred by these two clones, and with the relative metal-binding capacity of these two peptides that is predicted from their amino acid sequences.

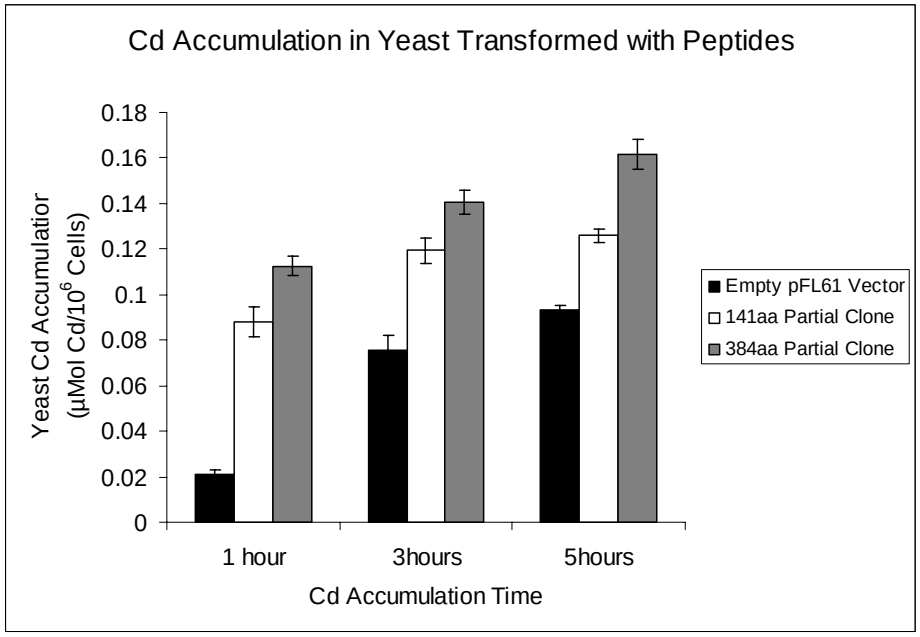


Figure 4.3 Cd accumulation in yeast cells.

Cd accumulation by yeast expressing the empty pFL61 vector (WT), compared with yeast expressing the 384 and 141 aa partial peptides from TcHMA4. Cd accumulation was conducted in liquid SD media supplemented with 20 µM $CdCl_2$. The error bars represent the mean of 4 replicate measurements $\pm$ the standard error of the mean.

Heavy metal tolerance in transgenic *Arabidopsis* plants expressing the C terminal HMA4 peptides.

In order to determine if these partial *TcHMA4* clones can increase metal tolerance in plants, transgenic *Arabidopsis* seedlings expressing these HMA4 partial peptides were assayed for shoot and root growth. We first looked at these parameters under normal conditions in the absence of heavy metals to see if the transformation event itself had any negative effects on plant growth. Our data indicated that both transformed and wild type plants grew equally well under control conditions, with very similar shoot biomass and root lengths (Figure 4.4). Subsequently shoot and root growth was assayed with the inclusion of 10μM Cd in the hydroponic growth solution. As seen in Figure 4.5, a moderate but statistically significant (20%) increase in shoot and root growth was observed in transformed plants compared with wild type plants; this response was seen in plants transformed with either the "long" or "short" peptides from the HMA4 C terminus, with the longer C terminus peptide conferring a somewhat greater tolerance to Cd. At a higher Cd level (30μM), a greater increase in Cd tolerance was seen, with shoot and root growth increasing 30-50% in the transgenic lines (Figure 4.6). Again, as was seen at the lower Cd level, the longer partial peptide conferred a somewhat greater degree of Cd tolerance.

Cd accumulation in in transgenic *Arabidopsis* plants expressing the C terminal HMA4 peptides.

Since one of the ultimate goals of this research is to create genetically modified plants that exhibit enhanced shoot heavy metal accumulation as a "proof of concept" for phytoremediation purposes, Cd accumulation in the shoots of transgenic *Arabidopsis* plants expressing the partial TcHMA4 peptides compared with wild type plants was examined. As seen in Figure 4.7, expression of both the "long" and "short"

partial TcHMA4 peptides conferred a significant (25-30%) increase in shoot Cd accumulation at both low (5 μM) and high (30 μM) Cd levels compared with wild type plants. Unlike the impact on metal tolerance, both peptides conferred a similar degree of enhanced Cd accumulation.

