ANCIENT CLASSES OF METALLOTHIONEINS IN *ARABIDOPSIS THALIANA*; DIFFERENTIAL METAL ION STABILIZATION, AND PLANT FUNCTION AS DETERMINED BY RNA INTERFERENCE AND OVEREXPRESSION

by

ANNE MARIE ZIMERI

(Under the Direction of Richard B. Meagher)

ABSTRACT

Metallothioneins (MTs) are small metal-binding proteins, typically less than 85 amino acids, which appear to be ubiquitous in eukaryotes and cyanobacteria. The *Arabidopsis* genome contains 9 putative metallothionein (MT) sequences with classical cysteine rich domains separated by spacer sequences. Phylogenic analysis of these and other plant MTs from numerous species identified four ancient classes (MT1-4) of MT sequences that predate the monocot-dicot plant group divergence 200 million years ago. The selective pressure that preserved these ancient classes of MTs in the plant genome is likely due to distinct functions, such as differential metal binding properties that provide protection from toxic metals and elevated levels of nutrient metals.

Contrary to the current tenet that MTs bind metals in accordance with the thiolate series, and consistent with the hypothesis that MTs have distinct functions, differential *in vivo* stabilization by metal ions was found among representatives of the four ancient MT classes. For example, based on protein stability, MT1 is best stabilized by cadmium followed closely by

copper and zinc. In contrast, MT2 is best stabilized by cadmium followed by arsenic, MT3 is best stabilized by zinc followed by cadmium, and MT4 is best stabilized by cadmium followed by copper.

RNA interference (RNAi) was used to disrupt translation of the entire *Arabidopsis* MT1 class to address its function in plants. Based on phylogenetic and protein stability data, it was hypothesized that suppression of all three MT1 class members would render *Arabidopsis* plants hypersensitive to cadmium. Plants with knocked down MT1 expression were found to be severely inhibited by cadmium as compared to wildtype. In addition, these lines accumulated less cadmium per gram of tissue than wildtype as determined by inductively coupled plasma mass spectrometry.

Based on the protein stabilization data for MT3, we wanted to determine if overexpression of MT3 would generate plants with increased zinc composition. Surprisingly, severe zinc sensitive phenotypes were observed in plants when zinc sulfate was added to germination media. However, plants germinated on normal growth media, then transferred to media supplemented with zinc, exhibited no growth or germination phenotypes, nor did they accumulate more zinc than wildtype controls. This suggests the mechanisms of sensitivity may be due to a maternal effect, and that mitochondria or chloroplasts are responsible for the observed phenotype.

In addition to phylogenetic, metal stabilization, and *in vivo* functional experiments, the characterization of *Arabidopsis* MTs in this body of work has identified candidate molecules toward both phytoremediation and nutrient enhancement ends. The field of phytoremediation uses both natural and engineered plants to detoxify the soil by both altering the state of the toxin to a less harmful form and/or by hyperaccumulating toxins that can be physically removed by

harvesting the plant. Molecules that bind specific metals, like MTs, have great potential for use in hyperaccumulating heavy metals in plant tissues. Genetically engineered plants that accumulate metals promise to improve agriculture as well. Crop plants that can accumulate nutrient metals, such as zinc, can benefit human health. In addition, plants engineered to better tolerate metals present in the soils can potentially expand geographic areas where plants can be grown.

INDEX WORDS: cadmium, zinc, thiol-reactive, metal tolerance, metal sensitivity, phytoremediation, nutrient metal, toxic metal, maternal effect, mitochondria

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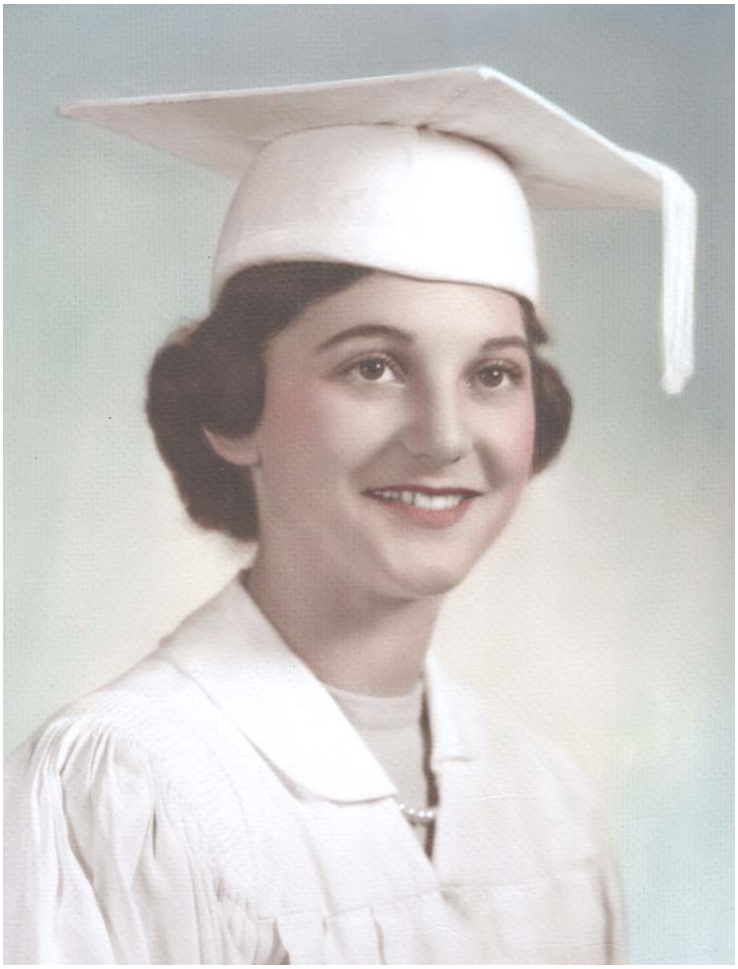
for my mother



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CHAPTER 1

INTRODUCTION

Metallothioneins (MTs) are small, metal-ion binding proteins that have a high cysteine content, are typically between 45 and 85 amino acids, and appear to be ubiquitous in eukaryotes and cyanobacteria. They were first discovered in equine kidney tissue in 1957 in early efforts to find a biological role for cadmium (Margoshes and Vallee, 1957). MT orthologs were subsequently identified in mice, which provided a model system that was more amenable to experimental and genetic manipulation. Early mouse experiments showed that in addition to cadmium, MTs have known affinities for other thiol-reactive metals such as copper and zinc (Nordberg and Kojima, 1979; Kagi and Kojima, 1987).

Altered expression of MT has been implicated in a number of human diseases, including cancer. MT levels are altered in cancerous tissues compared to healthy tissues (Koropatnick et al., 1995; Nagel and Vallee, 1995). For example, MTII is present at much higher levels in diseased breast tissue than in healthy tissue, in breast cancers where metastasis has already occurred. It has been shown that transforming the breast cancer cell line MCF7 with metallothionein MTII antisense DNA sequences down-regulated MT II protein expression and slowed the growth of these cells *in vitro* (Abdel-Mageed and Agrawal, 1997). Reduced or undetectable MTIII levels have been implicated in the neuronal disorder Alzheimer's Disease (AD). AD is characterized by neuritic plaques and neurocapillary tangles (Hardy and Davies, 1988). The MT associated with repair of neuronal damage, MT III, was originally named GIF (growth inhibitory factor) because it was found to have growth inhibitory activity in healthy

brain extracts (Uchida et al., 1991; Erickson et al., 1994). The MT III expression level is reduced in patients with AD (Yu et al., 2001). Mice with a knockout in MT III sustain greater neuronal damage from artificially induced seizures, but do not appear to contract any other neurological deficiencies that would clearly suggest it as a causative agent of AD (Aschner et al., 1997; Erickson et al., 1997).

In plants, MT levels are increased in diseased tissue as well as tissues subjected to common plant stresses such as high salinity, heat shock and wounding (Chevalier et al., 1995; Hsieh et al., 1995; Butt et al., 1998). MT induction also occurs when plants are exposed to metals (Snowden and Gardner, 1993; Chevalier et al., 1995). Based on the MT induction response to stress and metals, and mammalian data, suspected cellular roles for plant MTs include chaperoning nutrient metals, protecting cells from toxic metals, assisting metal transport and acting as intermediaries in redox reactions (Michalska and Choo, 1993; Jacob et al., 1998, 1998; Maret, 2000).

a. Biochemistry of metallothionein

Structural features of metallothionein:

The bulk of metallothionein studies have been biochemical in nature. Structural features of three mammalian MTs (rabbit, human, and rat liver MT) have been determined using a number of analytical and spectroscopic methods (Armitage et al., 1982; Otvos et al., 1982; Brouwer et al., 1995; Narula et al., 1995; Cook et al., 1998). ^{113}Cd nuclear magnetic resonance of rabbit liver MT showed that MT forms two distinct domains in the presence of metals (Otvos and Armitage, 1980) (Figure 1.1). The existence of the two domain formation was confirmed when the crystal structure for MT from rat liver was resolved in the presence of cadmium (Schultze et al., 1988). Robbins et al. (1991) subsequently defined the two domains as α-domain and β-

domain based on metal coordination by each region (Robbins et al., 1991). The β-domain is the N-terminal metal binding domain (residues 1-29) and the α-domain is the C-terminal metal binding domain (residues 33-61). Of the 7 divalent cadmium cations coordinated by MT, the β-domain coordinates three and the α-domain coordinates four. The sequences of residues 30 through 32, two of which are lysines, link these two domains.

Secondary and tertiary structures of MTs are not entirely dependent on their amino acid composition. Crystallographic studies showed apo-MT (metallothionein not yet associated with metals) to be a relatively structureless molecule. Analysis of apo-MT folding pathways has determined that metal ions are necessary for MT structure formation (Yang et al., 2001). Typically, the binding of metals to proteins that chelate metal ions increases the protein stabilization energy. The kinetic pathways of rabbit MT folding *in vitro* have shown that not only is metal required for MT secondary structure, but also that it occurs in a pH-dependent manner. The two domain MT structure formed more quickly at pH 7.2 (physiological pH) than at pH 8.4 (Ejnik et al., 2002). In rabbit liver, circular dichroism and magnetic circular dichroism data suggest the two domains bind cadmium over zinc preferentially at physiological pH and temperature (Stillman and Zelazowski, 1988). In addition, multidentate ligands, like those found in MTs, show greater binding strength than monodentate ligands, i.e., the stabilization increases with the number of metal ions bound (Arnold and Haymore, 1991). This structural dependence on metal ions has also been demonstrated in the Zn^{2+} binding site of alcohol dehydrogenase (Schneider et al., 1983), and in some oligomeric structures like the tertiary fold of the dimer interface of glucocorticiod receptor (Luisi et al., 1991). In addition, MTs are known to be highly reactive molecules as measured by their metal-ion exchange rate and the rapid oxidation of their sulfur groups (Quesada et al., 1996).

Metallothionein interdomain interactions:

Metallothionein metal-binding domains, though separated by a linker region, appear to be affected by interdomain interactions. For example, kinetic studies showed that the zinc transfer potential (the rate at which zinc is bound and released) was affected by alterations in the number of metal-binding domains present. Absorbance was monitored at 220 nm over a series of pH titrations to measure zinc release from MT as a whole, and from individual α- and β-domains. In whole molecule MT titrations, zinc was released more quickly as compared to the release from individual domain titrations (Jiang et al., 2000). These experiments suggest that interactions between the two domains alter the rate at which metal ions are exchanged. It remains to be determined how this cooperative interaction between domains affects MT function in the cell.

The metallothionein linker region importance:

Though mammalian and plant MTs are extremely disparate in both length and amino acid sequence, they all contain at least two lysine residues in their linker domain. In addition to domain interactions, the function of the two conserved lysines in the MT linker region were partially characterized. It was hypothesized that the charged lysine residues are essential to MTs because they assist in balancing excess negative charges created by metals that are tetrahedrally coordinated by cysteine residues in the α- and β- domains, and thereby stabilize overall MT structure (Cody and Huang, 1993). Prior data from crystal structure and circular dichroism showed that K31, a linker region lysine, interacts with the carbonyl groups of cysteine residues C19 and C21, and that K31 changes the net charge of an MT-zinc cluster from –3 to –2 (Pande et al., 1985; Robbins et al., 1991). To test the stabilization hypothesis, the linker region lysines in Chinese hamster MT2 were substituted with glutamate and glutamine residues. Yeast strains

with a knockout in one of their endogenous MTs were transformed with constructs that expressed the Chinese hamster MT with the linker region residue substitutions. The transformed yeast strains were tested for complementation of endogenous MT in the presence of metals. All strains grew as well as wildtype with the exception of the double (K30, 31Q) substitution. These strains, transformed with the double substitution, were sensitive to 300 μM cadmium whereas wildtype and single substitutions were not. These data support the hypothesis that the two lysine residues in the linker region are important for MT function (Cody and Huang, 1993).

b. Metallothioneins in the five kingdoms

Though there have been many reports on MT characteristics, they are from a variety of organisms and disciplines. Data was used from MTs across the five kingdoms to search for all possible *Arabidopsis* MT genes, and to form a more complete hypothesis for the function of plant MTs.

Mammalian metallothioneins:

Most mammals have one or two MT genes that encode a relatively well-conserved 61 amino acid protein with two cysteine-rich metal-binding domains separated by three amino acids, two of which are lysines (Arseniev et al., 1988). Characterization of the mouse (*Mus musculus*) MT gene has dominated the metallothionein literature since the early 1980's. There are four MT isovariants in mouse, which are clustered together on chromosome 8. These isovariants are between 60 and 68 amino acids in length and contain at least 19 cysteine residues. These cysteine residues are spaced in a highly conserved manner (Figure 1.2). The mouse MTs are expressed differentially in various tissues, during several developmental stages, and in

response to metals, steroids, and stress (Mayo and Palmiter, 1982; Mayo et al., 1982). It has been suggested that regulation of MTs in mouse is in response to Zn (II) requiring transcription factors (Robinson et al., 1993).

Metallothionein knockouts in mice have exhibited phenotypes that provide some insight into their function. Mouse MT1 and MT2 are both expressed throughout the body, including the central nervous system, and have been shown to be important for decreasing susceptibility to cadmium toxicity. When these two mouse MT genes are inactivated, exposure to 5 mg cadmium per kg body weight causes illness quickly, whereas wildtype mice show no adverse effects at this level of exposure (Michalska and Choo, 1993). These same MT1/ 2 null mice were more sensitive than wildtype to lead toxicity in the kidneys, despite the fact that the null mice accumulated three-fold less lead in this organ (Qu et al., 2002).

Mouse MTs have been connected to central nervous system damage and repair responses. When MT1 is overexpressed in mouse, inflammatory response to brain damage is reduced (Giralt et al., 2002). Mouse MT3, which is expressed mainly in the central nervous system, is homologous to human MTIII, which was originally named growth inhibitory factor (GIF) because it inhibits the survival of neurons in culture (Uchida et al., 1991). Mouse MT4, unlike MTs 1, 2, and 3, is not expressed in the brain and has not been extensively studied (Quaife et al., 1994).

Mouse MT promoters have been well characterized and contain many *cis*-acting and *trans*-acting upstream DNA elements. Many of these elements act to regulate both basal levels of MTs and stress-, metal- and steroid-induced levels of MTs. The mouse MT-1 gene promoter contains typical transcription factor IID recruitment elements such as the TATA box and 5' transcript initiator region. Upstream from the TATA box are a series of six metal response

elements (MREs a-f), which are responsible for both basal level and metal-induced expression of MT (Datta and Jacob, 1993; Samson and Gedamu, 1998). MREs act to induce MT expression in conjunction with the zinc activation of transcription factor MTF-1 (Westin and Schaffner, 1988; Ghoshal and Jacob, 2001). When zinc is not present, however, two enhancer binding proteins (C/BP-1, C/BP-2) bind MRE-c to allow basal level transcription (Aniskovitch and Jacob, 1998). Mouse MT and all mammalian MT promoters studied thus far contain at least one GC box that responds to constitutive transcription factors for basal MT transcription (Philipsen and Suske, 1999). No such promoter dissection has been performed on any plant MT genes to date.

Two subcellular localization patterns have been observed for MTs. Nuclear localization of MTs has been observed in both rats and humans (Banerjee et al., 1982; Woo and Lazo, 1997; Levadoux-Martin et al., 2001). This localization cannot be attributed to any obvious nuclear localization signals (NLS) in the protein sequence. Nor can it be attributed to other characteristics that drive proteins to the nucleus like multiple stretches of the basic amino acids arginine and histidine (Roberts et al., 1987). In rat, MT has been localized to the intermembrane space (IMS) of liver mitochondria, where it has a suspected role in the regulation of respiration. This link to respiration is due to MTs' known ability to bind and release zinc, which is required at several steps in the electron transport chain (Ye et al., 2001). Interestingly, Ye et al. showed that MT is not found in heart mitochondria. This suggests the mechanism for localization is specific to cell type.

Humans have eight expressed MT isovariants that not only respond to the typical mammalian induction factors, such as metal stress and steroids, but also appear to function in different tissue types and subcellular locales. MT1 and MT2 isovariants are expressed in almost all tissues both basally and in response to metals and stress (Kontozoglou et al., 1989;

Koropatnick et al., 1995; Nagel and Vallee, 1995). MT3 is expressed at high levels in the brain and at low levels in the pancreas, and MT4 is expressed only in the skin and tongue (Quaife et al., 1994; Ebadi et al., 1995). Some human MTs have been localized at the subcellular level to the nucleus. Despite being of a small enough size (6-8 kDa) to passively diffuse through intracellular membranes, there is a higher concentration in the nucleus compared to the cytoplasm. Active transport into specific intracellular compartments, like the nucleus, requires ATP. Yet, MT can be found in the nucleus of cells subject to ATP depletion (Woo et al., 2000). This suggests that MTs passively diffuse into the nucleus and are retained by another mechanism.

