MECHANISMS OF ZINC TOLERANCE IN THE HEAVY METAL HYPERACCUMULATOR *THLASPI CAERULESCENS*

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

Melinda Anne Klein

January 2007

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Melinda Anne Klein, Ph.D.

Cornell University, 2007

The main objective of this dissertation was to identify and examine the mechanisms conferring zinc tolerance used by the zinc and cadmium (Zn/Cd) hyperaccumulator, *Thlaspi caerulescens*, a plant that has been extensively studied for its altered physiology leading to increased metal tolerance and shoot metal accumulation. Three projects were carried out to address the question of extreme metal tolerance that is one of the hallmarks of this species.

The first project, isolation and characterization of a putative vacuolar zinc transporter, *MTP1*, found higher expression of this gene in *T. caerulescens* compared with the related non-accumulator, *T. arvense.* Additionally *MTP1* expression increased in response to increasing plant Zn status. When *MTP1* was expressed in yeast, Zn accumulation increased; however, no strong phenotype was seen in transgenic *Arabidopsis thaliana* overexpressing the *A. thaliana* homolog of *MTP1*.

In the second project, a *T. caerulescens* cDNA library was screened for genes conferring Zn tolerance to yeast when grown on high zinc media. A number of genes with widely divergent potential functions were identified including signaling, metabolic and protein regulation genes. These genes, when expressed in yeast, conferred either increased or reduced Zn accumulation, and several were chosen for whole plant studies. One specific gene of interest was *PKS4*, a serine-threonine kinase, which was associated with increased zinc accumulation relative to wild type yeast strains. Since other members in this kinase family have already been implicated

in plant abiotic stress responses, we examined the effect of altered *PKS4* expression *in planta* on heavy metal tolerance.

Finally, *T. caerulescens* suspension cell lines were created and characterized for the same metal hyperaccumulation traits exhibited in the whole plant (Zn/Cd tolerance, transport and accumulation). From this work, the increased metal tolerance and altered regulation of metal tolerance genes seen in plants was observed, indicating these are traits expressed at the cellular level. We are now able to stably transform these cell lines, which opens up future work testing candidate hyperaccumulation genes using these suspension cells lines.

BIOGRAPHICAL SKETCH

Melinda Klein was born in Long Beach, California in 1977 and was raised in Southern California by her parents Fred and Carolyn with her siblings, Kristina, Stephanie, Megan and Joe. Growing up, she gained an appreciation for the natural world through family camping trips that ranged throughout California and the greater Southwestern US. Her interest in science began in high school where she became involved in a number of research programs including a summer research program at California State University, Fullerton. Under the supervision of Dr. Steven Murray, she studied sea snail feeding preferences and presented her research at the American Junior Academy of Science meeting in the winter of her senior year. After graduating from La Habra High School in 1995, she attended UC Davis and conducted research with Dr. Alison Berry on the cytology of *Frankia spp*. nodules in *Datisca glomerata*. She spent the summer of her junior year studying fungal genetics in the laboratory of Dr. Olen Yoder and Dr. Gillian Turgeon at Cornell University. She graduated from UC Davis with a B.S. degree in Plant Biology with honors in the spring of 1999. She then moved to Ithaca and has been working in the lab of Dr. Leon Kochian as a graduate student in the field of Plant Biology examining mechanisms of zinc tolerance in *Thlaspi caerulescens* since the fall of 1999.

ACKNOWLEDGEMENTS

I sincerely thank my advisor Dr. Leon Kochian for the support, encouragement and advice he's provided through these past years. I also thank the members of my committee, Dr. Jim Giovannoni, Dr. Karl Niklas, and Dr. Jocelyn Rose, for the guidance provided over the course of this project.

I would like to thank all the members of the Kochian lab who have been such great colleagues and friends. Specifically, thanks to those members of the Thlaspi project that have provided encouragement, advice and assistance including – Dr. Debbie Letham, Dr. Nicole Pence, Matthew Milner, Laura Seib, Dr. Ashot Papoyan, Dr. Hitoshi Sekimoto, Dr. Hendrik Küpper, and Dr. Mitsuhiro Ueda. I would not have been able to get through this project without the help and support of the lab at large including Jon Shaff, Dr. Paul Mason, Dr. Lyza Maron, Dr. Miguel Piñeros, Dr. Owen Hoekenga, Dr. Jon Hart, Dr. Yihong Wang, Dr. Gökhan Hascisalihoglu, Dr. Li Li, Dr. Shan Lu, Dr. Jiping Liu, Dr. Dave Garvin, Elena DiMuzio. The technical assistance of Eric Craft, Randy Clark. Holly Manslank, Michelle Oakes, and Aliza Yair was greatly appreciated. I'd also like to thank Dr. Mike Rutzke for the seemingly thousands of ICP samples run for me over the years.

Thanks to the advice and guidance of those outside of my lab including Dr. Joyce van Eck whose guidance saved the suspension cell lines more than once, Mark Stoutemyer from Dr. Roger Innes Lab for providing and helping me maintain the suspension cell lines as well. Thanks to Dr. Kerry Pedley for assistance with the kinase project.

Finally, I couldn't have gotten through this endeavor without the help, love and support of my dear family and friends through these past years. Thank you so much.

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CHAPTER I

Introduction and Literature Review

Role of Zinc in Biology

Zinc (Zn) was first identified as an essential mineral nutrient for plant growth in 1926 by Sommer and Lipman (1926). Essential plant nutrients are defined as elements: 1) that are essential for completion of the plant life cycle; 2) whose function is irreplaceable by an alternate element; and 3) that are directly coupled to plant metabolism or a specific metabolic process (Arnon and Stout, 1939). As is the case for many plant micronutrients, Zn plays an essential role in the proper function of enzymes. Zn is taken up as a divalent cation and remains in that form as it acts in a structural, catalytic or co-catalytic capacity for many enzymes (Marschner, 1995). Zn does not change its oxidative state and thus, unlike many other transition metals, is not involved in oxidoreduction reactions. As extensively reviewed in Clarkson and Hanson (1980), the divalent Zn ion, with its small size and stable ionic charge, fulfills a role both as a structural and as an enzymatic cofactor when the plant requires stable metalloenzyme activity with a tetrahedral conformation.

In plants, Zn is essential for a wide range of physiological and biochemical processes. Zn is a necessary cofactor for RNA polymerase, ribosome structure and function, carbohydrate synthesis (for carbonic anhydrase, aldolase, and fructose 1,6-bisphosphatase activity) and DNA replication. Zn also limits oxidative degradation of auxin and is necessary to maintain membrane integrity (Cakmak and Marschner, 1988a, b; Cakmak et al., 1989; Falchuk et al., 1977; Ohki, 1976; O'Sullivan, 1970; Prask and Plocke, 1971).

Because its main role in plants is enzymatic, most plants only need ~20ppm Zn for adequate growth (Marschner, 1995). When grown on soils with high Zn concentrations, plants begin exhibiting toxicity symptoms at tissue Zn concentrations usually starting around 300 ppm Zn on a dry weight basis, although some species start to show toxicity symptoms at concentrations as low as 100 ppm (Ruano et al., 1988).

Elevated Zinc in Soils

While high concentrations of Zn naturally occur in some soils, most soils containing elevated Zn concentrations are the result of industrial contamination (Chaney, 1993), such as commercial areas where Zn was smelted or used in industrial processes. In fact, Zn has been found in the highest concentrations of all of the heavy metals found in industrial waste (Boardman and McGuire, 1990). Other sources of Zn contamination to the environment include long term pesticide applications on agricultural soils, rubber tire disposal and application of sewage sludge (He et al., 2005; Logan and Chaney, 1983; Smolders and Degryse, 2002). These contaminating sources are especially problematic when soil acidifies, as this causes Zn to become more soluble and mobile (Marschner, 1995). With increasing industrialization, heavy metal contamination of soils is becoming a greater problem and Zn is one of the most prevalent metal contaminants in industrialized countries (Nriagu and Pacyna, 1988).

Zinc Toxicity in Plants

The most noticeable visible symptom of Zn toxicity in plants is chlorosis in young leaves (Marschner, 1995). At toxic concentrations, Zn replaces other similar divalent cations including Fe, Mg and Mn. As these ions are necessary for the proper function of a number of photosynthetic enzymes, their replacement by Zn results in improper enzyme function, lower photosynthetic rates and photooxidative damage

(Van Assche and Clijsters, 1986a, b). When present at high concentrations, Zn can replace magnesium and ferrous (II) iron as all three have a similar ionic radius (Boardman and McGuire, 1990; Woolhouse, 1983 as referenced in Marschner, 1995). A report by Ruano et al. (1987) suggested that growth under higher Zn concentrations may also limit manganese uptake in roots, leading to Zn toxicity-induced manganese deficiency.

Zinc Tolerance in Plants

Plant Zn tolerance has been described as the ability to keep cytoplasmic Zn^{2+} activity levels within certain physiological limits when the total symplastic and apoplastic concentrations of Zn are high (Pence, 2002). The mechanisms used by plants to tolerate higher metal concentrations in the environment has been of interest for both naturally occurring and man-made sources of pollution leading to toxic concentrations of metals in the soil. As initially described in Tomsett and Thurman (1988), and further expanded upon in Marschner (1995), there are six main mechanisms of metal tolerance used by plants:

1. Cell wall binding
2. Cell wall - plasma membrane interface chelation
3. Limited transport across the plasma membrane
4. Active efflux out of the cytoplasm across the plasma membrane
5. Active efflux out of the cytoplasm into the vacuole
6. Cytoplasmic chelation by proteins, organic acids or inorganic compounds

Cell wall binding appears to play a role in Zn accumulation as a number of studies have shown that the plant apoplast can store a significant fraction of plant Zn (Küpper et al., 2000; Lasat et al., 1998; Nedelkoska and Doran, 2000). However

additional work shows that the cell wall does not accumulate Zn to the same extent as the vacuole (Vázquez et al., 1992). Cell wall-plasma membrane interface chelation has not been studied as a Zn tolerance mechanism because available techniques are not sensitive enough to quantify the affinity of plasma membrane sites for Zn binding.

The third mechanism, limited transport across the plasma membrane, has been characterized in plants at the molecular and physiological level with decreased expression of Zn transporters and decreasing rates of Zn uptake under conditions of increasing Zn concentration. The Zn transporter, *ZNT1*, first identified in *T. caerulescens*, encodes a putative plasma membrane Zn transporter that shows decreasing expression in both roots and shoots when grown under increasing Zn concentrations (Pence et al., 2000). This sensitivity to increasing Zn concentrations is also seen physiologically with a decrease in the V_{max} for Zn uptake in *T. caerulescens* roots as root and plant Zn status increases (Pence et al., 2000).

Active Zn efflux from the cytoplasm may play a limited role in plant tolerance because of the multicellular nature of plants. Most plants (e.g. non-accumulator plants) sequester excess Zn in the roots, restricting transport to the photosynthetically active shoots, as the photosynthetic machinery appears to be particularly sensitive to Zn and other heavy metals (Küpper et al., 2000). Heavy metal transporters that actively remove Zn from the cytoplasm when expressed in heterologous systems have been identified, most notably *HMA4*, which was shown to catalyze the efflux of zinc, cadmium, lead and copper from the cytoplasm (Hussain et al., 2004; Mills et al., 2003, 2005; Papoyan et al., 2004). However, based on higher transcription rates in roots relative to shoots, and promoter-reporter construct work, this transporter is thought to be localized to xylem parenchyma and thus involved in xylem loading, rather than participating in a more general role of cellular Zn efflux throughout the plant (Hussain et al., 2004; Papoyan and Kochian, 2004).

Chelators that can bind ionic Zn with high affinity and that may confer metal tolerance both in the cytoplasm and vacuole; include phosphate, organic acids, amino acids and proteins (usually those rich in Cys and His residues). Many studies have addressed where and how these compounds might play a role in the conference of metal tolerance. As described below, their location in the plant and their relative importance in conferring Zn tolerance vary widely.

Phosphate is the most common inorganic Zn ligand. Zn tolerance studies have found a correlation in roots, but not shoots, between increasing Zn and phosphate concentrations (Sarret et al., 2002; Zhao et al., 1998). Since this result is root specific, it is generally attributed to Zn-phosphate precipitation on the outer surface of the roots, rather than an internal mechanism to maintain low cytoplasmic Zn activity (Sarret et al., 2002; Zhao et al., 1998).

Organic acids are thought to play a role in metal chelation due to their negatively charged nitrogen, sulfur and oxygen ions, which are potent electron donors for metals such as Zn^{2+}. Malic and citric acid in particular have been suggested as Zn chelators, based on high concentration seen in plants that accumulate Zn (Godbold et al., 1984; Mathys 1977). However, more recent work by Shen et al. (1997) has questioned the role of malate as a Zn tolerance response in Zn hyperaccumulators, since high malic acid concentrations were found in both *Thlaspi caerulescens*, a Zn hyperaccumulator and in *T. ochroleucum*, a related non-hyperaccumulator, suggesting that this might be a general trait of the *Thlaspi* genera rather than a Zn tolerance-related trait. van Stevenick and colleagues (1994) found Zn-phytate globules present in the root elongation zone of various crop plants and proposed the formation of Zn-phytate complexes as a root Zn tolerance mechanism.

Two main families of metal binding proteins are associated with heavy metal chelation: phytochelatins (PCs) and metallothioneins (MTs). PCs are enzymatically

produced chains of γ-Glu-Cys subunits with a terminal Gly residue. Within plants, the number of subunits in PCs may vary greatly, with a chain length of up to 11 subunits and there can be a number of different terminal amino acids including Glu, Ser, Gln or β-Ala (Grill et al., 1985, 1986; Klapheck et al., 1994; Mehra and Winge, 1988; Meuwly et al., 1993). The creation of PCs is dependent on the transpeptidation activity of phytochelatin synthase (PCS), an enzyme whose activity is regulated by the presence and concentration of heavy metals (Loeffler et al., 1989; Rauser, 1995). This gene is usually expressed constitutively; however, induction of *PCS* gene transcription was seen following Cd^{2+} exposure in wheat roots and shoots, suggesting that some plants may regulate PCS activity at both the transcriptional and post-translational levels (Clemens et al., 1999; Ha et al., 1999). Activation of PCS enzyme activity is stimulated following exposure to a range of heavy metals, especially Cd^{2+} but also Cu^{2+}, Ag^{+}, Hg^{2+}, Zn^{2+} and Pb^{2+} (Clemens et al., 1999; Ha et al., 1999; Vatamaniuk et al., 1999). However, *pcs1* mutant lines of both Arabidopsis and the yeast *Saccharomyces pombe* only show sensitivity to arsenate and Cd^{2+}, not Zn^{2+} (Ha et al., 1999). PC-Zn complexes are relatively weak and PCs are not considered to play a major role in tolerance mechanisms for Zn (Leopold and Gunther 1997; Maitani et al., 1996). With regards specifically to PCs in the metal hyperaccumulator, *Thlaspi caerulescens*, Ebbs et al. (2002) found no differences in root or shoot PC levels in *T. caerulescens* in comparison with the related nonaccumulator, *T. arvense*, when both plants were grown on normal levels of Zn and elevated Zn or Cd.

Metallothioneins (MTs) are small, cysteine rich proteins that are synthesized via gene transcription/mRNA translation and are thought to play a role in metal tolerance. Common features of these proteins are their small size (4-8 kDa) and Cys-rich domains at the C and N termini (Hamer, 1986). Two different types of MTs are present in animals (Cobbett and Goldsbrough, 2002). Type I MTs appear to function

exclusively in Cd tolerance, and are considered to be functionally equivalent in plants to phytochelatins (Masters et al., 1994). In plants, only the type II MTs have been identified and these have been further parsed into four different groups, based on the arrangement of cysteine residues (Cobbett and Goldsbrough, 2002). The roles of MTs and MT-like proteins are still being determined as their small size and unstable nature in aerobic environments make them difficult to characterize their behavior (Kille et al., 1991). Through expression of MTs in heterologous systems and by studying changes in gene expression patterns, MTs have been linked to copper homeostasis, metal scavenging during tissue senescence and as regulators of cytoplasmic Zn activity (Garcia-Hernandez et al., 1998; Rauser, 1999; Robinson et al., 1996). Further work is necessary to better understand the function of these genes and their role in Zn homeostasis.

The final cellular metal tolerance mechanism, vacuolar compartmentation, is thought to contribute the largest degree of tolerance to Zn stress (Ernst et al., 1992; Vázquez et al., 1994). The storage of high Zn concentrations in vacuoles, rather than in cytoplasmic or apoplastic compartments, has been shown in a number of studies (Frey et al., 2000; Küpper et al., 1999).

Zn tolerance at the whole plant level is conferred through two very different strategies: Zn avoidance and Zn accumulation. Most plant species are avoiders and tolerate Zn by accumulating Zn in the roots, away from the photosynthetic shoots. These types of plants generally perform poorly on soils contaminated with high Zn concentrations. On the other hand, a subset of plants exist that accumulate high metal concentrations in their above-ground tissue to levels that are considered toxic to non-accumulator species. These plants, termed hyperaccumulators, are generally found in soils where elevated heavy metal concentrations occur both naturally and through anthropogenic metal contamination.

Zinc hyperaccumulation

Metal hyperaccumulator plants include over 400 species from 45 families (Brooks et al., 1977; Brooks, 1998). Generally, a hyperaccumulator species is defined as one where the above ground metal concentration is at least 100 times higher than 'normal' plant concentrations (Baker and Brooks, 1989). Zn, as stated earlier, is generally required at concentrations ranging from 20-100 ppm and is considered toxic at concentrations around 300 ppm dry weight (d.w.) (Marschner, 1995). Approximately 15 Zn hyperaccumulator species have been identified with naturally occurring above ground Zn concentrations greater than 10,000 ppm (1% d.w.) and levels in excess of 30,000 ppm have been measured when various species are grown hydroponically (Brown et al., 1995; Robinson et al., 1998; Shen et al., 1997).

The physiology of metal hyperaccumulating plant species is of interest at both a fundamental and applied level and there are many questions about the molecular and physiological mechanisms that confer metal hyperaccumulation. The fundamental question as to whether hyperaccumulation is exclusively determined at the cellular level, or requires more complex interactions in tissues and organs, is still poorly understood. By comparing the behavior of hyperaccumulator plants, which appear to exhibit an exaggerated metal deficiency response relative to non-accumulating plant species even as they hyperaccumulate that metal (Pence et al., 2000), the processes necessary for metal status signaling, metal uptake, the transport of metals through plants and the storage of metals by plants might be better understood.

On a more applied level, understanding mechanisms of metal tolerance and accumulation could be useful for remediating metal contaminated soils and improving transport of essential micronutrient metals to edible plant portions for animal and human nutrition. The use of metal hyperaccumlating plants as an inexpensive means to remediate metal contaminated soils has been proposed by a number of laboratories

(Chaney et al., 1997; Ebbs et al., 1997; Salt et al., 1998). However, this application has been limited by the small size and slow growth of most hyperaccumulating plant species. Thus, further research is needed to address the possibility of transferring the desirable metal accumulating traits to larger biomass plants. Studying the changes in metal transport and allocation observed in hyperaccumulating species might also lead to better understanding of how to enhance the micronutrient density of edible food crops. By understanding the processes leading to the movement of micronutrients such as Zn throughout the plant, the steps necessary for improving micronutrient seed density might be facilitated, which may help promote plant germination and early seedling growth on micronutrient deficient soils. Seeds with elevated levels of micronutrients could also improve the micronutrient status of humans from developing countries where most essential nutrients are derived from plants, a diet that is known to be poor in micronutrients, especially Fe and Zn (Guerinot and Salt, 2001; Grusak, 2002).

To better understand the mechanisms of Zn hyperaccumulation, the Zn/Cd hyperaccumulator *Thlaspi caerulescens* has been extensively studied and is considered a 'model' hyperaccumulator plant species (Assunção et al., 2003; Cobbett, 2003; Deniau et al., 2006). Leaf Zn concentrations have been characterized in the 10,000 ppm range for naturally occurring *T. caerulescens* plants and concentrations greater than 30,000 ppm Zn dry weight have been obtained in plants grown hydroponically (Brown et al., 1995; Robinson et al., 1998; Shen et al., 1997). Additionally, as a member of the Brassicaceae family, the study of *T. caerulescens* benefits from a close genetic relationship to *Arabidopsis thaliana* and all of the genetic resources available for this model plant species.

A number of physiological and molecular studies have been undertaken to examine the underlying mechanisms leading to increased tolerance and accumulation

that is associated with the hyperaccumulation phenotype. In *T. caerulescens* this is a result of physiological changes at the whole plant, organ and cellular levels and they are associated with root uptake and physiology, root to shoot transport and shoot physiology. These various regulatory points will be discussed with a focus on the known differences between the hyperaccumulator *T. caerulescens* and related non-hyperaccumulators.

Root physiology— There are a number of differences in the uptake and movement of Zn through the roots of hyperaccumulator plant species relative to non-hyperaccumulator species. In *T. caerulescens*, a 5-fold higher rate of Zn influx in roots was observed relative to the related non-hyperaccumulator *T. arvense* (Lasat et al., 1996) and significantly higher levels of transcription were seen for the putative plasma membrane Zn transporter *ZNT1* in the roots of *T. caerulescens* compared with *T. arvense* (Assunção et al., 2001; Pence et al., 2000). Additionally, the onset of *ZNT1* transcriptional downregulation by zinc in the growth medium occurred at significantly higher levels in *T. caerulescens* (50 μM) than *T. arvense* (1 μM), suggesting a decreased sensitivity to changes in plant Zn status (Pence et al., 2000). However, when the Zn content of high Zn-grown *T. caerulescens* roots was compared to *T. arvense* roots, significantly less Zn was detected in *T. caerulescens*, suggesting higher rates of translocation to the shoots and less storage of Zn in the roots of the hyperaccumulator (Lasat et al., 2000).

Root to shoot transport— The high rate of Zn uptake but low rate of Zn storage seen in the roots of *T. caerulescens* suggests that Zn transport from the roots through the xylem into the shoots is another important transport feature contributing to metal hyperaccumulation. As described above, more Zn is stored in the roots of non-accumulator species relative to hyperaccumulator species when plants are grown on high levels of Zn (Lasat et al., 2000). Furthermore, greater levels of Zn are stored in

the shoot tissue of the hyperaccumulator species compared to non-accumulators when both plants are grown on the same Zn concentrations. Lasat and colleagues (1998) demonstrated that *T. caerulescens* maintains a 5-10 fold higher Zn transport rate from roots to the shoot relative to *T. arvense*.

Shoot Physiology— Zn transport physiology in the shoot tissues of hyperaccumulating plant species differs significantly from non-hyperaccumulating plant species. Besides the obvious difference of high Zn concentrations present in the shoots of the hyperaccumulator, *T. caerulescens* must employ more effective tolerance mechanisms to allow the plant to grow with such a high Zn load. For example, at the tissue level, more Zn is stored in the non-stomatal epidermal cells of the leaves, which are not photosynthetic, thereby sequestering Zn away from the mesophyll where most leaf photosynthesis occurs (Küpper et al., 1999). At the cellular level, the vacuole is the location for most Zn accumulation by *T. caerulescens* (Cosio et al., 2004; Frey et al., 2000; Küpper et al., 1999; Ma et al., 2005). Work by Assunção and colleagues (2001) has shown high levels of transcription of the putative vacuolar transporter, *ZAT1*, in three different *T. caerulescens* accessions relative to non-hyperaccumulator species, suggesting that increased vacuolar Zn uptake might be due to an increased number of transporters rather than altered transporter behavior.

Questions Regarding Zinc Tolerance

With all that is known about Zn hyperaccumulating species, a great deal is still not known about the mechanisms of metal tolerance in heavy metal hyperaccumulators. As described above, physiological investigations have identified a number of metal transport differences in the roots, shoots and vascular tissue relative to non-hyperaccumulator species. Molecular work in hyperaccumulating species has provided additional confirmation of these differences, with higher levels of expression

seen for putative Zn transporter genes such as *ZNT1* and *ZAT*. Almost nothing is known about the regulatory and homeostatic processes associated with the hyperaccumulation phenotype. In yeast, the transcription factor ZAP1 has been shown to regulate Zn accumulation through the regulation of the expression of the genes encoding the high and low affinity Zn transporters, *ZRT1* and *ZRT2*, in response to changes in cellular Zn status (Zhao et al., 1998). No such system regulating Zn homeostasis has been identified in plants, but the large number of transporters with upregulated transcription relative to related non-hyperaccumulators suggests a coordinated level of regulation that is beyond the upregulation of a single transporter. Another focus of hyperaccumulation research should be to identify mechanisms for Zn tolerance at the cellular level. Efforts to identify the Zn chelators in the cytoplasm, vacuole or xylem in *T. caerulescens* have shown a correlation between Zn concentrations and histidine and organic acids (Lasat et al., 1998; Salt et al., 1999), but high concentrations of organic acids are also seen in other, non-accumulating *Thlaspi* species and do not appear to increase with increasing Zn concentrations (Shen et al., 1997; Tolra et al., 1996). Despite all the work conducted on *T. caerulescens* to date, little is known about metal tolerance mechanisms in this plant species.

Focus of the Present Study

The research presented in the following chapters examines the mechanisms of Zn tolerance used by *T. caerulescens*. Chapter II focuses on the putative vacuolar transporter, *TcMTP1*, including its isolation and characterization in studies with yeast and plants, in order to determine its role in Zn tolerance. Chapters III and IV examine a series of candidate Zn tolerance genes from *T. caerulescens*, identified from a functional screen of yeast Zn tolerance when a *T. caerulescens* cDNA library constructed in a yeast expression vector was transformed into wild type yeast. Finally,

in Chapter V, suspension cell lines from *T. caerulescens* were generated and characterized using physiological and molecular techniques to better understand the hyperaccumulation phenotype at the cellular level. The results from these studies provide a starting point for obtaining a more complete understanding of the Zn tolerance mechanisms employed by *T. caerulescens* in hyperaccumulation.

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CHAPTER II

Isolation and Characterization of *TcMTP1*

INTRODUCTION

The Cation Diffusion Facilitator (CDF) family is made up of a diverse set of metal transporters that are found in bacteria, yeast, plants and animals (Paulsen and Saier, 1997). Characteristic features of these proteins include six putative membrane spanning domains, a distinct N-terminal amino acid sequence specific to this family of transporters, a C-terminal cation efflux domain and, in eukaryotic members, a variable histidine rich region that could potentially bind heavy metals which is located between the fourth and fifth membrane spanning domains (Paulsen and Saier, 1997). Most characterized proteins in this family mediate efflux of heavy metals from the cytoplasm with variability in metal specificity and membrane localization (Haney et al., 2005 and references therein).

When this research project was initiated, limited information was available on this gene family. The yeast CDF transporters ZRC1 and COT1 had been identified and shown to play a role in vacuolar accumulation of Zn, Cd and Co in *Saccharomyces cerevisiae* (Conklin et al., 1992, 1994; Kamizono et al., 1989; Li and Kaplan, 1998). In Arabidopsis, the first CDF member, *ZAT* (Zn transporter of *Arabidopsis thaliana*, subsequently referred to as *AtMTP1*), was cloned and shown to have constitutive expression in young Arabidopsis seedlings at all Zn concentrations tested. Overexpression of this gene in Arabidopsis conferred increases in Zn tolerance and in Zn accumulation in roots (van der Zaal et al., 1999).

A gene such as *AtMTP1* that allows plants to accumulate Zn and could function in plant heavy metal tolerance through vacuolar sequestration is of interest to

our research program as it may allow us to elucidate the processes of Zn accumulation and sequestration. These processes are critical for the heavy metal extreme metal accumulation phenotype seen in the hyperaccumulator plant species, *Thlaspi caerulescens*. Thus the decision was made to search for and characterize the *AtMTP1* homolog in *T. caerulescens*. Following isolation of this gene, which was named *TcMTP1*, its expression was examined in *T. caerulescens* in comparison with the related non-accumulator, *T. arvense*, grown under a range of Zn concentrations. Additionally, its possible function was studied via heterologous expression of the *TcMTP1* full length coding sequence under the control of a constitutive promoter in yeast. Finally, overexpression lines in Arabidopsis were created and examined for effects on plant growth and Zn status in long-term growth studies.

METHODS AND MATERIALS

Cloning of TcMTP1. An alignment of four *A. thaliana* sequences for AtMTP1 and AtMTP1-like proteins available at the time from the NCBI database (cab88298, cab71901, aac95197, and aad11757) identified a region of consensus that was used to design a degenerate forward primer 5' CAGATGCAGCTCATTTGT/C TC/A/G TC 3' and reverse primer 5' ATGAGCTC/T/G CACATATGGGCT/G/A AT 3'. These primers were used to isolate a gene fragment with homology to *AtMTP1* from *T. caerulescens* (ecotype Prayon) shoot RNA through RT-PCR. The resulting gene fragment was used to screen a *T. caerulescens* (Prayon) cDNA library in the yeast-*Escherichia coli* shuttle vector pFL61 (Minet et al., 1992). A full length cDNA was identified from the screen, sequenced and designated *TcMTP1*.

Yeast Characterization. *S. cerevisiae* wild type strain DY1457 was transformed with *TcMTP1* in the pFL61 vector and grown on synthetic dextrose minimal media (SD media) which is made up of 0.67% bacto-yeast nitrogen base

without amino acids (Difco Laboratories, Sparks, MD), 2% glucose, 2% bacto-agar, 0.1% casamino acids, 20 mg/L adenine, 20 mg/L histidine, 20 mg/L tryptophan, and 30 mg/L leucine. For growth assays, yeast containing pFL61 or pFL61 with *TcMTP1* were grown overnight at 30ºC in SD media lacking agar and then subcultured into 10 mL SD media or SD media containing 1 mM or 2.5 mM $ZnSO_4$ at a final yeast density of OD_{600}=0.01. The yeast were then grown at 30ºC while being shaken at 120 rpm for 16 hours. At the end of 16 hours, the yeast density was determined by spectrophotometry at OD_{600}, spun at 2500 x g for 5 min, washed twice with 5 mL of 18 mΩ dH_2O and then the cells were transferred to quartz tubes for elemental analysis. The samples were digested with concentrated HNO_3 at 100ºC then further digested in nitric and hydrochloric acid at 200ºC until dry. Samples were resuspended in 5% HNO_3 and analyzed by inductively coupled argon-plasma emission spectrometry (ICP-AES; ICAP 61E Trace Analyzer, Thermo-Jarrell Ash, Waltham, MA) for Zn content.