Impact of expression of TcHMA4 peptides on plant response to Zn deficiency.

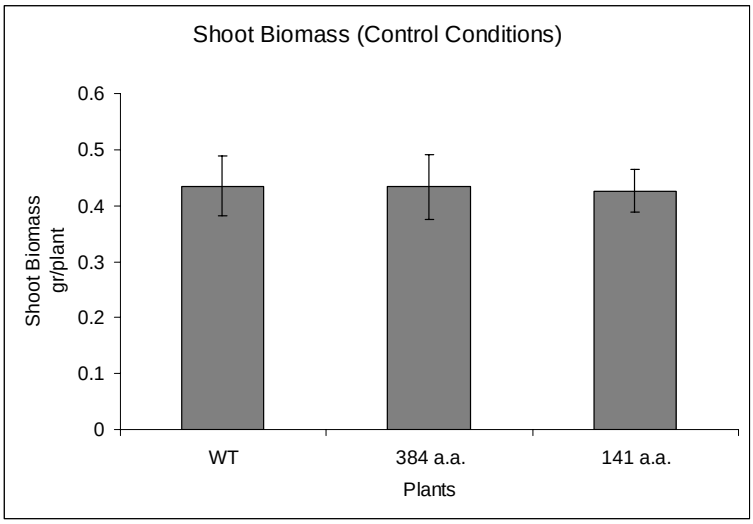
We were also interested in examining whether expression of these metal binding peptides could increase accumulation of the essential micronutrient, Zn, in the shoot and seed, with regards to both plant and human nutrition. To test this, both transgenic and wild type *Arabidopsis* seedlings were grown on full nutrient solution for their first 2 weeks after germination, and then transferred to the same media lacking Zn and grown until seed production. Subsequently, roots, stems, leaves and seeds were assayed for Zn content. It was found that for all the tissues analyzed, the transgenic lines accumulated significantly higher concentrations of Zn. As seen in Figure 4.8, the shoots and seeds of transgenic plants expressing either TcHMA4 peptide exhibited a much higher (approx. 50%) increase in Zn accumulation.

Discussion

The C-Terminus as a Metal-Binding Peptide: A Possible Role in Phytoremediation?

During our initial yeast screen for heavy metal tolerance genes, the two heavy metal ATPase clones that conferred Cd tolerance in wild type yeast were not the full length ATPase, but instead were partial *TcHMA4* clones. As seen in Figure 1, these two clones are 141 and 384 amino acids in length and both are from the C terminal

A



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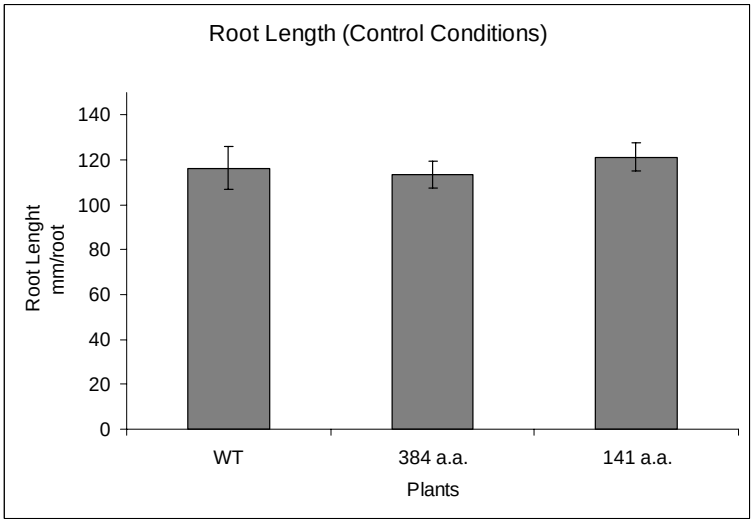
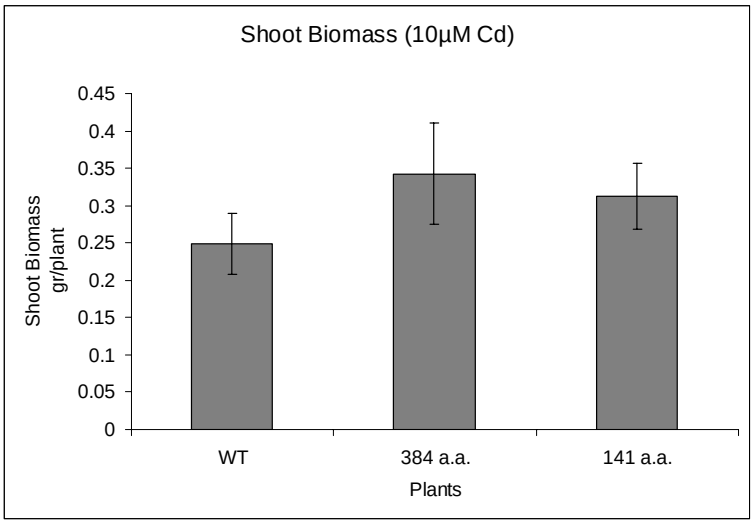