Some of the most compelling evidence for metallothionein function has been from *in vitro* mouse experiments. MT has been implicated as playing a role mitigating cellular zinc distribution, based on two key pieces of data from these experiments. First, the data that show MT is kinetically labile when bound to zinc. *In vitro* experiments showed that when MT bound only to Cd was mixed at physiological pH with MT bound only to zinc the MTs rapidly exchanged metals. A slight preference for zinc was found in the α-domain. The self-exchange rate of Cd was measured among 7 binding sites of MT bound only to cadmium, then subjected to the addition of cadmium (^{113}Cd) or zinc. Exchange of Cd among sites in the β-domain was more rapid, by several orders of magnitude, than the α-domain, but zinc was more rapidly exchanged overall (Otvos et al., 1987). Second, it has been shown that MTs are localized to both nuclei and to liver mitochondria, where cellular respiration has a high requirement for zinc (Ye et al., 2001). This supports the hypothesis that MTs assist in distributing zinc in the cell.

Metallothioneins are transported intracellularly. Zinc is absorbed through the gut in mammals from the surface of enterocytes, the cells that lines the small intestine. MT is induced

in these cells after ingestion of zinc (Hempe et al., 1991; Hempel et al., 1992). *In vitro* work in human intestinal cell lines has shown MTs can be secreted when bound to zinc (Moltedo et al., 2000). This suggests that metallothioneins may play a role in intercellular zinc transport.

Yeast metallothioneins:

The budding yeast, *Saccharomyces cerevisiae*, has three metallothioneins, CUP1, which has two isovariants CUP1-1 and -2, and CRS5. CUP1 was originally discovered in a genetic analysis where copper sensitive yeast cells were crossed with copper resistant strains of yeast. The resistance screen was followed by a targeted deletion screen. Strains with specific genes deleted become sensitive to copper (Hamer et al., 1985; Ecker and Davis, 1986). It was later found that not only is CUP1 necessary for copper tolerance in the cell, but it is also induced by copper (Thiele, 1988; Welch et al., 1989).

Copper resistant suppressor 5 (CRS5) was found in a suppressor screen for CUP1 mutants (Culotta et al., 1994). Although CRS5 only has 28% sequence similarity to CUP1, it lacks aromatic amino acids and is almost 30% cysteine, like other MTs (Figure 1.1). CRS5 and CUP1 are both under the control of the same copper trans-activator, ACE1, as shown by their dependence on an intact ACE1 locus (Culotta et al., 1994). CUP1-1 and CUP1-2 are both present in the cell at high copy number (http://yeastgfp.ucsf.edu/) (Huh et al., 2003). However, CRS5 is constitutively expressed at a much higher level than CUP1 (Hamer et al., 1985; Culotta et al., 1994).

A number of metallothionein regulatory sequences and molecules have been identified in yeast. Upstream of the yeast MT *CUP1* gene is a 39 base pair sequence that is copper responsive. The copper responsive upstream activation sequence (UAS_c) was shown to bind

Ace1 and Amt1, both trans-acting factors, in a copper dependent manner. In the presence of copper, Ace1 and Amt1 form a tetracopper center that allows for a confirmation change that promotes base-specific contacts that induce transcription (Furst, 1988; Thiele, 1988, Welch, 1989).

Cyanobacterial metallothioneins:

Several divergent MTs have been identified in cyanobacteria. These MTs differ dramatically from mammalian MTs in amino acid sequences, charge, total cysteine content, cysteine spacing patterns, and length of spacer sequences. In the cyanobacterium, *Synechococcus*, one MT has been found thus far. It is controlled by a locus on the chromosome that encodes the metallothionein, SmtA, and a putative *trans*-acting repressor, SmtB (Morby et al., 1993). The *smtA* gene is repressed in the absence of metals but is actively transcribed in the presence of cadmium and zinc (Huckle et al., 1993). The transcript encodes a 56 amino acid peptide that contains 10 cysteine residues. Like its mammalian counterparts, the *Synechococcus* MT crystal structure resolved a two-domain molecule separated by a small flexible linker region (Cook et al., 1998).

Higher plant metallothioneins:

In contrast to the simple gene clusters of MTs in mammals, higher plants have large, complex MT families (Robinson et al., 1993; Palmiter, 1998). Basal levels of most plant MTs are high and plant MTs have been identified from a variety of stress induced library screens, including, but not limited to, fungal infection, glucose starvation, and metal exposure (Snowden and Gardner, 1993; Chevalier et al., 1995; Murphy and Taiz, 1995; Butt et al., 1998). They are

similar in predicted structure and cysteine placement to MTs in other kingdoms, but their amino acid sequences are quite distinct. Most notable is linker region length and content. Plant MTs can have from 8 to 45 residues in linker regions and more than three lysine residues, whereas mammalian MTs have linker regions of between 3 and 5 amino acids, two of which are lysines.

Previous work in plants has identified several MTs, some of which have been grouped into two classes (MTI and MTII) that diverge in cysteine spacing patterns and spatial expression patterns at the whole plant level. One *Pisum sativum* (pea) MT was shown to increase metal tolerance when expressed in *Escherichia coli* and plants, and to be induced in response to metal stress (Evans et al., 1992). In *E. coli* expressing pea MT2, copper accumulation increased by eight-fold over wildtype and vector-only controls. Overexpression of this MT in *Arabidopsis* produced plants that germinated and grew in three-fold higher copper concentration than wildtype (Evans et al., 1992).

Genetic manipulation of plants to express mammalian MTs was explored, prior to the identification of the *Arabidopsis* MTs. In fact, the first mammalian protein expressed in plants was Chinese hamster MT in *Brassica campestra* (Lefebvre et al., 1987). Because it was a study in transgene expression, comprehensive tests were not performed to determine altered tolerance to metals and/or other plant stresses. However, in a later study, transgenic plants expressing mammalian MTs or tandem repeats of the mammalian MT α-domain were 10-fold more resistant to cadmium than wildtype (Pan et al., 1994).

In situ experiments show that endogenous expression of MT classes localized differentially at the organ level. MT1a and MT2a were expressed in phloem of most organs and trichomes, MT3 was predominantly expressed in the mesophyll, and MT4 expression was

restricted to seeds in both *Arabidopsis* and *Vicea faba* (Foley and Singh, 1994; Garcia-Hernandez et al., 1998; Guo et al., 2003).

c. Heavy metals

Definition and Biogeochemical Relevance of Heavy Metals

Historically, the term "heavy metal" has been used inconsistently in scientific literature and in the media. Metal elements have been considered "heavy" based on the density of the elemental form of the metal, specific gravity, atomic number and reactivity. For researchers with environmental and human health interests, the definition of heavy metals is expanded to include elements that can be toxic or poisonous to animals, and to aerobic and anaerobic processes, whether or not it is a dense metal. Based on this expanded definition, heavy metals include arsenic (As), chromium (Cr), cadmium (Cd), copper (Cu), mercury (Hg), lead (Pb), nickel (Ni), selenium (Se), thallium (Tl) and zinc (Zn). Although most heavy metals bind sulfur to some extent, a subset of this group is known as the thiolate series because of their different affinities for sulfur where Hg(II) has the greatest affinity followed by Cu(II), Cd(II), Pb(II), and Zn(II), with the lowest affinity.

The pools of thiol-binding heavy metals present on the earth's surface have varying origins and biological availabilities. Many heavy metals can exist as minerals in rock, ions dissolved in water, vapor, a variety of inorganic and organic compounds, and as bi-products and dust from manufacturing and mining industries (Fuge et al., 1993; Basta and McGowen, 2004). The presence of heavy metals, regardless of the source, poses an enormous health threat to those consuming water and vegetables from contaminated areas. Soil is the site of most food production and therefore the receptor of large quantities of fertilizers and pesticides, which can

contain heavy metals. It has been established for many years that vegetables, especially broccoli, and cauliflower, both of which are in the *Brassica* family, can accumulate large amounts of cadmium (Barman and Lal, 1994; Moral et al., 2002; Parveen et al., 2003; Finster et al., 2004).

Cadmium and human health:

Cadmium is a thiol binding metal found in the earth's crust bound to oxygen, chlorine or sulfur. All soils and rocks, including coal and mineral fertilizers, contain some cadmium. Cadmium is a contaminant in most zinc sources widely used in metal plating and is a by-product of coal mining and burning. It is often present in industrial discharges and enters water sources as such. The most common form of cadmium present in water sources is Cd(II). Cd(II) is soluble and can exist in high concentrations in upper aerobic surface layers of stagnant water, when cadmium is present in sediments at lower anaerobic layers (Holmes et al., 1974; Manahan, 1991).

Coal dust particles containing cadmium can enter the respiratory system and the cadmium can continually build up in the human body after years of low-level exposure. Cadmium particles that fall on the soil or into waterways can result in cadmium entering the food chain. Even when low levels of cadmium are in drinking water (5 ppb) and food (15 ppm), it accumulates in kidney and bone. Direct long-term effects of cadmium are kidney disease and fragile bone disease. In addition, cadmium can be biomagnified in the food chain by herbivores that eat plants that accumulate cadmium. One recent herbivory study reported willow (*Salix*) in southwestern Colorado, in the "ore-belt", accumulated 5 mg/kg cadmium. Adult ptarmigan birds in the ore-belt, which eat willow, accumulated up to 40-fold more cadmium than birds from other Colorado

areas. The birds also exhibited high mortality rates and lower bone calcium content, both of which are known to be associated with cadmium toxicity (Larison et al., 2000).

Occupational exposure to cadmium often occurs in high doses. Nickel-cadmium battery manufacturing poses the greatest risk for potential exposure followed by zinc smelting (cadmium is a contaminant in zinc ores), galvanization processes, cadmium containing pigment production and production of iron, steel and metal alloys. Higher doses of cadmium can cause more immediate physical symptoms, such as vomiting, diarrhea, and diseases such as irritable bowel syndrome and stomach disease. Breathing high levels of metallic cadmium (more than 100 $\mu g/m^3$ of cadmium fumes or 200 $\mu g/m^3$ as cadmium dust) can lead to a variety of respiratory effects. Metal fume fever and acute respiratory effects like tracheo-bronchitis can severely damage the lungs and often leads to death. Other chronic respiratory diseases such as emphysema, chronic bronchitis, and pulmonary fibrosis can arise from high cadmium exposure to the respiratory system. Ingestion of high levels of cadmium also causes osteomalacia, a painful bone disease, and severe kidney damage (Kjellstrom, 1992; Takebayashi et al., 2000; Jarup, 2002). Additionally, cadmium appears in those who smoke cigarettes at double the rate in non-smokers.

Zinc as a nutrient and as a toxin:

Some metals are required in the human diet and are critical for survival: zinc is one such metal. Zinc is known to be required for proper function of over 100 specific enzymes. It is often incorporated in cores of enzymes to allow for proper folding, based on its ability to form tetrahedral complexes with nitrogen, oxygen, and sulfur ligands (Vallee and Auld, 1990). For

example, alcohol dehydrogenase, carbonic anhydrase and superoxide dismutase all require zinc in order to have catalytic activity (Vallee and Auld, 1990).

More importantly, zinc is required for several transcription factors that contain a zinc finger motif, a DNA binding domain. Zinc finger motifs contain a small group of conserved amino acids that bind one zinc ion, and form an independent domain in the protein (Parraga et al., 1988; Bohm et al., 1997). The first transcription factor determined to require zinc was TFIIIA, which is required for RNA polymerase III to transcribe 5S rRNA genes. It has since been identified in several other transcription factors such as Sp1, which recognizes the GC boxes in housekeeping genes, and eIF2-beta which is involved in the recognition of initiation codons (Kuwahara et al., 1993; Kim et al., 1999).

When the recommended daily allowance (RDA) for zinc is not met, zinc deficiency symptoms, ranging from skin rashes to eye lesions to death, are manifested (Mills, 1989). The World Health Organization (WHO) reported in 2000 that after vitamin A deficiency, zinc deficiency was the leading cause of death from nutrient deficiency (Stephenson et al., 2000). Zinc deficiency is mainly due to inadequate intake or absorption of zinc from the diet, although loss of zinc during severe diarrhea contributes to zinc deficiency cases as well (Gracey, 1999). Severe zinc deficiency is characterized by short stature, hypogonadism, impaired immune function, skin disorders, and cognitive dysfunction (Arlette, 1983; Prasad, 1985; Evans, 1986). Using food availability data, it is estimated that zinc deficiency affects about one-third of the world's population. In total, 0.8 million deaths worldwide were attributed to zinc deficiency in 2001 (Stephenson et al., 2000).

Many diseases are associated with excess exposure to zinc as well. The clinical symptoms of zinc toxicosis, which occur after ingestion of 2 grams or more (on one occasion) of

zinc include: vomiting, diarrhea, red urine, icterus (yellow mucous membranes), liver failure, and kidney failure (Chirulescu et al., 1990; el-Kholy et al., 1990). In addition, excess exposure to zinc can cause zinc-induced hemolytic anemia, which destroys red blood cells (Tankanow, 1991).

Phytoremediation:

Phytoremediation is the use of plants to extract, remediate and remove heavy metal pollutants from the soil (Meagher, 2000). Unlike organic pollutants, which can be mineralized to harmless small molecules, heavy metals must be physically removed from the soil because they are elements and therefore, immutable. Prior to the advent of phytoremediation, the only methods of heavy metal remediation were containment, excavation and reburying, or ashing (Glass, 2000). Containment strategies include covering contaminated soil with several feet of uncontaminated soil, or removing contaminated soil to another location where it is deposited into a lined area and capped. Both containment strategies often continue to leach metals into the ground water below. Ashing involves the costly removal of contaminated soils prior to burning/ashing at high temperatures, and the ecology of the site is destroyed.

Once the potential of phytoremediation became apparent, many practical applications were developed. Rhizofiltration is a method whereby plants are used to filter contaminants from streams and marshes. One notable rhizofiltration experiment was its use in uranium and strontium cleanup near Chernobyl (Dushenkov, 1997). Volatilization of heavy metals is a method that applies to remediation of selenium and mercury, both of which can be converted to volatile forms. Already, plants have been engineered with bacterial genes *merA* and/or *merB* that catalyze the reduction of Hg(II) to Hg(0). These plants have been shown to efficiently convert

organic mercury to Hg(II), then to the less toxic and volatile Hg(0) significantly more than wildtype tobacco (Rugh, 1997; Heaton et al., 1998). Engineered hyperaccumulation of heavy metals in aboveground tissues for physical removal is a more recent advance in the field of phytoremediation. Plants engineered to accumulate arsenic have been shown to store up to 700 μg/g dry weight of arsenic, which is a 3-fold increase over wildtype plants (Dhankher et al., 2002). The search for molecules and pathways to sequester metals that have been transported to above ground tissues is a high priority for researchers in the field of phytoremediation. MTs have great potential to both transport and sequester heavy metals for phytoremediation purposes.

Functional characterization of metallothioneins in *Arabidopsis thaliana*:

In order to address the nature of an entire class of metallothioneins in plants, I first performed a genomic survey of higher plant MTs. Diverse MT sequences were found throughout the plant kingdom that fell into four ancient phylogenetic classes. Second, I needed to determine why there were four ancient *Arabidopsis* MT classes. I hypothesized that they behaved differently in the presence of different metals. To test for altered behavior in the presence of metals, an *in vivo* metal stability assay was developed in *E. coli*. A representative MT from each class was cloned and used to transform *E. coli*, which lacks endogenous MTs. It was found that MTs are quickly degraded in *E. coli* when thiol-reactive metals are absent. Therefore, transformed strains were exposed to specific metal ions to stabilize the cloned MTs. The stabilized MTs were then quantified and compared. It was found that MT3 was best stabilized by zinc, which is unique among the three other MT classes. MT classes 1, 2, and 4 were all best stabilized by cadmium. However, the degree of stabilization differed dramatically. MT2 was stabilized to a much higher level than MT1 or MT4. In addition, the metals that

stabilized MT classes 1, 2, and 4 after cadmium were different where, after cadmium, MT1 was stabilized by copper and zinc, MT2 was stabilized by arsenic, and MT4 was stabilized by copper.

The ancient nature, distinct sequence relatedness, and different metal binding stability in *E. coli* suggested different functional roles for *Arabidopsis* MTs. To test for a function in plants, and to determine whether the differential *in vivo E. coli* metal stability data collected for *Arabidopsis* MTs was biologically relevant in plants, I created plant lines that had reduced expression of all MT1 class members, using a RNA interference (RNAi) approach. In concordance with the *E. coli* stability data, MT1 (class MT1 was best stabilized by cadmium) knockdown lines were sensitive to cadmium as compared to wildtype plants. In addition, the knockdown lines accumulate less cadmium per dry weight tissue when grown on cadmium.

To test MT3 (class MT3 was best stabilized by zinc in *E. coli*) function in plants, I generated plant lines that constitutively overexpressed MT3, and exposed these plants to zinc and other thiol-reactive metals singularly and in combination. MT3 overexpression lines were defective in germination and had massively chlorotic cotyledons when seeds were exposed to excess zinc. This phenotype was overcome, however, when seeds were germinated on normal media for one week prior to exposure to excess zinc.

Based on biochemical, genetic and bioinformatics data of MTs from organisms across the five kingdoms, I hypothesized that the ancient classes of *Arabidopsis* MTs have been conserved, at least in part, because they have differential metal affinities and functions in the plant.

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Figure 1.1: The crystal structure of rat MT2 (PDB:4mt2) reveals a bi-lobed structure separated by a three amino acid linker region. Two of the linker region residues are lysines (lys30, lys31) shown in blue. The other linker residue, ser32, is shown in green. The β-domain contains nine cysteine residues, which act to coordinate three divalent cations; two zinc ions (shown in purple) and one cadmium ion (shown in orange). The sulfurs associated with each cysteine are highlighted in yellow. The α-domain contains eleven cysteine residues, which act to coordinate four divalent cadmium ions. A portion of the α-domain forms an α-helix (shown in gray) when in the presence of metal ions.