Northern Analysis. *TcMTP1* gene expression was examined in two different sets of *Thlaspi* tissues. The first set of tissues were roots and shoots from *T. caerulescens* plants grown hydroponically under greenhouse conditions for seven weeks in modified Johnson's solution (1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 5 μM Fe(III)-EDDHA (N,N'-ethylenediamine-di-(O-hydroxyphenylacetic acid)), 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM H_2MoO_4, 0.1 μM $NiSO_4$, and 1 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 5.5) and then were transferred to modified Johnson's solution containing either 1 μM (standard Zn level) or 500 μM Zn and grown for an additional 14 days before being harvested. The second group of *Thlaspi* tissue examined for *TcMTP1* gene expression included roots and shoots of *T. caerulescens* and *T. arvense* plants grown hydroponically on Zn-deficient (0 μM

$ZnSO_4$), Zn-sufficient (1 μM $ZnSO_4$) and Zn-excess (10 μM $ZnSO_4$ for *T. arvense*; 50 μM $ZnSO_4$ for *T. caerulescens*) solutions, which were based on the Johnson's nutrient solution described above .

Total RNA was isolated following the Trizol protocol (GibcoBRL/Invitrogen). 20 μg of total RNA was denatured, separated by glyoxal-agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham). Equal loading was confirmed both by spectrophotometric measurement of RNA concentrations and visually through ethidium bromide staining of ribosomal subunits. Membranes were UV crosslinked before hybridization. To examine *TcMTP1* gene expression, the original gene fragment identified from RT-PCR (*pTcS6*) was labeled with ^{32}P-dCTP with random hexamer primers and hybridized at 65ºC overnight in PerfectHyb Solution (Sigma Aldrich) with 10 μM salmon testes DNA blocking agent. Following hybridization, membranes were washed 2 times for 30 minutes at 65ºC in a low stringency wash solution (2xSSC, 0.1% SDS). After exposure to autoradiography film (Kodak), the membrane was stripped with boiling 0.5% SDS.

Generation and Characterization of AtMTP1 and TcMTP1 Overexpression Lines in A. thaliana. *AtMTP1* was isolated from Zn deficient *A. thaliana* (Col) root mRNA following single strand synthesis RT-PCR, using an oligo-dT primer. The *AtMTP1* cDNA was then amplified from the cDNA pool using the forward primer 5'- GGTTCTATTGAATTGGGTTTC -3' and the reverse primer 5'- TGCTGATACAAAAACCCTTCA -3'. An additional set of primers was designed to transfer the genes of interest into the *E. coli- Agrobacterium tumefaciens* shuttle vector pBAR1 (Holt et al., 2002; with addition of a 35S promoter in Hind*III*/Eco*RI* site at the start of the multiple restriction cloning site):
MTPpBAR 5'- 5'AT<u>CCCGGG</u>ATGGAGTCTTCAAGTC 3';
TcMTPpBAR 3' 5'AT<u>GGATCC</u>TCAGCGCTCGATTTGT 3';

AtMTPpBAR 3' 5'ATGGATCCTTAGCGCTCGATTTGTA 3'.

The first sixteen nucleotides between the *T. caerulescens* and Arabidopsis *MTP* genes are the same so the same 5' primer was used for the cloning of both genes. PCR products were separated on an agarose gel, fragment purified using a QIAEX II gel extraction kit (Qiagen) and cloned into the TOPO-TA vector (Invitrogen). The subsequent minipreps were used for subcloning into pBAR1. Primers included Eco*RI* and Xma*I* restriction sites (underlined) for directional cloning into pBAR1. The genes were digested with the Eco*RI* and Xma*I* enzymes and ligated into the corresponding sites in the pBAR1 vector. Sequence integrity was verified by sequencing. pBAR1 containing the *AtMTP1* and *TcMTP1* constructs was transferred to *A. tumefaciens* strain C58 via electroporation. Transformed *Agrobacterium* cells were selected from LB plates with 50 μg/L kanamycin and 50 μg/L rifampicin selection. A single colony was started in a 5 mL overnight LB kan/rif culture grown at 30ºC. The starter culture was used to inoculate 0.5 L of LB kan/rif overnight culture grown at 30ºC. Cells were then pelleted at 4000 x g for 20 min and resuspended in 1 L of dipping solution (50 g/L sucrose and 0.5 mL/L Silwet-L77). Flowering Arabidopsis (Col) plants were dipped in the dipping solution for 20 seconds per 4" pot, laid sideways to drain excess solution, covered with Saran Wrap and left in the dark overnight. The following day, plants were uncovered, straightened and returned to normal growing conditions. Seeds were harvested approximately four weeks following transformation. This protocol is based on the floral dip protocol of Clough and Bent (1998).

T_1 seeds were grown under greenhouse conditions in Metro Mix 360 soil (SunGro Horticulture Canada Ltd.) before treatment with gluphosinate ammonium (Finale, Farnam Co. Inc. Phoenix, Arizona) to screen for transformed plants. Seeds were collected from the resistant lines but no further work was done with the Arabidopsis plants expressing *TcMTP1* because of results from the overexpression

lines expressing *AtMTP1*. For the Arabidopsis plants expressing *AtMTP1*, the T_2 and T_3 generation seedlings were grown and tested via herbicide resistance for the presence of the transgene, followed by Northern analysis to confirm high levels of transgene expression (not shown). Once true breeding lines with high transgene expression were identified, they were grown hydroponically in modified Johnson's solution (1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 5 μM Fe(III)-EDDHA (N,N'-ethylenediamine-di-(O-hydroxyphenylacetic acid)), 1 μM $MnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM H_2MoO_4, 0.1 μM $NiSO_4$, and 1 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 5.5) in a range of Zn conditions (deficient: 0 μM $ZnSO_4$; sufficient: 1 μM $ZnSO_4$; and excess: 10 μM $ZnSO_4$) to look for phenotypic changes based on differences in the accumulation of Zn and other metals. Plants were grown in aerated nutrient solution for 28 days with solution changed every seven days. After 28 days of growth, roots were treated with a 10 minute, 5 mM $CaCl_2$ solution wash to remove root cell wall associated Zn, and then roots and shoots were harvested. Plant tissue was dried in 50ºC oven for at least one week. Dry weights were collected and samples were analyzed by ICP-AES for Zn content.

RESULTS

The degenerate primers designed from the gene sequence for *AtMTP1* and related members of this Arabidopsis gene family allowed us to successfully isolate a 730 bp fragment of a putative *T. caerulescens* homolog, designated *pTcS6*, which had 87% nucleotide homology to *AtMTP1* (Figure 2.1). This probe was then used to screen a *T. caerulescens* cDNA library which resulted in the isolation of a 1367 bp cDNA sequence (Figure 2.2). This sequence, designated *TcMTP1*, includes an 1191 base pair coding sequence. As depicted in Figures 2.2 and 2.3, the *TcMTP1* coding

Figure 2.1. Nucleotide sequence alignment by Clustal W of pTcS6 (probe of *TcMTP1*) and Arabidopsis *AtMTP1* sequence (At2g46800). Asterisks below indicate matching nucleotides.

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AtMTP1 ATGGAGTCTTCAAGTCCCCACCATAGTCACATTGTTGAGGTTAATGTTGGAAAATCTGAT 60
AtMTP1 GAAGAGAGAATAATTGTGGCGAGTAAAGTCTGTGGAGAAGCACCATGTGGGTTTTCAGAT 120
AtMTP1 TCTAAGAATGCTTCCGGGGATGCTCACGAACGCTCTGCTTCTATGCGGAAGCTTTGTATC 180
AtMTP1 GCCGTCGTGCTGTGTCTAGTGTTCATGAGTGTTGAAGTTGTTGGTGGGATTAAAGCCAAT 240

pTcS6                  CAGATGCAGCTCATTTGCTCTCTGACGTTGCTGCCTTTGCCATC 44
AtMTP1 AGTTTAGCTATATTAACCGATGCAGCTCATTTGCTCTCTGACGTTGCTGCCTTTGCTATC 300
                      * ************************************ ***

pTcS6  TCTCTCTTCGCCTTGTGGGCTGCTGGTTGGGAAGCGACGCCGAGGCAGACTTACGGGTTC 104
AtMTP1 TCCCTCTTCTCATTGTGGGCTGCTGGCTGGGAAGCGACTCCTAGGCAGACTTACGGGTTC 360
       ** ****** * ************** ************ ** ******************

pTcS6  TTCAGGATTGAGATCTTGGGAGCTCTTGTGTCTATCCAGCTCATTTGGTTGCTTACTGGG 164
AtMTP1 TTCAGGATTGAGATTTTGGGTGCTCTTGTATCTATCCAGCTCATTTGGTTGCTCACGGGT 420
       ************** ***** ******** ********************** ** **

pTcS6  ATCTTGGTTTATGAAGCCATATCAAGGCTTCTTACCGAGACCAGTGAGGTTAATGGATTC 224
AtMTP1 ATTCTGGTTTATGAAGCGATTATCAGAATTGTTACAGAGACCAGTGAGGTTAATGGATTC 480
       **  ************* **    **  ** **** ************************

pTcS6  CTTATGTTTGCTGTTGCTACGTTTGGTCTGCTGGTGAATATCATAATGGCTGTGATGCTT 284
AtMTP1 CTCATGTTTCTGGTTGCTGCCTTTGGTCTAGTGGTGAACATCATAATGGCTGTTCTGCTA 540
       ** ******   ****** * ********  ******* **************  ****

pTcS6  GGGCATGATCATGGTCATAGTCATGATCATGATCATG----------------------- 321
AtMTP1 GGGCATGATCATGGTCACAGTCATGGACATGGGCATGGCCACGGCCATGACCATCACAAT 600
       ***************** *******  ****  ****

pTcS6  ------------------------------------ATCACGATGATGGTCATGGTCAT 344
AtMTP1 CATAGCCATGGGGTGACTGTTACCACTCATCACCATCATCACGATCATGAACATGGCCAT 660
                                           ******** ***  ***** ***

pTcS6  AGTCATGGA---GAGGACAATCAGGATGAAGCTCATGGAGACGTTACTGAGCAGCTGTTG 401
AtMTP1 AGTCATGGTCATGGAGAGGACAAGCATCATGCTCATGGGGATGTTACTGAGCAATTGTTG 720
       ********    *  **  *  ** ** * ******** ** ***********  *****

pTcS6  GAGAAACCAAAG---CAGGA------GAAAGAGAAAAAGAAAAGGAACATCAATTTGCAA 452
AtMTP1 GACAAATCGAAGACTCAAGTCGCAGCAAAAGAGAAAAGAAAGAGAAACATCAATCTCCAA 780
       ** *** * ***   ** *        **********  ** ** ********* * ***

pTcS6  GGAGCTTATCTTCATGTTCTTGGTGATTCAATCCAGAGCGTTGGTGTTATGATTGGAGGA 512
AtMTP1 GGAGCTTATCTGCATGTCCTTGGGGATTCCATCCAGAGTGTTGGTGTTATGATTGGAGGA 840
       *********** ***** ***** ***** ******** ********************

pTcS6  GCTGCCATTTGGTACAACCCGAAATGGAAGATAATTGATCTGATCTGCACTCTTGCCTTT 572
AtMTP1 GCTATCATTTGGTACAATCCGGAATGGAAGATAGTGGATCTGATCTGCACACTTGCCTTT 900
       ***  ************ *** *********** * ************** *********

pTcS6  TCGGTTATCGTCTTGGGGACAACCATCAACATGATTCGAAACATTCTTGAAGTGTTGATG 632
AtMTP1 TCGGTTATTGTCCTAGGAACAACCATCAACATGATTCGCAACATTCTAGAAGTATTGATG 960
       ******** *** * ** ******************** ******** ***** ******

pTcS6  GAGAGTACGCCTAGAGAGATTGACGCTACAAAGCTCGAAAAGGGTTTGCTCGAAATGGAA 692
AtMTP1 GAGAGTACACCCAGAGAGATTGACGCCACAAAGCTCGAAAAGGGTTTGCTCGAAATGGAA 1020
       ******** ** ************** ********************************

pTcS6  GAAGTGGTGGCTGTTCATGAGCTTCACATATGGGCTAT                       730
AtMTP1 GAAGTGGTGGCTGTTCATGAGCTCCACATATGGGCTATCACAGTGGGAAAAGTGCTATTG 1080
       ********************** **************

AtMTP1 GCTTGCCATGTCAATATCAGACCAGAAGCAGATGCAGATATGGTGCTCAACAAGGTAATT 1140
AtMTP1 GATTACATCCGCAGGGAGTACAACATTAGTCATGTCACGATACAAATCGAGCGCTAA    1197
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cgactctctctcttctgATGGAGTCTTCAAGTCACATCATTGAGGTTAATGGAGGAA
GATCTGATGAAGAAAGAAGGGCTGTGGCAAGTAAAGTCTGTGGAGAGGCACCGTGTG
GGTTCTCAGATGCCAAGAATGTTTCAGGGGATACCAAAGAACGCAATGCTTCTATGC
GGAAGCTCTGTATCGCGGTGGTGTTATGTCTTGTGTTCATGAGCGTTGAAATCGTTG
GTGGAATCAAAGCCAATAGTTTGGCTATAATGACAGATGCAGCACATTTGCTCTCTG
ACGTTGCTGCCTTTGCCATCTCTCTCTTCGCCTTGTGGGCTGCTGGTTGGGAAGCGA
CGCCGAGGCAGACTTACGGGTTCTTCAGGATTGAGATCTTGGGAGCTCTTGTGTCTA
TCCAGCTCATTTGGTTGCTTACTGGGATCTTGGTTTATGAAGCCATATCAAGGCTTC
TTACCGAGACCAGTGAGGTTAATGGATTCCTTATGTTTGCTGTTGCTACGTTTGGTC
TGCTGGTGAATATCATAATGGCTGTGATGCTTGGGCATGATCATGGTCATAGTCATG
GTCATGGTCATGACCATGAAAACCATAGCCATGGGGTGACTGTTACCACTCATGATC
ATGATCCCACTCATGATCATGATCATGATCATGATCACGATGATGGTCATGGTCATA
GTCATGGAGAGGACAATCAGGATGAAGCTCATGGAGACGTTACTGAGCAGCTGTTGG
AGAAACCAAAGCAGGAGAAAGAGAAAAAGAAAAGGAACATCAATTTGCAAGGAGCTT
ATCTTCATGTTCTTGGTGATTCAATCCAGAGCGTTGGTGTTATGATTGGAGGAGCTG
CCATTTGGTACAACCCGAAATGGAAGATAATTGATCTGATCTGCACTCTTGCCTTTT
CGGTTATCGTCTTGGGGACAACCATCAACATGATTCGAAACATTCTTGAAGTGTTGA
TGGAGAGTACGCCTAGAGAGATTGACGCTACAAAGCTCGAAAAGGGTTTGCTCGAAA
TGGAAGAAGTGGTGGCTGTTCATGAGCTTCACATTTGGGCTATCACAGTGGGAAAGG
TTTTGCTTGCTTGCCATGTCAATGTTACACCACAAGCAGATGCAGATATGGTGCTCA
ACAAGGTGGTTGATTACATCCGCAGAGAGTACAATATCAGTCATGTAACCGTACAAA
TCGAGCGCTGAaagcaaagcaaagcaaaagctaagatcttgatgggtttttgtatc
agcattctcattatcaataatttttctacccttttgcactctatcattatcaata
aatttttctaccctactgcttaggtaaattggtataaaaaaaaaaaaaaaaaaag