Figure 4.4 Shoot and root growth of transgenic and wild type *Arabidopsis* under normal (- heavy metals) conditions biomass and root length.

A) Shoot biomass of *Arabidopsis* plants grown under normal nutrient conditions lacking heavy metal supplementation.

B) Root length of *Arabidopsis* plants grown under normal nutrient conditions lacking heavy metal supplementation.

A



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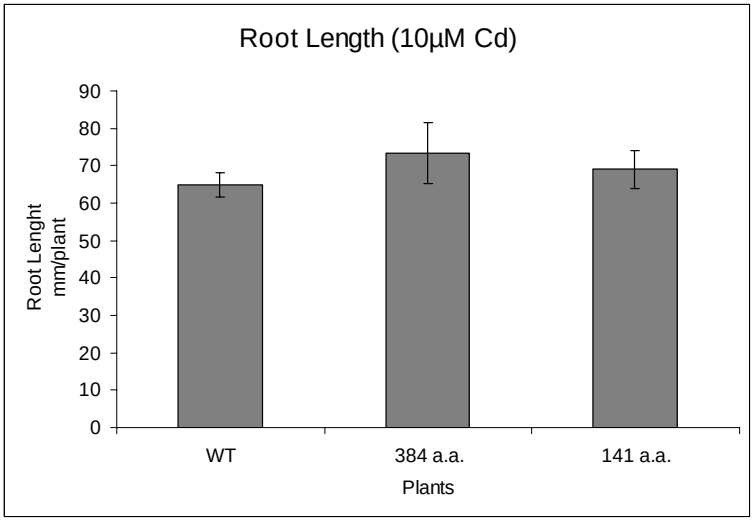
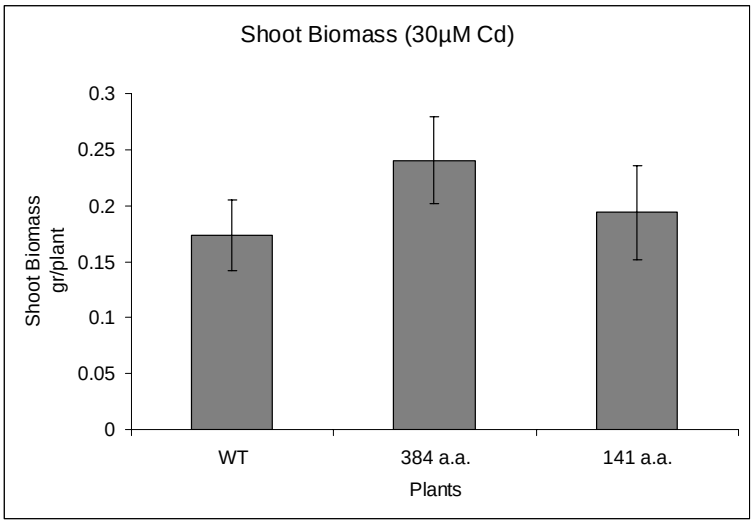


Figure 4.5 Shoot and root growth of transgenic and wild type *Arabidopsis* grown on a low level of Cd supplementation.

A) Shoot biomass of both wild type and transgenic *Arabidopsis* plants grown on nutrient solution containing 10µM Cd.