Figure 1.1

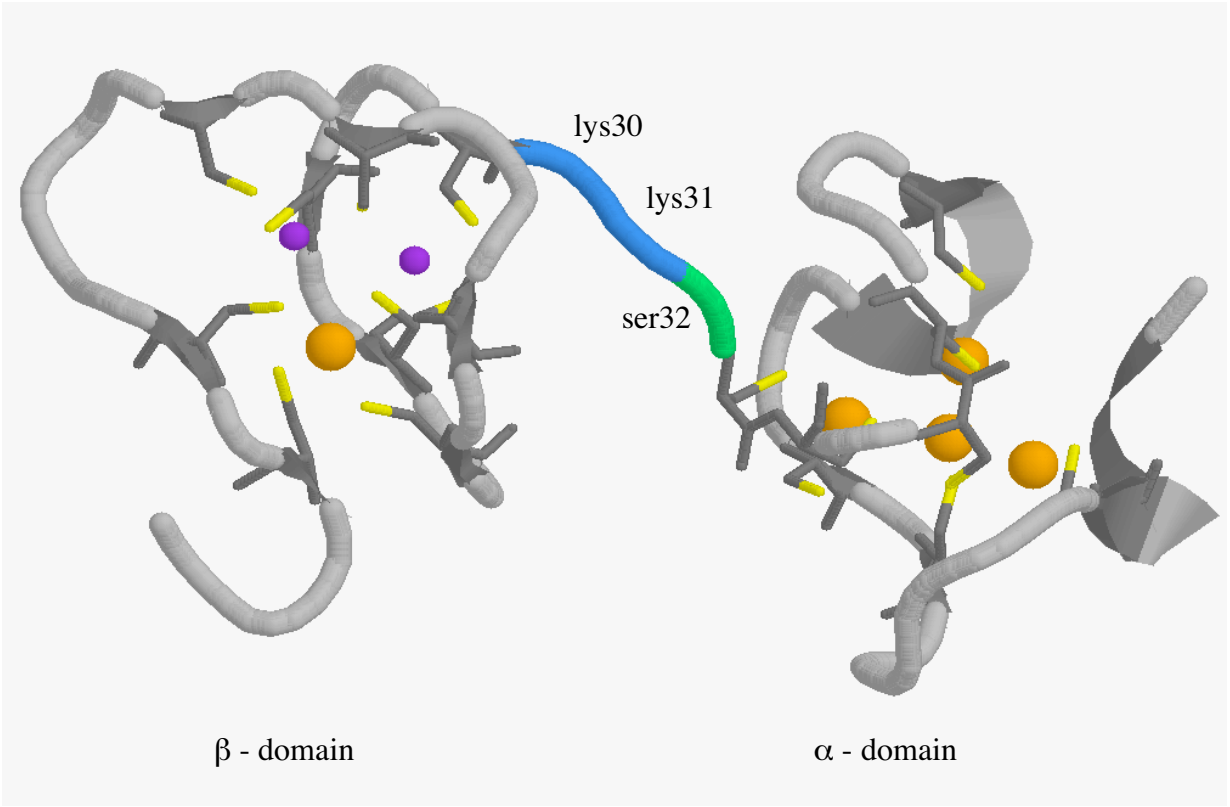

Figure 1.2: Alignment of the four mouse MTs (upper panel) and two yeast metallothioneins (lower panel). Cysteines (highlighted in red) are spaced similarly in all four mouse MTs, but not in the yeast MTs, although a similar number are present.

Figure 1.2

Mouse metallothioneins

```
M1 MDPN-CSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVC------KGAADKCTCCA
M2 MDPN-CSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGVAKCSQGCIC------KEASDKCSCCA
M4 MDPGECTCMSGGICIVGDNCKCTTCSVKTCRKSCCPCCPPGCAKCARGCIC------KGGSDKCS-CP
M3 MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ
```

Yeast metallothioneins

```
YCUP1 MFSELINFQNEGHECQCQCG-----SCKNNEQCQKSCSCPTG---CNS-DDKCPCGNKSEETKKSC-CSGK
YCRS5 MTVKICDCEGECCKDSCHCGSTCLPSCSGGEKCK--CDHSTGSPQCKSCGEKCKCETTCTCEKSKCNCEKC
```

CHAPTER 2

THE ANCIENT PLANT MT1 METALLOTHIONEINS ARE PREFERENTIALLY STABILIZED BY CADMIUM AND CONFER CADMIUM TOLERANCE

a. Introduction

Metallothioneins (MTs) are small, metal-ion binding proteins, typically between 45 and 85 amino acids in length, which appear to be ubiquitous in eukaryotes and cyanobacteria. In mammals, where they have been most thoroughly studied, MTs are expressed differentially in various tissues, at several developmental stages, and in response to metals, steroids, and stress (Mayo and Palmiter, 1982; Mayo et al., 1982). Most mammals have one or two MT genes that encode a relatively well-conserved 61 amino acid protein with two cysteine-rich metal-binding domains separated by three amino acids (Arseniev et al., 1988). The suspected cellular roles of mammalian MTs range from processing nutrient metals and protecting cells from toxic metals, to metal transport and acting as intermediaries in redox reactions (Michalska and Choo, 1993; Jacob et al., 1998; Maret, 2000).

Several divergent MTs have also been identified in cyanobacteria, protist algae, and higher plants. These MTs differ dramatically from mammalian MTs in amino acid sequence, charge, total cysteine content, cysteine spacing patterns, and length of linker domains. In addition, higher plants have large, complex MT families, unlike the simple gene clusters in mammals (Robinson et al., 1993; Palmiter, 1998). The plant and algal MTs exhibit beneficial metal binding and induction properties that may protect the organism from elevated levels of

both toxic (e.g. cadmium, mercury and arsenic) and nutrient (e.g. copper and zinc) metals. For example, in the marine brown alga *Fucus vesiculosus*, a 67-amino acid MT with 16 cysteine residues was found to be copper-inducible and bound both cadmium and copper *in vitro* (Morris et al., 1999). Work in plants has identified two MTs (MTI and MTII) that diverge in cysteine spacing patterns and spatial expression patterns at the whole plant level (Zhou and Goldsbrough, 1995). These MTs have been shown to bind metal *in vitro* in *E. coli,* increase metal tolerance in genetically manipulated plants, and to be induced in response to senescence and metal stress (Evans et al., 1992; Pan et al., 1993; Guo et al., 2003).

A genomic survey was performed to identify all MT genes in *Arabidopsis* using a number of bioinformatics tools. Nine MT genes were identified based on this analysis. Phylogenetic analysis identified four ancient classes of MTs that predate the monocot-dicot divergence, approximately 200 million years ago. In order to understand the relationships among the four groups, members within a group were compared to a consensus organismal tree. In particular, three of the four groups of MTs predate the divergence of monocot and dicots, and one group predates the divergence from gymnosperms.

Observations of the *in vivo* affinity of mammalian MTs to thiol-reactive ions were based primarily on the metal content of purified proteins, which often contain some zinc (one of the least thiol-reactive metals), and some cadmium, which is of moderate reactivity relative to copper and only a trace contaminant in the diet. It is hard to reconcile these data with *in vitro* biochemical experiments, which suggest that mammalian MT bound in accordance with the thiolate affinity of the metal ion, where zinc binds most weakly [i.e. Bi (III), Cu (I), Ag (I), Hg (II) > Cd (II) > Pb (II) > Zn (II)] (Hamer, 1986; Kagi and Schaffer, 1988). Therefore, one hypothesis of this study was that each ancient plant class of MTs, with its unique cysteine

spacing pattern and conserved amino acid sequences, would have a differential stability *in vivo* when in the presence of thiol-reactive metals.

In vivo stability assays were performed on *Arabidopsis* MT1a and mouse MT2 (mMT2), in *E. coli*. These data show that the *Arabidopsis* MT1a and mMT2 were most highly stabilized by Cd relative to the other metals tested. The remaining stabilization preferences, after cadmium, were different as well as the level of stabilization based on exposure to thiol-reactive metals. To test the MT stability data for biological relevance in plants, RNA interference (RNAi) was used to reduce the expression of all three members of *Arabidopsis* MT class 1. Consistent with the *E. coli* protein stability data, *Arabidopsis* RNAi knockdown lines showed the greatest sensitivity to cadmium as opposed to the other metals tested. Additionally, these RNAi lines accumulated less cadmium per gram of dry weight than wild-type plants.

b. Results

Bioinformatic Analysis of Plant Metallothioneins:

With the completion of the *Arabidopsis* genome sequence (Initiative, 2000), it was possible to access the entire MT gene family of one plant species. The *Arabidopsis* database (TAIR) and GenBank were searched using nucleotide and protein sequences of known MTs from a variety of plants and animal species. In addition, common MT cysteine spacing patterns were run through the TAIR Pat Match search program. Over 50 distinct plant DNA sequences were found, nine of which were in *Arabidopsis*. The *Arabidopsis* genes were spread amongst four of the five *Arabidopsis* chromosomes and occur both singularly and in clusters (Figure 2.1). Eight of the nine *Arabidopsis* MT genes were represented as expressed sequence tags (ESTs)

suggesting these eight sequences were functional genes. No ESTs were found for *MT1b*. The gene structure for each MT, including a hypothetical structure for *MT1b*, is shown in Figure 2.2.

In this study, we determined that the evolution of the four MT classes seen in *Arabidopsis* predates the major plant group divergences. According to an organismal tree, the major plant groups diverged as follows: monocots and dicots diverged 200 million years ago (mya), the gymnosperms diverged from the angiosperms 350 mya, and plants diverged from their protist green algal ancestors 400 mya (Cronquist, 1981; Bremer, 2000; Friis et al., 2000; Magallon and Sanderson, 2001; Sanderson and Doyle, 2001) (Figure 2.3).

Both neighbor-joining and maximum parsimony phylogenetic trees were constructed based on MT protein sequence alignments, using GCG software. Four distinct ancient classes of *Arabidopsis* MTs were found based on these phylogenies. The phylogenetic trees include all *Arabidopsis* MT classes, which grouped with MTs from other distant plant species within each class. This suggests MT classes arose before the divergence of the major plant groups, such as monocots from dicots and angiosperms from gymnosperms, as shown in the neighbor-joining amino acid tree shown in Figure 2.4. This concordance is consistent with the hypothesis that the MT classes are ancient and have been under strong selective constraint for more than 200 million years.

As an additional criterion to establish the relationship among MT classes, MTs were characterized based on cysteine spacing patterns in two or more of their cysteine rich (suspected functional) domains. We assigned number to the cysteine spacing patterns in each domain from all the MTs found in the database. The numerical assignment we gave to the cysteine spacing pattern found in nearly all mammalian MTs has been conserved for at least 270 million years and is shown (mMT2, ckMT) along with the four cysteine spacing patterns of representatives from

each *Arabidopsis* MT class in Figure 2.5 (Margulis and Schwartz, 1982; Kagi and Schaffer, 1988). The cysteine spacing patterns also support the existence of 4 ancient classes of plant MTs in *Arabidopsis*. For example, all MTs from class 1 share the same cysteine spacing pattern, and this pattern predates the monocot/dicot split because *Zea maize* and *Arabidopsis* have MTs with the identical cysteine spacing pattern. Based on three other distinct and completely conserved cysteine spacing patterns, MTs from the remaining three classes predate the monocot/dicot split, and one class, MT3, predates the angiosperm/gymnosperm split as in the phylogenic analysis. Using cysteine spacing patterns, MTs can be grouped into classes without having to use lengthy phylogenetic analysis.

Metallothioneins are differentially stabilized by thiol-reactive metals:

Previous experiments expressing MT sequences in *E. coli* used large carrier proteins to protect the MTs from degradation by bacterial proteases (Kille et al., 1991; Li et al., 2000). It was hypothesized that MTs were recognized as foreign and quickly targeted for degradation. Apo-MT (i.e. MT lacking metal) protein is unstructured until bound to metal ions (Ejnik et al., 2002) and, although present, is difficult to detect in mammalian cells as well (Yang et al., 2001). In preliminary studies, I observed that when *E. coli* expressed MTs fused to a small epitope tag, barely detectable levels of MTs were found in standard growth medium. Once sublethal levels of thiol-reactive metals were added to the medium, significant amounts of MT proteins were detected. Increased stability and the corresponding increased levels of MT protein, in the presence of different thiol-reactive metals, were assumed as indicators of metal binding affinity because thiol-binding metals might stabilize plant MT structure in *E. coli*. To test this prediction, a representative of *Arabidopsis* MT class 1 was selected for analysis. Class member

MT1a was selected because it was represented most often as an EST in the GenBank database, and was easily cloned from an *Arabidopsis* whole plant cDNA library (Figure 2.6). The clone was expressed in *E. coli* under the control of an IPTG-inducible Lac promoter. The well-characterized mouse MT2 coding sequence (mMT2) was cloned for comparison. Mouse MT2 was shown to have affinities for cadmium, copper and zinc, and we wanted to test the validity of our assay with this known MT. Each sequence contained an identical, small 9-amino acid hemagglutinin (HA) epitope tag fused, in frame, to the amino terminus to allow for quantitative comparison of protein levels among classes (Figure 2.6).

Barely detectable levels of either MT were found after induction with IPTG in standard bacterial medium. However, MTs reached a new, much higher steady state level after the concomitant addition of metal ion and inducer as shown in Figure 2.7. At 15 minutes following induction and addition of at least one metal ion, cells were producing measurably higher levels of MT proteins and maximum levels were reached in 60 to 75 minutes. Independent experiments showed that higher protein levels were not achieved for any of the MTs with further incubation beyond 75 minutes. In order to standardize protein results among MT1a, mMT2, and various metals, the protein levels of each MT were quantified 60 minutes post-induction in the presence of 50 μM levels of five thiol-reactive metal ions (As, Cd, Cu, Hg, Zn) and Fe. These quantifications were normalized to a standard curve derived from a known dilution series of HA epitope run on each gel (Figure 2.7). Signal intensities were normalized to standards containing 0.2 ng and 2 ng of cross-linked HA peptide control run on each gel in order to compare the absolute levels of different MTs and experiments and experiment repetitions.

Northern blot comparisons of RNA levels verified that metals do not greatly affect MT transcript levels (Figure 2.8) and therefore metal ions must be affecting MTs at the protein level.

Additionally, bacterial cultures expressing MTs and grown in medium containing 50 μM of the metals tested were plated in a dilution series to be certain that metals at a 50 μM concentration did not inhibit colony growth (Figure 2.9). *Arabidopsis* MT1a had the following relative binding affinities based on protein stabilization: Cd(II) > Cu(II), As(III), Hg(II) > Zn(II). The quantitative data from several repetitions of this experiment for *Arabidopsis* MT1a and mouse stabilization data are shown in Figures 2.10 and 2.11.

RNA interference of class 1 metallothioneins:

To verify the biological relevance of our stability assay and to elucidate a possible function of class 1 metallothioneins in *Arabidopsis*, the expression of all three MT class 1 genes was reduced by targeting MT1 RNA for degradation using an RNA interference (RNAi) approach. The coding sequence of all three class 1 *Arabidopsis* MTs is highly conserved, as shown in Figure 2.12, which allowed a single RNAi construct to collectively target the three MT1 mRNAs for degradation. In order to determine the effectiveness of knocked down expression of class 1 MTs in RNAi lines, mRNA steady state levels were examined. RNA was extracted from transgenic plant lines as well as wild-type controls to examine mRNA levels of each of the three MT1 class members. Transgenic lines had reduced mRNA levels of MT1a as determined by Northern blotting (Figure 2.13a). MT1b and MT1c mRNAs were not clearly detectable on Northern blots in both wild-type and RNAi lines. This result was consistent with the lower number of ESTs observed for MT1c relative to MT1a in the GenBank and TAIR databases. RT-PCR results showed reduced levels of MT1a mRNA and undetectable levels of MT1c mRNA in RNAi lines as compared to substantial amounts of these two in the wild-type controls. RT-PCR failed to detect MT1b transcripts in wild-type or in RNAi lines (data not

shown). Profilin 1 and MT2 mRNA levels were not reduced in any of the RNAi samples, they served as internal controls for comparisons among RT-PCR samples and different plant lines (Figure 2.13b).

Eight transgenic T_2 generation RNAi lines, which segregated as if a single transgenic insert were present, were studied. All RNAi lines exhibited varying degrees of cadmium sensitivity relative to wild-type when grown on MS plates containing 50 μM cadmium chloride. Seven of the 8 RNAi lines germinated, but grew at a slower rate with fewer lateral roots than wild-type (Figure 2.14). One line, Ri-7, began to germinate, then died after 3 days on 50 μM cadmium chloride. Ri-7 germinated and grew on 30 μM cadmium chloride, although plants were much smaller than wild-type and severely chlorotic (Figure 2.15). All lines grew as wild-type on medium lacking cadmium. Lines grown on medium with 250 μM zinc sulfate or 50 μM mercury chloride grew as wild-type as well (data not shown).

To assess the mechanism of MT sensitivity, the levels of cadmium accumulation were determined in three of the RNAi knockdown lines; Ri-3, Ri-4, and Ri-7. Plant lines were germinated on 30 μM cadmium chloride, which is a sublethal dose for both wild-type and all MT1 RNAi knockdown lines. Fresh weights of transgenic lines, in comparison to wild-type, grown at this concentration are shown in Figure 2.16. As determined by inductively coupled plasma spectroscopy (ICP), RNAi lines Ri-3, Ri-4 and Ri-7 accumulated at least 5 times less cadmium than wild-type in both shoots and roots, where endogenous MT1 is highly expressed (Figure 2.17). These RNAi lines accumulated less than two-fold as much zinc as wild-type, but they accumulated similar amounts of arsenic, copper and iron, which correlates with the *E. coli* stabilization data (Figure 2.10).

c. Discussion

The *Arabidopsis* MTs can be divided into four distinct classes based on analysis of sequence phylogenies and cysteine spacing patterns. At a minimum, all the MT classes predate the divergence of monocots and dicots. One theory to explain the preservation of the ancient plant MT classes is that the function of each class of MT protein isovariants is critical to the plant. In support of this explanation, a dramatic difference in cadmium accumulation was found between wild-type *Arabidopsis* and plants with MT class 1 RNA knocked down, in the presence of cadmium. Perhaps plants have functional classes of MTs to specifically target metals for either nutrient uptake followed by transport to aboveground tissues, or to bind toxic metals in vacuoles or for transport via various pumps into vacuoles. For example, many plant nutrient pumps can also bind Cd. AtNramp transports both iron and cadmium into the plant (Thomine et al., 2000). MT1 and a monocot MT1 homolog are expressed at higher levels in the root than in above-ground tissues and perhaps can deal with the cadmium brought into the plant via this nutrient pump (Snowden and Gardner, 1993).