Figure 2.2. *TcMTP1* cDNA sequence with coding sequence capitalized

sequence shares 83% nucleotide and 84% amino acid homology with *AtMTP1*. The *T. caerulescens* homolog also exhibits the features indicative of the CDF family of membrane transporters, including six membrane spanning domains, a CDF family specific N-terminal amino acid sequence, a C-terminal cation efflux domain, and a variable histidine-rich region between the fourth and fifth membrane spanning domains (Figure 2.3). During the course of this research, Assunção and colleagues (2001) published a characterization of *TcMTP1* isolated from the La Calamine ecotype of *T. caerulescens* which they named *ZAT1*. Comparison of these two sequences identified a single nucleotide difference in the coding sequence which leads to an aspartate to glycine amino acid substitution at residue 85. This amino acid

```
TcMTP1 MESSS----HIIEVNGGRSDEERRAVASKVCGEAPCGFSDAKNVSGDTKERNASMRKLCI 56
AtMTP1 MESSSPHHSHIVEVNVGKSDEERIIVASKVCGEAPCGFSDSKNASGDAHERSASMRKLCI 60
       *****    **:*** *:*****  ***************:**.***::**.********

TcMTP1 AVVLCLVFMSVEIVGGIKANSLAIMTDAAHLLSDVAAFAISLFALWAAGWEATPRQTYGF 116
AtMTP1 AVVLCLVFMSVEVVGGIKANSLAILTDAAHLLSDVAAFAISLFSLWAAGWEATPRQTYGF 120
       ************:***********:******************:****************

TcMTP1 FRIEILGALVSIQLIWLLTGILVYEAISRLLTETSEVNGFLMFAVATFGLLVNIIMAVML 176
AtMTP1 FRIEILGALVSIQLIWLLTGILVYEAIIRIVTETSEVNGFLMFLVAAFGLVVNIIMAVLL 180
       *************************** *::************ **:***:*******:*

TcMTP1 GHDHGHSHGHGH----DHENHSHGVTVTTHDHDPTHDHDHDHDHDDGHGHSHGEDNQDEA 232
AtMTP1 GHDHGHSHGHGHGHGHDHHNHSHGVTVTTHHHH--------HDHEHGHSHGHGED-KHHA 231
       ************    **.***********.*.        ***:.**.*.**** :..*

TcMTP1 HGDVTEQLLEKPK---QEKEKKKRNINLQGAYLHVLGDSIQSVGVMIGGAAIWYNPKWKI 289
AtMTP1 HGDVTEQLLDKSKTQVAAKEKRKRNINLQGAYLHVLGDSIQSVGVMIGGAIIWYNPEWKI 291
       *********:*.*     ***:************************** *****:***

TcMTP1 IDLICTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIW 349
AtMTP1 VDLICTLAFSVIVLGTTINMIRNILEVLMESTPREIDATKLEKGLLEMEEVVAVHELHIW 351
       :************************************************************

TcMTP1 AITVGKVLLACHVNVTPQADADMVLNKVVDYIRREYNISHVTVQIER 396
AtMTP1 AITVGKVLLACHVNIRPEADADMVLNKVIDYIRREYNISHVTIQIER 398
       **************: *:**********:*************:****
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Figure 2.3. Protein (amino acid) alignment between TcMTP1 and AtMTP1 (At2g46800). Features of these proteins include membrane spanning domains (shaded regions), the N-terminal CDF signature sequence (double underline), C-terminal cation efflux domain (single underline) and variable histidine rich region (bold text region). "*" below sequence alignment indicates fully conserved residues, ":" indicates strongly conserved residues and "." indicates weakly conserved residues.

substitution is also present in AtMTP1 and in alternate *T. caerulescens* accessions of this gene (NCBI nucleotide accessions: AY311490.1, AY483146.1) and is most likely a minor allelic difference.

When the *pTcS6* gene fragment was used in Northern analysis to examine *TcMTP1* expression in *T. caerulescens* and *T. arvense* plants grown at deficient, sufficient and excess Zn levels, a significantly higher expression was observed in both roots and shoots from *T. caerulescens* (Figure 2.4A). *T. caerulescens* plants grown on sufficient (1 μM) and high (500 μM) Zn concentrations show increasing gene

expression with exposure to increasing Zn levels and higher gene expression is seen in shoots relative to roots (Figure 2.4B). Comparison of the sequence for the *pTcS6* probe used to determine *TcMTP1* gene expression and the *T. arvense TaMTP1* sequence from the NCBI database (sequence AY483145) show a nucleotide similarity of 78% according to Clustal W alignment (MegAlign, DNASTAR Inc.). In comparison, the *pTcS6* probe shares 90% similarity to *TcMTP1* for the region specified.

When wild type yeast was transformed with the *TcMTP1* coding sequence in the pFL61 plasmid under the control of the constitutive phosphoglycerate kinase promoter and grown to mid-log phase in SD media, or SD media supplemented with 1 mM or 2.5 mM Zn, expression of *TcMTP1* conferred a significant increase in Zn accumulation (Figure 2.5A) and a significant decrease in growth (Figure 2.5B) in comparison with a control yeast line containing the empty pFL61 vector.

The *A. thaliana AtMTP1* overexpression lines, when grown on Zn deficient, Zn sufficient, and high Zn hydroponic media for 28 days, showed no consistent or significant difference between the control and the overexpression lines with regards to Zn content or plant growth expressed as root or shoot dry weight (Figures 2.6 and 2.7). While some plant lines showed an increase in root and shoot Zn content and shoot growth when grown under Zn deficient conditions, these differences were not consistent among the lines.

DISCUSSION

The *TcMTP1* gene, cloned via screening of a *T. caerulescens* cDNA library is nearly identical in DNA sequence to the other versions of the *TcMTP1* gene in the NCBI database (AY311490.1, AY483146.1, AF275750.1) with only minor allelic differences, indicating that the correct gene was cloned. To date, twelve CDF family

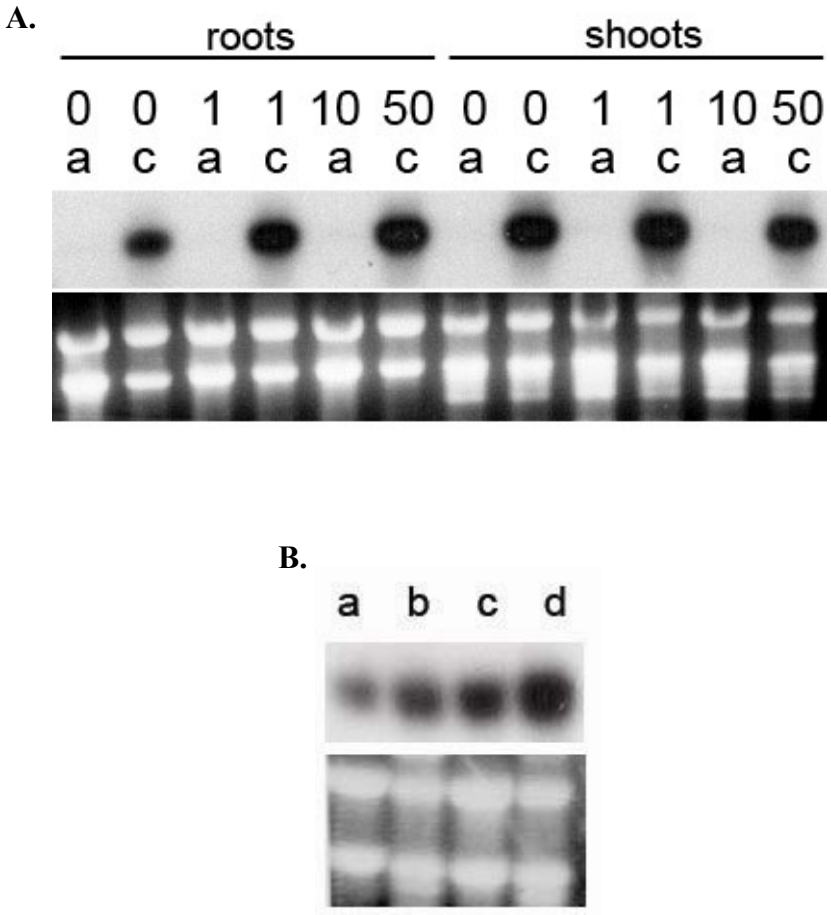

Figure 2.4. *TcMTP1 g*ene expression patterns in *Thlaspi*. A) *TcMTP1* expression in *T. caerulescens* (c) and *T. arvense* (a) roots and shoots for plants grown on deficient (0μM), sufficient (1 μM) and excess (10 μM for *T. arvense*; 50 μM for *T. caerulescens*) Zn levels. The number in the figure specifies the Zn concentration. B) *TcMTP1* expression in *T. caerulescens* roots (*a, b*) and shoots (*c, d*) grown on sufficient (1 μM; *a, c*) and excess (500μM; *b, d*) Zn levels.

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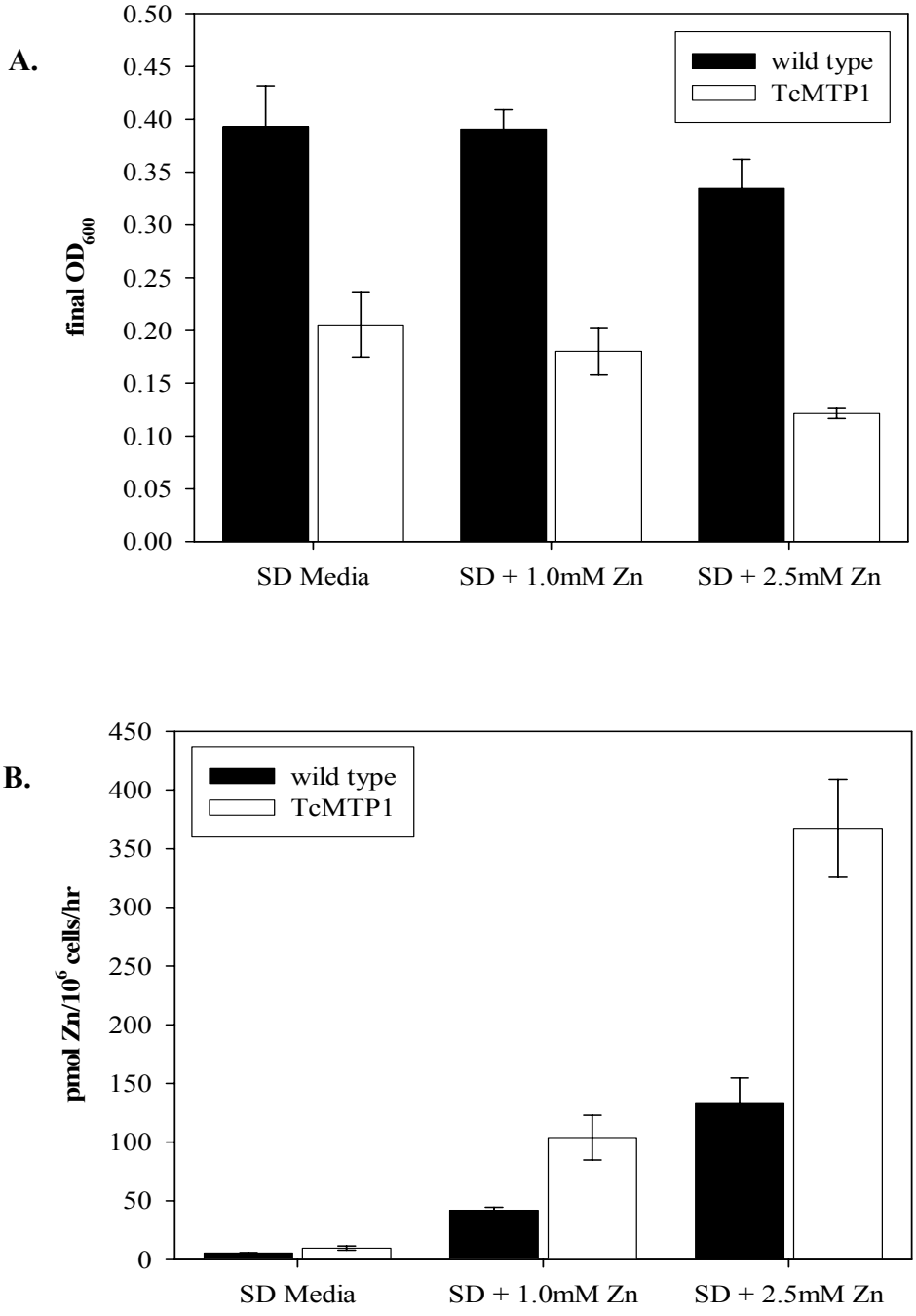

Figure 2.5. Zn accumulation (A) and growth rate (B) of *S. cerevisiae* containing the empty pFL61 vector (wild type) or *TcMTP1* in pFL61. Cells were grown for 16 h in SD media alone or SD media supplemented with 1 mM or 2.5 mM Zn.

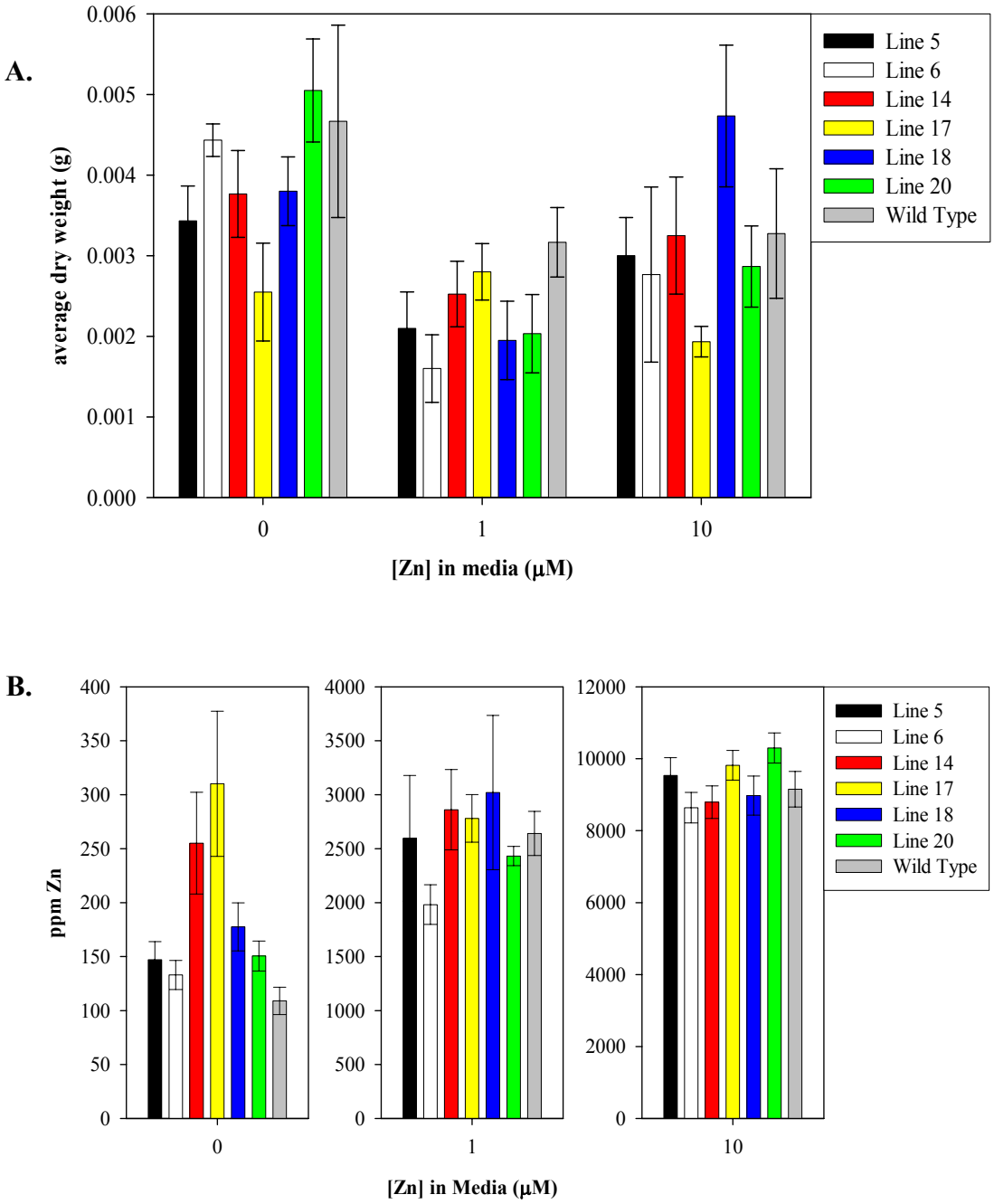

Figure 2.6. Effect of *AtMTP1* overexpression on root growth (A) and root Zn content (B) in 28 day old *A. thaliana* plants. Lines 5, 6, 14, 17, 18 and 20 are all independent transformations. Wild type is untransformed *A. thaliana* (Col).

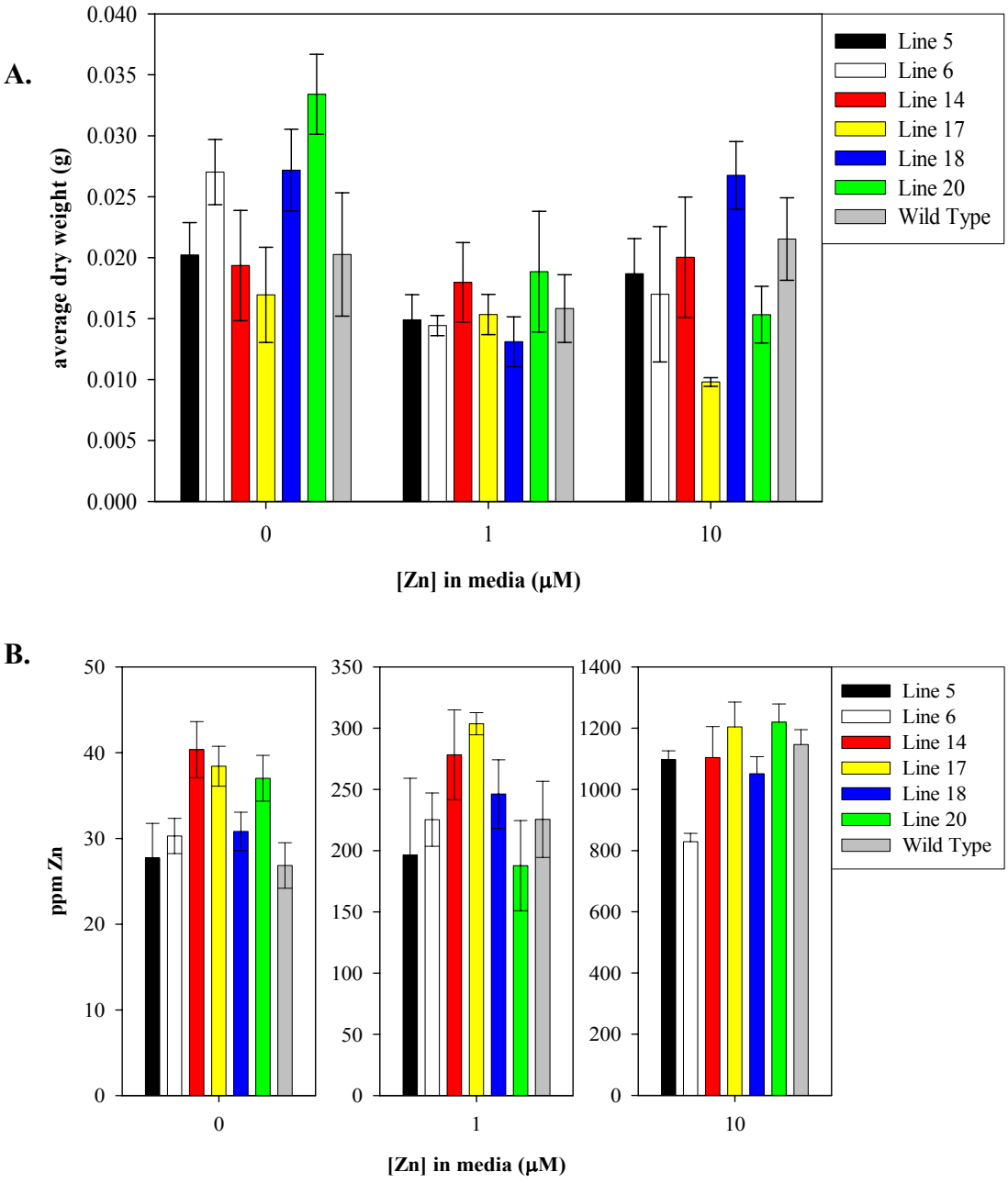

Figure 2.7. Effect of *AtMTP1* overexpression on shoot growth (A) and shoot Zn content (B) in 28 day old *A. thaliana* plants. Lines 5, 6, 14, 17, 18 and 20 are all independent transformations. Wild Type is untransformed *A. thaliana* (Col).

members have been identified in Arabidopsis (plants T database: http://plantst.genomics.purdue.edu/; Delhaize et al., 2003). As an additional confirmatory measure, a sequence alignment at the nucleotide level between *TcMTP1* and the twelve *AtMTP* genes present in the Arabidopsis genome was performed and *TcMTP1* most closely aligned with *AtMTP1* (alignment not shown).

The low level of *MTP1* gene expression seen in *T. arvense* relative to *T. caerulescens* is similar to results from other studies that looked at expression of this gene in *T. caerulescens, A. halleri* or *T. goesingense* relative to related non-hyperaccumulating plant species (Assunção et al., 2001; Becher et al., 2004; Dräger et al., 2004; Persans et al., 2001). A number of genes have been shown to be upregulated in metal hyperaccumulating plant species relative to related non-hyperaccumulators so it is not surprising that this pattern is also seen with another Zn transporter (Becher et al., 2004). All published studies of *MTP1* gene expression in non-hyperaccumulating plant species have found no differences in expression in response to changes in plant Zn status (Kobae et al., 2004; van der Zaal et al., 1999). Persans et al. (2001) found no difference in *TgMTP1* expression after treatment with a range of nickel concentrations but did not examine the effect of Zn. Assunção et al. (2001) saw higher levels of *TcMTP1* gene expression in *T. caerulescens* shoots relative to roots but observed a limited change in expression in response to growth under different Zn concentrations; however the highest Zn treatment examined was only 10 μM. Dräger et al. (2004) found an increase in *MTP1* expression in response to increasing Zn status in roots of *A. halleri* when plants were grown in 1, 100 and 300 μM Zn for four days, but did not see an increase in *MTP1* expression in shoots. However, this may have been due to the short (4 d) time period of exposure to these elevated Zn levels. In contrast, this study shows higher levels of *TcMTP1* gene expression in both the roots

and shoots of plants grown on 500 μM Zn for two weeks, compared with plants grown on lower Zn levels.

Yeast Characterization. Most of the functional analyses of plant *MTP*s to date have used metal sensitive mutant *S. cerevisiae* lines lacking the vacuolar Zn transporters COT1 and/or ZRC1, to show that the introduction of *MTP* genes from various plant species can increase yeast metal tolerance (Becher et al., 2004; Blaudez et al., 2003; Dräger et al., 2004; Kim et al., 2004; Persans et al., 2001). On the other hand, Bloss et al. (2002) was unable to show increased Zn tolerance in *S. cerevisiae* mutant strains but did demonstrate increased metal tolerance in a *S. pombe* strain lacking functional ZRC1 when *AtMTP1* was expressed. In our work, wild type yeast was used to see if the expression of *TcMTP1* resulted in an increased uptake and tolerance of Zn when the transgenic yeast were grown in liquid culture. As seen in Figure 2.5, there was a substantial increase yeast Zn uptake by yeast lines expressing *TcMTP1*. However, this increase is matched by a decrease in yeast growth, suggesting that the increased yeast Zn uptake is associated with decreased Zn tolerance. The simplest explanation for this finding is that in yeast, TcMTP1 was expressed in the plasma membrane and mediated increased Zn uptake that resulted in elevated Zn toxicity.

Future work with this gene in yeast should focus on a more detailed characterization of the transporter. Kim et al. (2004) suggested that *TgMTP1* mediates Zn efflux from the cytoplasm across the plasma membrane, but the results presented here, at least for yeast, suggests that the transporter mediates Zn influx and not efflux. Another study by Dräger and colleagues found that the *A. halleri* homolog of MTP1 localized to the vacuolar membrane (2004). More detailed influx and efflux studies with a variety of metals would provide a better understanding of the role for the *TcMTP1* transporter.

Characterization of AtMTP1 and TcMTP1 overexpression lines in A. thaliana. As we are interested in the mechanisms underlying Zn transport and accumulation and the role of the MTP transporters in these processes, the effect of overexpressing *AtMTP1* in transgenic *A. thaliana* lines in response to growth in a range of Zn concentrations over a long term period was examined. No significant effect of the transgene was seen over the long term as shown in Figures 2.6 and 2.7.

There is still a great deal of information that is not known about this gene in hyperaccumulator plant species. *MTP* expression patterns suggest some degree of responsiveness to changes in plant Zn status (mostly at the high Zn range) but no tissue specific localization information currently exists for *MTP* expression. This project was hampered by the lack of a reliable transformation system in *T. caerulescens*. However, if this technical issue is overcome, expression of *TcMTP1* promoter/GFP fusions in *T. caerulescens* plants would be very informative.

One of the practical goals of studying Zn hyperaccumulation with regard to phytoremediation is to create large biomass plants that accumulate heavy metals to the same extent as *T. caerulescens*. Research with plant *MTP* genes has shown that modifying expression of *MTP* in Arabidopsis (both overexpression and reduced expression) appears to have a direct effect on Zn homeostasis (Kobae et al., 2004; van der Zaal et al., 2001). This effect on plant Zn status following altered gene expression in transgenic plants has not been seen for other micronutrient/heavy metal transporters. For example, *IRT1* in the ZIP family of micronutrient transporters is regulated at the transcriptional, translational and post-translational levels (Connolly et al., 2002). Thus, even when high levels of *IRT1* transcripts were present, low levels of IRT1 protein were observed, presumably due to metal sensing mechanisms regulating IRT1 protein abundance. These additional regulation mechanisms mean that if a whole plant level effect overexpression of the *IRT1* gene has to include modifications

to the protein itself before an effect is seen at the whole plant level. This complication does not appear to be present with the MTP family since modification of *MTP* transcript abundance led to changes in Zn status (Kobae et al., 2004; van der Zaal et al., 2001).

In conclusion, the study described here focused on the isolation and characterization of the *MTP1* transporter in both yeast and plants. There was increased Zn accumulation when *TcMTP1* is constitutively expressed in yeast, but no effect on Zn accumulation was seen in transgenic plants overexpressing *AtMTP1* following growth in hydroponic media containing Zn levels between 0 and 10 μM Zn. Future work should focus on the growth of overexpression lines on higher Zn concentrations and perhaps decreasing exposure time to see if overexpression of the *TcMTP1* gene in Arabidopsis can result in changes in Zn homeostasis and physiology. The determination of tissue and cell-specific localization of *MTP1* gene and protein expression in both hyperaccumulator and non-hyperaccumulator plant species would also be of interest in further dissecting the role of this gene in metal accumulation.

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CHAPTER III

Functional Analysis in Yeast of Heavy Metal Tolerance Genes from *Thlaspi caerulescens*

INTRODUCTION

The zinc (Zn) and cadmium (Cd) hyperaccumulation phenotype seen in *Thlaspi caerulescens* is due to a number of physiological differences related to the accumulation and tolerance of these heavy metals. Previous studies have focused on understanding differences between *T. caerulescens* and the related non-accumulating species, *T. arvense*, at the organ and whole plant levels. In these studies, radiotracer flux approaches using $^{65}Zn^{2+}$ compared Zn transport in *T. caerulescens* and *T. arvense*, and indicated there are increased rates of Zn transport from the external solution into roots of *T. caerulescens*, a greater rate of Zn loading into the xylem and subsequent transport from the roots to the leaves, and greater Zn transport into leaf cells (Lasat et al., 1996, 1998). Based on these physiological studies, it was speculated that the difference in Zn uptake in *T. caerulescens* roots can be attributed to higher expression of plasma membrane Zn transporters (Lasat et al., 1996). Molecular studies have confirmed these results, showing higher gene expression of the Zn/Cd transporter *ZNT1* in both roots and shoots of *T. caerulescens* (Pence et al., 2000). In Chapter II, *TcMTP1*, a vacuolar Zn transporter, was also shown to be more highly expressed in *T. caerulescens* relative to *T. arvense*. Finally, high levels of Zn accumulation in *T. caerulescens* leaf cells have been localized to non-photosynthetic, non-stomatal epidermal cells (Frey et al., 2000; Kupper et al., 1999). These studies demonstrate the range of adaptations present in hyperaccumlators that appear to contribute to accumulation of high levels of Zn and Cd, while preventing cytoplasmic toxicity.

From the physiological and molecular data previously presented in the literature for metal hyperaccumulation in *T. caerulescens*, it appears that the hyperaccumulation phenotype is dependent on two different mechanisms to deal with high levels of heavy metals. A version of metal avoidance was seen in the root and stem tissue, as Zn and Cd are rapidly moved radially through the root and up the stem in the xylem, thus Zn appears to be primarily moving in the apoplasm in these tissues. Therefore, even when *T. caerulescens* plants are grown at high Zn concentrations, the amount of Zn accumulating in most cells of the root and stem remains relatively low. This transported Zn ends up accumulating in leaf epidermal and, to a lesser extent, mesophyll cells (Frey et al., 2000; Küpper et al., 1999; Ma et al, 2005). The molecular basis for this behavior is unknown. Although a number of genetic and genomic-based studies have been undertaken to better understand metal hyperaccumulation in *Thlaspi*, including the construction of EST libraries, QTL analysis and transcript profiling using microarrays (Assunção et al., 2006; Hammond et al., 2006; Plessl et al., 2005; Rigola et al., 2006), no candidate genes of interest identified in these screens have been characterized. In order to identify potential *T. caerulescens* genes involved in Zn tolerance, a *T. caerulescens* cDNA library was generated in a yeast expression vector and screened in the yeast, *Saccharomyces cerevisiae*, by growing the transformed yeast on levels of Zn that are normally toxic to wild type yeast. Through this screen, a number of genes that confer Zn tolerance to yeast were identified. The Zn tolerance genes identified, based on their sequence annotation, represent a range of different protein families, and may be implicated in Zn tolerance mechanisms involving both avoidance and sequestration. Furthermore, characterization of these genes in both yeast and transgenic Arabidopsis plants is also presented, in order to begin to better understand how some of these genes might be involved in the extreme metal tolerance exhibited by *T. caerulescens*.

METHODS AND MATERIALS

Yeast Growth Conditions. For the yeast tolerance screen, the wild type yeast strain DY1457 (MATα *ade6 can1 his3 trp1 ura3*) was transformed with either the empty yeast expression vector pFL61 (Minet et al., 1992) or a *T. caerulescens* cDNA library in the pFL61 vector. The *T. caerulescens* cDNA library was constructed as described by Pence (2002), and was made from poly A^+ RNA from root and shoot tissue from *T. caerulescens* seedlings grown under both Zn deficient and Zn replete conditions. Yeast were grown on a synthetic dextrose minimal media supplemented with 0.1% casamino acids, adenine (20 mg/L), histidine (20 mg/L), tryptophan (20 mg/L), leucine (30 mg/L) and this media will subsequently be referred to as SD media. SD media plates also contained 2% w/v agar (Difco Laboratories, Sparks, MD).

Library Screen and Identification of Putative Zn Tolerance Genes. Since an initial screen of *S. cerevisiae* strain DY1457 containing the empty pFL61 vector determined that yeast growth was severely limited when grown on SD plates supplemented with 8.0 mM $ZnSO_4$, this concentration was chosen to screen the *T. caerulescens* cDNA library for increased Zn tolerance. Following initial identification of Zn tolerant lines, yeast strains were re-streaked on fresh high Zn SD plates to confirm tolerance. The pFL61 vectors containing the *T. caerulescens* genes of interest were isolated and a multiple restriction enzyme digest was run to identify unique genes. Those cDNAs considered unique were then sequenced to determine their nucleotide sequence, which was then compared to the *A. thaliana* sequence data base at NCBI.

Yeast Characterization. To characterize the role of the putative tolerance genes, growth rates and Zn accumulation in the yeast lines expressing specific *T. caerulescens* genes were determined by growth in a liquid culture made up of either SD media alone or SD media supplemented with 2.5 mM $ZnSO_4$. This lower Zn

concentration was chosen because in liquid media, the 8 mM Zn concentration used on agar plates completely abolished yeast growth. All yeast lines were grown overnight at 30ºC in SD media and then sub-cultured into 10 mL of SD media or SD media containing 2.5 mM $ZnSO_4$ at an initial yeast density of $OD_{600} = 0.01$. Subsequently, the yeast were grown on a rotary shaker at 120 rpm at 30ºC for 16 hours. At the end of 16 hours, the yeast density was determined, the yeast cultured centrifuged at 2500 x g for 5 min, washed twice with 5 mL of 18 mΩ dH_2O, and then the cells were transferred to quartz tubes for elemental analysis. The samples were digested with concentrated HNO_3 at 100ºC, and then further digested in nitric and hydrochloric acid at 200ºC until dry. Samples were resuspended in 5% HNO_3 and analyzed with an inductively-coupled plasma trace analyzer emission spectrometer (ICP-AES; model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) for Zn content.

Thlaspi Seedling Growth Conditions. *T. caerulescens* and *T. arvense* plants were grown as previously described by Pence (2002). Briefly, *T. caerulescens* (ecotype Prayon) and *T. arvense* seeds were germinated and seedlings grown in modified Johnson's nutrient solution containing: 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 5 μM Fe(III)-EDDHA (N,N'-ethylenediamine-di-(O-hydroxyphenylacetic acid)), 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM H_2MoO_4, 0.1 μM $NiSO_4$, and 1 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 5.5. The plants were grown in a greenhouse without artificial light. The nutrient solution was constantly aerated and replaced weekly. Due to the greater growth rate of *T. arvense* relative to *T. caerulescens*, *T. caerulescens* plants were grown hydroponically in modified Johnson's solution for 50 days, while *T. arvense* plants were grown for 40 days, before the start of the Zn treatments. Zn treatments included standard Johnson's solution with 0 μM $ZnSO_4$ (Zn deficient), 1 μM $ZnSO_4$ (Zn-sufficient), or 10 μM $ZnSO_4$ for *T.*

arvense and 50 μM $ZnSO_4$ for *T. caerulescens* (high Zn) treatments. After 14 days of growth at these Zn concentrations, plants were harvested, separated into root and shoot tissue and flash frozen in liquid N_2.

Northern Analysis. Transcript abundance for candidate Zn tolerance genes was examined in *T. caerulescens* and *T. arvense* root and shoot tissue from plants exposed to the range of Zn concentrations described above. Total RNA was isolated following the Trizol protocol (GibcoBRL/Invitrogen). 20 μg of total RNA was denatured, separated by glyoxal-agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham). Equal loading was confirmed both by spectrophotometric measurement of RNA concentrations and visually through ethidium bromide staining of ribosomal subunits. Membranes were UV crosslinked before hybridization. To examine gene expression, the original gene fragments identified in the tolerance screen were labeled with ^{32}P-dCTP with random hexamer primers and hybridized at 65ºC overnight in PerfectHyb Solution (Sigma Aldrich) with 10μM salmon testes DNA blocking agent. Following hybridization, membranes were washed 2 times for 30 minutes at 65ºC in a low stringency wash solution (2 x SSC, 0.1% SDS). After exposure to autoradiography film (Kodak), the membrane was stripped with boiling 0.5% SDS.

Transgenic Arabidopsis Plants with Modified Expression of Candidate Zn Tolerance Genes. Arabidopsis knockout lines containing T-DNA inserts from *Agrobacterium tumefaciens* in or near the genes of interest were identified from the SIGnAL T-DNA Express database (http://signal.salk.edu/cgi-bin/tdnaexpress) and ordered through the Arabidopsis Biological Resource Center (ABRC). The lines obtained were grown on soil under general greenhouse conditions and leaves were collected to identify homozygous lines by PCR-based insertional analysis. Homozygous lines containing the T-DNA insert were identified through a PCR based

screening method, and grown to test for potential differences in Zn accumulation in the root and shoot. Plants were grown hydroponically in modified Johnson's solution with 1 μM $ZnSO_4$ for three weeks with weekly solution changes and constant aeration. At the beginning of week four, the Zn concentration in the modified Johnson's solution was changed to 0, 1 or 10 μM $ZnSO_4$. At the end of one week, roots were treated with a 10 minute, 5 mM $CaCl_2$ solution wash to remove root cell wall associated Zn, and then roots and shoots were harvested and analyzed for metal content via ICP-AES as described above.

RESULTS

A yeast tolerance screen was conducted to look for *T. caerulescens* genes that conferred Zn tolerance to yeast when grown under high Zn conditions. Initial experiments determined that the growth of wild type *S. cerevisiae* strain DY1457 containing the empty expression vector pFL61, was severely limited when grown on SD media containing 8mM $ZnSO_4$. Yeast were then transformed with a cDNA library from *T. caerulescens* and the transformed yeast cells were grown on the high Zn plates. Through this screening effort, thirty-five independent lines were initially identified with the ability to sustain growth superior to the wild type yeast on the high Zn SD plates. These yeast strains were re-streaked onto fresh plates to confirm growth on high Zn and then the vectors containing the *T. caerulescens* genes were isolated. A restriction enzyme digest was run to identify duplicate clones and the unique *T. caerulescens* cDNAs were sequenced. Sequenced genes from eighteen unique constructs were compared to the *Arabidopsis thaliana* genome in the NCBI database. Eleven sequences that could not be linked through any known plant or yeast pathways to a heavy metal tolerance or accumulation function were not considered further but their closest Arabidopsis homologs are listed in Table 3.1. The remaining seven

sequences that were studied further are listed in Table 3.2 with their closest Arabidopsis homolog and the percent identity at the nucleotide and protein level.

A series of yeast and plant-based experiments were performed to further examine the role of these seven putative Zn tolerance genes. Of these genes, yeast lines overexpressing *PKS4* exhibited similar Zn tolerance (Figure 3.1A) but accumulated significantly higher concentrations of Zn relative to control yeast cells (containing the empty vector; Figure 3.1B). Consequently, this gene was the focus of more detailed research efforts and will be discussed at length in Chapter IV. The remaining genes are discussed below.

To look at the function of a specific candidate gene, overexpression lines in yeast and Arabidopsis T-DNA knockout lines were studied and results will be described below on a per gene basis. The first study of these tolerance genes was a preliminary effort that examined Zn accumulation and yeast growth rates in liquid culture relative to yeast expressing the empty vector. Growth rates were determined after sixteen hours of growth in SD media containing 2.5 mM $ZnSO_4$. Based on these results and their gene expression patterns in *T. caerulescens* and the related non-hyperaccumulator, *T. arvense*, a second yeast experiment was run for a subset of these genes, this time with replicates and in both standard SD media and standard SD media with 2.5 mM $ZnSO_4$.

Plant-based examination of these tolerance genes focused on analysis of their gene expression in wild type plants as well as the Zn sensitivity in transgenic T-DNA knockout lines for the genes of interest. Patterns of gene expression were examined by Northern analysis in roots and shoots of *T. caerulescens* and *T. arvense*, after growth in a range of Zn concentrations (Zn deficient, Zn sufficient and high Zn). Finally, homozygous T-DNA knockouts for the specific candidate genes in *Arabidopsis* plants were grown in hydroponic media with the same three Zn regimes

Table 3.1. *T. caerulescens* genes that confer Zn tolerance in yeast but were not further studied due to a lack of obvious connection to Zn stress**.**

Clone	Description of closest *Arabidopsis thaliana* match	Gene
2-1	TYKY for NADH:ubiquinone oxidoreductase	At1g79010
3-2	Unknown Protein	At5g62750
4-2	poly adenine tail only	n/a
5-1	putative aspartate tRNA ligase	At4g26870
8-1	5'utr of At1g73820	At1g73820
11-1	60S ribosomal protein L31	At5g56710
11-5	Oxygen-evolving enhancer protein 3 precursor-like protein	At4g21280
15-1	ubiquinol--cytochrome-c reductase-like protein	At5g13430
19-1	photosystem II 10 kD polypeptide precursor (psbH)	At1g79040
20-6	ATPase beta and epsilon subunit fusion	AtCg00480
17-1	extensin like protein	At1g12090

Table 3.2. *T. caerulescens* genes that confer Zn tolerance in yeast and were further studied.

Clone #	Length (nt)	Full Length	Closest At match	Gene #	Protein Acc#	% NT Identity	% Protein Identity
18-1	1241	82%	putative protein kinase (*PKS4*)	At4g30960	NP_194825	87	94
1-1	716	45%	glutamate ammonium ligase (glutamine synthetase- GS1)	At3g17820	NP_188409	89	95
6-1	699	yes	synbindin homolog (*SYL*)	At5g02280	NP_195848	95	100
10-1	957	yes	putative ubiqutin conjugating enzyme E2 (*UBC6*)	At2g46030	AAY44847	91	94
24-7	474	yes	putative ubiquitin extension protein (*UBQ5*)	At2g47110	NP_566095	91	99
4-3	1149	yes	14-3-3-like protein GRF14 phi (general regulatory factor 4)	At1g35160	NP_564453	89	97
3-1	1277	yes	*SRO4* (similar to RCD One 4)	At3g47720	NP_190356	80	68

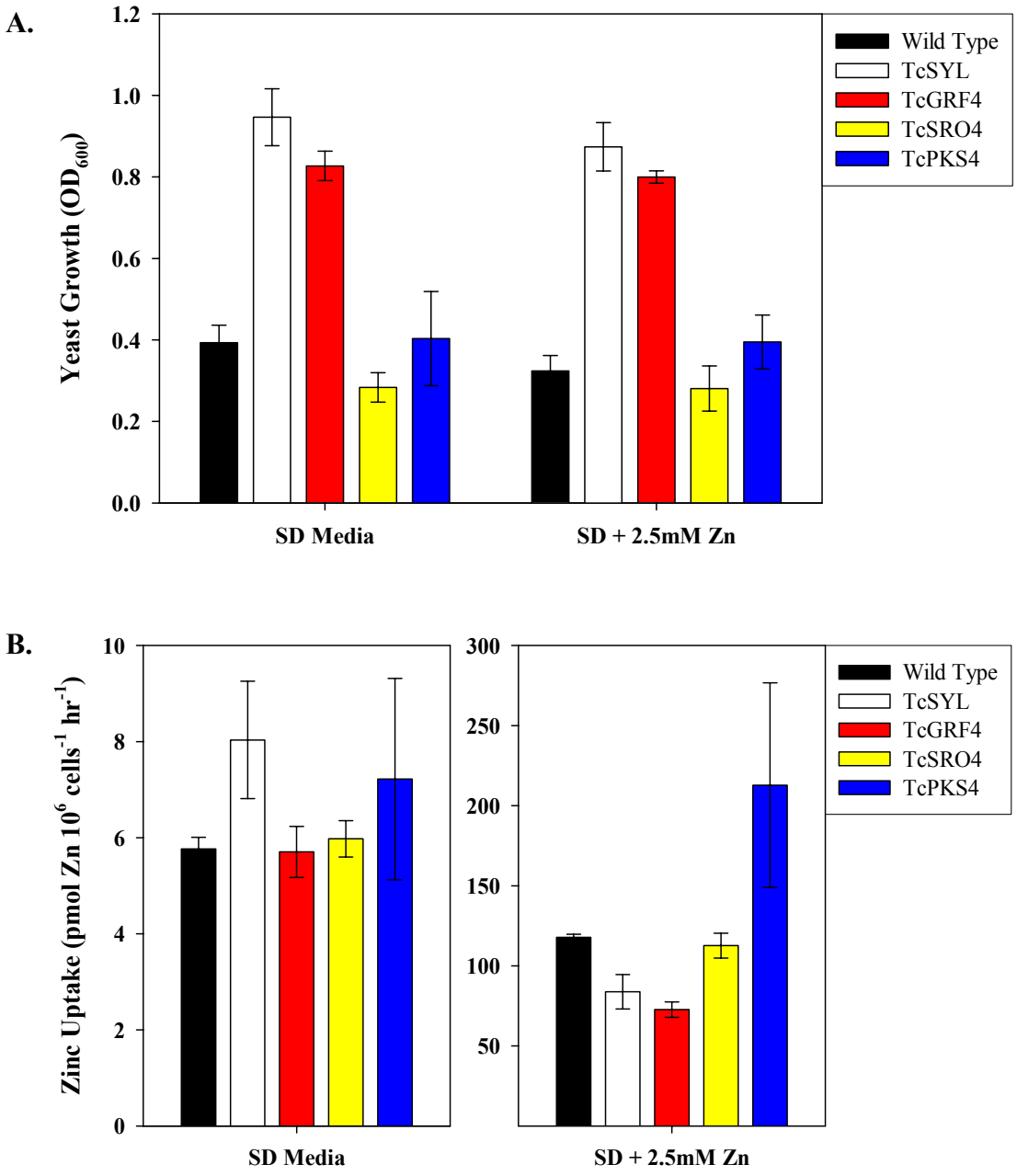

Figure 3.1. Yeast growth (A) and Zn uptake (B) for *S. cerevisiae* lines expressing the specified candidate Zn tolerance genes. Yeast growth was determined by measuring the O.D. at 600 nm after 16 h of growth on standard and high Zn (2.5 mM) liquid media. Zn uptake for *S. cerevisiae* lines was determined by ICP-AES analysis as described in the Materials and Methods. Lines expressing the candidate *T. caerulescens* Zn tolerance genes include: Similar to RCD One 4 (SRO4), General Regulatory Factor 4 (GRF4), Synbindin-Like Protein (SYL), and Protein Kinase 4 (PKS4) compared with yeast expressing the empty pFL61 vector (wild type).

described above to examine the effect of the specific gene knockout on Zn tolerance and accumulation.

Glutamine Synthetase

Of the thirty-five genes originally identified in the screen, this gene was independently isolated as conferring yeast Zn tolerance twelve times. The version expressed in yeast was not full length, and the 160 amino acid open reading frame matched the C-terminal of the full length *Arabidopsis* glutamine synthetase with five amino acid substitutions. The effect of high Zn concentrations on growth and Zn accumulation in yeast expressing this cDNA were examined and shown in Figures 3.2A and 3.2B. As seen in these figures, the yeast exhibited significantly greater growth and accumulated much less Zn relative to yeast containing the empty vector suggesting that this gene promotes Zn tolerance through a Zn exclusion mechanism. The expression of this gene in *T. caerulescens* and *T. arvense* showed higher transcript abundance in roots relative to shoots for both species but did not show a response to changing plant Zn status (Figure 3.3). Glutamine is an amino acid with the capacity to chelate Zn; however, a mechanism by which enhanced glutamine synthesis would confer Zn tolerance via Zn exclusion is not obvious. Possibly the yeast cells expelled glutamine, which then chelated extracellular Zn and prevented its uptake. Because it was difficult to relate glutamine synthesis to an efficient Zn tolerance mechanism in plants, further investigation was not conducted on this gene.

Ubiquitin Conjugating Enzyme (UBC6)

Ubiquitin conjugating enzymes (E2) mediate the transfer of a thioester-linked ubiquitin from a ubiquitin-activating enzyme (E1) to either a ubiquitin ligase (E3) or

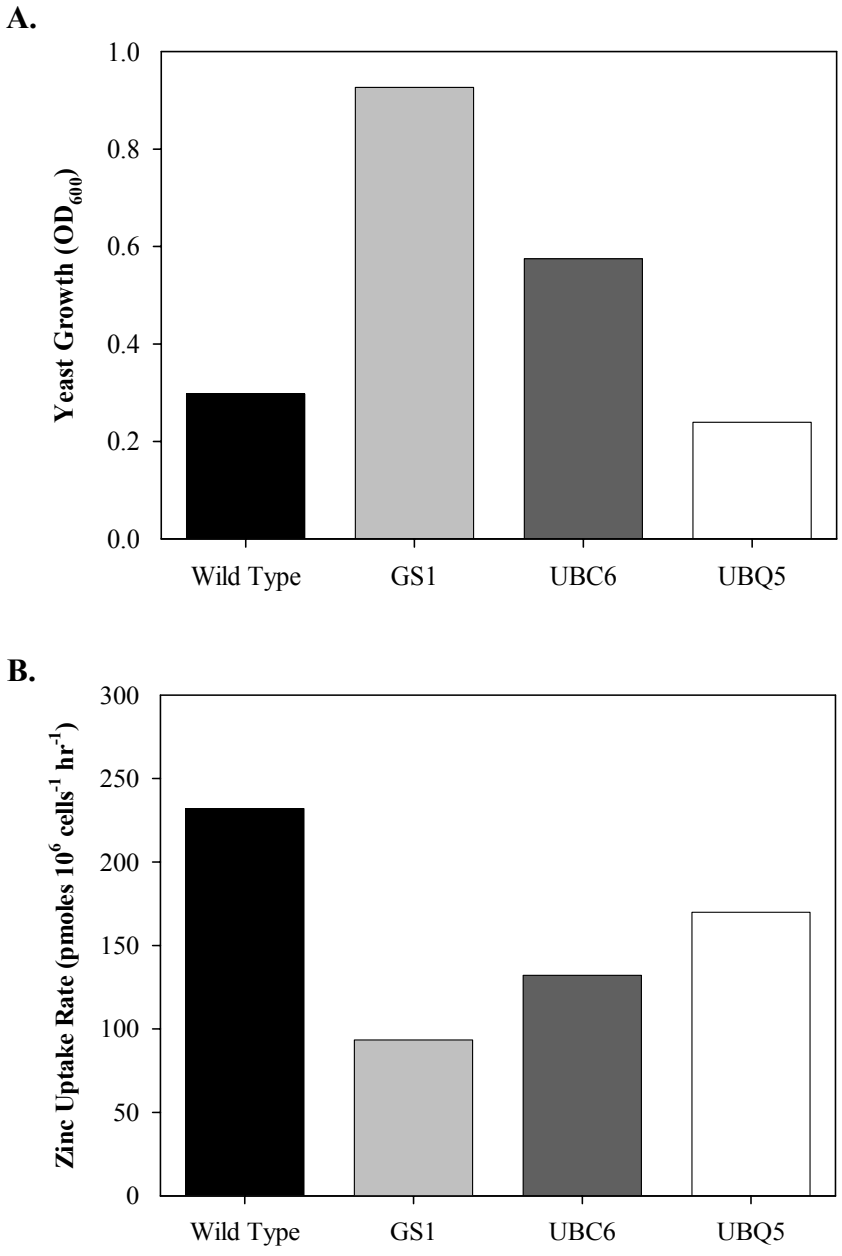

Figure 3.2. Yeast growth (A) and Zn uptake (B) for *S. cerevisiae* lines expressing the specified candidate Zn tolerance genes. Yeast growth was determined by measuring O.D. at 600 nm after 16 h of growth on high Zn (2.5 mM) liquid media for *S. cerevisiae*. Zn uptake for *S. cerevisiae* lines were determined by ICP-AES analysis as described in Materials and Methods. Yeast lines expressed either the empty pFL61 vector (wild type), or the candidate *T. caerulescens* Zn tolerance genes glutamine synthetase (GS1), ubiquitin conjugating enzyme E2 (UBC6), and ubiquitin extension protein (UBQ5).

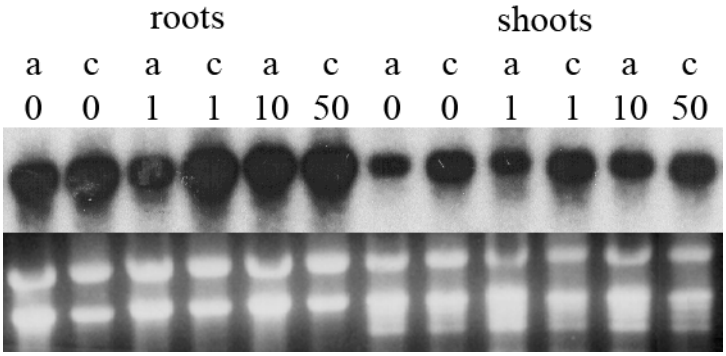

Figure 3.3. Northern analysis for the *T. caerulescens* glutamine synthetase gene in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Plants were grown on hydroponic media containing 0, 1, and 10 or 50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

directly to a substrate protein marked for modification with E3 (for a review, see Hellmann and Estelle, 2002). The *T. caerulescens* homolog of the Arabidopsis E2 conjugating enzyme UBC6 was identified in our tolerance screen. This gene shares 91% identity with the Arabidopsis ortholog both at the nucleotide and protein levels. In Arabidopsis, UBC6 has been identified as an E2 enzyme involved in senescence and floral expression (Watts et al., 1994). In yeast, the cells expressing this *Thlaspi* E2 enzyme exhibited greater Zn tolerance and lower Zn accumulation relative to control yeast expressing the empty vector (Figure 3.2B). The expression patterns for this gene in *T. caerulescens* and *T. arvense* showed high and constitutive expression in the roots of both species (Figure 3.4). It is interesting that in roots and shoots, this gene is more highly expressed in *T. caerulescens* compared with *T. arvense*. This may be significant as it has previously been found that a number of metal transporters that are possibly involved in hyperaccumulation are more highly expressed in *T. caerulescens* compared with related non-accumulators (Assunção et al., 2001; Pence et al., 2000).

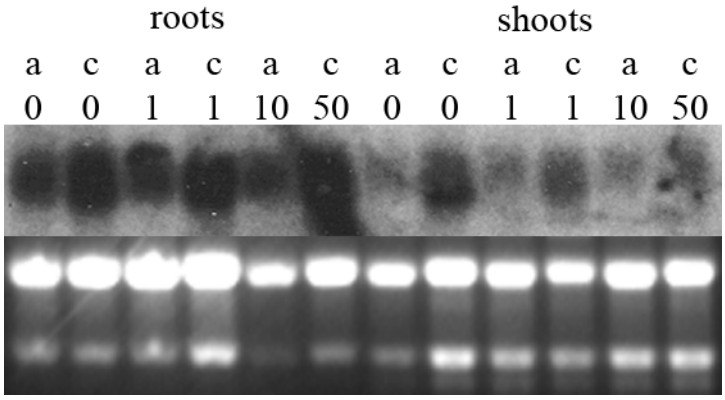

Figure 3.4. Northern analysis of the *T. caerulescens* gene encoding ubiquitin conjugating enzyme E2 (*UBC6*) in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, and 10 or 50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

Ubiquitin Extension Protein (*UBQ5*)

The gene encoding an ubiquitin extension protein identified in this tolerance screen is most similar to *UBQ5* in Arabidopsis (99% identity between *Thlaspi* and Arabidopsis proteins). In Arabidopsis, *UBQ5* is a chimeric gene that encodes a monomeric ubiquitin-40S ribosomal subunit fusion protein. In yeast, this gene most closely matches a highly conserved ubiquitin-ribosome fusion protein that acts as a chaperone, where the presence of the ubiquitin is necessary for proper ribosomal biogenesis (Finley et al., 1989). In previously published work in Arabidopsis, higher levels of expression are seen in growing tissues and meristems (Callis et al., 1990). Examination of gene expression in the *Thlaspi* species showed higher levels of expression in *T. arvense* shoots relative to *T. caerulescens*, especially for plants grown on 1 and 10 μM Zn (Figure 3.5). In roots, transcript levels for both species showed a moderate decrease in expression with increasing Zn concentrations, and higher expression levels were seen in *T. caerulescens*. Two bands were present in the