B) Root length of *Arabidopsis* plants of both wild type and transgenic *Arabidopsis* lines grown on nutrient solution containing 10µM Cd.

A



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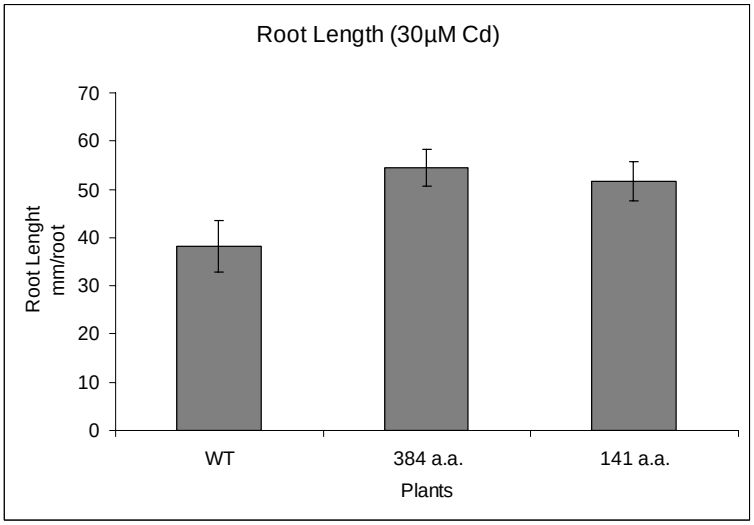

Figure 4.6 Shoot and root growth of transgenic and wild type *Arabidopsis* grown on a high level of Cd supplementation.

A) Shoot biomass of both wild type and transgenic *Arabidopsis* plants grown on nutrient solution containing 30µM Cd.

B) Root length of *Arabidopsis* plants of both wild type and transgenic *Arabidopsis* lines grown on nutrient solution containing 30µM Cd.

region of the TcHMA4 protein, which is predicted to reside in the cytoplasm and contains numerous heavy metal-binding amino acids. From their predicted amino acid sequence, it is clear that the clones lack any membrane-spanning domains and are too small to function as a metal transporter. The only explanation for the increased Cd tolerance conferred by these partial clones is that they are functioning as Cd-binding ligands in the yeast cytoplasm. Our yeast metal tolerance and accumulation experiments with these clones verified this hypothesis, as expression of both clones conferred a high degree of Cd tolerance (considerably greater than the Cd tolerance associated with the full length TcHMA4) and Cd hyperaccumulation (four to six-fold increase in yeast Cd accumulation after 1 hr). The longer, 384 amino acid clone harbors both the long, his-9 repeat as well as the numerous cysteine pairs and single histidine residues of the TcHMA4 C terminus, while the shorter 141 amino acid peptide lacks the poly-histidine tail as well as several of the cysteine-cysteine repeats. There was a clear correlation between the number of metal-binding motifs and the degree of metal tolerance and accumulation conferred by the two peptides, with the longer peptide conferring significantly higher Cd tolerance and Cd hyperaccumulation in yeast.

It has previously been suggested that the cysteine dipeptides and histidine-rich domains in heavy metal ATPases are involved in heavy metal binding (Solioz et al 1996, Williams et al 2000), and the findings presented here confirm these speculations for the TcHMA4 protein. Based on the association constants for the binding of heavy metals to di-cysteine and di-histidine residues (NIST stability constant of metal complexes database), the histidine and cysteine repeats, particularly in the longer peptide, should provide a large number of high affinity binding sites for a range of heavy metals, including Cd, Pb, Hg, Zn, and Cu. Therefore, the findings in yeast led

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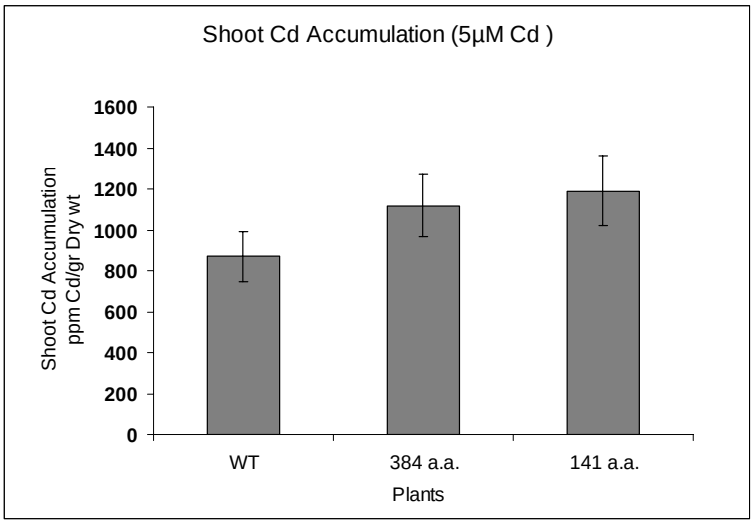