Cadmium toxicity is often caused when cadmium is substituted at a core in enzymes requiring other metals such as zinc or copper. This substitution can cause the enzyme to be inactive. Though MTs are known to bind nutrient metals, the release of these, such as zinc and copper, is favored over the release of cadmium (Jiang et al., 1998), thus favoring incorporation of zinc and copper into enzymes as opposed to cadmium. More damaging to the plant cell, however, is the decrease in chloroplast density caused by exposure to cadmium. Thiol binding metals, such as cadmium can inhibit proper electron transport in the chloroplast thereby suffocating the plant (Baryla et al., 2001). Here, we show that *Arabidopsis* class 1 MTs are best stabilized, in *E. coli*, by cadmium and may therefore be involved in sequestering this heavy metal

in the cell. Once MTs are absent from plant cells, cadmium may be free to poison electron transport, disrupt enzymes requiring metal cations at their core, and interfere with chloroplast replication.

Consistent with cadmium toxicity response phenotypes, plant lines with RNAi knockdown constructs targeting all three MT1 class members were severely inhibited on doses of cadmium that were sublethal to wild-type plants. RNAi lines that were able to germinate and grow on 50 μM cadmium were chlorotic as well, perhaps further indicating that cadmium toxicity is related to chloroplast replication.

Plants maintain a delicate balance of essential heavy metal elements, such as copper, zinc and nickel at micronutrient levels while excluding toxic metals such as cadmium, arsenic and mercury. Their mechanisms for regulating metal concentrations are largely unknown, though a few important classes of proteins have been identified. Plant MTs are one such class suspected to be mechanistically important for the regulation of these optimal metal concentrations. Worldwide, soil composition is extremely diverse with many natural and agricultural soils having toxic levels of both nutrient and other metals (Adriano, 1986) that pressure plants to maintain non-toxic intracellular metal concentrations. Metallothioneins are likely candidates for proteins involved in maintaining plant metal homeostasis.

d. Materials and Methods

Bacterial strains, plant ecotypes, and plasmid vectors:

E. coli bacterial strains Top10F (Invitrogen), BL21(DE3) (Novagen) and *Agrobacterium tumefaciens* strain C58 were used to host plasmids Bluescript SK^{++}(Strategene), pET15b (Novagen) and pBIN19 (An et al., 1988), respectively. *Arabidopsis thaliana* Columbia ecotype

was used in all plant experiments. Plants grown on half-strength Murishige and Skoog (MS) medium (Murashige and Skoog, 1962) phytagar (8g/l) plates were incubated in growth chambers at 24°C with 12 hour light/12 hour dark cycles.

Bacterial protein preparation:

Vector-only control and MT constructs were in IPTG inducible pET 15b vectors transformed into host strain BL21(DE3) (Novagen). Five ml overnight cultures were grown at 37°C in the presence of ampicillin (100μg/l). LB medium (500 ml) was inoculated with 1 ml of each overnight culture. Cultures were grown to OD_{600} of 0.5 followed by addition of IPTG to 0.2 mM and metal salts to 50 μM. One hour after induction, 7 ml of each culture were harvested and added to cold Tris•Magnesium buffer (10 mM Tris, 5 mM $MgCl_2$, 2 mM NaN_3) followed by centrifugation and resuspension in lysis buffer (10 mM Tris, 5 mM $MgCl_2$, 0.3 mg/ml lysozyme). The resuspension was frozen in liquid nitrogen, thawed, then re-frozen in a 37°C water bath three times before a final spin and collection of the supernatant.

Approach to steady state:

Bacterial cultures expressing MTs were grown in the presence of 0.2 mM IPTG inducer with and without 50 μM cadmium. Each bacterial culture was grown to early log phase (OD_{600}= 0.5) before induction and treatment with 50 μM metal for 0, 15, 30, 60, and 75 minutes. Extracts were resolved on 13% SDS-PAGE gels and blotted to Immobilon-P^{SQ} 0.1 micron membranes (Millipore Corporation, Bedford, MA). Western blots were probed with monoclonal HA antibodies (Covance, Princeton, NJ) and quantified relative to HA standard peptide using NIH Sci-Image software (http://rsb.info.nih.gov/nih-image/).

Colony growth assay:

Bacterial cultures were grown and induced for 60 minutes as in the approach to steady state experiments. One μl of each culture and 10-fold dilutions thereof, were spotted onto LB medium. Plates were incubated at 37°C for 16 hours.

Northern Blots:

Bacterial RNA extractions were performed as described in O'Hara et al. Plant RNA was extracted with RNeasy kits (Qiagen). RNA was electrophoresed in 7% (v/v) formaldehyde, 1% agarose gels. RNA was transferred to nylon membranes (ICN Biotrans) by capillary blotting. Twenty-five ng of DNA was used in a reaction with random hexanucleotide primers and α-dATP^{32} (Roche Random Prime Kit) to make the probe. Blots were prehybridized in sealed vessels in hybridization buffer (250 mM NaCl, 20 mM sodium phosphate (pH 7), 2.5 mM EDTA, 5% SDS, 0.5% dry milk, 0.5 mg/ml tRNA, 5 mg/ml salmon sperm DNA) at 65°C for 2 hours before addition of probe. Blots were then incubated with probe at 65°C for 20h. Prior to exposure in

Phosphor Imager cassettes (Molecular Dynamics), blots were washed for 2-3 hours in a low salt wash buffer (40 mM sodium phosphate (pH 7), 1% SDS, 1 mM EDTA) with buffer changes every hour.

RNA interference:

The coding regions of all three MT1 class members were aligned using PileUp software (Genetics Computing Group, Wisconsin). There was significant homology in the majority of the gene (Figure 2.12). The RNAi construct included 200 bp of antisense MT1a RNA (beginning

with the start codon and ending with nt 200) followed by a 800 bp non-coding of the GUS gene as a spacer region and ending with 200 bp of MT sense sequence complimentary to the antisense sequence at the 5' end of the construct. Primers designed to amplify both the sense and antisense strands were used to amplify these regions for subsequent overlap extension PCR (OE-PCR) (Pawloski et al., submitted). The final OE-PCR was cloned into binary vector pBIN19 (An et al., 1988).

Transgenic plant lines:

Arabidopsis siliques were vacuum infiltrated with *Agrobacterium tumefaciens* that contained RNAi constructs in binary vector pBIN19. T1 generation seeds were sterilized using chlorine gas for 4.5 h and plated on MS medium containing kanamycin (35mg/l). Seedlings surviving selection were transplanted to soil for T2 seed collection. Seeds were sown on MS medium containing kanamycin to screen for the transgene. Kanamycin resistant plantlets were grown to set seed for T2 generation experiments. Wild-type seeds (Columbia with T-DNA insertion of the kanamycin resistance marker in an actin gene) were plated as controls.

RT-PCR: RNA from WT and RNAi lines of *Arabidopsis* was reverse transcribed into cDNA using the Thermo-script kit (Invitrogen). cDNA template was added to reactions at either 1 ng, 0.1 ng or .01 ng. Primers specific to each MT class member and profilin (prf) primers (Table 2) were used to amplify MT genes and *prf* control genes. PCR reactions were resolved on 1.5% agarose gels followed by ethidium bromide staining.

Inductive Coupled Plasma Mass Spectroscopy (ICP):

Plants grown on MS medium plates with kanamycin (35mg/L) and 30 μM cadmium chloride were harvested at 28 days. Tissues were dried at 70°C for 48 h. A nitric acid/perchloric acid (7:1) mix was added at 5 ml per gram dry weight. Tissues were acid digested in EPA glass vials (Enviroware) at room temperature for at least 48 h. Samples were diluted 5-fold in water and agitated at room temperature for 24 h. Samples were centrifuged at low speed to remove undigested debris. Supernatants were retrieved for ICP analysis.

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Table 2.1

MT1 sense primer	
MT1 sense	agt tgc gag aag aac tac aac aag gag t
MT1 antisense primers	
MT1a 3A	tag acc ata ata att
MT1b 3A	agc tta gaa gag ttg
MT1c 3A	tag tta cct tta ctc att a
MT2 primers	
MT2-s	gat gtt cca gat tac gct tct tgc tgt gga gga aac
MT2-a	ctt gca ggt gca agg
Profilin primers	
prf1-5'S5	aat aaa caa ttt aca tac cat tac
prf1-3'N1	aat caa aac tca ata cat atg gag a

Table 2.2: RT-PCR primers specific for each member of MT class 1 were used to determine transcript levels. Profilin 1 and MT2 primers were used in control reactions. All primers are listed 5' to 3'.

Figure 2.1: The *Arabidopsis* metallothioneins are spread amongst four of the five chromosomes. They occur both singularly (MT4a, MT2a, MT3, MT2b, MT1b) and in clusters (MT1a, MT1c and MT4b, MT4c). The MT chromosome and locus numbers from TAIR (The Arabidopsis Information Resource http://www.arabidopsis.org/agi.html) are as follows: MT1a (At1g07600), MT1c (At5g07610), MT4a (At2g23240), MT4b (At2g42000), MT4c (At2g42000), MT2a (At3g09390), MT3 (At3g15353), MT2b (At5g02380), MT1b (At5g56800/At5g56790). Two MT4 genes, MT4b and MT4c, overlap and have the same locus number. MT1b has not been annotated in the TAIR database and is in the intergenic region of the two loci listed above.

Figure 2.1

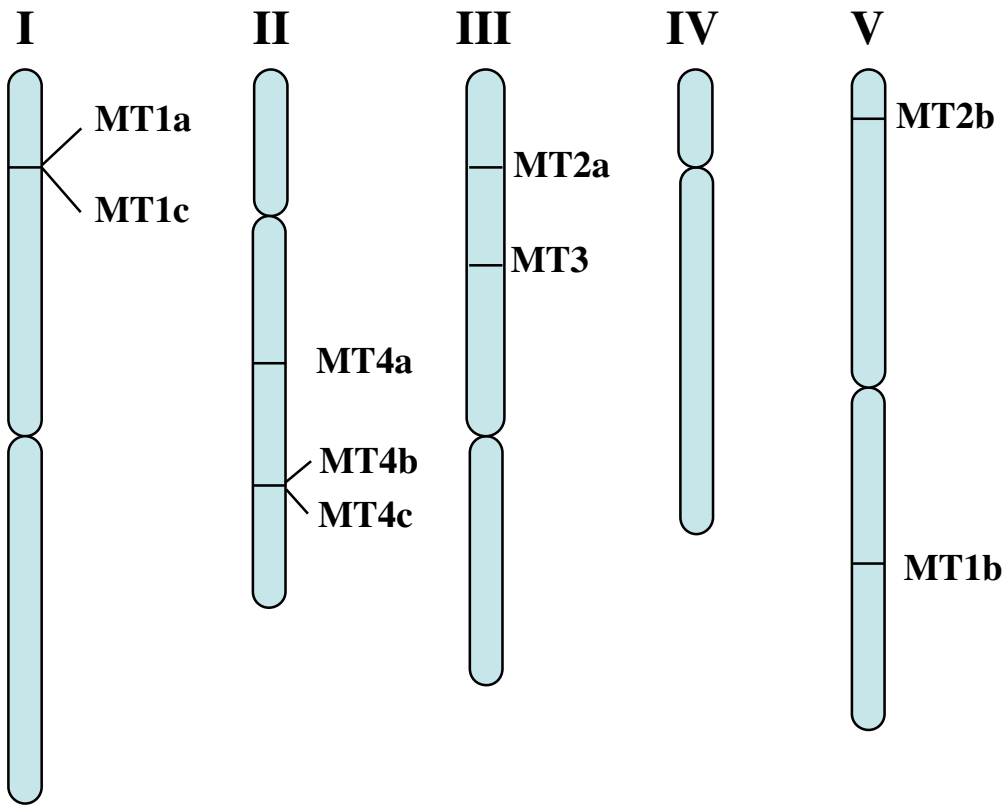


Figure 2.2 Gene structures of the *Arabidopsis* metallothioneins. Gray boxes represent untranslated regions, dark gray boxes represent coding regions, and black lines represent introns. All structures are listed from 5′ to 3′.

Figure 2.2

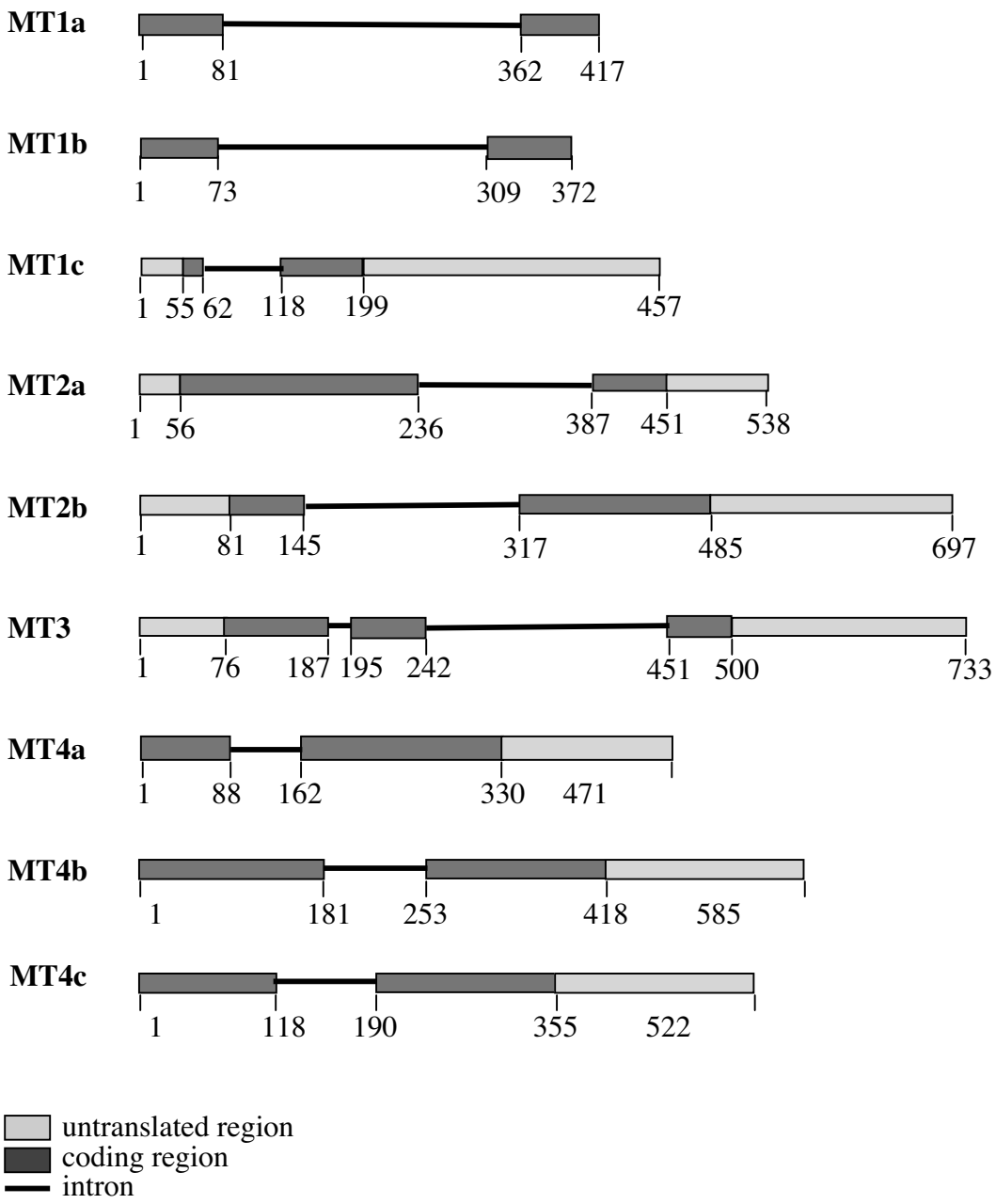

MT1a
1
81
362
417
MT1b
1
73
309
372
MT1c
1
55
62
118
199
457
MT2a
1
56
236
387
451
538
MT2b
1
81
145
317
485
697
MT3
1
76
187
195
242
451
500
733
MT4a
1
88
162
330
471
MT4b
1
181
253
418
585
MT4c
1
118
190
355
522
untranslated region
coding region
intron

Figure 2.3: Consensus organismal tree, based on rRNA and morphological data, showing the major plant groups. Divergence in millions of years is noted at each branch point and is as follows: monocots from dicots, 200 million years ago (mya); angiosperms (includes monocots and dicots) from gymnosperms, 350 mya; higher plants (includes angiosperms and gymnosperms) from green algae, 400 mya; green algae from cyanobacteria, 1000 mya.