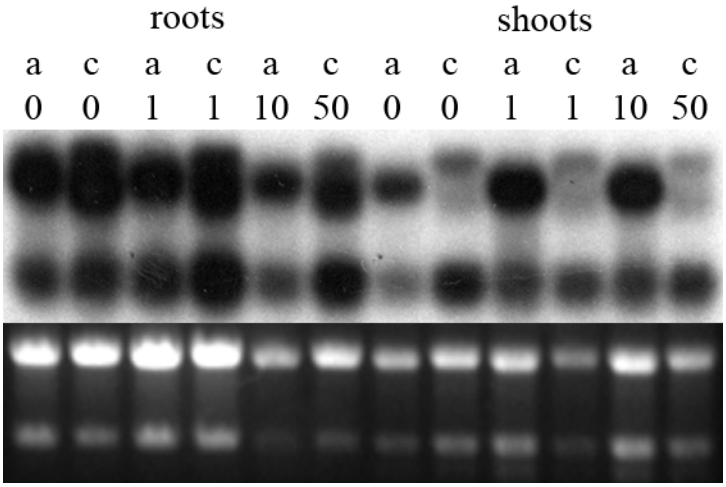

Figure 3.5. Northern analysis for the *T. caerulescens* gene encoding ubiquitin extension protein (UBQ5) in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, and 10 or 50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls. The second band visible is unbound ubiquitin.

Northern blots in Figure 3.5 and the lower band is thought to be a transcript for ubiquitin without the ribosomal protein. In the yeast tolerance screen, expression of this protein in yeast resulted in a small decrease in Zn tolerance and lower levels of Zn accumulation (Figures 3.2A, B). Based on these results, it would appear that this gene does not actually confer an increase in yeast Zn tolerance and therefore, no further work was conducted on this gene.

Synbindin-like Protein

The next *T. caerulescens* tolerance gene to be examined shares 100% identity at the protein level with an Arabidopsis gene of unknown function, At5g02280. This gene shows some similarity to synbindin, a protein from rat brains that coimmunoprecipitates with syndecan-2, a heparin sulfate proteoglycan that may play a

role in the organization of cell-surface structures and signaling (Ethell et al., 2000). Based on this gene's similarity to synbindin, it was named *TcSYL* for Synbindin-Like protein in *Thlaspi caerulescens*. When the action of this gene was examined in yeast under high Zn conditions, the yeast grew better and accumulated less Zn relative to the control yeast strain (Figure 3.1A, B). This gene was also expressed to a much higher level in *T. caerulescens* shots and roots relative to *T. arvense*, and the gene was expressed more strongly in roots (Figure 3.6). It does not appear that the expression of this gene changes in response to changing plant Zn status.

GRF4 – A 14-3-3 like protein

The 14-3-3 proteins are so named because they were originally identified and numerically labeled in a protein classification project based on the electrophoretic mobility and column fractionation of bovine brain proteins (Moore and Perez, 1967). Subsequent work on this protein family has found family members in all eukaryotes (Robinson et al., 1994). Within Arabidopsis, the 14-3-3 protein family contains fifteen family members, thirteen of which are known to be expressed (Rosenquist et al., 2001; Wu et al., 1997). While 14-3-3 proteins share a common function that involves binding to a phosphorylated protein and regulating activity of that protein through subsequent changes in conformation, the processes and enzymes affected by 14-3-3 proteins in plants vary widely, and include nitrate reductase (Roberts 2000; Weiner and Kaiser, 1999), glutamine synthase (Moorhead et al., 1999), and TATA-box binding proteins (Pan et al., 1999; Roberts, 2000). The specific isoform identified from *T. caerulescens* shares 97% protein identity with the Arabidopsis GRF4 14-3-3 protein, also known as the phi (φ) isoform. This protein has been shown to promote

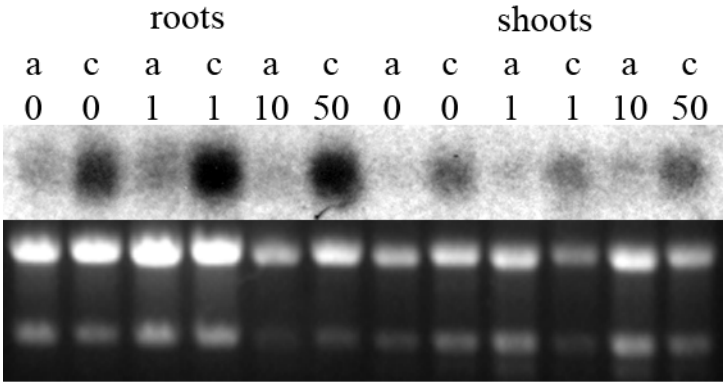

Figure 3.6. Northern analysis for the putative synbindin-like (*SYL*) gene isolated from *T. caerulescens*, in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, and 10 or 50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

activation of the H^+ATPase, AHA2, through interactions with the C-terminal end of AHA2 (Baunsgaard et al., 1998; Jahn et al., 1997; Rosenquist et al., 2000).

Yeast cells expressing this gene exhibited higher Zn tolerance and significantly lower levels of Zn accumulation compared with the control Zn strain (Figures 3.1 A, B). Northern analysis did not reveal any striking features regarding relative expression in the two *Thlaspi* species or in response to changing plant Zn status (Figure 3.7). To investigate the role of this gene *in planta,* a true breeding Arabidopsis T-DNA knockout line for this gene (Salk line 088321), which has a T-DNA insert in the second intron, was isolated. A very noticeable phenotype of reduced plant growth was seen in lines containing this insert (Figure 3.8); however this phenotype was not apparent in succeeding generations suggesting reversion to the wild type phenotype. Another Salk line (001867) has a T-DNA insert in the 5' UTR of GRF4. True breeding homozygous lines containing the insert were identified and grown

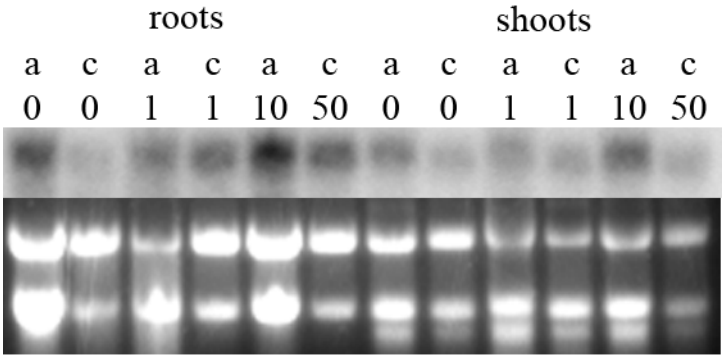

Figure 3.7. Northern analysis of the14-3-3 φ isoform (homolog of *GRF4*) isolated from *T. caerulescens* in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, and 10 or50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

hydroponically under a range of Zn concentrations, but no significant differences in growth or plant Zn accumulation were seen between the T-DNA line and wild type plants (Figure 3.9).

SRO4

The final *T. caerulescens* gene identified in our tolerance screen and warranting further study encodes a protein of unknown function that shares 68% protein identity and is most closely related to the Arabidopsis *SRO4* gene. This gene is similar to *RCD1*, or Radical-Induced Cell Death1 gene, which has been independently identified four different times in four different oxidative stress related screens (Belles-Boix et al., 2000; Fujibe et al., 2004; Lin and Heaton, 2001; Overmyer et al., 2000). In the study by Belles-Boix et al. (2000), RCD1 was called CEO1 (Clone Eighty-One) and they showed through a yeast two-hybrid screen that CEO1 interacts with putative DNA-binding proteins. Subsequent work by Ahlfors et al. (2004) determined that RCD1/CEO1 is part of a small gene family in plants that includes five more genes,

Figure 3.8. Shoot growth of the Arabidopsis T-DNA knockout line for the Arabidopsis homolog of *TcGRF4* (plant on the left marked with black circle), in comparison with wild type shoot growth for the plant on the right.

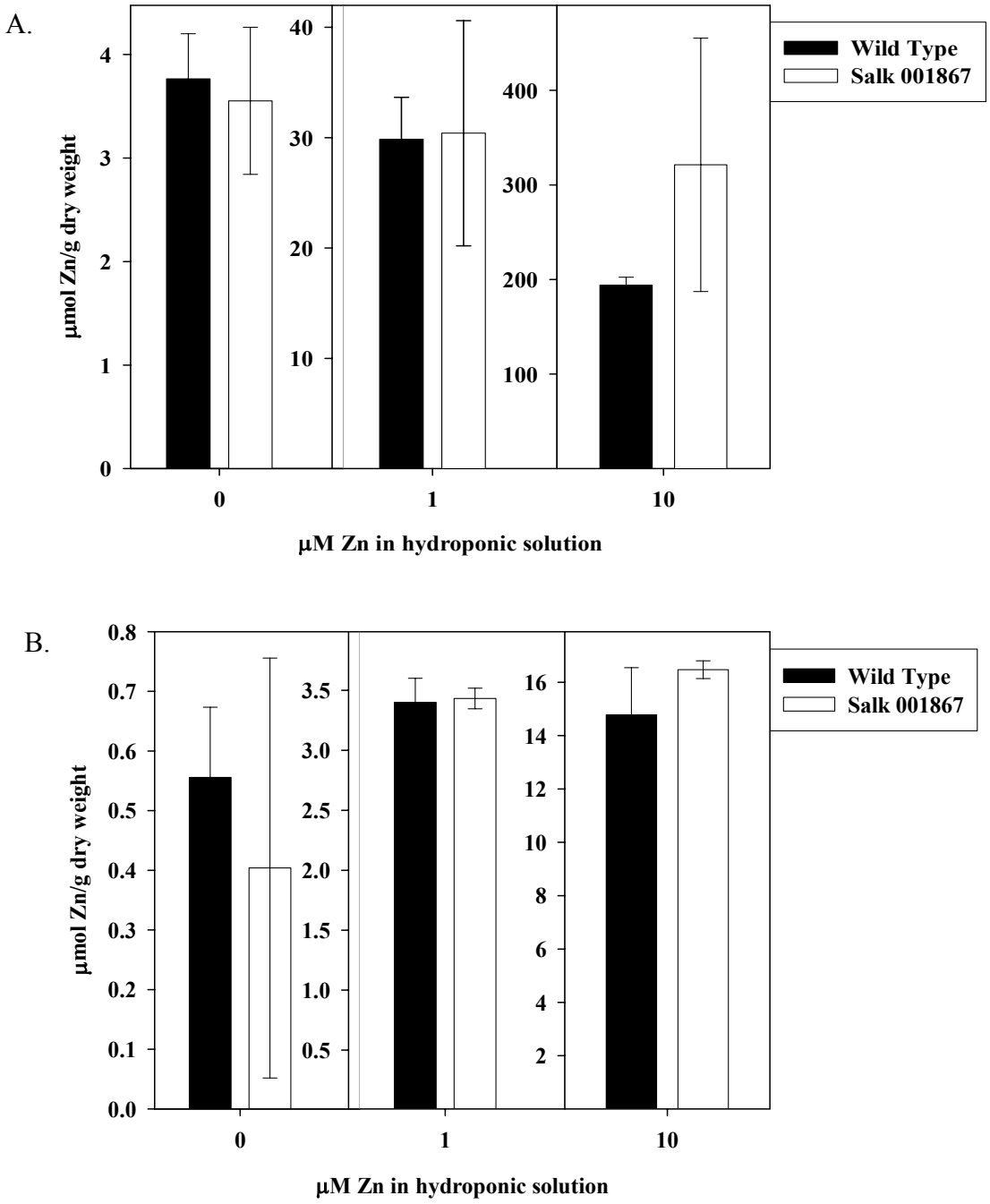

Figure 3.9. Zn concentrations in roots (a) and shoots (b) of 4 week old wild type *Arabidopsis thaliana* and a second T-DNA knockout line for *AtGRF4* (Salk line 001867) after 1 week of growth in a nutrient solution with Zn concentrations of 0, 1 and 10 µM $ZnSO_4$. Uptake is expressed on a per gram dry weight basis (µmol metal g^{-1} dry weight). Bars indicate standard error of the mean.

SRO1-5. Of these genes, *RCD1* and *SRO1* are the only family members that contain both the WWE domain (named for the conserved tyrosine and glutamate amino acid residues) and the PARP domain, a conserved catalytic domain involved in the covalent attachment of ADP-ribose from NAD+ to a limited number of DNA binding proteins leading to decreased DNA affinity (Marchler-Bauer et al., 2005). The other SRO genes (2-5) contain only the putative PARP domain (Ahlfors et al., 2004). The WWE domain is thought to play a role in protein ubiquitination and ADP ribosylation (Aravind 2001). SRO5 has salt stress dependent gene expression (Borsani et al., 2005). The function of any of the other SRO genes, including SRO4, has not yet been determined.

In our studies, expression of the *T. caerulescens SRO4* gene in yeast resulted in small decreases in yeast growth under both standard and high Zn conditions (Figure 3.1A). On the other hand, yeast expressing *SRO4* accumulated somewhat higher amounts of Zn when grown on standard SD media, but not when grown on high Zn media (Figure 3.1B).

Expression of *SRO4* in both *Thlaspi* species was only detected in *Thlaspi* roots with higher expression in *T. caerulescens* compared with *T. arvense*, and differences in transcription levels were seen with changes in Zn status (Figure 3.10). Expression of this gene is strongly induced by Zn deficiency, and its expression decreases dramatically as plant Zn status is increased. The tissue specificity of *SRO4* expression as well as the correlation between Zn status and gene expression suggests this gene may play a role in zinc signaling in roots. Arabidopsis SALK lines with a T-DNA insert in the 5'UTR of the Arabidopsis homolog for this gene were identified. Following generation of homozygous T-DNA knockout lines for this gene, both wild type and transgenic knockout lines were grown on Zn deficient, standard Zn, and high Zn hydroponic media (0, 1 and 10 μM $ZnSO_4$, respectively). As seen in Figure 3.11,

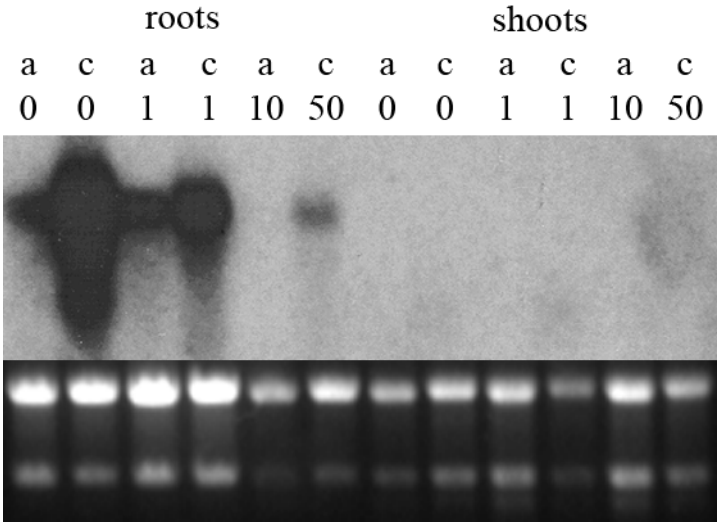

Figure 3.10. Northern analysis of the *SRO4* gene isolated from *T. caerulescens* in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient, and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, or 10/50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

no significant differences were seen in Zn accumulation in roots or shoots between the knockout line and wild type plants grown on high Zn media. The effectiveness of the T-DNA in inhibiting gene transcription was not determined and as this insert was in the 5' UTR, may not have completely eliminated gene transcription. These findings for *T. caerulescens SRO4* suggest that further study into the function of this gene should clarify the role this gene plays in Zn nutrition and homeostasis.

DISCUSSION

When yeast are exposed to high concentrations of Zn, the standard responses include reduced transcription of yeast plasma membrane Zn transporter genes as well as increased endocytosis of Zn transporter proteins from the plasma membrane (Eide, 2003). Under these conditions, it is not unexpected that a number of endocytotic-

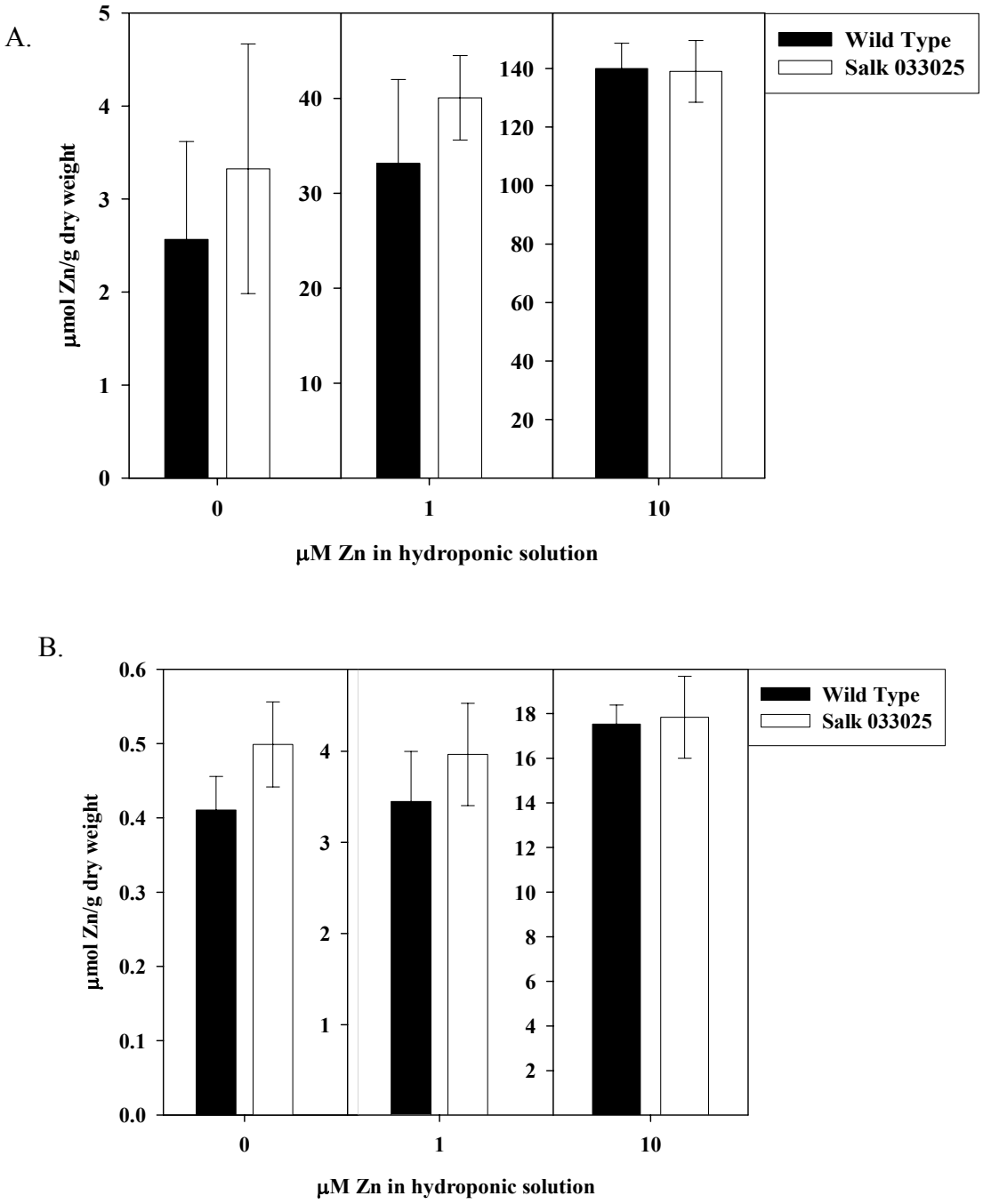

Figure 3.11. Zn accumulation in roots (a) and shoots (b) of wild type *Arabidopsis thaliana* seedlings and a T-DNA knockout line for the Arabidopsis homolog of *SR04*, after 1 week of growth in a nutrient solution with Zn concentrations of 0, 1 and 10 µM $ZnSO_4$. Uptake is expressed on a per gram dry weight basis (µmol Zn g^{-1} dry weight). Bars indicate standard errors of the mean.

related genes were identified under the high Zn conditions employed in this tolerance screen. These findings suggest that plants may also employ endocytosis in response to high Zn to recycle Zn transporters. The ubiquitin conjugating enzyme and the ubiquitin extension protein are both associated with protein degradation and constitutive expression of these genes would most likely help in the removal of Zn transporters under the conditions examined. Because these proteins did not show a significant difference in plant gene expression under varying Zn concentrations, they were not studied further.

The role of glutamine synthetase in regulating Zn homeostasis is not clear. While this gene comprised almost 1/3 of the genes identified in the screen, and glutamine synthetase has been identified in a *T. caerulescens* microarray experiment as highly expressed under low Zn conditions (Plessl et al, 2005), no link between its function in nitrogen assimilation and Zn homeostasis has been determined, and a correlation between these two processes is not obvious. The low Zn accumulation seen in yeast expressing the *Thlaspi* glutamine synthetase has led to the speculation here that increased glutamine synthesis might result in increased efflux of this potential Zn ligand, which could reduce Zn entry into the cell in a manner similar to plant root aluminum tolerance mechanisms that use the exudation of aluminum-binding organic acids to exclude Al from the root tip (Kochian et al, 2004).

Another gene that also appears to promote Zn tolerance through reduced Zn accumulation is the *synbindin-like* or *SYL* gene. *SYL* genes have been linked to membrane signaling and vesicle transport in mice and unlike the ubiquitin genes, expression of the *SYL* gene in *Thlaspi* appears to show some response to changes in plant Zn status, with gene expression increasing with plant Zn status in the roots. Genes that reduce Zn accumulation are of interest considering most of the Zn in *T. caerulescens* accumulates in epidermal leaf cells. Therefore, it would be expected that

as Zn moves along its transport pathway from the root to the final storage in the leaf epidermis, cells along this pathway would not sequester Zn. While it is too early to speculate about the role of this gene in *T. caerulescens*, the high expression of this gene in *T. caerulescens* relative to *T. arvense* and the observed Zn specific changes in gene expression led us to create Arabidopsis lines overexpressing *SYL*. These lines are currently available but have not yet been characterized.

The isolation of a gene encoding a 14-3-3 protein in this metal tolerance screen was somewhat unexpected, however this gene could be acting as a general stress response since this protein has been shown to interact with the plasma membrane H^+-ATPase (AHA2) (Rosenquist et al., 2000). The yeast data indicating this *Thlaspi* protein causes lower yeast Zn accumulation allows us to speculate that the constitutive expression of the 14-3-3 may activate a yeast H^+-ATPase, leading to a larger proton gradient which could drive H^+/Zn^{2+} antiport at the plasma membrane and thus enhanced Zn efflux. It would be interesting to see the effect of overexpression of this *Thlaspi* gene in transgenic Arabidopsis plants. The Salk line harboring the T-DNA knockout in the Arabidopsis homolog of this gene showed severely reduced growth in some of the homozygous lines, but this trait was not stable and was not present in succeeding generations, possibly due to splicing of the T-DNA insert present in the intron over time.

In subsequent studies, particular focus rested on *TcSRO4*. Related family members SRO5 and RCD1 have been implicated in the reduction of salt and reactive oxygen stress and these genes appear to interact with DNA binding domains. Not only did constitutive expression of *TcSRO4* lead to increased yeast Zn accumulation, but expression of the gene *in planta* is specific to roots and is expressed under Zn deficiency conditions. This gene was selected as a candidate for further study. Towards that end, a number of transgenic lines of Arabidopsis overexpressing this

gene have been generated, and further characterization of their physiology should help unveil the role of this gene *in planta*.

Previous yeast-based metal tolerance screens were based on metal-sensitive yeast mutants lacking a metal transporter involved in tolerance, and thus complementation of those mutants with plant cDNA libraries usually identified functional metal transporters. In this screen wild type yeast was used which is more metal tolerant, which allowed a wide net to be cast in terms of tolerance genes. This enabled the identification of a number of genes not previously associated with metal tolerance. Just as tolerance mechanisms are divided into avoidance and sequestration mechanisms, the genes identified here could fall into similar categories. Besides genes that are more peripherally linked to Zn tolerance, genes that should be further studied to better understand the molecular basis for the hyperaccumulation phenotype in *T. caerulescens* have also been identified.

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CHAPTER IV

Identification and Characterization of a Protein Kinase from *Thlaspi caerulescens* with a Potential Role in Zinc Tolerance

INTRODUCTION

Plants need to tightly regulate their nutrient status in the face of varying environmental conditions in order to properly grow and reproduce. In order for plants to obtain enough nutrients for basic biological functions without accumulating toxic levels of a specific nutrient, a number of regulating mechanisms are utilized. Under low nutrient status, increased production of specific transporters, activation of transporter activity through protein-protein activation and release of metal ligands into the rhizosphere are all general mechanisms used by plants to increase nutrient uptake (Clemens et al., 2002). Under conditions of high nutrient availability, plant cells respond with decreased transcription of transporter genes, the repression of transporters by protein-protein interactions and/or the endocytosis of transporters to remove them from the membrane where they function (Hall, 2002).

One of the well studied pathways mediating salt stress in Arabidopsis is the *salt overly sensitive* (SOS) pathway. This pathway was first identified through an EMS mutagenized *Arabidopsis thaliana* population screened for salt sensitivity (Liu and Zhu 1997; Wu et al., 1996; Zhu et al., 1998). Three members of this pathway that help confer salt tolerance include SOS1, a Na^+/H^+ antiporter; SOS2, a protein kinase and SOS3, a calcineurin B-like protein (Liu et al., 2000; Liu and Zhu, 1998; Shi et al., 2000). In this pathway, following activation due to salt stress-induced increases in cytoplasmic Ca^{2+}, SOS3 activates SOS2 by binding to the autoinhibitory region known as the FISL motif (Guo et al., 2001). Once activated, the SOS3/SOS2 protein

complex phosphorylates and activates SOS1, leading to the transport of Na^+ of salt from the cytoplasm via the plasma membrane localized SOS1 transporter (Quintero et al., 2002).

The SOS2 gene is one member of the 25 member protein kinase (PKS) family in Arabidopsis. This family is alternatively referred to as the CBL-interacting protein kinase (CIPK) family and is a subset of the plant specific SnRK3 kinase group (Hrabak et al., 2003). Other kinases in the PKS family have been implicated in signaling associated with a range of abiotic stressors, including abscisic acid (Gong et al., 2002a; Guo et al., 2002; Kim et al., 2003a), glucose signaling (Gong et al., 2002b), cold, light, salt and sugar signaling (Kim et al., 2003b).

As previously mentioned in Chapter III, a Zn tolerance screen of a *T. caerulescens* cDNA library in yeast identified a member of the PKS family, the previously uncharacterized *PKS4/CIPK6/At4g30960*. The isolation and subsequent molecular and physiological characterization of *TcPKS4* in *T. caerulescens* is described here. This involved investigation of *TcPKS4* gene expression in response to changes in *T. caerulescens* Zn status, as well as examining the effect of expression of *TcPKS4* in yeast and transgenic Arabidopsis plants on Zn tolerance.

METHODS AND MATERIALS

Yeast Growth Conditions. For the yeast tolerance screen, the wild type yeast strain DY1457 (MATα *ade6 can1 his3 trp1 ura3*) was transformed with either the empty yeast expression vector pFL61 (Minet et al., 1992) or the pFL61 vector harboring different *T. caerulescens* cDNAs. The *T. caerulescens* cDNA library was constructed as described by Pence (2002), and was made from poly A^+RNA from *T. caerulescens* seedling roots and shoots grown under both Zn deficient and Zn replete conditions. Yeast were grown on a synthetic dextrose minimal media supplemented

with 0.1% casamino acids, adenine (20 mg/L), histidine (20 mg/L), tryptophan (20 mg/L), leucine (30 mg/L) and will subsequently be referred to as SD media. SD media plates also contained 2% w/v agar (Difco Laboratories, Sparks, MD).

Library Screen and Identification of TcPKS4. An initial screen with the *S. cerevisiae* strain DY1457 containing the empty pFL61 vector determined that yeast growth was severely limited when grown on 8.0 mM $ZnSO_4$, so this concentration was used to screen a cDNA library from *T. caerulescens* for genes conferring increased Zn tolerance. Following initial identification of Zn tolerant yeast lines, yeast strains were restreaked on fresh plates to confirm the tolerance phenotype. Then the pFL61 vector containing this specific *T. caerulescens* cDNA was isolated, sequenced and named *sTcPKS4* for shortened *T. caerulescens PKS4*. As the *TcPKS4* gene identified was not full length, the gene fragment was used to screen a *T. caerulescens* (Prayon) cDNA library and a matching full length cDNA was identified from the screen, sequenced and designated *TcPKS4.*

Isolation of AtPKS4 *and Variants.* A full length *AtPKS4* was derived from Zn deficient *Arabidopsis thaliana* (Col) root tissue mRNA following single strand synthesis RT-PCR, using an oligo-dT primer. The *AtPKS4* cDNA was then amplified from the cDNA pool using the forward primer 5'-AT<u>GCGGCCGC</u>ATGGTCGGAGCAAAACCGGTGGA-3' and the reverse primer 5'-AT<u>GCGGCCGC</u>TCAAGCAGGTGTAGAGGTCCAGA-3'. The gene specific sequence came from the NCBI database (http://www.ncbi.nlm.nih.gov/) for the closest identified Arabidopsis homolog: At4g30960. The region underlined in both primers is the Not*I* restriction site for the adaptors used for subcloning into pFL61, following the initial cloning into the TOPO-TA vector (Invitrogen). Two variant forms were made to examine the effect of modifying or removing previously identified functional domains in PKS4 in yeast and plants. The first variant was a truncated *AtPKS4*

(*sAtPKS4*) form and was made by the construction of a new 5' primer 5'-ATGCGGCCGCATGGCGAGCAAATCGAA-3' along with the same 3' primer used to isolate the full length *AtPKS4*. The second variant was of a full length *AtPKS4* with a threonine to aspartate amino acid change at aa182 (*AtPKS4-T182D*), amplified by PCR where two primers, pks4T182D 5': 5'-GTCATGACACTTGTGGAACTCCGGCTT-3' and pks4T182D 3': 5'-GTCATGAAGAAGTCCGTCTTGTTTCAA-3' were used to modify the ACA codon to GCA, leading to the amino acid change. Fragments were digested using Bsp*HI* and Not*I* to move the fragments into pFL61. Proper sequence orientation and modifications were confirmed by nucleotide sequencing.

Yeast Characterization. To characterize the effect of this gene on yeast Zn physiology, yeast containing the empty vector (pFL61) or the pFL61 vector with the *PKS4* variants including *sTcPKS4*, *TcPKS4*, *sAtPKS4*, *AtPKS4* and, in a later experiment, *AtPKS4T182D*, were tested for growth and Zn accumulation in liquid SD media containing a range of Zn concentrations. Yeast were grown overnight at 30ºC in SD media and then subcultured into 10 mL of SD media or SD media containing 1 mM or 2.5 mM $ZnSO_4$ at an initial yeast density of $OD_{600} = 0.01$. Yeast were grown at 30ºC while being shaken at 120 rpm for 16 hours. At the end of 16 hours, the yeast density was determined by spectrophotometry at OD_{600}, spun at 2500 x g for 5 min, washed twice with 5 mL of 18 mΩ dH_2O, and then the cells were transferred to quartz tubes for elemental analysis. The samples were digested with concentrated HNO_3 at 100ºC and then further digested in nitric and hydrochloric acid at 200ºC until dry. Samples were resuspended in 5% HNO_3 and analyzed for mineral element content using an axially viewed inductively-coupled plasma emission spectrometer (ICP-AES; model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) for Zn content.

Since the initial work in yeast in Chapter III suggested that PKS4 increased yeast Zn accumulation, yeast lines with non-functional vacuolar Zn transporters were obtained from Dr. David Eide (University of Wisconsin, Madison) and used to further examine the function of the full length *AtPKS4*. The effect of *AtPKS4* on yeast strains lacking functional ZRT3, ZRC1 and COT1 Zn transporters were examined. The yeast strains tested included the parental strain CM100 (MATα can1 his3 leu2 trp1 ura3) and mutant strains CM101 (MATa can1 his3 leu2 trp1 ura3-52 zrt3::KAN^R), CM102 (MATα can1 his3 leu2 trp1 ura3-52 zrc1::HIS3) and CM103 (MATα can1 his3 leu2 trp1 ura3-52 cot1::URA3). Since both the CM103 yeast strain and the pFL61 vector use URA resistance for the selectable marker, *AtPKS4* was moved into pESC-LEU (Stratagene, La Jolla, CA) using Not*I* restriction sites available in both the gene and vector. The pESC-LEU strain uses a GAL10 promoter and so all experimental work on this strain required growth of yeast on galactose instead of glucose. Yeast studies examining growth of the mutant lines with empty vector and with vector containing *AtPKS4* in response to a range of Zn conditions in SD media followed the experimental procedure listed above.

Plant Growth Conditions. Plant growth was carried out as described by Pence (2002). Briefly, *T. caerulescens* (Prayon) and *T. arvense* plants were germinated and grown in modified Johnson's nutrient solution which consists of 1.2 mM KNO_3, 0.8 mM $Ca(NO_3)_2$, 0.2 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4$, 50 μM KCl, 12.5 μM H_3BO_3, 5 μM Fe(III)-EDDHA (N,N'-ethylenediamine-di-(O-hydroxyphenylacetic acid)), 1 μM $MnSO_4$, 1 μM $ZnSO_4$, 0.5 μM $CuSO_4$, 0.1 μM H_2MoO_4, 0.1 μM $NiSO_4$, and 1 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 5.5. The plants were grown in a greenhouse without artificial light. The nutrient solution was constantly aerated and replaced weekly. Due to the greater growth rate of *T. arvense* under the standard greenhouse conditions used, *T. caerulescens* and *T. arvense* plants were grown

hydroponically in modified Johnson's solution for 50 and 40 days, respectively, before the start of the Zn treatment. Zn treatments included the standard modified Johnson's solution with Zn-deficient (0 μM $ZnSO_4$), Zn-sufficient (1 μM $ZnSO_4$) or Zn-excess (10 μM $ZnSO_4$ for *T. arvense*; 50 μM $ZnSO_4$ for *T. caerulescens*) treatments. After 14 days of growth, plants were harvested, separated into root and shoot tissue and flash frozen in liquid N_2.

Northern Analysis. *TcPKS4* gene expression was examined in *T. caerulescens* and *T. arvense* root and shoot tissue. Total RNA was isolated following the Trizol protocol (GibcoBRL/Invitrogen). 20 μg of total RNA was denatured, separated by glyoxal-agarose gel electrophoresis and transferred to a nylon membrane (Hybond N^+; Amersham). Equal loading was confirmed both by spectrophotometric measurement of RNA concentrations and visually through ethidium bromide staining of ribosomal subunits. Membranes were UV crosslinked before hybridization. To examine *TcPKS4* gene expression, the gene fragment originally identified (*sTcPKS4*) was labeled with ^{32}P-dCTP with random hexamer primers and hybridized at 65ºC overnight in PerfectHyb Solution (Sigma Aldrich) with 10 μM salmon testes DNA blocking agent. Following hybridization, membranes were washed 2 times for 30 minutes at 65ºC in a low stringency wash solution (2 x SSC, 0.1% SDS). After exposure to autoradiography film (Kodak), the membrane was stripped with boiling 0.5% SDS.

Generation and Characterization of PKS4 *Modified Expression Lines in* A. thaliana. The following genes were transformed into *Arabidopsis thaliana* (Col) for overexpression lines: *AtPKS4*, *AtPKS4T182D* and *TcPKS4*. An additional set of primers were designed to transfer the genes of interest into the *E. coli- Agrobacterium tumefaciens* shuttle vector pBAR1 (Holt et al., 2002; with addition of a 35S promoter in Hind*III*/Eco*RI* site at the start of the multiple restriction cloning site):

AtPKS4 5' 5' -ATGGATCCATGGTCGGAGCAAAAC- 3';

AtPKS4 3' 5' -TTCTAGATCAAGCAGGTGTAGAG- 3';

TcPKS4 5' 5' -ATCCCGGGATGGGGAAGAGAAAA- 3';

TcPKS4 3' 5'- TTCTAGATCAAGCAGGAGTAGAC- 3'.

PCR products were separated on an agarose gel, fragment purified using a QIAEX II gel extraction kit (Qiagen) and cloned into the TOPO-TA vector (Invitrogen). The subsequent minipreps were used for subcloning into pBAR1. Primers included Bam*HI* and Xba*I* or Xma*I* and Xba*I* restriction sites (underlined) adaptors for directional cloning of Arabidopsis and *Thlaspi PKS4* genes (respectively) into pBAR1 (Holt et al., 2002; with addition of 35S promoter in Hind*III*/Eco*RI* site at the start of the multiple restriction cloning site). The genes were digested with their respective restriction enzymes and ligated into the corresponding sites in the pBAR1 vector. Sequence integrity was verified by sequencing. pBAR1 containing the *AtPKS4*, *AtPKS4T182D* and *TcPKS4* constructs was transferred to *A. tumefaciens* strain C58 via electroporation. Transformed Agrobacterium cells were selected from LB plates with 50 μg/L kanamycin and 50 μg/L rifampicin selection. A single colony was started in a 5 mL overnight LB kan/rif culture grown at 30ºC. The starter culture was used to inoculate 0.5 L of LB kan/rif overnight culture grown at 30ºC. Cells were then pelleted at 4000 x g for 20 min and resuspended in 1 L of dipping solution (50 g/L sucrose and 0.5 mL/L Silwet-L77). Flowering Arabidopsis (Col) plants were dipped in the dipping solution for 20 seconds per 4" pot, laid sideways to drain excess solution, covered with Saran Wrap and left in the dark overnight. The following day, plants were uncovered, straightened and returned to normal growing conditions. Seeds were harvested approximately 4 weeks following transformation. This protocol is based on the floral dip protocol of Clough and Bent (1998).

T_1 seeds were planted and treated with gluphosinate ammonium to screen for transformed plants (Finale, Farnam Co. Inc.), and seeds were collected from the resistant lines. No further work was done with the Arabidopsis plants expressing *TcPKS4* due to time constraints and because of the results from the work in yeast. For the Arabidopsis plants expressing *AtPKS4* and *AtPKS4T182D*, the T_2 and T_3 generation seedlings were grown out and tested via herbicide resistance for the presence of the transgene, followed by Northern analysis to confirm high levels of transgene expression (not shown). Once true breeding lines with high transgene expression were identified, they were grown hydroponically in modified Johnson's solution containing a range of Zn concentrations (deficient: 0 μM $ZnSO_4$; sufficient: 1 μM $ZnSO_4$; and excess: 10 μM and 50 μM $ZnSO_4$) to look for phenotypic changes based on differences in the accumulation of Zn and other metals. Plants were grown in aerated nutrient solution for 28 days with the growth solution changed every seven days. After 28 days of growth, roots were treated with a 10 minute, 5 mM $CaCl_2$ solution wash to remove root cell wall associated Zn, and then roots and shoots were harvested. Plant tissue was dried in 50ºC oven for at least one week. Dry weights were collected and samples were analyzed by an axially viewed inductively-coupled plasma emission spectrometer (ICP-AES; model ICAP 61E trace analyzer, Thermo Electron, San Jose, CA) for Zn content.

Another set of transgenic Arabidopsis plants were grown on MS plates to determine short term effects of growth on increasing Zn concentrations. Seeds were treated with a 3% bleach solution, washed and vernalized for 2 d at 4ºC and then planted out on MS plates containing 1x Murashige and Skoog salts (Sigma-Aldrich) 1% sucrose, 0.3 g/L MES, pH to 5.8 with KOH, 2.2 g/L gelrite gellan gum (Sigma-Aldrich). Four different media were made containing no additional Zn, 0.25 mM, 0.50 mM or 1.00 mM $ZnSO_4$ added. Zn was added prior to adjusting the pH of the media.

After vernalization, seedlings were grown in a growth chamber with 16h/8h light regimen, 21ºC/20ºC for seven days to look for short term effects of elevated Zn concentrations on growth and appearance of the Arabidopsis plant lines.

RESULTS

Screening of a T. caerulescens *cDNA Library and Identification of* TcPKS4. A 1241 nucleotide fragment was isolated as a candidate Zn tolerance gene in yeast following a functional screen for growth on high Zn in yeast transformed with a *T. caerulescens* cDNA library (see Chapter III for experimental details). This cDNA was not full length and lacked a start codon, but when thirty-eight nucleotides (including the Not*I* restriction site) from the pFL61 vector DNA was included just upstream of the partial *Thlaspi* cDNA was included, a 1077 nucleotide coding sequence with a start codon was created (Figure 4.1). According to a BLAST search (http://www.ncbi.nih.gov/), this gene most closely matched the Arabidopsis serine-threonine kinase *PKS4* and was therefore named *sTcPKS4*.

To obtain the full length coding sequence, a *T. caerulescens* (Prayon) cDNA library was screened with the *sTcPKS4* clone. From this screen, a 1338 nucleotide gene that matched *sTcPKS4* was identified. This clone, named *TcPKS4*, shares 94% protein level homology to the AtPKS4 protein and the alignment between the full length versions of both genes is shown in Figure 4.2. Both the *T. caerulescens* and Arabidopsis versions of *PKS4* contain the characteristic N-terminal kinase domain and a C-terminal regulatory domain (Figure 4.2).

Isolation of PKS4 variants. The *sTcPKS4* originally isolated from the tolerance screen was 87 amino acids shorter than the full length TcPKS4 protein, and the first eighteen amino acids in that sequence were from the plasmid DNA. However, this truncated version of the gene still appeared to confer tolerance,

atggggaaagagaaaagaaaaaaattgatcGCGGCCGCCAGTGTGGTGGAATTCCTTGTAC
GTGGCGGCGAATTATTCGCCAAGGTCGCTAAAGGAAGGCTACGCGAGGAC
GTGGCGCGAGTTTACTTCCAGCAATTGATCTCCGCCGTCGATTTCTGCCAC
AGCCGGGGAGTTTATCACCGGGATCTGAAACCGGAGAATCTGTTGTTGGA
CGAGGAAGGGAATCTCAAGGTGACTGATTTCGGTCTCTCTGCATTCACTGA
GCATTTGAAGCAAGACGGGCTTCTCCACACAACTTGTGGAACTCCGGCGT
ATGTTGCGCCGGAGGTTATTCTGAAGAAAGGATACGATGGAGCGAAGGCG
GATCTGTGGTCTTGCGGCGTTATCCTCTTTGTGCTGCTCGCTGGGTATTTGC
CGTTTCAGGATGATAATTTGGTGAACATGTATCGGAAGATCTACAGAGGA
GATTTCAAATGTCCTGGATGGCTTTCCTCCGACGCGAGAAGGCTCGTGACG
AAGCTTCTGGATCCGAATCCGAATACCCGAATCACCATCGATAAGGTCAT
GGATTCAACCTGGTTTAAGAGAACCGCAGCGAGATCCAAAAACGAACCAA
TCACGACGACAACCGCTGAAGTTGCGGCTGAGGATCCTGATTTCTCCGTGC
ACAAGTCAAAGGAAGAGACGGAGACGCTAAACGCGTTTCATATAATCGCG
TTATCTGAAGGGTTTGATCTGTCGCCGCTGTTCGAGGAGAAGAAGAAAGA
GGAGAAGAGAGAGATGAGATTCGCGACTTCACGTCCGGCGAGCAGCGTGA
TCTCGAGCTTGGAAGAGGCGGCGAAAGTTGGGAACAAGTTCGATGTTAGG
AAGAGCGAGAGTAGAGTGAGGATCGAAGGGAAACAGAATGGGCGGAAAG
GGAAATTGGCGGTGGAGGCGGAGATATTCGCGGTGGCTCCGTCGTTCGTC
GTGGTGGAGGTGAAGAAAGATCACGGAGACACTCTTGAGTACAACAACTT
TTGCAGTACTGCTCTTAGACCAGCTCTCAAGGACATTCTCTGGACGTCTAC
TCCTGCTTGA

Figure 4.1. Coding sequence of original partial sequence for the *TcPKS4* cDNA. The lower case letters denote vector sequence with the underlined Not*I* site indicating the start of gene specific region.

therefore a similar truncated version of *AtPKS4* was created to see if this difference was specific to *T. caerulescens PKS4*. The *AtPKS4* gene was truncated to a start codon at amino acid 90 and this construct was transferred to yeast in order to test the effect of a truncated kinase in Zn tolerance tests. The alignment of the full length and truncated TcPKS4 and AtPKS4 proteins is shown in Figure 4.3.

Research by other laboratories examining the biochemical function of PKS family members found that modifying the threonine (T) to aspartate (D) in the activation loop (shown with asterisk in Figure 4.2) results in a constitutively active kinase (Gong et al., 2002a; Gong et al., 2002b; Gong et al., 2002c; Gong et al., 2002d; Guo et al., 2001). Based on these studies, an *AtPKS4-T182D* version of *AtPKS4* was