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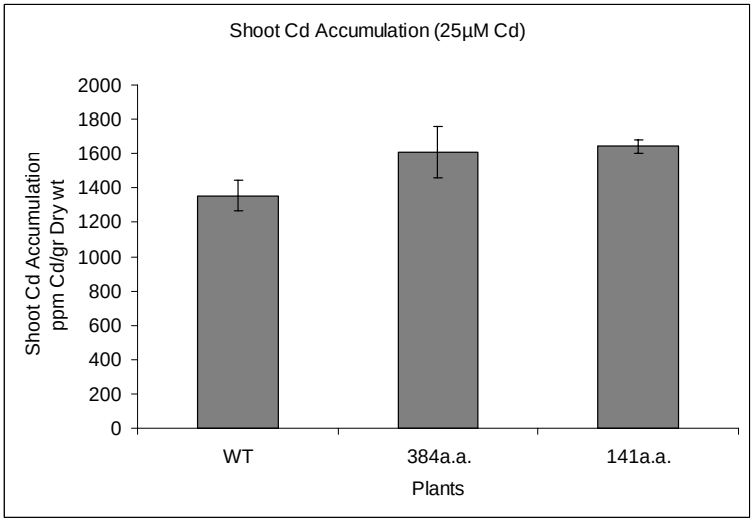
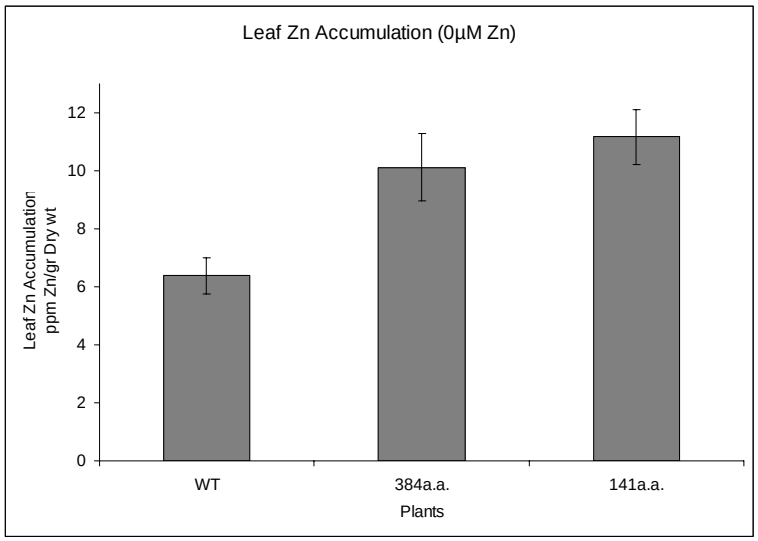


Figure 4.7 Shoot Cd accumulation.

A) Accumulation of Cd in shoots of both wild type and transgenic *Arabidopsis* plants grown on nutrient solution containing 5µM Cd.

B) Accumulation of Cd in shoots of both wild type and transgenic *Arabidopsis* plants grown on nutrient solution containing 25µM Cd.