Figure 2.3

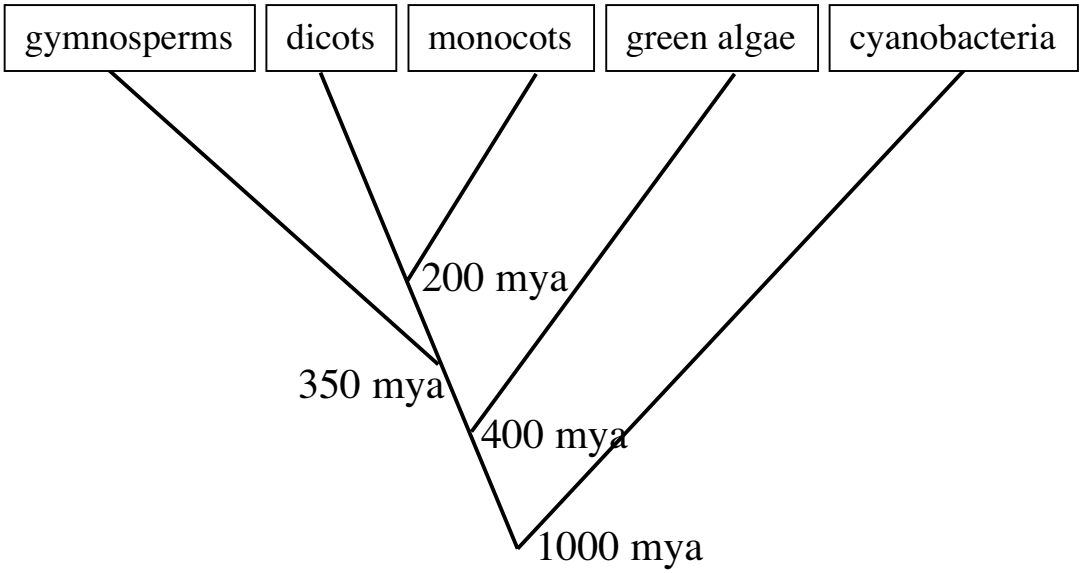

gymnosperms
dicots
monocots
green algae
cyanobacteria
200 mya
350 mya
400 mya
1000 mya

Figure 2.4: Plant Metallothionein Amino Acid Sequence Tree: A representative neighbor-joining (NJ) tree shows that the four classes of MT protein sequence in *Arabidopsis* separate into four clades. All neighbor-joining (NJ) trees generated support the existence of four clades of *Arabidopsis* MTs. No single tree is statistically favored to support one exact topology over the nine other trees generated. Underline = monocots (angiosperms), standard font = dicots (angiosperms, **bold face** = gymnosperms. *Arabidopsis* sequences are in the format MTX. Accession numbers: MT1 (MT1a BE844990; MT1c N38326; Bn, *Brassica napus* U20236; Zm, *Zea Mays* X82186; Hv, *Hordeum vulgare* D50641; Os1, *Oryza sativa* Q40633; MT1b U11254); MT4 (MT4a X92116; MT4b AAB63543; MT4c Z32602; Pet, Petunia hybrid AF049937; Gm, *Glycine max* AF004808; Zm, *Zea mays* U10696, Ta, *Triticum aestivum* X68290; Os4 *Oryza sativa* AY572960); MT2 (Br, *Brassica rapa* D78491; Bc, *Brassica campestris* L31940; MT2b AY037263; MT2a AF324665; Ca, *Coffea arabica* U11423; *Nicotiana glutinosa* U46543; Fs, *Fagus sylvatica* AJ130886; Os2 *Oryza sativa* D89931; Ec, *Eichornia crassipes* AJ010160; Le, *Lycopersicon esculentum* Z68309); MT3 (Fa, *Fritillaria agrestis* AAB95219; Os 3, *Oryza sativa* 3 T03438; Md, *Malus domestica* O24059; Fv *Fragaria vesca* AJ01444; Cp, *Carica papaya* Y08322; Gh, *Gossypium hirsutum* AF118230; Ad *Actinidia deliciosa* L27811; MT3 R84113; **Pa**, *Picea abies* U91997; **Pm**, *Picea menziesii* U55051; **Pg**, *Picea glauca* L47746).

Figure 2.4

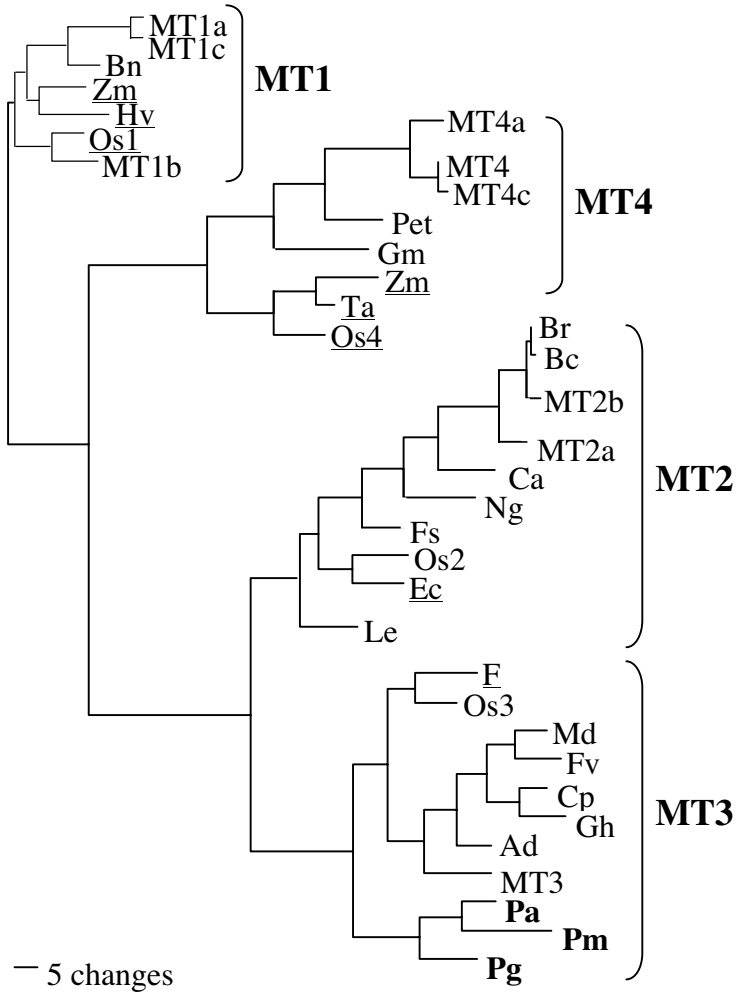

MT1a
MT1c
Bn
Zm
Hv
Os1
MT1b
MT1
MT4a
MT4
MT4c
MT4
Pet
Gm
Zm
Ta
Os4
Br
Bc
MT2b
MT2a
Ca
Ng
Fs
Os2
Ec
Le
MT2
F
Os3
Md
Fv
Cp
Gh
Ad
MT3
Pa
Pm
Pg
MT3
5 changes

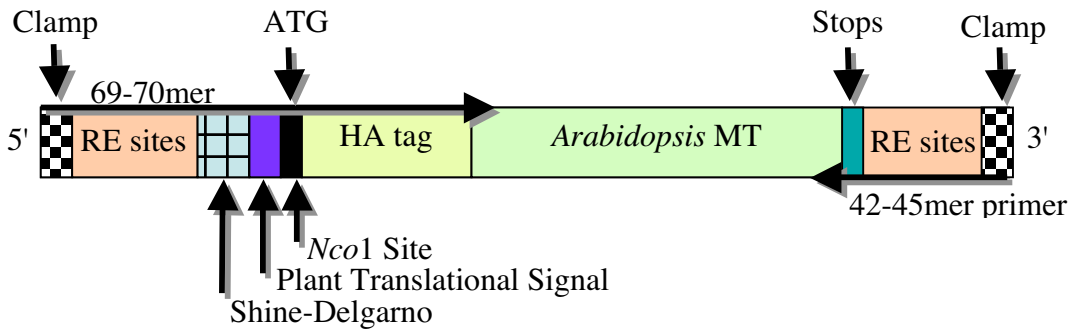
Figure 2.5: Conserved cysteine spacing patterns indicate the existence of four classes of plant metallothioneins. Each cysteine-rich domain was given a numerical assignment by numbering the first amino acid following a cysteine as one (1), counting to the next cysteine, and assigning that number. The example domain given is the N-terminal domain of MT1. The patterns are listed for the four classes of plant MTs, mouse (mMT2), and chicken (ckMT). The single difference in cysteine spacing between mammals and birds is underlined. Dotted lines represent the linker regions that separate the domains.

Figure 2.5

Class	Size (a.a.)	Cysteine Spacing Pattern
MT1	45	2 4 2 4 2 … 3 2 4 2 4 2
MT2	81	1 4 2 4 2 4 3 … 2 4 2 3 2
MT3	68	3 2 6 … 2 4 2 3 2
MT4	84	4 2 4 6 2 … 2 4 2 3 2 … 2 4 2 3
mMT2	61	2 6 2 4 2 3 2 3 … 1 2 1 4 3 4 2 7 2 1
ckMT	63	2 6 2 4 2 3 2 3 … 1 2 1 4 3 4 2 8 2 1

Figure 2.6: *Arabidopsis* cloning cassette and primers. Primers for the 5' end of the MTs included a six base pair clamp, three restriction enzymes (RE) sites (*Xba*1, *BamH*1, *Nco*1) and sequence that encodes for a nine amino acid HA (hemagglutinin) epitope tag and at least 18 base pairs specific to MT. Within the 5' primer were also a consensus plant translation signal and a Shine-Delgarno ribosome binding site. Primers for the 3' end included at least 12 base pairs of sequence homologous MT1a, followed by two stop codons, two RE sites (*Xho*1, *BamH*1) and a six base pair clamp.

Figure 2.6



5' oligo

MT1a5S tcggtctctagaggatccatgggatacccatacgatgttccagattacgctgcagattctaactgtgga

3' oligo

MT1a3A aacacgctcgaggagctcaagctttcatcaacagttacagtt

Figure 2.7: Class 1 MT1a is stabilized *in vivo* by thiol-reactive metal ions. **A.** Western blot of *Arabidopsis* MT1a expressed in bacteria with and without 50 μM $CdCl_2$. Graph of relative protein levels from several replicates of the western blots. **B.** Western blot of dilution series of HA peptide that was run on each gel for normalization purposes.

Figure 2.7

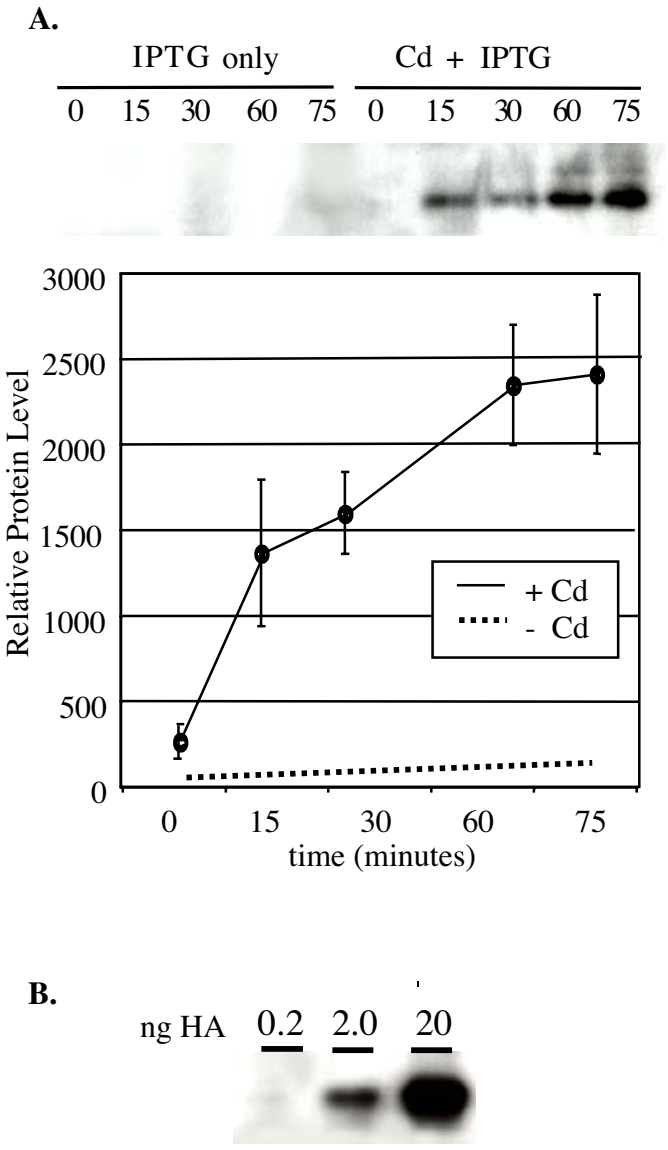

A.
IPTG only
Cd + IPTG
0 15 30 60 75 0 15 30 60 75
3000
2500
2000
1500
1000
500
0
Relative Protein Level
+ Cd
- Cd
0 15 30 60 75
time (minutes)
B.
ng HA 0.2 2.0 20

Figure 2.8: Northern blots of bacterial extracts treated with 50 μM of six metal ions show that metal treatment does not greatly affect transcript levels of MT1a (top panel). Blots were probed with bacterial 16S ribosomal RNA as loading controls (bottom panel). * RNA from cells expressing MT2a was used as a negative control. ∅ = no metal treatment.

Figure 2.8

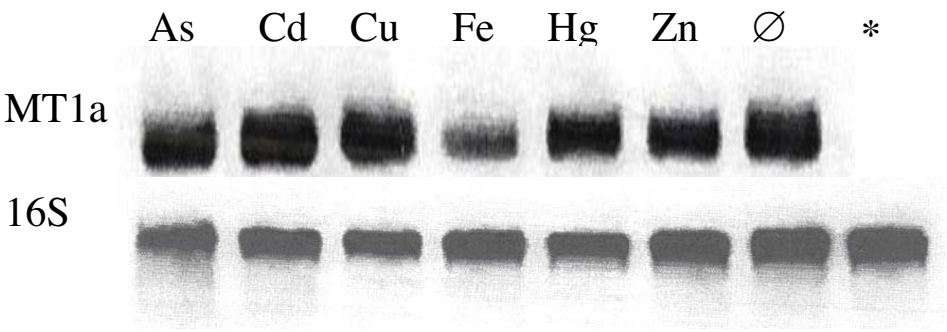
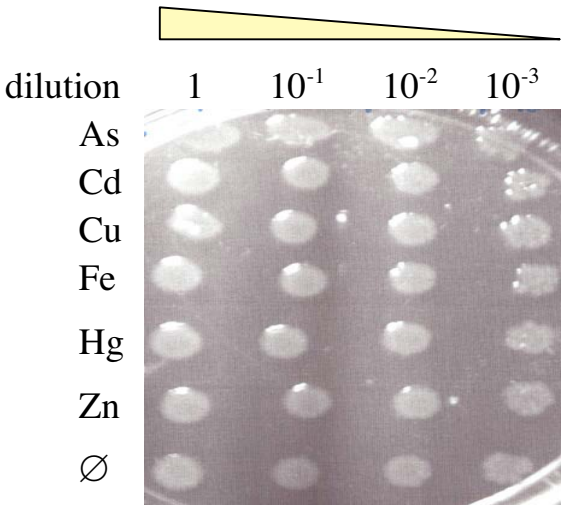


Figure 2.9: Growth of bacterial cultures expressing MTs was not inhibited by the metal concentrations used for stability assays (50 μM) as shown by colony dilutions. Bacterial cultures were grown to an OD_{600} of .5, induced with IPTG, and were treated with metal ions as in the stability assays. Prior to harvesting for protein extraction, dilutions of the culture were plated on Luria Broth medium to compare colony growth to a wild-type control (bacteria containing empty vector). ∅ = no metal treatment.

Figure 2.9



MT1a

Figure 2.10: *In vivo* metallothionein stabilization of MT1a as a function of six specific metal ions. Data was collected from western blots probed with mono-HA antibody and normalized to HA peptide. Data representing 3 independent replicates normalized to HA levels and a representative western blot of bacterial extracts expressing a representative from *Arabidopsis* class MT1 (MT1a) are shown. ∅ = no metal treatment.

Figure 2.10

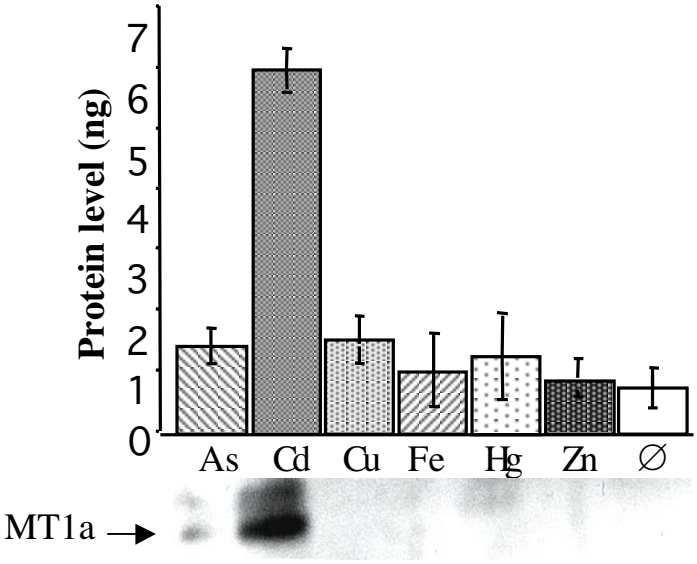


Figure 2.11: Stabilization assay performed on mouse MT2 (mMT2). As expected, mMT2 was best stabilized by cadmium and copper. Representative western blot of bacterial extracts expressing a mouse MT2 and data representing 3 independent replicates normalized to HA levels are shown. Blots were probed with mono-HA antibody.

Figure 2.11

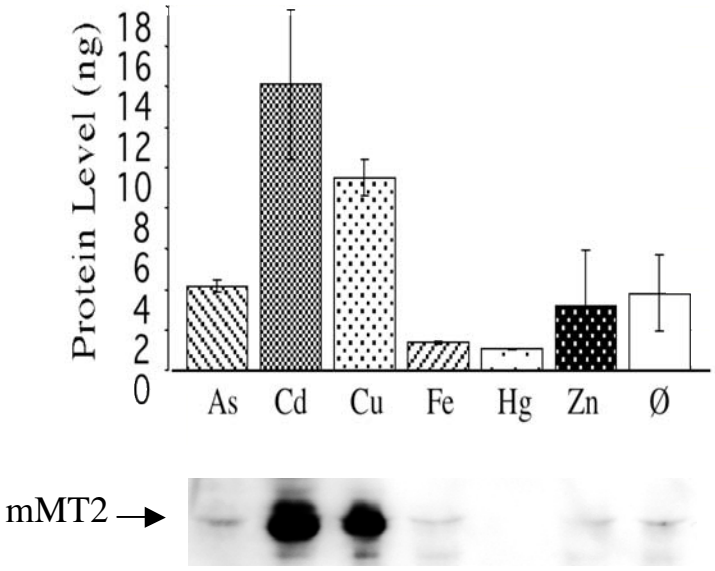


Figure 2.12: Alignment of coding region of the three *Arabidopsis* MT1 class members. The entire region shown was used in RNAi constructs to target endogenous RNA for degradation. Nucleotide differences are highlighted in gray.