```
TcPKS4  MVGAKPVKMVENEFAGGVSTGLLHGRYELGRLLGHGTFAKVYHARNVATGKSVAMKVVGK 60
AtPKS4  MVGAKP---VENGSDGGSSTGLLHGRYELGRLLGHGTFAKVYHARNIQTGKSVAMKVVGK 57
        ******   ***   ** ***************************: ************

TcPKS4  EKVVKVGMVEQIKREISVMRMVKHPNIVELHEVMASKSKIYFAMELVRGGELFAKVAKGR 120
AtPKS4  EKVVKVGMVDQIKREISVMRMVKHPNIVELHEVMASKSKIYFAMELVRGGELFAKVAKGR 117
        *********:**************************************************

TcPKS4  LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 180
AtPKS4  LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 177
        ************************************************************
            *
TcPKS4  GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 240
AtPKS4  GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 237
        ************************************************************

TcPKS4  RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIDKVLDSTWFKRTAARSKNEPITTTTAEV 300
AtPKS4  RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIEKVMDSPWFKKQATRSRNEPVAAT-ITT 296
        *********************************:**:**.***: *:**:***:::*   .

TcPKS4  AAEDPDFSVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 360
AtPKS4  TEEDVDFLVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 356
        : ** ** ************************************************

TcPKS4  SSLEEAAKVGNKFDVRKSESRVRIEGKQNGRKGKLAVEAEIFAVAPSFVVVEVKKDHGDT 420
AtPKS4  SSLEEAARVGNKFDVRKSESRVRIEGKQNGRKGKLAVEAEIFAVAPSFVVVEVKKDHGDT 416
        *******:****************************************************

TcPKS4  LEYNNFCSTALRPALKDILWTSTPA 445
AtPKS4  LEYNNFCSTALRPALKDIFWTSTPA 441
        ******************:******
```

Figure 4.2. Protein sequence for *Thlaspi* and Arabidopsis PKS4. The activation domain is marked by the solid line and bracketed by the conserved DFG and APE amino acid motifs in bold, the conserved threonine modified to aspartate for creation of a constitutively active kinase, is marked with an asterisk, the conserved FISL Motif for SOS3 binding is marked with a dashed line, with the FISL amino acids marked in bold letters. "*" below sequence alignment indicates fully conserved residues, ":" indicates strongly conserved residues and "." indicates weakly conserved residues.

created through PCR-induced mutation to examine the effect of this amino acid change on PKS4 function in yeast and plants.

Yeast Characterization. To examine the effects of *PKS4* on Zn metabolism, yeast expressing *PKS4* was grown in media containing a range of Zn concentrations. Both shortened and full length variations of *PKS4* from both *T. caerulescens* and

```
sTcPKS4  ------------------------------------------------------------MGK 3
TcPKS4   MVGAKPVKMVENEFAGGVSTGLLHGRYELGRLLGHGTFAKVYHARNVATGKSVAMKVVGK 60
sAtPKS4  ------------------------------------------------------------
AtPKS4   ---MVGAKPVENGSDGGSSTGLLHGRYELGRLLGHGTFAKVYHARNIQTGKSVAMKVVGK 57


sTcPKS4  EKRKK---------------LIAAASVVEF---------------LVRGGELFAKVAKGR 33
TcPKS4   EKVVKVGMVEQIKREISVMRMVKHPNIVELHEVMASKSKIYFAMELVRGGELFAKVAKGR 120
sAtPKS4  --------------------------------MASKSKIYFAMELVRGGELFAKVAKGR 27
AtPKS4   EKVVKVGMVDQIKREISVMRMVKHPNIVELHEVMASKSKIYFAMELVRGGELFAKVAKGR 117
                                                    ***************

sTcPKS4  LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 93
TcPKS4   LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 180
sAtPKS4  LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 87
AtPKS4   LREDVARVYFQQLISAVDFCHSRGVYHRDLKPENLLLDEEGNLKVTDFGLSAFTEHLKQD 177
         ************************************************************

sTcPKS4  GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 153
TcPKS4   GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 240
sAtPKS4  GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 147
AtPKS4   GLLHTTCGTPAYVAPEVILKKGYDGAKADLWSCGVILFVLLAGYLPFQDDNLVNMYRKIY 237
         ************************************************************

sTcPKS4  RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIDKVMDSTWFKRTAARSKNEPITTTTAEV 213
TcPKS4   RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIDKVLDSTWFKRTAARSKNEPITTTTAEV 300
sAtPKS4  RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIEKVMDSPWFKKQATRSRNEPVAAT-ITT 206
AtPKS4   RGDFKCPGWLSSDARRLVTKLLDPNPNTRITIEKVMDSPWFKKQATRSRNEPVAAT-ITT 296
         *********************************:**:**.***: *:**:***:::*   .

sTcPKS4  AAEDPDFSVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 273
TcPKS4   AAEDPDFSVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 360
sAtPKS4  TEEDVDFLVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 266
AtPKS4   TEEDVDFLVHKSKEETETLNAFHIIALSEGFDLSPLFEEKKKEEKREMRFATSRPASSVI 356
         : ** ** ************************************************

sTcPKS4  SSLEEAAKVGNKFDVRKSESRVRIEGKQNGRKGKLAVEAEIFAVAPSFVVVEVKKDHGDT 333
TcPKS4   SSLEEAAKVGNKFDVRKSESRVRIEGKQNGRKGKLAVEAEIFAVAPSFVVVEVKKDHGDT 420
sAtPKS4  SSLEEAARVGNKFDVRKSESRVRIEGKQNGRKGKLAVKAEIFAVAPSFVVVEVKKDHGDT 326
AtPKS4   SSLEEAARVGNKFDVRKSESRVRIEGKQNGRKGKLAVEAEIFAVAPSFVVVEVKKDHGDT 416
         *******:****************************:**********************

sTcPKS4  LEYNNFCSTALRPALKDILWTSTPA 358
TcPKS4   LEYNNFCSTALRPALKDILWTSTPA 445
sAtPKS4  LEYNNFCSTALRPALKDIFWTSTPA 351
AtPKS4   LEYNNFCSTALRPALKDIFWTSTPA 441
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Figure 4.3. Protein alignment of full length and truncated PKS4 proteins from *T. caerulescens* and *A. thaliana*. "*" below sequence alignment indicates fully conserved residues, ":" indicates strongly conserved residues and "." indicates weakly conserved residues.