A



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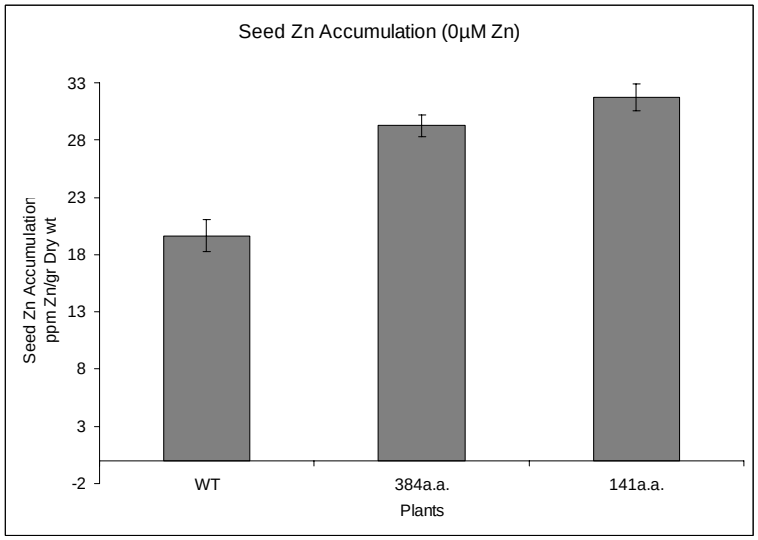

Figure 4.8 Zn accumulation in response to Zn deficiency conditions.

A) Accumulation of Zn in the shoots of both wild type and transgenic *Arabidopsis* plants in response to Zn deficiency (growth on 0 Zn for 45 days).

B) Accumulation of Zn in the seeds of both wild type and transgenic *Arabidopsis* plants grown under 0 Zn treatment in response to Zn deficiency (growth on 0 Zn for 45 days).

us to hypothesize that these two peptides, when expressed to a high level in plants, may confer significant increases in heavy metal tolerance and accumulation and thus may have useful applications with regards to enhancing the phytoremediation potential of plants via biotechnology.

Expression of TcHMA4 C terminus peptides in transgenic *Arabidopsis* plants.

These partial *TcHMA4* clones were expressed in *Arabidopsis thaliana* in order to study their potential for environmental applications, including the creation of plants with increased heavy metal tolerance and enhanced metal hyperaccumulation, and also for plants that may have the ability to alter their response to deficiencies of the essential micronutrient Zn, which is often a limiting micronutrient for both plants and humans (Ghandilyan et al 2006; Welch et al 2004). After identifying a number of homozygous T_3 lines with high expression of both transgenes, these lines were used to characterize the physiology of heavy metal tolerance and accumulation in the shoots for phytoremediation purposes, and the accumulation of Zn in shoots and seeds for studies relating to enhanced plant and human Zn nutrition

Tolerance to heavy metals is an important trait to consider for the creation of genetically modified plants for phytoremediation purposes. First, control experiments (normal nutrient solution) were conducted to demonstrate that transformed plants have about the same root growth and shoot biomass as wild type *Arabidopsis* plants. This information is important because it shows the transformation event itself did not have any negative effects on plant growth. Growth of transformed and wild type *Arabidopsis* plants on a lower Cd level (10μM) as well as a more toxic Cd level (30 μM) showed that the transformed plants produced greater shoot biomass and root growth than did wild type plants. Both transformants ("shorter" and "longer" TcHMA4 partial sequences) were able to tolerate high concentrations of Cd in the

environment better than the wild type *Arabidopsis* plants. As in yeast, it appears that expression of the longer C terminal peptide conferred a slightly greater degree of Cd tolerance, which is consistent with the larger number of metal-binding amino acids harbored within this peptide. These data clearly suggest that the introduction of these TcHMA4 peptides into other plant species has the potential to increase overall plant metal tolerance

Enhanced accumulation of heavy metals in the above ground biomass is the ultimate goal in creating plants that can be useful for phytoremediation purposes. Our data from yeast expressing the partial *TcHMA4* sequences clearly indicates that both short and long sequences are able to function as ligands for heavy metals. The subsequent experiments with transgenic *Arabidopsis* expressing the same peptides verified that *in planta*, these peptides also can work to enhance shoot Cd accumulation. While the moderate 20-30 % increases in shoot Cd accumulation seen here for transgenic *Arabidopsis* plants are certainly not large enough increases to make a direct contribution for real world phytoremediation purposes, these findings are a promising first step as a "proof of concept" for subsequent research aimed at creating genetically modified plants for commercial phytoremediation purposes.