Figure 2.12

```
MT1a ATGTGACTCTTGCAGTTGCGAGAAGAACTACAACAAGGAGTGCGACAAC
MT1b ATGTGATTCGTGCAGTTGCGAGAAGAACTACAACAAGGAGTGTGATAAC
MT1c ATGTGTGTGGTGCAGTTGCGAGAAGAACTACAACAAGGAGTGCGACAAC

MT1a TGTAGCTGTGGATCAAACTGCAGTTGTGGGTCAAACTGTTACTGTTGAT
MT1b TGTAGCTGTGGATCAAACTGCAGTTGCGGGTCAAGCTGTTACTGTTGAA
MT1c TGTAGCTGTGGATCAAACTGTA----------CTGTTACTGTTGAT

MT1a GAAATTATTA---TGGTCTAAAATCATATATATGGCAGAAAAATTGGGG
MT1b GAAATTATCAGCATGGTCTCAAATCA----------AGGAAAATTGGGA
MT1c GAAATTATTA---TGGTCTAAAATCATATATATGGCAGAAAAATTGGGG

MT1a AAAATATGTGTTTTTATGCTAAGAGAT---GTGTGTGTTGTTGAATAAA
MT1b AAAAAATGTGTTTTTATGCTAAGAGAT-CTTTGTGTAT-GTTGAATAAA
MT1c AAAATATGTGTTTTTATGCTAAGAGAT---GTGTGTGTTGTTGAATAAA

MT1a GACGTGACCGTTGTGTTGCGTATCAACTCTTC-TAAG-CTTTGACTT
MT1b GACTTGGC-ATTGTGTTGCGTAATGAGTAAAGGTAAC-TATTAAGCT
MT1c GACGTGACCGTTGTGTTGCGTATCAACTCTTC-TAAGCTTTTGACTT
```

Figure 2.13: RNAi lines have lower levels of MT1 transcripts than wild-type controls. **A.** Northern blot probed with the 3'UTR of MT1a. Control blot probed with actin. **B.** RT-PCR shows reduced or undetectable levels of MT1 class member transcripts in RNAi lines. Profilin (prf) was used as a cDNA template input control. MT2 was used as a control to show that RNAi constructs were specific to MT1.

Figure 2.13

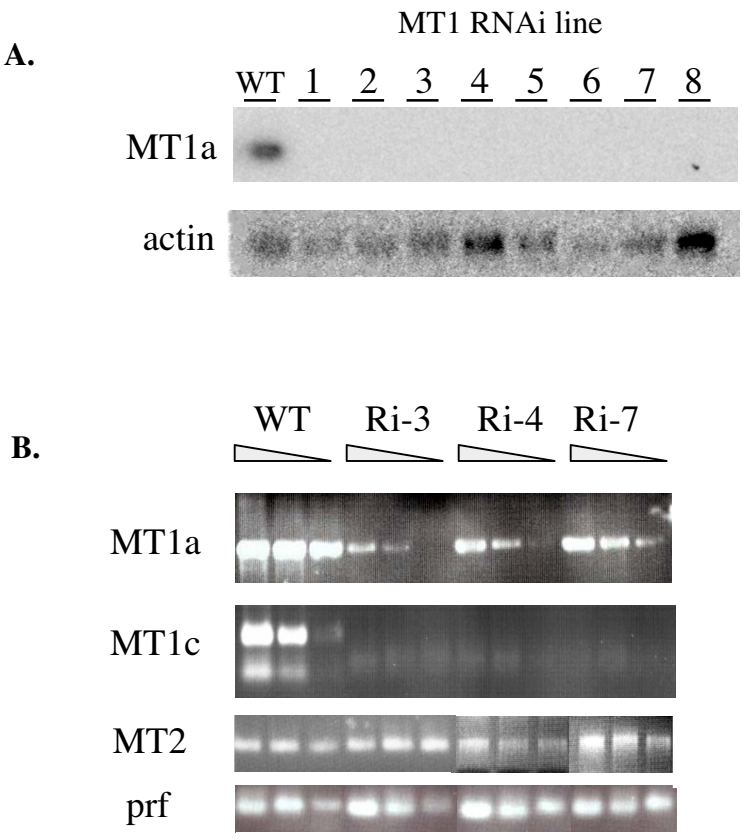

A.
MT1 RNAi line
WT 1 2 3 4 5 6 7 8
MT1a
actin
B.
WT Ri-3 Ri-4 Ri-7
MT1a
MT1c
MT2
prf

Figure 2.14: Plant lines with MT1 class transcripts knocked down show growth phenotypes on cadmium. Two week-old *Arabidopsis* wild-type (WT) and RNAi knockdown line (MT1-Ri-3, MT1-Ri-4) seedlings grown on MS (left panel) and MS + 50 μM $CdCl_2$ (right panel).

Figure 2.14

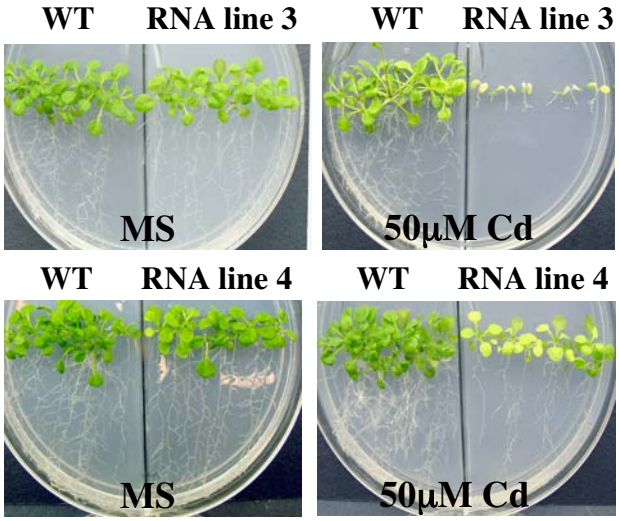

WT
RNA line 3
WT
RNA line 3
MS
50µM Cd
WT
RNA line 4
WT
RNA line 4
MS
50µM Cd

Figure 2.15: MT class 1 knockdown line Ri-7 showed a growth phenotype on cadmium. Week-old seedlings from RNAi line 7 did not germinate on 50 μM $CdCl_2$, but grew on 30 μM $CdCl_2$, although they were smaller and more chlorotic than wild-type.

Figure 2.15

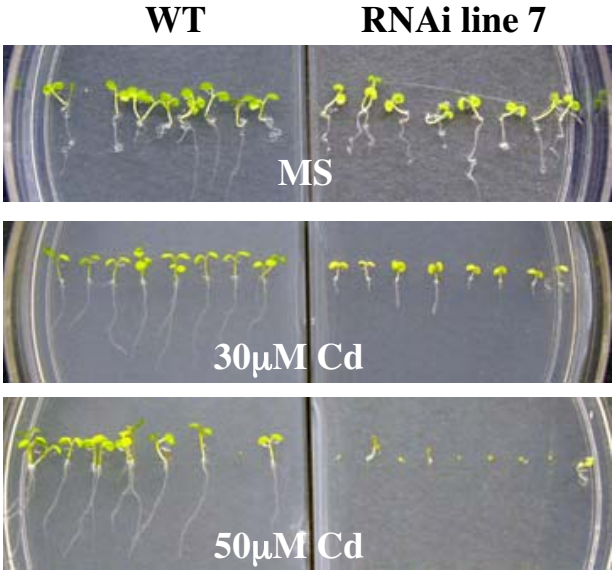


Figure 2.16: MT1 RNAi lines accumulated less fresh weight than wild-type when grown on media spiked with cadmium. Fresh weight data was taken from above ground organs and tissues of plants grown for 24 days on MS plates with 30μM $CdCl_2$. n = number of plants

Figure 2.16

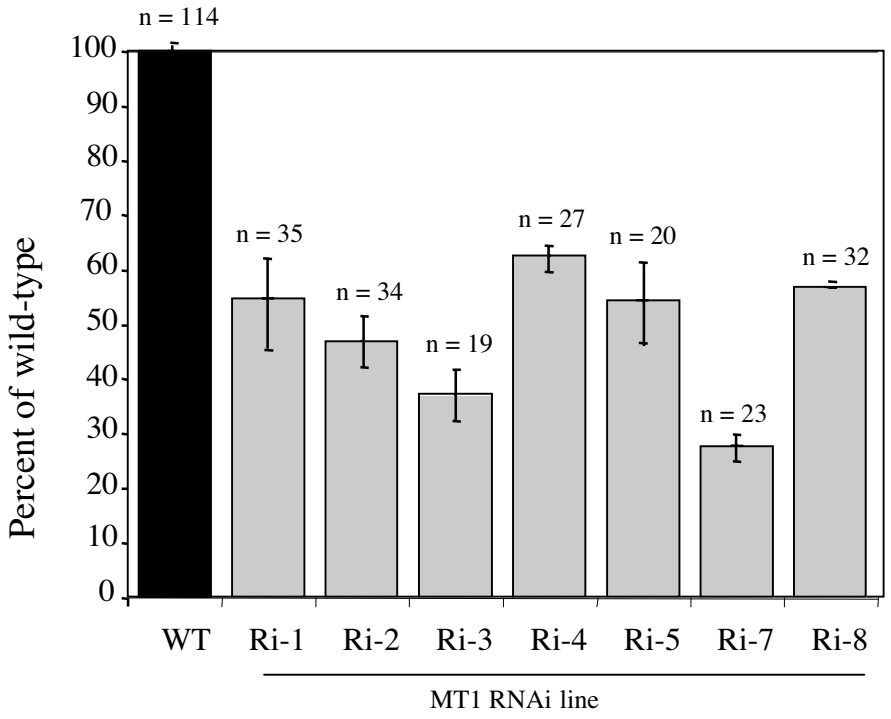
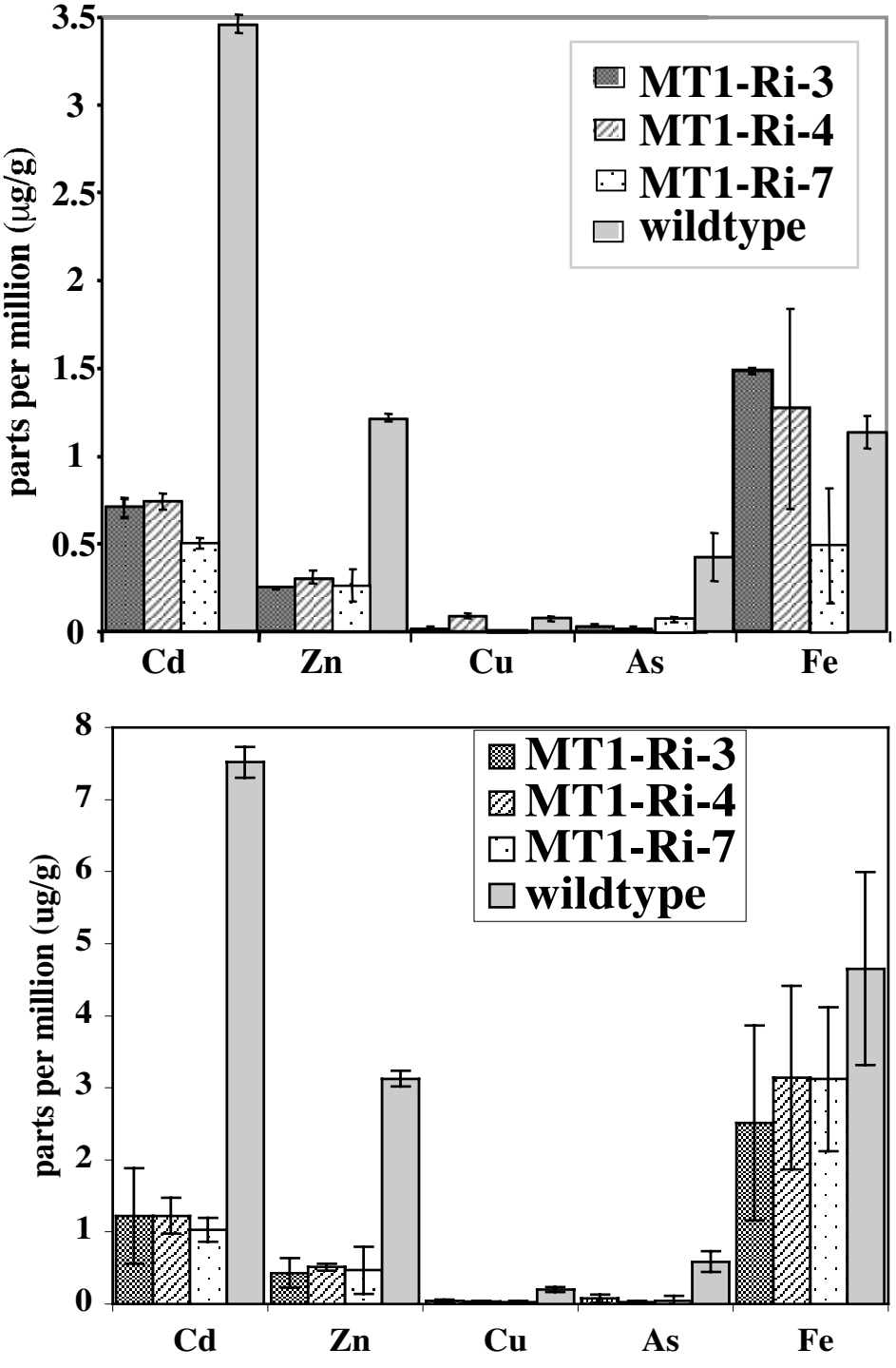

n = 114
100
90
80
70
60
50
40
30
20
10
0
Percent of wild-type
n = 35
n = 34
n = 19
n = 27
n = 20
n = 23
n = 32
WT
Ri-1
Ri-2
Ri-3
Ri-4
Ri-5
Ri-7
Ri-8
MT1 RNAi line

Figure 2.17: ICP data on WT and two RNAi knockdown lines grown in the presence of 30 μM $CdCl_2$ shows that lines knocked down in MT class 1 expression accumulated less cadmium, zinc, and arsenic than wild-type in both shoots (top panel) and roots (bottom panel).

Figure 2.17


parts per million (μg/g)
3.5
3
2.5
2
1.5
1
0.5
0
MT1-Ri-3
MT1-Ri-4
MT1-Ri-7
wildtype
Cd
Zn
Cu
As
Fe
parts per million (ug/g)
8
7
6
5
4
3
2
1
0
MT1-Ri-3
MT1-Ri-4
MT1-Ri-7
wildtype
Cd
Zn
Cu
As
Fe

CHAPTER 3

OVEREXPRESSION OF *ARABIDOPSIS THALIANA* METALLOTHIONEIN CLASS 3 CAUSES MATERNALLY INHERITED T_2 SEED GERMINATION PHENOTYPES IN THE PRESENCE OF ZINC

a. Introduction

Plant metallothioneins (MTs) are small, low molecular weight peptides that have been implicated in plant metal ion and nutrient homeostasis, and a variety of stress responses including responses to oxidative damage, heat shock, and high salinity (Taiz, 1984; Evans et al., 1992; Chevalier et al., 1995; Dunaeva and Adamska, 2001; Navabpour et al., 2003). Plant MTs are extremely cysteine rich, up to 30%, and have two or three metal binding domains separated by a linker region, based on predictions from the known structures of mammalian and invertebrate MTs (Armitage et al., 1982; Otvos et al., 1982; Brouwer et al., 1995; Narula et al., 1995; Cook et al., 1998).

The *Arabidopsis* genome contains nine MT-like sequences that assort into four ancient classes, all with classic cysteine-rich domains separated by spacer sequences. MT class 3 (MT3) has one gene member in *Arabidopsis*, whereas class 1, class 2 and class 4 each have several members (Figure 2.4, Chapter 2). Class 3 MTs ares represented as expressed sequence tags (EST) multiple times in the database in both angiosperms and gymnosperms suggesting strong selective pressure to preserve and express this MT isovariant for at least 350 million years, when those two main plant groups diverged (Cronquist, 1981; Bremer, 2000; Sanderson and Doyle, 2001). Protein sequences of MT3 can differ

by many amino acids across species, but they all retain the same cysteine spacing pattern in the N-terminal (CXXCXCXXXXXC) and the C-terminal (CXCXXXCXCXXCXC) cysteine rich domains. The same is true for MT classes 1, 2, and 4, where they all retain the same cysteine spacing patterns within a class (Figure 2.5, Chapter 2).

In vivo stability assays were performed on three classes of *Arabidopsis* MTs in *E. coli*. These data showed that *Arabidopsis* MTs have either unique protein stability patterns or are stabilized to different levels based on exposure to thiol-reactive metals. Of all the metals examined (i.e., As, Cd, Cu, Fe, Hg, Zn), *Arabidopsis* MT3 was most strongly stabilized by zinc. This is unique from classes 1, 2, and 4, which were most strongly stabilized by cadmium. To further elucidate a possible function of MT class 3 metallothioneins in plants, the MT3 gene was overexpressed in *Arabidopsis*, and the resulting plants were challenged with either excess cadmium or zinc.

b. Results

MT class 3 is best stabilized by zinc over other thiol-reactive metal ions *in vivo* in *E. coli*:

To determine metal stability *in vivo* for *Arabidopsis* MTs, protein stability assays were performed on class MT3 and a representative from classes 2 and 4 expressed *in vivo* in *E. coli* in the presence of various metal ions, as described in Chapter 2. Class members MT2a, and MT4a were selected because they were represented most often as ESTs in the GenBank and TAIR databases. MT2a and MT3 cDNA were PCR amplified from an *Arabidopsis* whole plant cDNA library using the primers listed in Table 3.1. MT4a, which is expressed mainly in seeds, was assembled synthetically using a templateless PCR strategy (Figure 3.1) (Kawashima et al., 1992; White and Rivin, 1995). Each was then cloned and expressed in *E. coli* under the control of an IPTG-inducible Lac promoter. Based on the *in vivo E. coli* stabilization assay (see Chapter 2), the *Arabidopsis* MTs had the following relative binding affinities based on protein stabilization:

MT2a, Cd(II) > >> As (III) > Hg(II) > Cu(II), Zn(II); MT3, Zn(II) > Cd(II) > As(III) > Cu(II), Hg(II), Fe(II); and MT4a, Cd(II) > Cu(II) > As(III), Hg(II), Zn(II) (Figure 3.2).