Arabidopsis were examined. A second, subsequent experiment examined the effect of the threonine to aspartate modification in *AtPKS4* relative to controls (expressing the empty pFL61 vector) or yeast expressing the full length *AtPKS4*. The high Zn treatment chosen in these experiments in liquid culture was 2.5 mM, as the level used to screen for Zn tolerance on agar plates (8 mM) was too high in liquid culture since the agar complexes a significant fraction of the Zn. After 16 hours of growth on the high Zn media (SD + 2.5 mM $ZnSO_4$), all of the PKS4 variants from both *Thlaspi* and Arabidopsis (except the full length AtPKS4) showed enhanced Zn tolerance and Zn accumulation, compared to the control yeast (Figures 4.4 and 4.5). Therefore, these differences in Zn accumulation by yeast conferred by either PKS4 protein are not determined by the presence of the first 100 amino acids of the protein, and it does not appear that the PKS4 from *T. caerulescens* confers any additional Zn tolerance or accumulation.

A second experiment was performed examining Zn uptake and yeast growth in lines expressing either the standard *AtPKS4* or a modified version with a threonine to aspartate substitution (T182D). Since this threonine residue has been identified as an active phosphorylation site in a number of PKS family members during autophosphorylation, replacing threonine with the negatively charged aspartate mimics the charge of a phosphorylated threonine, thus leading to protein activation (Gong et al., 2002a, 2002b, 2002c). However, no differences in yeast Zn tolerance and accumulation were seen in yeast expressing the mutant PKS4-T182D protein indicating that, at least in yeast, autophosphorylation did not produce any additional effects (data not shown).

One potential explanation for the increased Zn tolerance seen in yeast is stimulation of a vacuolar Zn transporter, which could increase the amount of Zn sequestered in the vacuole away from the more Zn sensitive cytoplasm. To test this

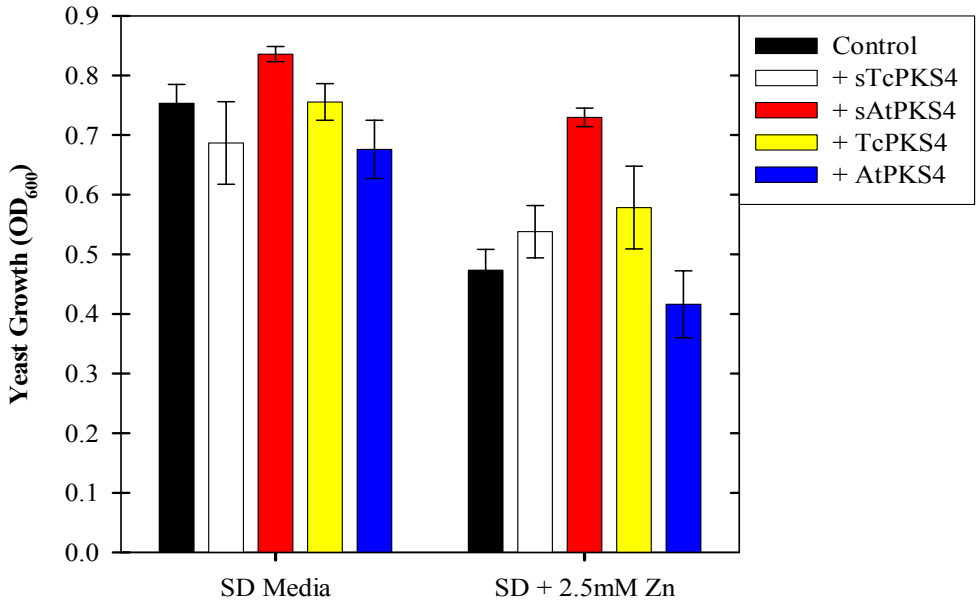

Figure 4.4. Growth of yeast expressing the empty pFL61 vector (control) and different *TcPKS4* and *AtPKS4* variants on both standard media (SD) and standard media supplemented with high Zn.

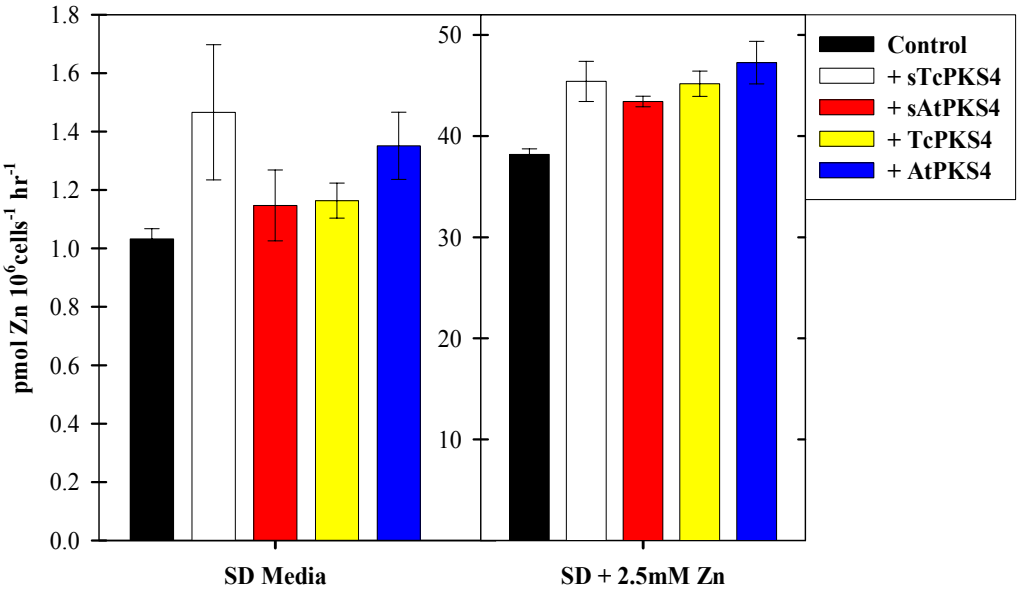

Figure 4.5. Zn accumulation in *S. cerevisiae* expressing variants of *PKS4* from Arabidopsis and *T. caerulescens*, compared with control yeast expressing the pFL61 vector.

hypothesis, yeast lines that were defective in several different vacuolar Zn transporters were obtained from Dr. David Eide at the University of Wisconsin, Madison, and the effect of the expression of *PKS4* on the growth and Zn accumulation of these mutant lines was studied. The strains examined included CM100 (a parental strain and the control line for this experiment), CM101 (a ZRT3 deficient line), CM102 (a ZRC1 deficient line) and CM103 (a COT1 deficient line). ZRT3 is a member of the ZIP family that has been shown to remobilize Zn from the vacuole when yeast are grown under Zn limited conditions (MacDiarmid et al., 2002). ZRC1 and COT1 are members of the CDF family of Zn transporters and both have been shown to move Zn from the cytoplasm into the vacuole (Conklin et al., 1992, 1994; Kamizono et al., 1989; Li and Kaplan 1998).

Yeast were grown as described previously on SD media or, for the high Zn media, the SD media was supplemented with 1 mM $ZnSO_4$ due to increased metal sensitivity of the mutant lines when grown on 2.5 mM $ZnSO_4$. All of the mutant lines expressed either the empty pFL61 vector or the vector harboring *AtPKS4*. As seen in Figures 4.6 and 4.7, all three mutant lines harboring just the pFL61 vector exhibited the Zn sensitivity phenotype (reduced growth and increased Zn accumulation in 1 mM Zn) that was characteristic of these lines. However, when these lines expressed *AtPKS4*, Zn tolerance in the *zrt3* and *cot1* mutant lines reverted to that of the wild type yeast, and at least for *zrt3*, the increased Zn accumulation associated with increased Zn sensitivity in this mutant line was reduced to levels seen in wild type yeast (Figure 4.6 and 4.7). When *AtPKS4* was expressed in the *zrt3* and *cot1* lines, both lines were more Zn tolerant than the wild type CM100 yeast strain. In the *zrc3* line, expression of *AtPKS4* had no effect on Zn sensitivity, and although it did decrease Zn accumulation was less than in the *zrc1* line without *AtPKS4*, the Zn accumulation was still much greater than in wild type yeast.

Northern Analysis. Constitutive and relatively similar levels of *TcPKS4* expression is seen in both roots and shoots of both *T. caerulescens* and *T. arvense* (Figure 4.8). Furthermore, *TcPKS4* expression did not respond to changes in plant Zn status. This is similar to previous work conducted by Guo and colleagues (2001) who found constitutive expression of *AtPKS4* in roots and shoots when Arabidopsis plants were exposed to high levels of NaCl (100 mM and 200 mM NaCl).

Generation and Characterization of Transgenic A. thaliana *Lines with Altered Expression of PKS4*. Based on the studies in yeast, the possible role of *AtPKS4* in *A. thaliana* was examined by altering its expression, both in overexpression lines and in dsRNAi based *AtPKS4* knockouts. Both the *AtPKS4* and *AtPKS4-T182D* genes were placed under constitutive expression with the 35S promoter and incorporated into *A.thaliana* (Col) through *Agrobacterium tumefaciens*-based transformation. dsRNAi lines were obtained as a gift from Dr. Yan Guo in the laboratory of Dr. Jian-Kang Zhu.

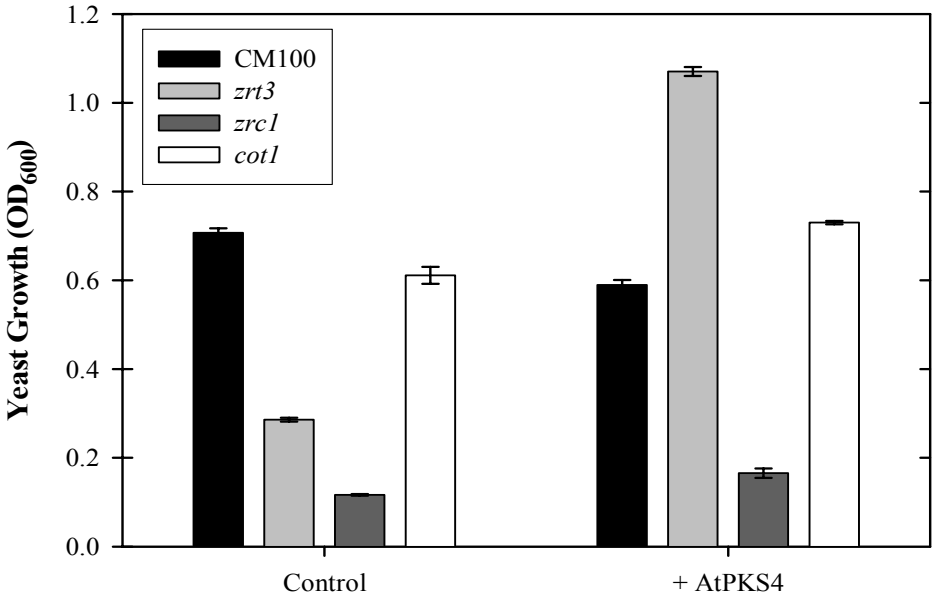

Figure 4.6. Growth of mutant *S. cerevisiae* lines where the specific vacuolar Zn transporters, ZRT3, ZRC1, and COT1, are lacking but *AtPKS4* or the empty pFL61 vector (control) are expressed.

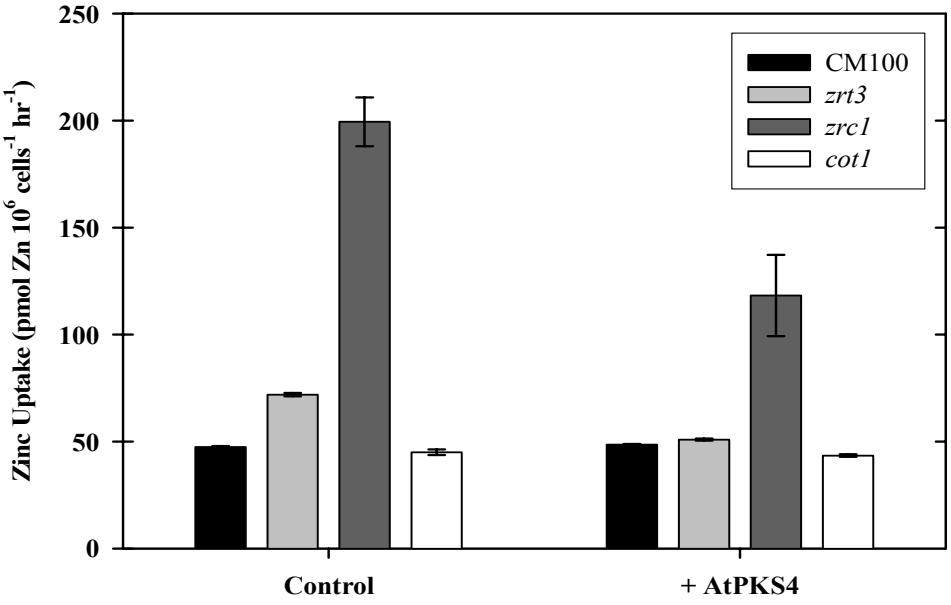

Figure 4.7. Zn accumulation in mutant *S. cerevisiae* lines where the specific vacuolar Zn transporters, ZRT3, ZRC1, and COT1, are lacking but either *AtPKS4* or the empty pFL61 vector (control) are expressed.

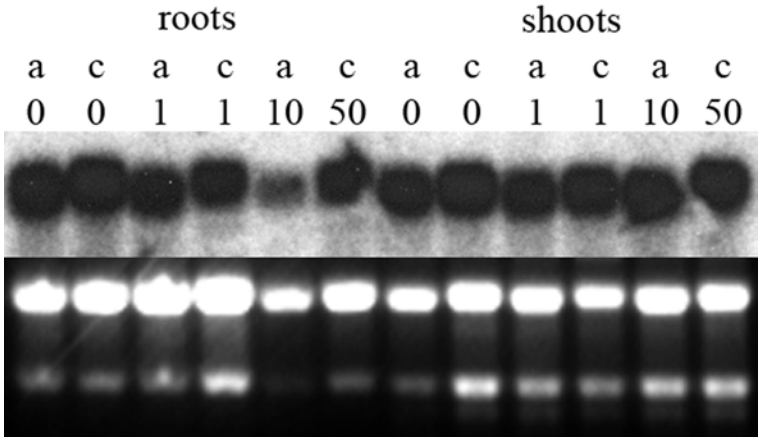

Figure 4.8.
Northern analysis of *TcPKS4* gene in *T. arvense* (a) and *T. caerulescens* (c) roots and shoots grown under Zn-deficient, Zn-sufficient and high Zn conditions. Seedlings were grown on hydroponic media containing 0, 1, or 10 or 50 μM $ZnSO_4$. The ribosomal RNA bands are shown as loading controls.

The dsRNAi lines were analyzed for gene expression via Northern analysis and those lines lacking expression of *AtPKS4* were used for subsequent growth experiments. Plants were grown hydroponically for four weeks on our standard Johnson's nutrient solution ([Zn] = 1 μM) for the first two weeks and then grown on a modified Johnson's solution with Zn concentrations of 0, 1 and 10 μM Zn for two more weeks to look for effects of altered *AtPKS4* expression of plant growth. As seen in Figures 4.9 and 4.10, no consistent differences are seen in either shoot or root growth, or Zn accumulation. However, when plants overexpressing *AtPKS4-T182D* were grown on MS agar plates or MS agar plates supplemented with 0.25 mM Zn, the transgenic lines grew to a larger size relative to the wild type plants (Figures 4.11 and 4.12). While an increase in shoot growth was seen for the lines overexpressing *AtPKS4-T182D* these lines did not show changes in shoot Zn content when grown on standard or high Zn

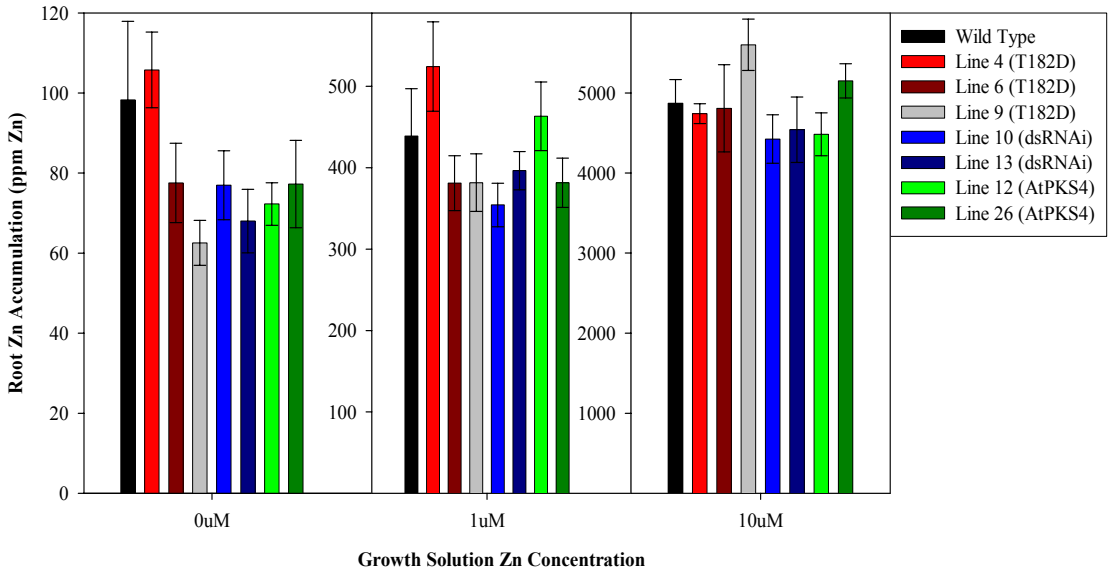

Figure 4.9. Zn concentration in roots of wild type *A. thaliana,* homozygous overexpression (T182D, AtPKS4) and knock out (dsRNAi) lines after two weeks of growth in a nutrient solution with Zn concentrations at 0, 1, and 10 μM $ZnSO_4$.

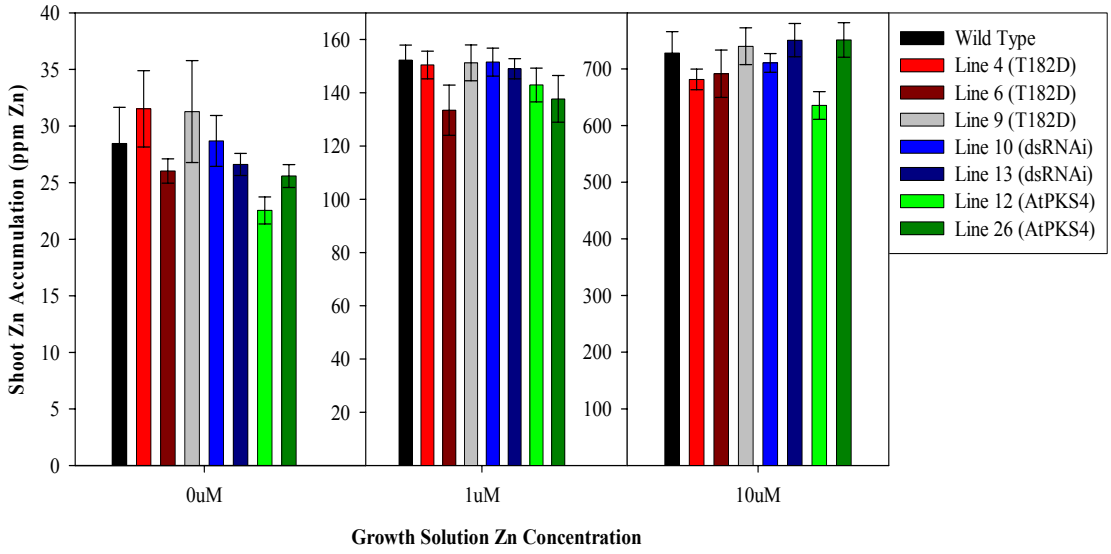

Figure 4.10. Zn concentration in shoots of wild type *A. thaliana,* homozygous overexpression (T182D, AtPKS4) and knock out (dsRNAi) lines after two weeks of growth in a nutrient solution with Zn concentrations at 0, 1, and 10 μM $ZnSO_4$.

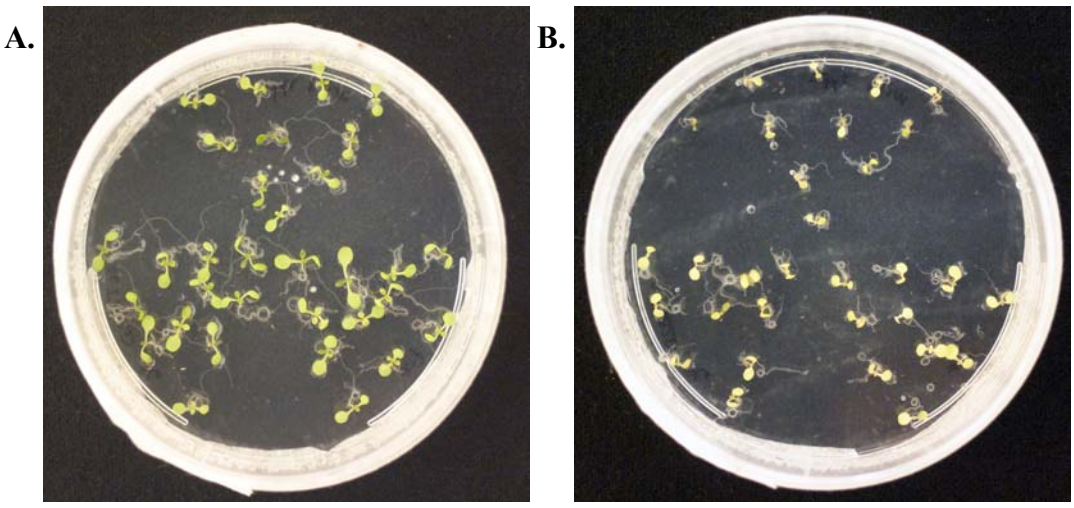

Figure 4.11. Wild type and transgenic *Arabidopsis thaliana* seedlings grown on: A) Standard MS media, and B) MS media supplemented with 0.25 mM $ZnSO_4$ as described in the Materials and Methods. The specific Arabidopsis lines are, moving clockwise from the top: wild type, overexpression line AtPKS4-T182D line 9, and overexpression line AtPKS4-T182D line 6

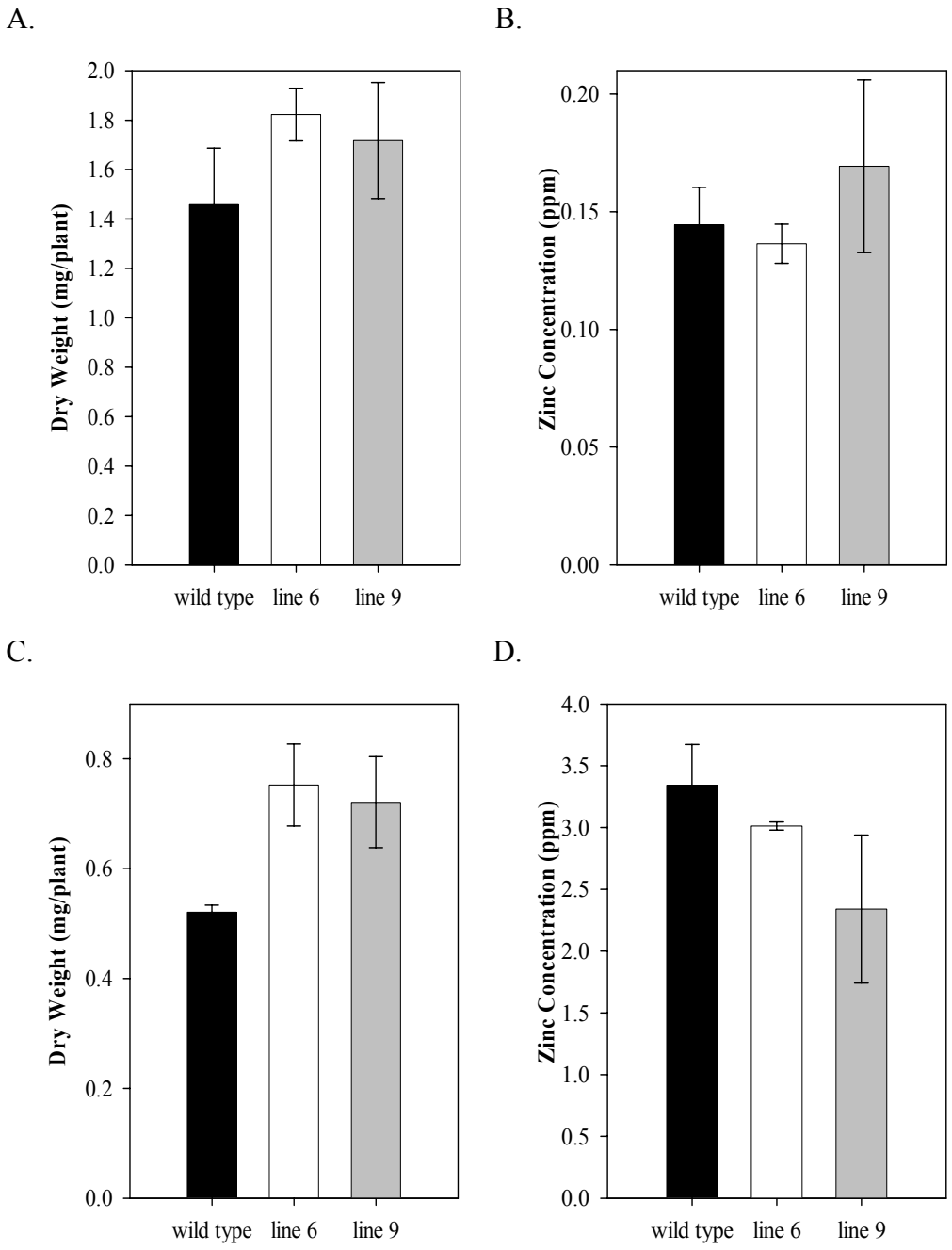

Figure 4.12. Comparison of shoot dry weights (A & C) and Zn concentrations (B & D) for both wild type Arabidopsis plants and transgenic plants expressing *AtPKS4-T182D* (lines 6 and line 9). Plants were grown on MS plates without (A & B) or with (C & D) an additional 0.5 mM $ZnSO_4$ added.

media (Figure 4.12). This increased shoot growth was not seen in the *AtPKS4* overexpression or dsRNAi lines (pictures not shown).

DISCUSSION

The SOS pathway has been a major area of focus for salt tolerance research recently. Since the identification of the three original genes, SOS1, SOS2 and SOS3, the list of processes known to be affected by this pathway has expanded and now includes a range of abiotic stresses. In Arabidopsis, along with the role of limiting salt stress that is mediated by SOS2 (Quintero et al., 2002), SOS2 family members have been implicated in abscisic acid (Gong et al., 2002a; Guo et al., 2002) and glucose signaling (Gong et al., 2002b). Additionally, related family members in rice have been implicated in cold, light, salt and sugar signaling (Kim et al., 2003). SOS2 has also been shown to activate another SOS1 family member, namely CAX1, a calcium/H^+ antiporter (Cheng et al., 2004). The SOS1 transporter activated by SOS2 belongs to the Cation Antiporter family, which constitutes a large family of antiporters present in both yeast and plants, mediating the transport of a number of cations including Na^+, K^+, Mg^{2+} and Mn^{2+} (Mäser et al., 2001). This suggests that the SOS1 and SOS2 families affect a range of abiotic stressors. Additionally, *MHX1*, a Zn/Mg/Fe transporter, is a member of the SOS1 family and increased expression of this transporter has been seen in the Zn hyperaccumulator, *A. halleri* (Elbaz et al., 2006; Shaul et al., 1999). The range of abiotic stressors linked to the SOS2 family and the wide range of cation transport seen in SOS1 family members make this a potential metal tolerance pathway of interest. With the identification of *PKS4* in our Zn tolerance screen, research efforts focused on examining the effect of this gene on Zn accumulation and tolerance. Our initial work in yeast show that when *PKS4* is expressed in yeast, both an increase in Zn tolerance and accumulation are observed,

suggesting increased tolerance through Zn sequestration in the vacuole. There have been studies showing increased kinase gene expression following metal stress (Chuang et al., 2000; Yeh et al., 2004), but as far as we know, no studies to date have shown a link between kinase expression and increased Zn tolerance. The connection of this gene family to heavy metal tolerance is a new observation but reasonable considering the similar transport and homeostasis issues that link salt and heavy metal stress.