Possible role of TcHMA4 peptides in enhancing plant and human Zn nutrition

Zn deficiency is a serious nutritional problem both for plants and humans. It has been estimated that 30% of the world's soils are Zn deficient for crop production (Takkar et al 1993; Welch et al 1991), and along with Fe deficiency, Zn deficiency is one of the most serious worldwide problems in developing countries, who obtain most of their micronutrients from seed crops. Hence, the possibility that these peptides could be used to enhance Zn acquisition in plants that could have an impact on both plant and human Zn nutrition was investigated. Expression of both of these TcHMA4

peptides conferred a significant increase in Zn accumulation in all plant tissues, especially in the shoot and seed. These findings showing an increased Zn accumulation in edible parts of the plants, especially in the seeds, may open up an avenue of future research aimed at creating genetically modified plants that will have higher nutritional value for humans, and possible may be able to better tolerate low levels of available Zn in the soil for plant production. Thus, the transformation of agriculturally important crops such rice with these peptides from the TcHMA4 protein may be useful in improving the micronutrient value of these crops. One problematic issue that will need to be addressed however, is how to enhance Zn accumulation in edible plant parts without increasing Cd accumulation. Hence it will be necessary to conduct careful field trials with plants expressing these peptides to determine if they will preferentially accumulate Zn from normal, agricultural soils that are usually low in Cd.

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CHAPTER V

Concluding Remarks and Future Work

Heavy metal pollution is becoming an increasingly significant problem throughout the world. More and more studies show the risks to human health associated with exposure to heavy metals such as Pb and Cd. Therefore it is important to develop technologies that will effectively clean the polluted areas, one of which may be the use of terrestrial plants for phytoremediation. By transforming non-hyperaccumulator plants with peptides derived from *TcHMA4* gene to serve as metal-binding ligands, as well as with the entire *TcHMA4* gene targeted to the xylem, it may be possible to engineer plants that will be able to tolerate and flourish on soils intoxicated with heavy metals and also accumulate large concentrations of heavy metals in the easily harvestable, above-ground biomass. After a number of croppings with these transgenic plants, the widespread soil contamination is transferred to and concentrated in the harvested shoot biomass, which then undergoes a volume reduction process (e.g., ashing) and the heavy metal containing plant ash is ultimately stored in a landfill designed for metal contaminants. This approach is much more environmentally friendly and is much less expensive than current, engineering-based technologies which include capping the contaminated site with asphalt or concrete, or removing all of the topsoil of a contaminated site for storage in a landfill.

Possible improvements to the system.

As mentioned in Chapter IV, the expression of the partial *TcHMA4* sequences in transgenic *Arabidopsis* plants was done under the control of 35S promoter; thus these peptides were expressed throughout the plant. However, one of the hallmarks of

metal hyperaccumulator plants such as *Thlaspi caerulescens* is that they do not accumulate the heavy metals in the roots, but rather efficiently translocate them into the shoots where efficient detoxification and sequestration mechanisms are operating. Therefore, expression with tissue-specific promoters that localize peptide expression in the shoots may help to further enhance shoot metal accumulation.

Alternatively, expression of these peptides specifically in the root may be useful for another goal. Because heavy metal accumulation in food crops is the primary avenue of entry of heavy metals into the food chain, it may be possible using these peptides to engineer food crops that that will sequester heavy metals found in agricultural soils in the roots, and thus minimize their translocation to the edible plant parts of the above-ground biomass. Tissue specific expression systems are available for plant transformation. Tehryung et al (2005) describe an elegant root-specific transformation system that they have termed the repressor-operator gene complex, or ROC. This system is based on a dual transformation system where a light induced component represses the expression of the second transformant, hence insuring that the target transgenic protein is expressed only in the roots. There are also available shoot specific promoters which are based on the RUBISCO small subunit light sensitive promoter (Dhanker et al 2002).

Combinatorial expression to further enhance shoot metal accumulation

One of our future goals is the transformation of non-accumulator plants with a combination of the full length *TcHMA4* ATPase using the endogenous promoter to target the xylem cells where this transporter is normally expressed, in combination with shoot-specific expression of the peptides from the C terminus of the TcHMA4 protein. This may enable us to engineer plants that will have the extremely active xylem metal loading seen in hyperaccumulator plants, with ligand "trapping" of the

transported metals in shoot cells. The combinatorial expression of both of these peptides in a high biomass plant may help us generate a good candidate for the phytoremediation of heavy metalcontaminated soils.

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