Metallothionein class 3 overexpression plant lines:

To test the MT3 stabilization data for biological relevance in plants, *Arabidopsis* plants overexpressing MT3 were generated and tested for tolerance to several thiol-reactive metals. Overexpression lines were generated by transforming plants with *Agrobacterium tumefaciens* containing a binary vector with MT3 cloned behind a constitutive (CaMV35S) promoter. T_1 generation transgenic plant lines were first selected on kanamycin. Selected lines were allowed to set seed to obtain T_2 generation plants. Of fourteen T_2 lines, three segregated at a 3:1 ratio on selection medium, suggesting the parental T_1 plants were hemizygous for a single T-DNA insert. Overexpression of the MT3 gene was verified using RT-PCR (Figure 3.3). When the three properly segregating T_2 lines (MT3-2B, MT3-10B, MT3-13) were plated on medium containing 250 μM zinc, which has 10-fold greater zinc content than half-strength MS medium control plates, all seeds were slow to germinate, produced smaller plants and had chlorotic cotyledons (Figure 3.4). The lack of segregation of the zinc sensitivity of these lines, on high zinc, was the first indication that the overexpression of MT3 affected the seed because the seed and was perhaps due to a maternal influence. The maternal organelles present in the T_2 seed endosperm all have been developed in the presence of a copy of the MT3 overexpression transgene. In contrast, MT3 overexpression lines were all slightly more resistant to 100 μM Cd than wildtype. Although when grown on cadmium the overexpression seedlings were chlorotic, as wild-type plants, they accumulated 4-fold more biomass in above ground tissue (Figure 3.5).

Considering that the endosperm environment might cause the zinc sensitive phenotype, the phenotype in the 2N plant was examined by performing seedling transfer experiments. Seeds

were germinated on half-strength MS medium for 7 days, at which time wild-type and overexpression lines were visually indistinguishable. Seedlings were then transferred to half-strength MS medium supplemented with either 250 μM zinc or 50 μM cadmium. One week after transfer, there were no obvious phenotypic differences between wildtype and overexpression lines on either metal (Figure 3.6).

It was hypothesized that excess MT3 in the cell would allow hyperaccumulation of zinc over other thiol-binding metals. To determine if MT3 overexpression lines accumulate more zinc than cadmium, seeds were germinated and grown for two weeks on half-strength MS media with both 25 μM cadmium and 25 μM zinc. MT3 overexpression lines grown on these plates grew as wild-type. According to inductively coupled plasma mass spectroscopy (ICP) data, the zinc to cadmium ratio in the MT3 plants was significantly higher than in wildtype, but zinc accumulation did not surpass cadmium accumulation. The quantitative data from several repetitions of this experiment are shown in Figure 3.7.

c. Discussion

The preservation of ancient plant MT classes may be due to distinct metal ion specificities in their cysteine rich domains. In support of this explanation, a dramatic difference was found between the metal ion stability of MT3, which is best stabilized by zinc, and the other MTs. In addition, although the other three classes were most well stabilized by Cd, slight differences were found among these remaining classes in their preferences stabilization by copper. This differential stabilization by metal suggests that each MT class has a distinct functional role based on its metal ion specificity.

In plants, it has been shown that an MT2 homolog in *Vicia faba* (bean) were expressed at higher levels in leaves than roots (Foley and Singh, 1994). Perhaps MT2 affinity for these metals provides photosynthetic machinery and cells with protection against the toxicity of thiol-reactive metals such as cadmium and arsenic. Consistent with this idea, chlorophyll destruction occurs when cadmium binds to chlorophyll molecules instead of magnesium. Cadmium may also interfere with chloroplast replication (Baryla et al., 2001), stomata function and some Calvin cycle enzymes (Steffens, 1990).

Although Cd and Cu stabilized MT4a, it was the least stabilized, and accumulated the lowest protein levels of any MT tested. MT4 is the only MT class with three domains and is represented only as an EST in dry seed libraries, whereas other MT class members are represented from a variety of libraries prepared from diverse tissues, organs and stress conditions. Therefore MT4 is unlikely to function as a generalized stress response to high intracellular metal concentrations. The broad specificity of MT4 may be beneficial to seed germination, when nutrient metal ions, like copper, are shuttled for assembly into metallo-enzymes.

The favored stabilization of MT3 by zinc is in contrast to the data from *in vitro* biochemical experiments that suggest mammalian MT binds metals in accordance with thiol's affinity for metal ions, where zinc binds most weakly (Bi (III), Cu (I), Ag (I), Hg (II) > Cd (II) > Pb (II) > Zn (II)) (Hamer, 1986; Kagi and Schaffer, 1988). To determine both a possible function and to apply our observation that MT3 is best stabilized by zinc, we overexpressed MT3 protein in transgenic *Arabidopsis* plants. Although the MT3 overexpression plant lines did not accumulate a large excess of zinc, they did have a significant increase in their zinc to cadmium ratio, and they exhibited a germination phenotype when challenged with high zinc medium. One possibility is that excess MT3 in the seed, as shown by ICP, affects the ability to germinate

because MT3 binds all of the zinc in the seed, rendering it kinetically unavailable for processes involving zinc-requiring enzymes, such as those involved in electron transport. Alternatively, overproduction of MT3 commandeers all of the available sulfur for incorporation into the MT3 thiol rich amino acid sequence.

More likely, however, is that MT3 allows excess zinc to be available for electron transport in the maternally inherited organelles, mitochondria and chloroplasts. Prior *in vitro* work in mouse has demonstrated that when excess free zinc is available, it is bound to MT where it is imported into the inter mitochondrial space (IMS), where it inhibits respiration (Ye et al., 2001). The zinc sensitivity of the seeds from MT3 overexpression lines represents the first *in vivo* evidence that supports the association of MT and mitochondria respiration inhibition from excess MT-supplied zinc. The maternal effect found by the lack of segregation of the MT3 phenotype in T_2 generation transgenic lines is consistent with poisoning of cytoplasmic organelles, like the mitochondria and chloroplast, both of which are maternally inherited from the egg cell cytoplasm. Of the two, mitochondria handle most of the electron transport in seed until the seed leaves emerge and are exposed to sunlight. Cadmium, on the other hand, is not as labile when bound to MT as zinc (Otvos et al., 1987). When MT3 overexpression lines are germinated on cadmium, the excess MT3 bound to cadmium may protect the developing embryo from cadmium toxicity.

The effect on maternally inherited organelles, occurring in the 2n seed coat, the 3n endosperm or the 2n embryo, would explain why the T_2 seeds do not segregate in the expected 3:1 ratio for MT3 related phenotypes, i.e., the transgene has a dominant effect on germination. MT3 is expressed at basal levels in above ground tissues and root tips under normal growth conditions, but expression has not been found in the seed (Guo et al., 2003). Notably, only one *Arabidopsis* MT

class, MT4, has been found solely in the developing seed (Kawashima et al., 1992; White and Rivin, 1995). MT4 is best stabilized in *E. coli* by copper and cadmium. Perhaps the expression of this class MT4 exclusively during this developmental time reduces the kinetic availability of zinc in the maternal organelles in the seed. Again, this may be why MT3 overexpression lines germinated on cadmium are slightly more robust than wildtype possibly because cadmium is not required for seed development and the sequestration of cadmium can only benefit plant germination.

Beyond germination, plants may have functional classes of MTs to specifically target metals for either nutrient uptake followed by transport to aboveground tissues, or to bind toxic metals in vacuoles, or for transport via various pumps into vacuoles. For example, the strong preference of MT3 for Zn(II) over all other metal ions examined suggests it may play a role throughout the plant in zinc processing. Zinc is essential for the structure of many cellular proteins. In fact, up to 3% of gene products identified in fully sequenced genomes are zinc binding proteins, likely because large amounts of zinc are needed for metabolic homeostasis (Wood et al., 2002). Specific cellular functions of metal processing in plants may be allocated to ancient classes of MT3 proteins with distinct zinc ion specificity.

d. Materials and Methods

Bacterial strains, plant ecotypes, and plasmid vectors:

E. coli bacterial strains Top10F (Invitrogen), and *Agrobacterium tumefaciens* strain C58 were used to host plasmids pBluescript SK^{++}(Strategene) pBIN19 (An et al., 1988). *Arabidopsis thaliana* Columbia ecotype was used in all plant experiments. Murishige and Skoog (MS) (Murashige and Skoog, 1962) phytagar plates were incubated in growth chambers at 24°C with 12 hour light/12 hour dark cycles at 22°C.

Bacterial protein preparation:

Vector-only controls and MT constructs were in IPTG inducible pET 15b vectors transformed into host strain BL21(DE3) (Novagen). Five ml overnight cultures were grown at 37°C in the presence of ampicillin (100 μg/l). LB medium (500 ml) was inoculated with 1 ml of each overnight culture. Cultures were grown to OD_{600} of 0.5 followed by addition of IPTG to 0.2 mM and metal salts to 50 μM. One hour after induction, 7 ml of culture were harvest and added to cold Tris•Magnesium buffer (10 mM Tris, 5 mM $MgCl_2$, 2 mM NaN_3) followed by centrifugation and resuspension in lysis buffer (10 mM Tris, 5 mM $MgCl_2$, 0.3 mg/ml lysozyme). The resuspension was frozen in liquid nitrogen, thawed in a 37°C water bath, then refrozen three times before a final spin and collection of the supernatant.

RT-PCR:

RNA from WT and RNAi lines of *Arabidopsis* was extracted with Plant RNeasy (Qiagen), then reverse transcribed into cDNA using the Thermo-script kit (Invitrogen). Primers specific to MT class 3 and Profilin 1 (*prf*) primers (Table 3.1) were used to amplify MT genes and *prf* control genes. PCR reactions were resolved on 1.5% agarose gels followed by ethidium bromide staining.

Transgenic plant lines:

Arabidopsis siliques were vacuum infiltrated with *Agrobacterium tumefaciens* that contained overexpression constructs in binary vector pBIN19. T_1 generation seeds were sterilized using chlorine gas for 4.5 hours and plated on half-strength MS media containing kanamycin (35mg/l). Seedlings surviving selection were transplanted to soil for T_2 seed

collection. Seeds were sown on half-strength MS medium containing kanamycin (35mg/l) to screen for the transgene. Fifteen kanamycin resistant plantlets were grown to set seed for T_2 generation experiments. Wild-type seeds Columbia were plated as controls.

Inductive Coupled Plasma Mass Spectroscopy (ICP):

Plants grown on half-strength MS medium plates with 250 μM zinc, or 25 μM zinc and 25 μM cadmium chloride were harvested above the roots. Tissues were dried at 70°C for 48 h. A nitric acid/perchloric acid (7:1) mix was added at 10 ml per gram dry weight. Tissues were acid digested in EPA glass vials (Enviroware) at room temperature for at least 48 h. Samples were diluted 5-fold in water and agitated at room temperature for 24 h. Samples were centrifuged at low speed to remove undigested debris. Supernatants were analyzed with ICP.

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Table 3.1

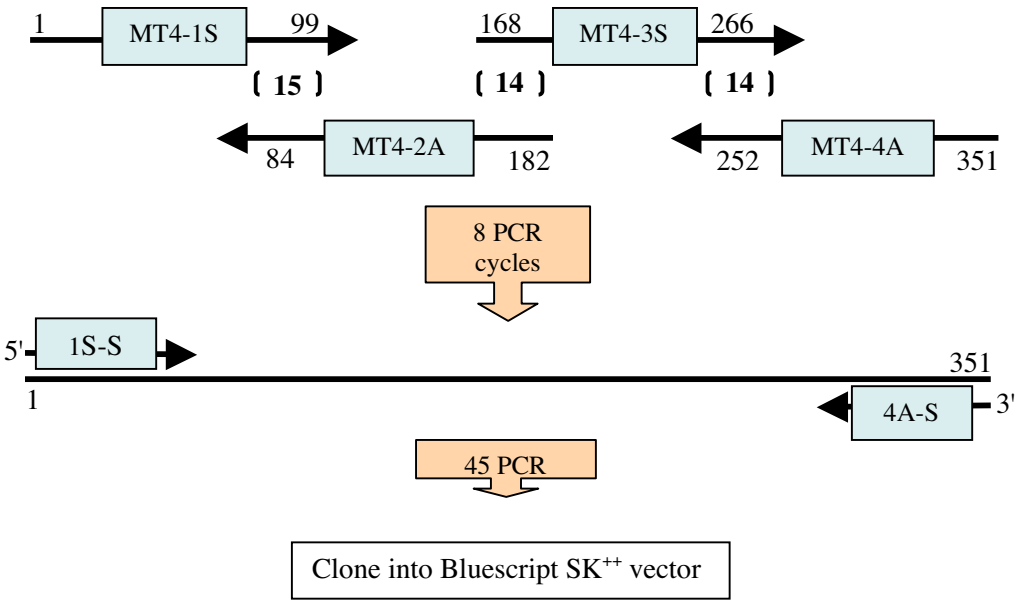
5′ oligo	
MT2a5S	tcggtctctagaggatccatgggatacccatacgatgttccagattacgcttcttgctgtggaggaaact
MT3_5S	tcggtctctagaggatccatgggatacccatacgatgttccagattagcttcaagcaactgcggaagc
3′ oligo	
MT2a3A	aacacgctcgaggagctcaagctttcatcacttgcaggtgcaagg
MT3_3A	aacacgctcgaggagctcaagctttcattagttggggcagca

Table 3.1: Oligonucleotide primers use to amplify MT2a and MT3 from *Arabidopsis* whole plant cDNA library. The 5′ primer contains a 6 base pair clamp, several restriction enzyme sites, and codes for the 9 amino acid HA epitope tag as well as sequences specific to each MT. The 3′ primer contains MT sequences, restriction enzyme sites and a 6 base pair clamp. Primers are listed 5′ to 3′.

Figure 3.1: Templateless PCR strategy and PCR primers for the amplification of MT4. **A.** Four primers that overlapped by at least 14 base pairs (MT4-1S, MT4-2A, MT4-3-S, MT4-4A) were mixed and run in eight PCR cycles. A portion of the eight-cycle PCR product was used as a template for two shorter primers (1S-S, 4A-s). The final product was cloned into pBluescript SK^{++} using the same cloning strategy as for MT2, and MT3. **B.** Primers for templateless PCR are listed.

Figure 3.1

A.



B.

Primer	Sequence (5'- 3')
MT4-1S	tcg gtc tct aga gga tcc atg gga tac cca tac gat gtt cca gat tac gct gca gac aca ggc aaa gga agt gca agc gct agc tgc aac gat cgt tgt
MT4-2A	atg ctt cgc tca tca tct tgc acc tgc aag att cgc cac cgg gac atg gag aag ggc agc cac aac gat cgt tgc agc tag cgc ttg cac ttt cct tg
MT4-3S	atg agc gaa gca tct ggt ggg gat caa gag cac aac acg tgt cca tgt ggg gag cac
MT4-4A	aac acg ctc gag gag ctc aag ctt cta agc agc gca agt ggc aca ggt gca gcc ctc
1S-S	tcg gtc tct aga gga tcc atg gga tac cca
4A-S	aac acg ctc gag gag ctc aag ctt cta agc

Figure 3.2: *In vivo* metal stabilization assay shows that representatives of each MT class are differentially stabilized in the presence of six thiol-reactive metal ions. Representative western blots are shown for each MT tested. Bar graphs represent quantitative data from multiple replicates normalized to HA protein. Both MT2a and MT4a were best stabilized by cadmium, while MT2a was stabilized to much higher levels. MT3 is best stabilized by zinc, closely followed by cadmium.

Figure 3.2

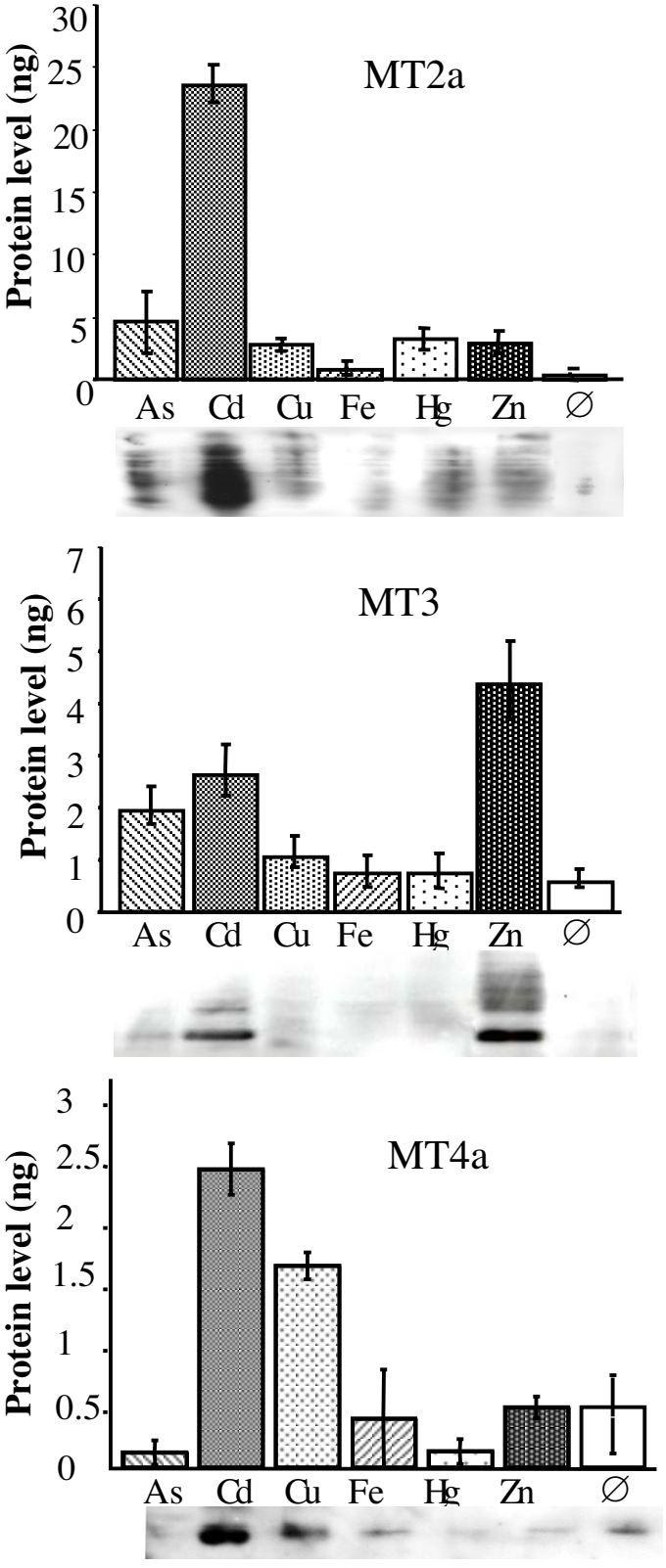

MT2a
Protein level (ng)
30
25
20
15
10
5
0
As
Cd
Cu
Fe
Hg
Zn
∅
MT3
Protein level (ng)
7
6
5
4
3
2
1
0
As
Cd
Cu
Fe
Hg
Zn
∅
MT4a
Protein level (ng)
3
2.5
2
1.5
1
0.5
0
As
Cd
Cu
Fe
Hg
Zn
∅

Figure 3.3: Expression of MT3 transcript level was higher in overexpression lines MT3-2B, -10B, and -13, as shown by RT-PCR. **A.** MT3 transcripts (254 bp) present in overexpression lines. Profilin 1 (prf1) was used a cDNA template control and is shown to the right of each overexpression line. Triangles above each gel represent the input level of cDNA, where for MT3 reactions input cDNA is 1 ng, 0.1 ng, and 0.025 ng and prf 1 reactions input cDNA is 0.5 ng, 0.1 ng, 0.01 ng. **B**. PCR was performed on genomic DNA using MT3 primers, as a control, and the 637 base pair genomic introns containing band is shown in the lower panel.