There are a number of questions that remain following the identification and characterization of this gene. It was curious that in yeast, this shortened version of this kinase provided the same increase in Zn tolerance and accumulation as the full length version. The C terminal contains the kinase activity domains; however it is not clear if the entire region is necessary for proper kinase function. The insensitivity of yeast to the constitutively active version of *AtPKS4-T182D* relative to the unmodified *AtPKS4* is also of interest because yeast was used as a heterologous system. A complementary system to the *SOS* series of genes have not been identified in yeast. Whether the activity of this kinase in yeast is due to a SOS-like system that has not yet been identified, or whether this kinase might be acting on cellular components that differ from those in plants, is not known.

To better understand the effect of this gene in yeast, a series of tolerance and growth assays were performed with mutant yeast strains. To date, the only characterized vacuolar Zn transporters in *Saccharomyces cerevisiae* are ZRT3, COT1 and ZRC1. As previously mentioned ZRT3 is a member of the ZIP transporter family and plays a role in Zn remobilization from the vacuole to the cytoplasm (MacDiarmid et al., 2002). ZRC1 and COT1 are Zn transporters in the Cation Diffusion Facilitator (CDF) family that has been shown to play a role in Zn influx from the cytoplasm to the vacuole (Conklin et al., 1992, 1994; Kamizono et al., 1989; Li and Kaplan 1998).

The Arabidopsis SOS1 transporter belongs instead to the Cation/H^+ antiporter family, which has members in *S. cerevisiae*, but those genes that have been characterized have not been linked to vacuolar heavy metal transport (Mäser et al., 1999). Work on the SOS family has not identified the region of interaction between the kinase domain and transporters, leaving the mechanism of interaction unknown. Our work with the yeast mutant lines suggests that there may be interactions between other transporter families and this kinase. Experiments in *S. cerevisiae* looking at the effect of this gene in various mutant lines suggest that the COT1 transporter may be affected by the presence of the kinase as that mutant line was the only line that did not show a significant difference in growth rate or Zn accumulation between lines containing the empty vector or constitutively expressing *AtPKS4*. In this specific experiment there was only a limited effect of this gene on the wild type yeast strain, while in all the other experiments where *PKS4* was expressed in wild type yeast, an increase in tolerance and accumulation was seen.

We conducted two different sets of experiments looking at the effect of modifying the expression of the *PKS4* kinase, including repression or overexpression, on Zn tolerance and accumulation in transgenic Arabidopsis plants. The first set of experiments used one month old plants that were then grown for one week on a range of Zn concentrations. This approach did not yield any significant differences in root and shoot growth or Zn concentration between the wild type plants, and transgenic plants that either were overexpressing the non-modified *AtPKS4* or the 'constitutively active' *AtPKS4 T182D*, or transgenic lines where *PKS4* expression was reduced by dsRNAi. One possible shortcoming of the first experiment is that the one month period of growth on adequate Zn followed by the relatively short exposure (one week) to Zn deficient or excess conditions may not have been long enough to result in an altered phenotype. A potentially more effective approach to study the effect of altered

Zn status on plant growth and Zn accumulation is with short term studies using seedlings grown on agar plates containing nutrients and different Zn levels. The results of a preliminary experiment run with wild type plants and transgenic lines overexpressing *AtPKS4 T182D* grown on standard media and media supplemented with high Zn. As seen in Figures 4.11 and 4.12, the *AtPKS4 T182D* lines grew significantly more dry weight shoot biomass than wild type plants on high Zn media. It is of interest that there was no significant difference in the shoot Zn accumulation between the overexpression and wild type lines, despite the enhanced Zn tolerance in the overexpression lines. This might be due to the fact that in the overexpression lines where expression is driven by the 35S promoter, the gene is expressed in all tissues and cells. Hence, more Zn might have been stored in the root vacuoles of the transgenic lines, allowing for increased Zn tolerance without increased Zn accumulation in the shoots. It is clear that further work, perhaps based on hydroponic studies using MS media, or shorter term studies with the more physiologically relevant modified Johnson's nutrient solution should be conducted.

In summary, we describe here the isolation and characterization of a kinase implicated in moderating Zn tolerance in both yeast and plants. While a number of further studies are necessary for a better elucidation of the role of this gene *in planta*, the initial work presented here in both yeast and in plants suggests the existence of a new pathway in plants that plays a role in metal tolerance. It is possible that manipulation of this pathway once elucidated, may be useful for developing more metal tolerant plants for the phytoremediation of heavy metal contaminated soils.

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CHAPTER V

Investigation of Heavy Metal Hyperaccumulation at the Cellular Level: Development and Characterization of *Thlaspi caerulescens* Suspension Cell Lines

INTRODUCTION

The heavy metal hyperaccumulation phenotype seen in *Thlaspi caerulescens* is the result of physiological differences at the whole plant, organ and cellular levels relative to non-hyperaccumulator plant species (Küpper et al., 1999; Lasat et al., 1996, 1998). These differences lead to the accumulation of as much as 40,000 ppm Zn and 10,000 ppm Cd dry weight in shoot tissues. Most non-hyperaccumulator plant species only accumulate 100-300 ppm Zn and 0.1-10 ppm Cd foliar concentrations (see Kochian et al. 2002 and references therein). These differences in Zn and Cd content relative to non-hyperaccumulators is due, in part, to enhanced metal transport at a number of different sites within the plant, including the root surface, xylem loading and reabsorption of xylem-born metals by leaf cells (Lasat *et al*. 1996, 1998). These differences are also seen at the molecular level with higher expression of metal transporter genes in both roots and shoots of *T. caerulescens* relative to *T. arvense* (Assunção et al. 2001; Pence et al. 2000). For example, expression of the Zn/Cd transporter, *ZNT1*, is much greater in roots and shoots of *T. caerulescens* than in *T. arvense* and down-regulation of *ZNT1* expression by increased plant Zn status requires accumulation of much higher levels of Zn in *T. caerulescens* plants (Pence et al., 2000). *TcMTP1*, a vacuolar metal efflux transporter, also shows significantly higher levels of gene expression in *T. caerulescens* relative to related non-hyperaccumulators (Assunção et al., 2001).

At the organ level, a number of studies have examined Zn/Cd accumulation within leaves of *T. caerulescens*. In a study by Cosio et al., (2005), the *T. caerulescens* ecotypes Ganges and Prayon were grown hydroponically with ^{109}Cd included in the hydroponic media to allow visualization of Cd accumulation within the plant via autoradiography. In this study, Cd was found to be localized to the leaf margins in both ecotypes with additional Cd accumulation seen as spots across the adaxial and abaxial sides of the leaf blade. The distribution of Cd remained unchanged over the thirty day exposure period, suggesting that Cd preferentially accumulates in specific regions of the leaf and does not remobilize (Cosio et al., 2005). Other studies of heavy metal storage in *T. caerulescens* foliar tissue have determined that Zn and Cd are sequestered in the apoplast and, to a greater extent, in a soluble form within the vacuoles of non-photosynthetic epidermal cells (Frey et al., 2000, Küpper et al., 1999, Ma et al., 2005). Physiological investigations have shown that at high Zn concentrations, Zn influx into leaf protoplasts was significantly greater in *T. caerulescens* protoplasts compared to those from *T. arvense* leaves (Lasat et al., 1998), while for the Cd accumulating *T. caerulescens* ecotype, Ganges, pre-exposure of plants to Cd resulted in enhanced leaf protoplast Cd influx (Cosio et al., 2004).

Most studies examining the physiological activity of *T. caerulescens* at the cellular level have used protoplasts (Cosio et al., 2004; Lasat et al., 1998; Ma et al., 2005; Marques et al., 2004; Piñeros and Kochian, 2003). While these are attractive systems for single cell studies, the release of protoplasts from differentiated tissue or organs is highly perturbative and probably results in significant alterations in cellular transport physiology. Other attempts to examine heavy metal tolerance have used suspension cell lines derived from non-tolerant species grown under heavy metal stress to create tolerant cell lines (Al: Conner and Meredith 1985, Devi et al., 2001, Martinez-Estevez et al., 2003; Ni: Gabbrielli et al., 1995, Nakazawa et al., 2004, Saito

et al., 2005; Cr/Ni: Samataray et al., 2001; Mn: Petolino and Collins 1984; Cu/Zn: Kishinami and Widholm 1987). While these studies produced suspension cell lines with improved metal tolerance, the resulting physiological changes may not represent the mechanisms of accumulation and tolerance used by metal hyperaccumulators. Work examining Zn accumulation in suspension cell lines of *Nicotiana plumbaginifolia* found evidence for metal-induced increased tolerance due to increased production of citrate (Kishinami and Widholm, 1987). Constitutively elevated concentrations of organic acids have been noted by a number of researchers in studies examining Zn tolerance in *T. caerulescens* (Boominathan and Doran, 2002; Shen et al., 1997; Tolra et al., 1996). Work by Shen et al., (1997) found high but variable levels of citrate and malate in both roots and shoots of the related non-accumulator *T. ochroleucum* as well as in *T. caerulescens*, suggesting that this might be a general trait of *Thlaspi* and not linked to enhanced metal tolerance and hyperaccumulation.

In this study, the creation and characterization of *Thlaspi caerulescens* suspension cell lines is reported. The physiological and molecular responses of these undifferentiated cell lines relative to an *A. thaliana* suspension cell line were examined through both short and long term physiological experiments in response to a range of Zn and Cd concentrations.

METHODS AND MATERIALS

Creation of Suspension Cell Lines

T. caerulescens (Prayon) seeds were sterilized with a 5% bleach solution for 5 minutes, washed with sterile 18 MΩ H_2O and then sown on either low Zn or high Zn Murashige and Skoog (MS) plates made up of MS salts and vitamins, 3% sucrose, 0.7% phytagar (Gibco BRL), 0.5-2.0 mg/L 2,4-D, 0-1.0 mg/L kinetin with pH 5.8

(adjusted with KOH). The total Zn concentration (30 μM) in the low Zn media came from the Zn included in the MS salts. To produce high Zn MS plates, an additional 270 μL/L of 1M $ZnSO_4$ solution was added to the media (final Zn concentration of 300 μM). Seeds were incubated in the dark at 25ºC until callus formation occurred. Two months after sowing, calli produced from seeds (embryos) or seedlings (cotyledons or roots) were divided and vigorous calli were selected to be subcultured onto fresh plates. Media used for calli growth was the same as that used in calli-inducing plates except the agar was increased to 0.8%. One month after the first subculture, large and vigorous callus aggregates were selected and transferred to liquid culture media. The liquid culture media was identical to the media used for the MS plates without agar. Suspension cells were regularly subcultured over a six month period before the start of experiments. *Arabidopsis thaliana* (Ler) suspension cells were provided by the laboratory of Dr. Roger Innes (Indiana University).

Characterization of Suspension Cell Lines

Culture Conditions. Both *Thlaspi* and Arabidopsis suspension cells were grown on modified MS media of the following compositions: *Thlaspi* high Zn media (TcZn) consisted of MS salts and vitamins, 3% sucrose, 1 mg/L 2,4-D, 1 mg/L kinetin, and 270 μL/L 1 M $ZnSO_4$ for a final Zn concentration of 300 μM, pH 5.8; *Thlaspi* low Zn media (TcMS) consisted of MS salts and vitamins, 3% sucrose, 1 mg/L 2,4-D, and 0.5 mg/L kinetin, pH 5.8, with a final Zn concentration of 30 μM; Arabidopsis media (AtMS) consisted of MS salts, Gamborg vitamins, 3% sucrose and 1 mg/L 2,4-D, pH 5.7, with a final Zn concentration of 30 μM. All suspension cell cultures were shaken at 120 rpm at 20-24ºC in the dark using a rotating shaker. Unless otherwise stated, all treatments were in 30mL of media in 125 mL Erlenmeyer flasks. At harvest, cells were vacuum filtered through Whatman #40 paper in a

Büchner funnel, washed briefly with 18 MΩ dH_20 and then with a wash solution of 5 mM $CaCl_2$ and 3% sucrose. The fresh weight from each flask was determined after filtration, cells were lyophilized, the dry weight was determined and then cells were then transferred to quartz tubes for metal analysis by ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry).

Time Course Growth Curves. Growth curve studies were run with a starting cellular concentration of 2.5 mg fresh weight cells/mL. Cells from replicate flasks were harvested every three days starting on day 0 and continuing until day 24 and the cell lines were harvested and handled as outlined above.

Long Term Zn and Cd Tolerance. Long term studies examining tolerance and accumulation of Cd and Zn were initiated at a cell density of approximately 3.3 mg fresh weight cells/mL and grown for 10 days. Cell lines were treated with a range of Zn and Cd concentrations (30-1500 μM Zn and 0-50 μM Cd) with filter-sterilized heavy metal stocks added directly to flasks following media autoclaving to avoid metal precipitation.

Short Term Cd Influx and Efflux. TcZn and AtMS suspension cell lines for both experiments were subcultured in 250 mL Erlenmeyer flasks in 80 mL total volume and an initial cell density of approximately 3.3 mg fresh wt cells/mL. On day 6, cells for Cd efflux measurements were treated with Cd (10 μM $CdCl_2$ final concentration) to begin a twenty hour exposure period prior to efflux treatment. Subsequently, the Cd efflux experiments were initiated by vacuum-filtering the cells, rinsing them with 25 mL of Cd-free suspension cell media and then resuspending them in 60 mL of Cd-free suspension cell media. 10 mL aliquots were taken at specified times, vacuum filtered using Whatman #40 filter paper in Büchner funnels, washed with 10 ml of 5 mM $CaCl_2$, collected, frozen and lyophilized. Dry weights were determined and the samples were analyzed by ICP-AES for Cd content. Cd influx

experiments were conducted on day seven with the zero time point taken immediately before Cd treatment. After exposure of cells to the medium containing 10 μM $CdCl_2$, 10 mL samples were collected at the specified time points to determine Cd uptake rates. The Cd influx was terminated by vacuum filtering the cells, washing them with 10 mL of 5 mM $CaCl_2$, after which they were vacuum filtered and lyophilized. Dry weights were determined and samples were analyzed by ICP-AES for Cd content.

Molecular Characterization. TcZn and AtMS suspension cells were subcultured into 250 mL Erlenmeyer flasks in 80 mL of total culture volume and an initial cellular concentration of approximately 3.3 mg fresh wt cells/mL. The *Thlaspi* (TcZn) and Arabidopsis (AtMS) suspension cells were grown in either low, standard or high Zn media or standard media containing 10 μM Cd (Zn concentration for low, standard and high Zn TcZn media: 30 μM, 300 μM and 1000 μM Zn, respectively; Zn concentration for low, standard, and high Zn AtMS media: 30 μM Zn with 120 μM EDTA, 30 μM, and 300 μM Zn, respectively). Suspension cells were harvested on day seven by vacuum filtration through Miracloth (Calbiochem, San Diego, CA), washed once with 18 MΩ H_2O and then flash frozen in liquid N_2. RNA was isolated using Trizol following the manufacturer's directions (Invitrogen, Carlsbad, CA), except that for RNA isolation from *T. caerulescens* cells 1.2 M NaCl and 0.8 M citrate with equal volume isopropanol was added to prevent sugar precipitation. Isolated RNA was treated with DNAaseI Amplification grade (Invitrogen, Carlsbad, CA) and then run on a Qiagen RNAeasy column (QIAGEN Inc., Valencia, CA). Semi-Quantitative RT-PCR with 500 ng of total RNA was then performed using the Invitrogen One Step RT-PCR kit following manufacturer's directions. The primer concentration was 10 μM for each gene specific primer set (Table 5.1). Primers were designed for homologous regions of the genes of interest and designed to amplify regions ranging from 300 to 1000 bp. Initial control reactions showed that under the

chosen conditions, all PCR products were in the linear amplification range (data not shown). The experiments were repeated using three biological replicates and duplicate RT-PCR experiments were conducted for each gene with each biological replicate.

RESULTS

Creation of Suspension Cell Lines

To date, there have been no published attempts to produce calli or suspension cells from *Thlaspi* species. Murashige and Skoog (MS) and Linsmaier and Skoog (LS) media have previously been used to successfully produce calli from other *Brassica* species including *A. thaliana* (Encina et al., 2001; Jain et al., 1988; Toriyama et al., 1987). *T. caerulescens* calli and suspension cells were produced from seeds and seedlings germinated on MS media under both low and high Zn concentrations. Calli were subcultured and then those cells showing vigorous growth were transferred to liquid MS media for long term maintenance of suspension cell lines. High Zn grown *T. caerulescens* calli showed greater fresh weight production relative to cell lines grown under low Zn conditions.

Characterization of Suspension Cell Lines

Suspension Cell Growth Rates. Following creation of the *T. caerulescens* suspension cell lines, a time course experiment was performed to examine the rates of growth for *T. caerulecens* cells in standard MS media (TcMS) and Zn supplemented MS media (TcZN) relative to Arabidopsis suspension cells grown on standard MS media (AtMS). The time course experiments were started with an initial cell density of 75 mg fresh wt per 30 mL media and spanned 24 days. As seen in Figure 5.1A, Arabidopsis suspension cell lines produced significantly higher fresh weight biomass

Table 5.1 Forward and reverse specific primers used for the RT-PCR amplification of the Zn transporter cDNAs, the size of the amplified fragment and annealing temperatures.

Gene	Primer Sequences	Fragment Size	PCR cycle	$T_{annealing}$
HMA4	(fwd) 5'-GAAGAAGTTGAAGTAGATGAG-3' (rev) 5'-GATTGCTGGAGTATAGTACTGAGAACATTT-3'	332	33	58
MTP1	(fwd) 5'-AGGCAGACTTACGGGTTCTTCA-3' (rev) 5'-TCCTCCAATCATAACACCAAC-3'	503	20	58
ZNT1	(fwd) 5'-ATCCTCTGTGATGCTGGCGAATC-3' (rev) 5'-CAGGGCTATGCGAGTTGAAAGA-3'	924	25	58
Actin	(fwd) 5'-GAAGAACTACGAGCTACCTGATG-3' (rev) 5'-GATCCTCCGATCCAGACACTGTA-3'	320	22	58

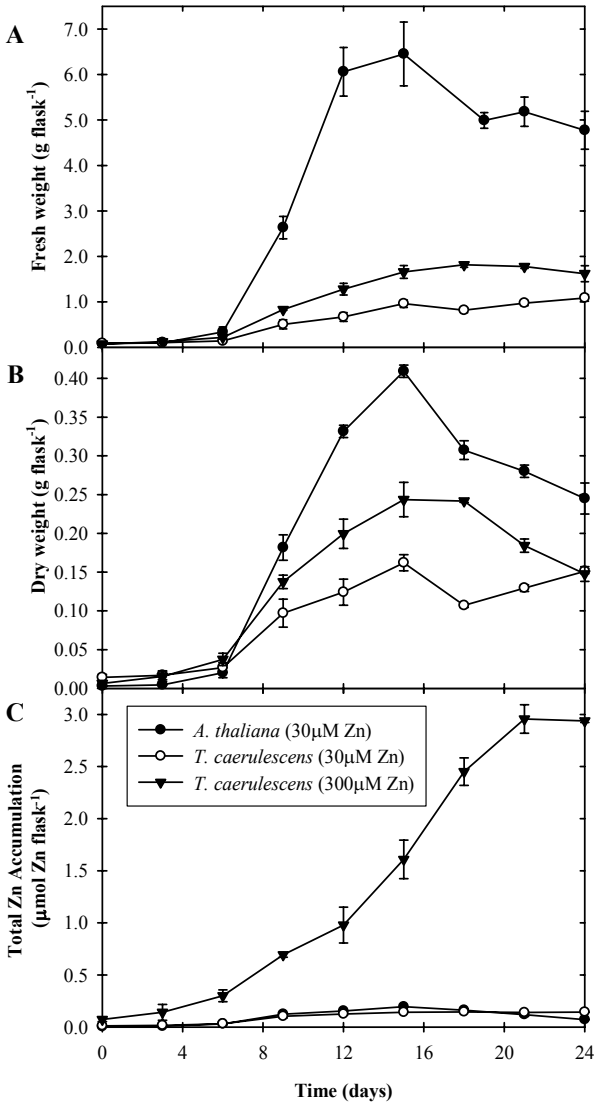

Figure 5.1. Time course for growth and metal (Zn) accumulation in suspension cell lines of *Thlaspi caerulescens* and *Arabidopsis thaliana*. For the growth and accumulation data depicted here, suspension cell lines were grown in liquid MS media as described in the Materials and Methods. Arabidopsis media (AtMS) had a total Zn concentration of 30 µM while the Zn concentration in the *T. caerulescens* media was 30 or 300 µM Zn for the TcMS and TcZn media lines, respectively. **A)** Suspension cell growth over time as measured by fresh weight increase for the Arabidopsis cell line grown on standard MS media and for *T. caerulescens* cell lines grown on standard MS media or on MS media supplemented with $ZnSO_4$ to a final Zn concentration of 300 µM. **B)** Suspension cell growth over time as measured by dry weight increase. All the conditions are as described in the legend for Figure 5.1A. **C)** Time course for suspension cell Zn accumulation during the course of the 24 day growth experiment. Cellular growth and Zn accumulation were determined on a per flask basis for each set of suspension cells on the particular day. All data points are the mean ± SE (n=3-4)

compared to either *T. caerulescens* line, although the difference was less based on dry weights (Figure 5.1B). For the cell lines examined, the TcMS cells grew the slowest. When the total Zn concentration was at 300 μM for TcZN cells, the growth rate increased although it still was somewhat slower than the growth of the AtMS suspension cells on 30 μM Zn. When total cellular Zn accumulation over the course of the 24 day experiment was determined, the TcMS and AtMS cell lines grown on 30 μM Zn exhibited similar, low rates of total Zn accumulation, while the TcZN cells grown on a much higher Zn concentration (300 μM Zn) not surprisingly accumulated much more Zn (Figure 5.1C). These differences in growth rate and Zn accumulation among the three lines suggest that limited Zn availability and subsequent lower levels of Zn accumulation might be a limiting factor in the growth of *T. caerulescens* on standard MS growth media and possibly the Zn supplemented media as well.

Long Term Growth in Response to a Range of Zn Concentrations. When TcZN and AtMS suspension cells were grown for 10 days in MS media with Zn concentrations varying from 30 μM to 1.5 mM Zn, the TcZN cells exhibited no significant difference in growth rate even when grown on 1.5 mM Zn, while the growth of AtMS suspension cells was inhibited at all Zn concentrations greater than 30 μM (Figure 5.2A). AtMS cells grown on 1.5 mM Zn showed a 96% reduction in growth. The TcZN cells accumulated considerably less Zn on a per weight basis since for example, the Zn concentration in TcZn suspension cells was approximately 30% that in AtMS cells at Zn concentrations higher than 300 μM (Figure 5.2B). However, because the TcZn cells are more Zn tolerant and grow better at the high Zn concentrations, the total cellular Zn accumulation per flask was higher in the TcZn cells at the two highest Zn concentrations (1 and 1.5 mM Zn). As seen in Figure 5.2C, at 1.5 mM Zn, the TcZN cells and the AtMS cells accumulated 7.8 μmol Zn per flask, and 3.2 μmol Zn per flask, respectively.

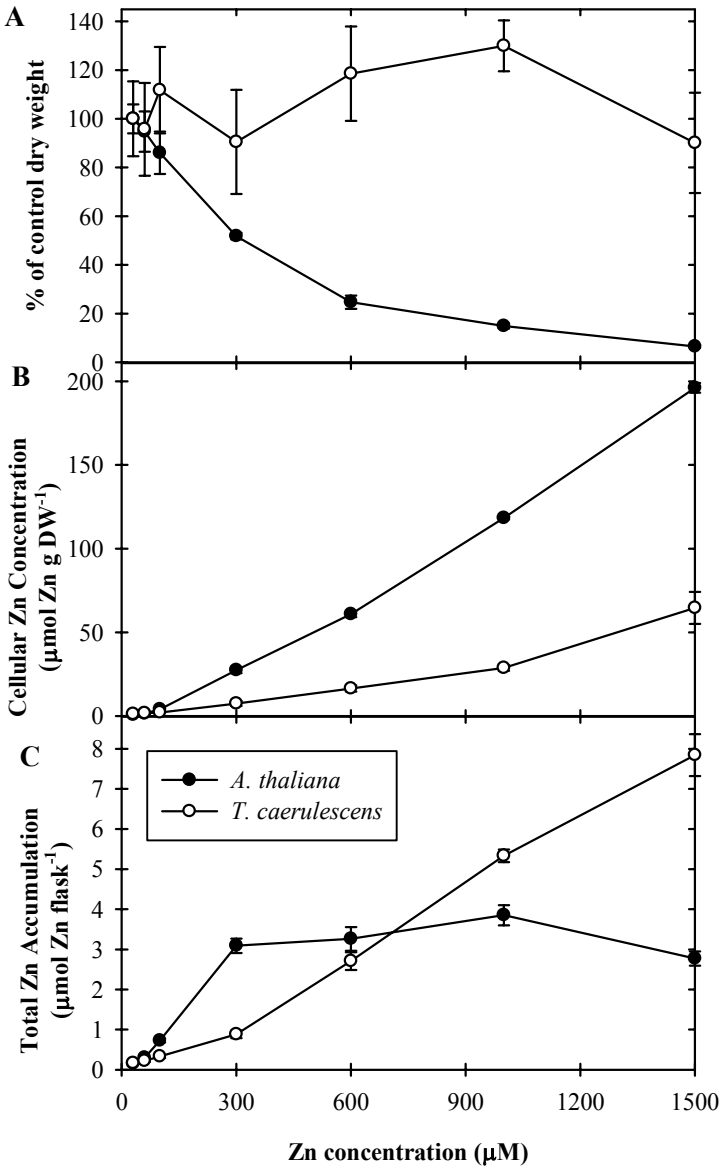

Figure 5.2. The influence of increasing Zn concentration in the liquid growth media (ranging from 30 to 1500 µM Zn) on cell growth and Zn accumulation for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells. A) Suspension cell growth presented as the % of biomass (dry weight) accumulation at the specific Zn concentration relative to growth on the standard MS media where the Zn concentration was 30 µM. B) Cell Zn accumulation as determined by changes in suspension cell Zn concentration for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells in response to growth on 30 to 1500 µM Zn. C) Total Zn accumulation for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells in response to growth on 30 to 1500 µM Zn based on the amount of Zn accumulated on a per flask basis. All data points are the mean ± SE (n=2-3)

Long Term Growth in Response to a Range of Cd Concentrations. When grown for 10 days in MS media containing Cd concentrations between 0 and 50 μM, both TcZN and TcMS suspension cells exhibited a significant increase in growth in response to increasing Cd levels, while the Arabidopsis suspension cells (AtMS) showed a significant inhibition of growth (>50% inhibition at 50 μM Cd; Figure 5.3A). At the higher Cd concentrations, the Arabidopsis suspension cells accumulated significantly greater amounts of Cd than either *T. caerulescens* line (Figure 5.3B). Even with the decrease in biomass, the AtMS cells accumulated greater total amounts of Cd on a per flask basis compared to either *T. caerulescens* line (Figure 5.3C).

Short Term Cd Influx and Efflux. Based on the findings presented in Figures 5.2 and 5.3, it appears that the Arabidopsis suspension cell line accumulates more Zn and Cd than do the suspension cells derived from the hyperaccumulator, *T. caerulescens*. This could be due to an increased Cd influx, decreased efflux, or a combination of both in the Arabidopsis cell line. To test these hypotheses, both short term Cd accumulation and efflux were determined for the TcZN and AtMS suspension cell lines. In Figure 5.4A, the time course of cellular Cd accumulation over the first 5 minutes was determined. From these data, it appears that similar initial rates of Cd influx are seen in the *T. caerulescens* and Arabidopsis suspension cells. For the Cd efflux experiments, both suspension cell lines were allowed to accumulate Cd for 20 h prior to the initiation of the Cd efflux experiment. At the start of the efflux period, the AtMS cells had accumulated 177 ± 4 nmol Cd/g DW while the TcZN cells had accumulated 131 ± 4 nmol Cd/g DW. After a 5 min efflux period into Cd free media, the Arabidopsis cells had maintained a Cd concentration of 168 ± 2 nmol Cd/g DW(a loss of 5%) while the *Thlaspi* cells exhibited a significantly greater Cd efflux, with a loss of 30% of their total accumulated Cd after the 5 min efflux period (a final cellular Cd concentration of 92.5 ± 5 nmol Cd/g DW) (Figure 5.5).

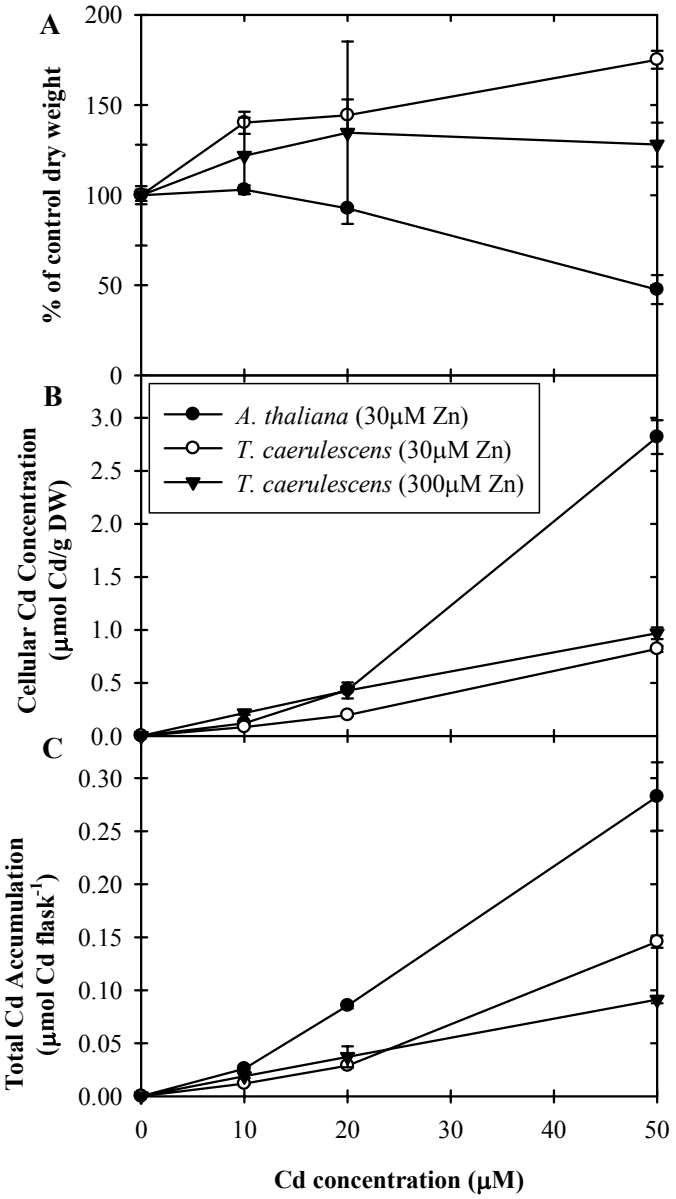

Figure 5.3. The influence of increasing Cd concentration in the liquid growth media (ranging from 0 to 50 µM Cd) on cell growth and Cd accumulation for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells. A) The influence of increasing Cd concentrations on suspension cell growth presented as the % of biomass (dry weight) accumulation at the specific Cd concentration relative to growth on the standard MS media without Cd. B) Cell Cd accumulation as determined by changes in suspension cell Cd concentration for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells in response to growth on 0 to 50 µM Cd. C) Total Cd accumulation for *T. caerulescens* (TcZn) and Arabidopsis (AtMS) suspension cells in response to growth on 0 to 50 µM Cd based on the amount of Cd accumulated on a per flask basis. All data points are the mean ± SE (n=2-3)

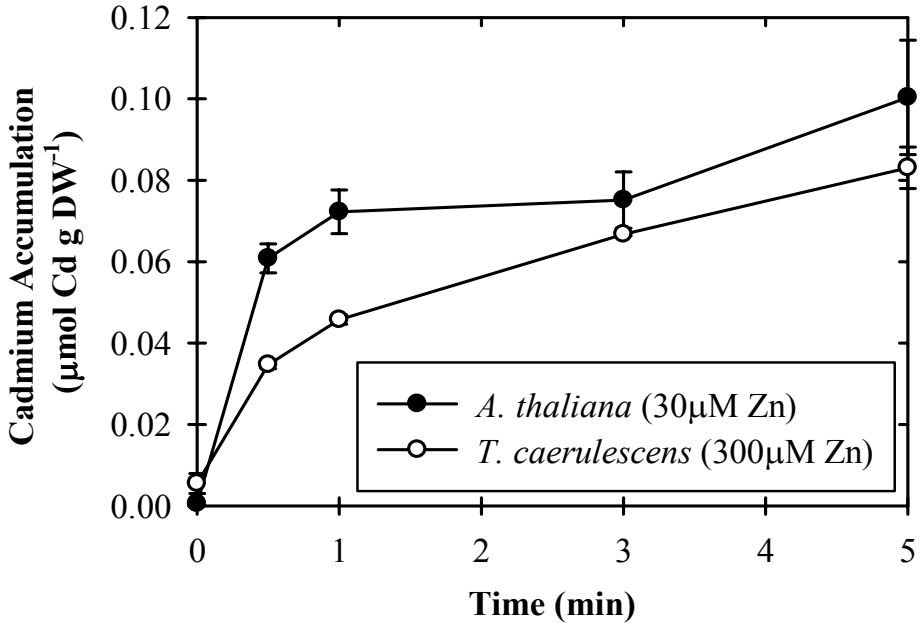

Figure 5.4. Time course for Cd influx from *T. caerulescens* and Arabidopsis suspension cells. Seven day old cells growing on TcZn or AtMS media (for *T. caerulescens* or Arabidopsis cells, respectively) were sampled just before addition of Cd to the uptake solution, and $CdCl_2$ was added to a final concentration of 10 µM. 10mL cell aliquots were then taken each minute for the 5 min uptake period. At the specified time points, sampled cells were vacuum filtered, desorbed to remove cell wall Cd, dried, weighed and Cd content was determined by ICP-AES. Three separate biological flasks were quantified for each line. Data points represent mean of time points taken from three different flasks ± SE (n=3)

Transporter Gene Expression in the Two Cell Lines. In these studies of transporter gene expression in response to changes in cellular Zn (ranging from Zn deficiency to high Zn concentrations) and Cd status, it was first necessary to determine conditions under which Zn deficiency could be imposed. To do this, the GeoChem PC speciation program (Parker *et al*. 1995) was used to analyze the effect of EDTA (ethyleneditetraacetic acid) as a Zn^{2+} chelator to lower the Zn^{2+} activity level and create a medium that would impose Zn deficiency conditions. It was determined that an EDTA concentration of 120 µM would lower the Zn^{2+} activity in the MS media to levels approximately 10% of the activity levels seen in standard MS media, an activity

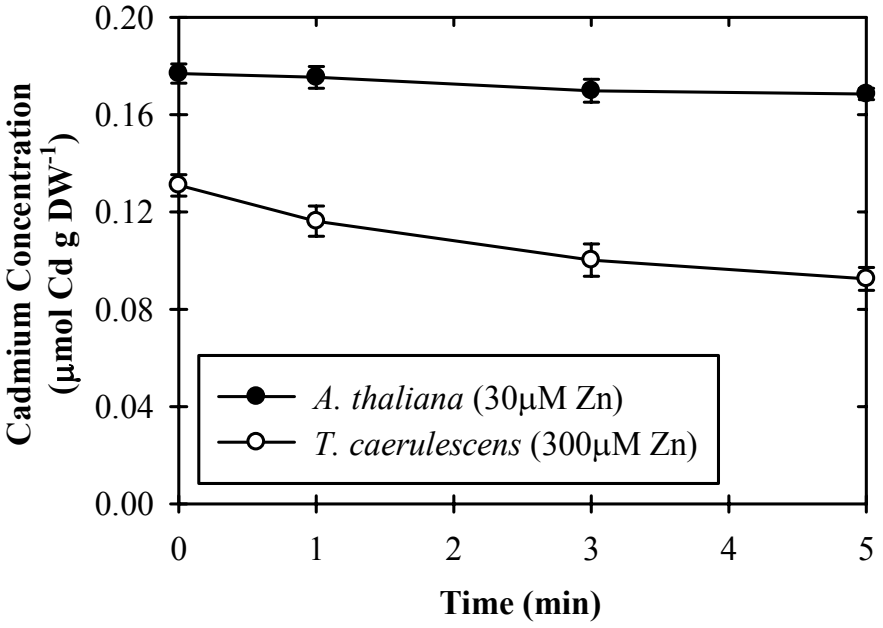

Figure 5.5. Time course of Cd efflux from *T. caerulescens* and Arabidopsis suspension cells. After a 20 h incubation in TcZn or AtMS media (for the *T. caerulescens* or Arabidopsis cells, respectively) containing 10 µM $CdCl_2$, the 7 day old cells were washed then resuspended in Cd free MS media and 10 mL aliquots were taken at each minute over the 5 min efflux period. Aliquoted cells were vacuum filtered, briefly washed to remove solution adhering to the cells, dried, weighed and Cd content was determined by ICP-AES. Three separate biological flasks were quantified for each line. Data points represent mean of time points taken from three different flasks ± SE (n=3)

level that should impose Zn deficiency conditions . Using this medium, it was found that Arabidopsis suspension cell growth was reduced by 70% and total Zn accumulation by 50%, compared to cells grown on standard MS (results not shown). Based on these results, EDTA was added to a final concentration of 120 µM to create a low (deficient) Zn medium for Arabidopsis suspension cells.

To examine the transporter transcription levels of these *T. caerulescens* suspension cells, the expression of three previously studied Zn transporters implicated in Zn hyperaccumulation and tolerance for *T. caerulescens* was examined: the P-type ATPase *HMA4*, the CDF family member *MTP1* and the ZIP family member, *ZNT1*.

To limit differential hybridization, PCR primers were designed to exactly match the *T. caerulescens* and the *A. thaliana* (Ler) coding sequences for each transporter as well as the coding sequences for an actin gene from each species, which was used as a control.

Based on the semi-quantitative RT-PCR results shown in Figure 5.6, both constitutive and Zn status-dependent differences were seen in the expression of these metal transporters in *T. caerulescens* and *A. thaliana* suspension cells. *HMA4* showed high transcription levels independent of cellular Zn or Cd status in the TcZN cell lines compared with the AtMS cells. Expression of *MTP1* also showed higher constitutive expression in TcZN suspension cells relative to AtMS cells; both sets of *MTP1* expression patterns were not affected by metal (Zn and Cd) status. Both *HMA4* and *MTP1* were also found to be highly expressed in the intact *T. caerulescens* plant compared with related nonaccumulator species (Assunção et al., 1999; Papoyan and Kochian, 2004). The expression of *ZNT1*, a member of the ZIP transporter family was found to be highly expressed in *T. caerulescens* suspension cells, and expression in both species was repressed as cellular Zn status increased. As expression of the actin gene, used as an internal standard, was significantly greater in *A. thaliana* cells, when *ZNT1* expression was normalized to actin expression, the upregulation of *ZNT1* expression was even more pronounced in TcZN relative to AtMS suspension cells than that depicted in Figure 5.6. High expression of *ZNT1* under low to sufficient Zn conditions in *T. caerulescens* plants compared with *T. arvense* has been previously documented (Pence et al., 2000). Overall, the expression patterns for these three genes in suspension cells followed the general expression patterns previously determined in *T. caerulescens* and *A. thaliana* plants and showed high expression of key genes in *T. caerulescens* compared with non-accumulator plant species that has been commonly observed in whole plants is also seen at the cellular level.

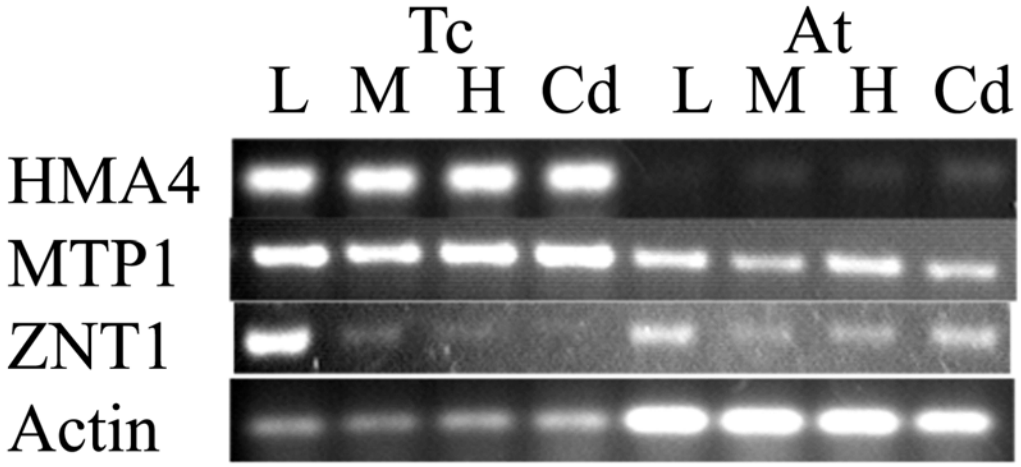

Figure 5.6. Semi-quantitative RT-PCR of *T. caerulescens* (TcZN) and *A. thaliana* (AtMS) suspension cells grown in MS media that varied in Zn or Cd. Low Zn treatment was growth on standard SD media with a Zn concentration of 30 μM, Medium Zn treatment was growth on SD media containing 300 μM Zn, and High Zn treatment was growth on SD media containing 1000 μM Zn. For the Cd treatments, 10 μM $CdCl_2$ was included in the growth solution. See Materials and Methods for details concerning the media composition. The number of PCR cycles run to compare expression between *T. caerulescens* and Arabidopsis was 33 for HMA4, 20 for MTP1; 25 for ZNT1 and 22 for actin. For each of two biological replicates collected, RNA was isolated from 80 mL of suspension cells grown in a 250 mL flask for 7 days. RNA from each biological replicate was used to run three replicate PCR reactions. The images shown are representative of the results obtained from each biological replicate.

DISCUSSION

In this study, suspension cell lines were developed for the metal hyperaccumulator, *Thlaspi caerulescens*, in order to investigate whether the extreme metal tolerance/accumulation phenotype seen in this plant is a characteristic seen at the single cell level, or whether hyperaccumulation requires additional functional coordination of different cells, tissues and organs in the plant. From the physiological and molecular studies conducted here comparing suspension cell lines derived from *T. caerulescens* and the related non-accumulator, *A. thaliana*, it is clear that a number of

the whole plant traits are expressed at the cellular level, including increased tolerance to Zn and Cd, and a higher Zn requirement for growth. It has previously been demonstrated that intact plants of *T. caerulescens* are "Zn inefficient", in that they require more Zn for normal biomass production compared with the related non-accumulator plant, *T. arvense*, (Ozturk et al., 2003). This high Zn requirement is also seen at the cellular level in the current study, comparing undifferentiated suspension cells for *T. caerulescens* and Arabidopsis. As seen in Figure 5.1, when grown on standard MS media, where the total Zn concentration is 30 μM (a Zn^{2+} activity of approximately 0.37 nM), the Arabidopsis suspension cells grew better than the *T. caerulescens* cell lines that had been previously subcultured on low Zn (30 μM) or high Zn (300 μM) media. When the total Zn concentration in the liquid MS media was increased to 300 μM, the *T. caerulescens* cell lines showed improved growth, which was similar to, but still not as rapid as, the Arabidopsis cell line growth on 30 μM Zn. These findings suggest that there are fundamental differences in Zn homeostasis in *T. caerulescens* relative to non-hyperaccumulating plant species. This is also suggested from the Zn accumulation data shown in Figure 5.1, as on the low Zn media with a Zn concentration of 30 μM, both the Arabidopsis and *Thlaspi* cell lines had similar, low Zn accumulation that remained unchanged after approximately twelve days of growth (Figure 5.1C). However, when the *Thlaspi* cell lines were grown on high Zn media, Zn accumulation by the TcZN cells increased until day 21 and this accumulation was approximately 50-fold greater than the accumulation of either the TcMS or AtMS lines grown on low Zn. This constant increase in Zn accumulation seen by the TcZN line until day 21, suggesting that Zn present in the media continues to be taken up by the cells even after the cells enter the stationary growth phase.

The long term studies examining cell growth and Zn/Cd accumulation in response to Zn levels in the MS media, ranging from 30 μM to 1.5 mM Zn, or Cd

concentrations ranging from 0 to 50 μM, showed that the *T. caerulescens* cell lines are more heavy metal tolerant than the Arabidopsis cell line (Figures 5.2 and 5.3). Even at the highest Zn concentration of 1.5 mM, the *T. caerulescens* suspension cells showed no significant decrease in growth while the Arabidopsis cell line was strongly inhibited, with a greater than 90% inhibition of cell growth. In contrast, in response to Cd, the *T. caerulescens* cell lines exhibited a stimulation of growth that was as high as 50-75% at the highest Cd concentration (50 μM), while in response to 50 μM Cd, growth of the Arabidopsis suspension cell line was inhibited by more than 50% (Figure 5.3).

With regards to heavy metal transport in intact *T. caerulescens* plants, it has been well documented that there are a number of changes in metal transport at different sites in the plant, compared with the related non-accumulator, *T. arvense*. Lasat et al., (1996; 1998) showed that for Zn transport these transport alterations in *T. caerulescens* include: 1) greater Zn uptake into the root; 2) less Zn accumulation in the vacuole of root cortical cells, which presumably provides a large "mobile" pool of Zn for radial movement to the root xylem; 3) greater xylem loading of Zn and associated larger xylem transport to the shoot; and 4) larger Zn uptake into leaf cells, which presumably reflects uptake into leaf epidermal cells, where a significant fraction of the Zn is stored.

Interesting differences in Zn and Cd transport were also observed in *T. caerulescens* suspension cells compared with the Arabidopsis cell line. As depicted in Figures 5.2 and 5.3, individual *T. caerulescens* cells exhibited a significantly lower Zn and Cd accumulation, which does not match with the expectation that cells from a hyperaccumulator would accumulate more, not less, of these heavy metals. As the long term Zn and Cd accumulation studies reflect a net flux that is due to metal influx minus efflux, these differences in net Zn and Cd accumulation were examined in more

detail via short term Cd accumulation and efflux studies over the first 5 min of uptake and efflux. This would allow a comparison of unidirectional Cd influx and efflux in the two cell lines. The short term time course for Cd accumulation (Figure 5.4) indicated that the time course is biphasic, with an initial rapid uptake over the first minute, which presumably is dominated by Cd entry and binding in the cell wall (see, for example, Hart et al., 1998). Subsequently, over the next four minutes a slower linear phase of Cd uptake was seen which is presumed to be dominated by Cd influx across the plasma membrane (Hart et al., 1998). Based on this analysis, it appears that there might be a slight increase in Cd influx for Arabidopsis relative to *T. caerulescens* suspension cells. However, there is a much bigger difference in Cd efflux between Arabidopsis and *Thlaspi* suspension cells (Figure 5.5), as *T. caerulescens* suspension cells had released nearly 30% of the Cd accumulated prior to the efflux period, while the Arabidopsis cell line only lost approximately 5% of the accumulated Cd.

When gene expression is compared between the two cell lines, the high expression of *HMA4* in the *T. caerulescens* cells is striking and may offer an explanation for the decreased metal accumulation in the *Thlaspi* suspension cells. It has been shown in several studies that HMA4 in Arabidopsis and *T. caerulescens* is a plasma membrane transporter that mediates the efflux of Zn, Cd and other heavy metals (Hussain et al., 2004; Papoyan and Kochian, 2004). In whole plants, HMA4 is expressed in the root vasculature and it has been suggested that it is involved in xylem loading of metals. This leads to the suggestion that the suspension cells derived here from *T. caerulescens*, while expected to be undifferentiated, reflect the transport properties of xylem parenchyma cells. It is also possible that a general characteristic of *T. caerulescens* cells that are not located at critical sites for metal uptake such as the root and leaf epidermis are to exclude metals from the cell. The lower rates of Zn and Cd accumulation combined with the greater short term Cd efflux rates and higher

constitutive levels of *HMA4* expression (Figure 5.6) in these *T. caerulescens* cells does suggest a general tendency towards excluding Zn and Cd along with the ability to tolerate higher concentrations. Considering that high levels of Zn/Cd accumulation only appear to occur in specific *T. caerulescens* epidermal and mesophyll cells, the ability to take up the heavy metals from the soil and transport them to storage cells requires that a great number of intermediary cells in the transport pathway from the root to the leaf epidermis do not retain the Zn or Cd, but rather keep it in a mobile pool to facilitate the very efficient root-to-shoot metal transport. Non-accumulator species such as Arabidopsis, typically sequester heavy metals in the root to prevent the excess heavy metal from reaching the shoot and leaves. One of the most sensitive processes negatively affected by heavy metals is photosynthesis and it appears that non-accumulators limit metal availability to photosynthetic apparati by root sequestration, or by sequestration in the stem of the shoot. The Zn and Cd transport properties of the Arabidopsis suspension cells appears to reflect this feature.

Recent research efforts have attempted to make comparisons of gene expression associated with metal tolerance and accumulation on a genome wide level in metal hyperaccumulating species relative to related non-accumulator species. Using an Affymetrix *A. thaliana* microarray, transcript profiling of roots and shoots of the hyperaccumulators *Arabidopsis halleri* and *T. caerulescens* or *A. thaliana* and *T. arvense* showed that there was a high expression of a number of metal tolerance and accumulation genes in the hyperaccumulator species relative to the non-hyperaccumulator species (Becher et al., 2004; Weber et al., 2004; Hammond et al., 2006). Metal transporters, including members of the ZIP, NRAMP, CDF and P-type ATPase families were found to be upregulated in these studies in the hyperaccumulators. This altered regulation of gene expression seen at the whole plant level is also present at the cellular level, as evidenced by the upregulation of

transporter genes in the *T. caerulescens* suspension cell lines presented here. Understanding the factors involved in the transcriptional regulation of candidate hyperaccumulation genes, including the roles of promoter elements and transcription, will be an important future area of investigation.

The use of *T. caerulescens* in genetic studies as a model metal hyperaccumulating plant species has been limited by a number of factors including a lengthy life cycle, a long period of vernalization to induce flowering, low fertility, and a low transformation efficiency (Assunção et al., 2003; Peer et al., 2003). One possible method to better understand the role and contribution of specific genes to heavy metal hyperaccumulation may be through the transformation of suspension cells and, when necessary, regeneration of plants from transformed tissue culture cells for studies *in planta*. The transformation potential of these suspension cells is currently being examined by our laboratory and preliminary findings suggest that it will be possible to stably transform *T. caerulescens* suspension cells. Using this suspension cell system, it may be possible to determine the role of various candidate genes for heavy metal hyperaccumulation and tolerance through investigating the effect of altering their expression (both elevating and suppressing) on cellular metal tolerance/transport and for the study of *cis* and *trans* factors that regulate gene expression. Transformation of suspension cells has been previously reported (Menges and Murray, 2004) and *Arabidopsis halleri* plants regenerated from calli were shown to maintain their heavy metal accumulation phenotype (Dal Corso et al., 2005). All of these findings from this report and other laboratories suggest the feasibility of such an approach.

In conclusion, in this study the creation and characterization of *T. caerulescens* suspension cells was examined. From this investigation, it was found that many of the traits associated with metal hyperaccumulation in intact *T. caerulescens* plants are also

expressed at the cellular level. These include a greater requirement of Zn for normal growth, greater tolerance to the heavy metals Zn and Cd, altered cellular transport of heavy metals, and high expression of certain key metal transporter genes. Furthermore, these *T. caerulescens* suspension cell lines may be a useful system for the manipulation of expression of candidate hyperaccumulation genes, in order to test their role in the extreme metal hyperaccumulation phenotype; the hallmark of *Thlaspi caerulescens*.

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CHAPTER VI

Concluding Remarks and Future Work

The main objective of this dissertation was to examine the mechanisms of Zn tolerance used by the Zn/Cd hyperaccumulator, *Thlaspi caerulescens*. Three projects using different strategies were carried out to address the question of extreme metal tolerance that is one of the hallmarks of hyperaccumulator plant species. The thesis research involved: 1) the isolation and characterization of a putative vacuolar Zn transporter from *T. caerulescens*; 2) screening a *T. caerulescens* cDNA library in yeast for genes that conferred growth on a high Zn media that is toxic to wild type yeast; and 3) creating and characterizing *T. caerulescens* suspension cell lines to examine metal hyperaccumulation traits exhibited in single cells (Zn and Cd tolerance, transport and accumulation). This work has provided new insight into the tolerance mechanisms used by *T. caerulescens*.

It has been theorized that if metal hyperaccumulation is the result of upregulation of only one gene, strong upregulation of a foliar vacuolar Zn transporter could limit the amount of available Zn for cytoplasmic activity and 'draw' Zn up through the plant (Chardonnes et al., 1999). This would explain the higher shoot concentrations of Zn, the lower Zn levels in roots and higher Zn flux rates through the roots and xylem. This theory is supported by the higher basal Zn concentrations necessary to avoid Zn deficiency symptoms in *T. caerulescens* (Ozturk et al., 2003).

With this in mind, the first project undertaken was the isolation and characterization of the putative vacuolar transporter gene, *MTP1*, from *T. caerulescens*. In this study it was shown that *MTP1* is expressed at a much higher

level in *T. caerulescens*, compared with the related non-accumulator, *T. arvense*, and *MTP1* expression increased in response to increasing plant Zn status. When *MTP1* was expressed in yeast, Zn accumulation increased. While a strong phenotype was not seen in the transgenic *Arabidopsis thaliana* plants overexpressing the *A. thaliana* homolog of *MTP1*, there are a number of possible reasons for this result. The lack of tissue-specific expression in the overexpression lines and the length of time the plants were exposed to a range of Zn concentrations may have allowed the plants to reacclimate and other transporters to compensate for the *AtMTP1* overexpression.

The lack of a significant effect following *AtMTP1* overexpression in Arabidopsis on Zn uptake and the growth response to low and high Zn could be due to the low Zn concentrations chosen for this set of experiments. Future work should address this possibility by growing the transgenic Arabidopsis lines at higher Zn concentrations. The choice of 10 μM Zn for the high Zn treatment now seems to be on the low side relative to other published work, where Arabidopsis overexpression lines for *MTP1* in poplar and Arabidopsis used Zn levels as high as 2.5 mM (Blaudez et al., 2003; van der Zaal et al., 2001). However, it should be noted that those plants were grown on agar medium supplemented with a very high ionic strength MS media, so that the actual Zn^{2+} activity was much lower than 2.5 mM (but probably still much higher than 10 μM). Likewise, it would be of interest to repeat the growth studies at higher Zn concentrations with the *TcMTP1* expressing lines that were generated after the current study was completed, and thus were not characterized.

The next project undertaken to understand Zn tolerance in *T. caerulescens* was a screen of a cDNA library in wild type yeast grown on high levels of Zn that were toxic to wild type yeast. The purpose of this screen was to identify novel *T. caerulescens* Zn tolerance genes. A number of genes with widely divergent potential functions were identified from this screen, including signaling, metabolic and protein

regulation genes. These genes, when expressed in yeast, resulted in either higher or lower rates of Zn uptake, suggesting this approach identified genes that may function in different cells and tissues within the plant where either high or low Zn accumulation is necessary for the overall hyperaccumulation phenotype in *T. caerulescens* plants.

For these candidate tolerance genes, the findings suggest several different mechanisms leading to improved yeast Zn tolerance both with and without increased Zn accumulation. The genes encoding glutamine synthetase and a 14-3-3 protein were of interest as genes limiting Zn uptake or mediating Zn exclusion in yeast. Additional work focusing on the role of glutamate as a compound involved in exogenous Zn binding and exclusion is of moderate interest and could be pursued through analysis of the yeast liquid media for glutamate effluxed from transgenic yeast expressing the *Thlaspi* glutamine synthetase gene. Because the focus of this project was on mechanisms of Zn tolerance that result in enhanced cellular Zn accumulation, further study of this gene was not continued. Knock out lines of the closest Arabidopsis homolog of the 14-3-3 isoform were identified and the resultant phenotype suggested that this gene is of interest for its role in cellular energetics, rather than in a Zn specific response. The two remaining genes of interest that mediated higher rates of Zn accumulation when expressed in yeast relative to wild type yeast were *TcSRO4* and *TcPKS4*. *TcSRO4* is a gene of unknown function with a very sensitive Zn-dependent expression pattern in roots. The *RCD/SRO* family, which includes *TcSRO4*, has been linked to oxidative stress and while this gene was not further characterized, further examination of this gene for its link with Zn tolerance should be a productive area of study. The final gene that showed increased Zn accumulation in yeast was a *SOS2* like kinase- *PKS4*. This gene conferred a higher rate of Zn accumulation in yeast suggesting that it may activate a vacuolar metal transporter as has been seen with other family members, thus providing improved Zn tolerance. The research on this gene *in*

planta yielded less definitive findings; however the application of the yeast two-hybrid split ubiquitin system which allows the study of protein-protein interaction with membrane proteins, to look for the target proteins for PKS4 in both plants and yeast is of interest considering the number of metal transporters that have been linked to PKS family members.

The third project looked at the involvement of metal tolerance and transport mechanisms in the hyperaccumulation trait at the cellular level via development and characterization of *T. caerulescens* suspension cell lines in comparison with cell lines for the related non-accumulator, *Arabidopsis thaliana*. From this work it was determined that at the cellular level, *T. caerulescens* exhibits some of the same metal-related traits seen in intact plants, including increased metal tolerance, decreased Zn efficiency and altered metal accumulation. Interestingly, the *T. caerulescens* cell lines accumulated less Zn and Cd than did Arabidopsis cell lines. Increased metal storage in *T. caerulescens* is only localized to very specific cell types, primarily the leaf epidermal cells and this hyperaccumulator is noted for its ability to efficiently translocate the metal along the long distance pathway. Thus, it might be expected that for most of the cell types in *T. caerulescens* other than leaf epidermal cells, it might be advantageous not to sequester the metal but exclude it from the cells, thus facilitating transport to the leaf. The research with *Thlaspi* suspension cells indicates that these cell lines might be an excellent model system to genetically modify and identify factors that may play a role in Zn tolerance and transport and that underlie the hyperaccumulation phenotype. Very recently, work in our lab has shown that these cell lines are amenable to genetic transformation (data not shown). Because of *T. caerulescens*' long generation time, genetically modifying suspension cells and testing the effect of alterations in gene expression using the Zn accumulation and tolerance assays described in Chapter V, could be a productive path to understanding the role of

specific genes in Zn accumulation and tolerance. Additionally, regeneration of genetically modified *T. caerulescens* plants from suspension cell lines may be a quicker and more efficient means of stable plant transformation than the floral dip method recently developed in the Kochian lab for *Thlaspi* transformation. The suspension cell lines characterized here have a great deal of potential and future efforts should take advantage of the regenerative and transformative powers of these cell lines to further explore the mechanisms of Zn and Cd tolerance and accumulation seen in *T. caerulescens*.

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