Figure 3.3

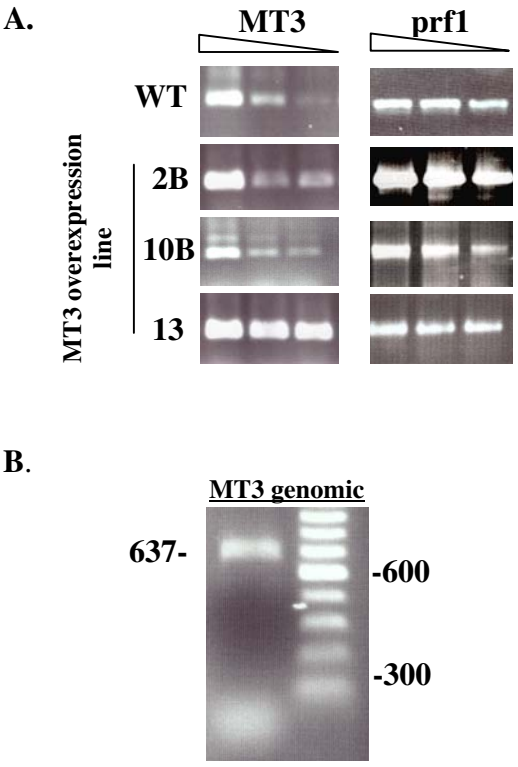

A.
MT3
prf1
WT
2B
10B
13
MT3 overexpression line
B.
MT3 genomic
637-
-600
-300

Figure 3.4: *Arabidopsis* plant lines overexpressing MT3 have phenotypes on both 100 μM cadmium and 250 μM zinc. Transgenic plants grew as wildtype on half-strength MS media (top panel).

Figure 3.4

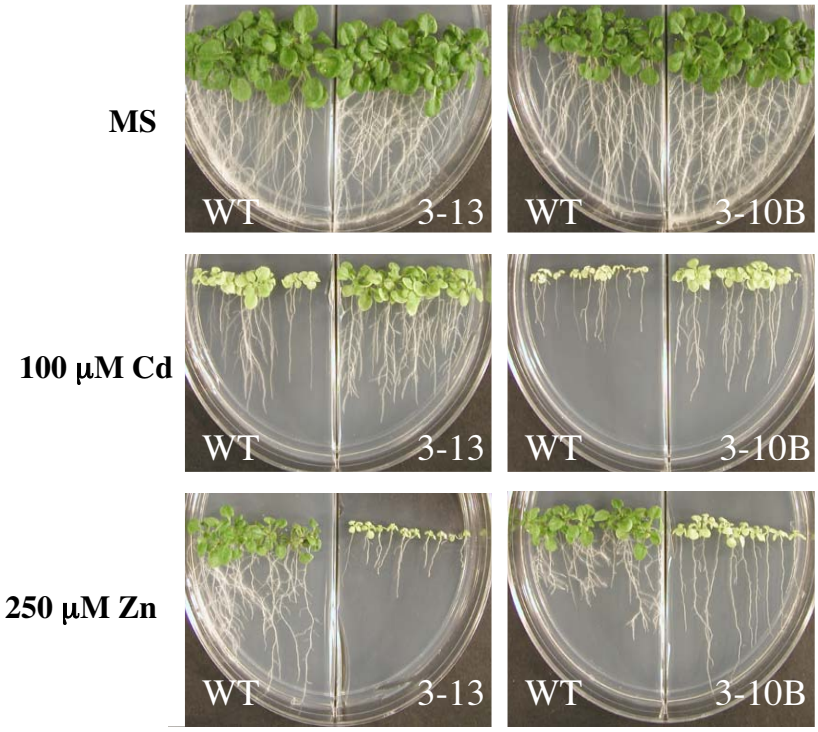


Figure 3.5: *Arabidopsis* transgenic lines overexpressing MT3 have higher fresh weights, after two weeks, when germinated on cadmium and lower fresh weights when germinated on zinc, as compared to wild-type controls.

Figure 3.5

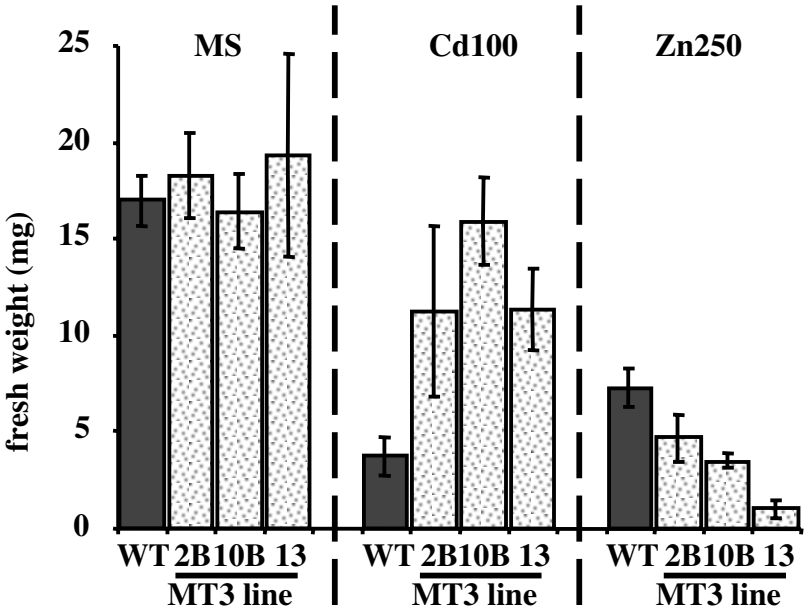

MS
Cd100
Zn250
fresh weight (mg)
25
20
15
10
5
0
WT 2B10B 13
MT3 line
WT 2B10B 13
MT3 line
WT 2B10B 13
MT3 line

Figure 3.6: Seedlings germinated on half-strength MS for one week, followed by transfer to media spiked with either 50 μM Cd, 250 μM zinc, or no metal germinated and grew as wild-type.

Figure 3.6

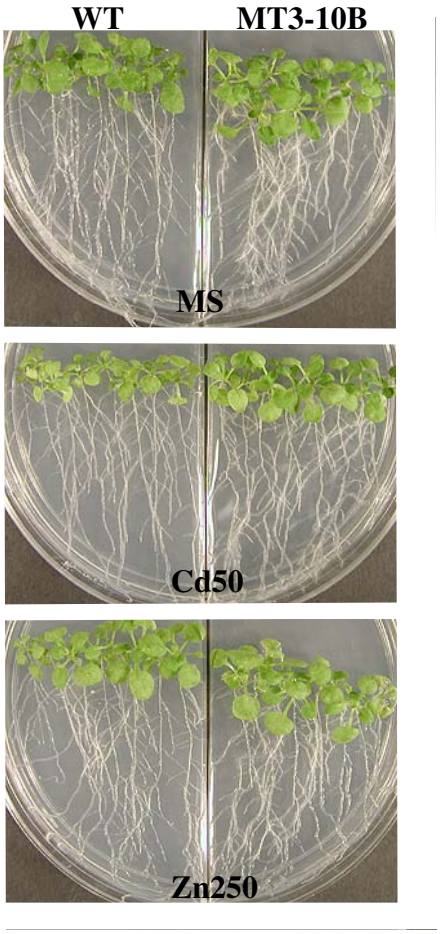
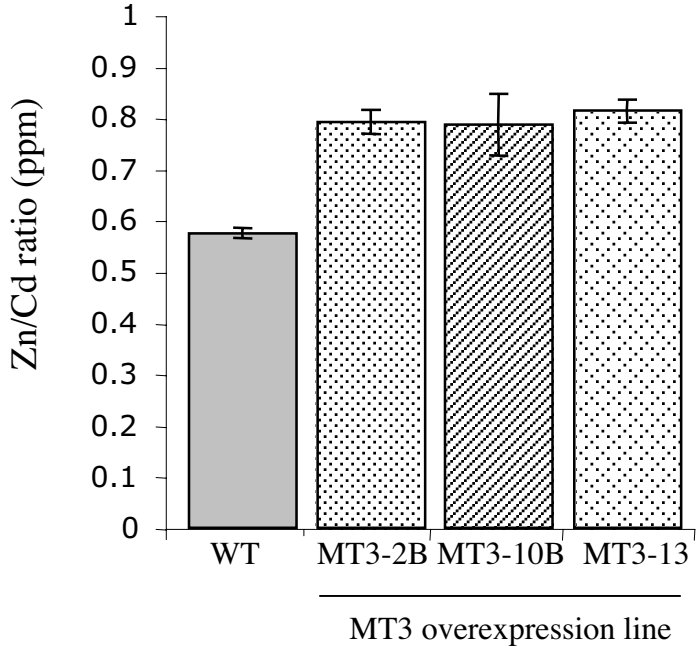


Figure 3.7: The ratio of Zn to Cd in MT3 overexpression lines is significantly greater than the ratio in wildtype, when plants were germinated and grown for 24 days on half-strength MS media spiked with both 25 μ M Cd and 25 μ M zinc.

Figure 3.7


Zn/Cd ratio (ppm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
WT
MT3-2B
MT3-10B
MT3-13
MT3 overexpression line

CHAPTER 4

DISCUSSION

In this study, it was determined that the evolution of the *Arabidopsis* MT classes is concordant with the organismal tree and at a minimum predates the divergence of monocots and dicots. There have been several organismal plant phylogenic trees generated with similar overall topology based on morphology and 18S and 26S ribosomal RNA sequences. The majority of the phylogenies suggest the evolution of the major extant plant family and algal progenitors in the same chronological order (Cronquist, 1981; Jones and Luchsinger, 1986). Based on this consensus organismal tree, the major plant groups diverged as follows; monocots and dicots diverged 200 million years ago (mya), the gymnosperms diverged from the angiosperms between 350-400 mya, and plants diverged from their protist green algal ancestors 450 mya. The *Arabidopsis* MTs segregate into four distinct classes, based on phylogenic data, all of which are at least 200 million years old because neighbor-joining phylogenic trees grouped *Arabidopsis* MTs with MTs from other species, including monocots and gymnosperms.

Cysteine spacing patterns for each class member were given numerical assignments as another criteria for determining the ancient nature of MT classes. The segregation of four classes of *Arabidopsis* MTs is supported by cysteine spacing pattern analysis because class members from distant species all had the same cysteine spacing pattern. Classification of MTs using cysteine spacing patterns is simpler than phylogenies because as new sequences are added to the sequence databases, the spacing pattern can be used to determine the MT class to which it belongs, rather than generating a new set of neighbor-joining phylogenic trees.

The strong selective constraint that maintained the same cysteine spacing patterns in each MT class is likely due to distinct MT functions in the cell. One obvious distinct function may be that the ancient cysteine spacing patterns among plant MT classes have distinct metal-ion specificities. In support of this explanation, four unique patterns of stabilization were found in *E. coli* expressing a representative member of each of the four ancient classes of *Arabidopsis* MTs using an *in vivo* stabilization assay as described in Chapter 2. Differences in stabilization were likely selected for because of specific relationships between metals and the plant, including stresses and nutrient requirements. For example, biogeochemical exposure to cadmium directly causes stress to the plant. Whereas, heat shock and herbivory causes indirect metal stress to the plant because injured and apoptotic cells can release metals thereby exposing surrounding tissue to elevated levels of metal ions. The plant cell may use MTs as much needed metal-binding molecules in stress response pathways.

Further, plants may have functional classes of MTs to specifically target metals for either nutrient uptake followed by transport to aboveground tissues, or to bind toxic metals in vacuoles or for transport via various pumps into vacuoles. The strong preference of *Arabidopsis* MT3 for zinc over all other metal ions examined suggests it may play a role throughout the plant in zinc processing. Zinc is essential for the structure of many cellular proteins. A large portion of gene products identified in fully sequenced genomes (~3%) are zinc binding proteins and these large amounts of zinc are needed for homeostasis (Wood et al., 2002). This accounts for the serious consequences of dietary zinc deficiency. It was shown in animal cells that rat liver MT regulates mitochondrial (mt) respiration rates through its role transporting Zinc into the mt intermembrane space (Ye et al., 2001). These specific cellular functions of metal processing in plants may be allocated to ancient classes of MT3 proteins with distinct zinc ion specificity.

Consistent with our data showing *Arabidopsis* MT2a is stabilized by cadmium and copper at levels four times greater that *Arabidopsis* MT1a, it has been shown that MT2a expression provides greater protection than MT1a to yeast against toxic levels of these two metal ions (Zhou and Goldsbrough, 1994). In plants, it has been shown that *Arabidopsis* MT2a and a MT2 homolog in *Vicia faba* (bean) were expressed at higher levels in leaves than roots (Foley and Singh, 1994). Perhaps MT2 affinity for cadmium and copper ions provides photosynthetic machinery and cells with protection against the toxicity of these and other thiol-reactive metals such arsenic. Consistent with this idea, chlorophyll is destroyed when cadmium is bound in chlorophyll molecules instead of magnesium. Cadmium may also interfere with chloroplast replication (Baryla et al., 2001), stomata function, and some Calvin cycle enzymes (Steffens, 1990).

Although cadmium and copper stabilized MT4a, it had the lowest protein levels of any MT tested. MT4 is the only MT class with three domains and is represented as an EST only in dry seed libraries, whereas other MT class members are represented from a variety of libraries prepared from diverse tissues and organs. Therefore, MT4 is unlikely to function as a generalized stress response to high intracellular metal concentrations. Perhaps MT4 requires other plant proteins for stabilization and its broad specificity is beneficial to seed germination, when nutrient metal ions, like copper, are shuttled for assembly into metallo-enzymes.

Toxic plant responses to cadmium are caused by several different mechanisms related to the high thiol-reactivity of divalent cadmium, Cd(II). Cadmium toxicity is often caused when Cd(II) is substituted in enzymes requiring other related metals such as Zn(II) or Cu(II) at their core, which can produce inactive forms of these enzymes or even cause them to function less efficiently (Andersson et al., 1981; Cha et al., 1996; Ubbink et al., 1996; Buchko et al., 2000).

Divalent cadmium can also inhibit proper electron transport in the mitochondria (Miccadei and Floridi, 1993; Fern et al., 1996). Specifically damaging to the plant cell, however, is the decrease in chloroplast density caused by exposure to cadmium. Additionally, plants suffering from cadmium toxicity exhibit smaller cell size (Baryla et al., 2001). The cadmium sensitivity found in RNAi lines suppressing MT1 is likely due to excess cadmium in the cytoplasm causing damage to key enzymes. *Arabidopsis* MT1 and a monocot MT1 homolog (Snowden and Gardner, 1993) are expressed throughout the plant, though both are at higher levels in the root than in aboveground tissues. *Arabidopsis* class 1 MTs are stabilized best by cadmium and may therefore be involved in detoxifying this heavy metal from both root and shoot tissues. Once MTs are absent from plant cells the cadmium is free to poison electron transport, disrupt enzymes requiring metal cations at their core, and interfere with chloroplast replication.

By targeting mRNA from all members of a class, redundancy in the function of class members can be addressed. The fact that different metal ions stabilize the plant MT classes differentially suggests that each MT class has a distinct functional role. Initial work characterizing the *Arabidopsis* MT1 and MT2 class sequences showed that a representative of each could suppress the copper and cadmium sensitivity of a yeast MT deletion (*cup-1Δ*). However, the specific affinity of these proteins for cadmium and copper over other metals was not explored.

Consistent with cadmium toxicity, plant lines knocked down for MT1 class protein expression via RNA interference were hypersensitive to cadmium, even at low concentrations of cadmium that had little inhibitory effect on the growth of wildtype plants. RNAi lines that were able to germinate and grow on 50 μM cadmium chloride were highly chlorotic suggesting that the cadmium toxicity was related to chloroplast replication or function. It is possible that in the

plant lines knocked down for expression of the MT1 isovariants, excess cadmium may remain unchelated and free to poison electron transport, disrupt the maturation of zinc requiring enzymes, and interfere with chloroplast replication.

Plants maintain a delicate balance of essential heavy metal elements, such as copper, zinc, and nickel, at micronutrient levels, while excluding toxic metals such as cadmium, arsenic, and mercury. Their mechanisms for regulating metal concentrations are largely unknown, and plant MTs are suspected to be mechanistically important to this process. Our data support such a role for MT1 class proteins. MT1 class isovariants may be required to protect *Arabidopsis* plants from the toxic effects of the heavy metal cadmium, but not mercury or arsenic. Consistent with these data is the preferential stabilization of the MT1a isovariant in the presence of cadmium *in vivo* in *E. coli*, and lowered zinc content of MT1 knockdown plant lines. Thus, MT1 proteins may also act as carriers of nutrient metal ions and as supported by the lower zinc content of MT1 knockdown lines.

One role MTs are suspected to play in metal detoxification of plant cells is to simply sequester thiol-reactive heavy metals to prevent their intrusion into metal requiring enzymes and electron transport systems. Though MTs are known to bind nutrient metals, such as zinc and copper, the release of these is a favored reaction over the release of cadmium (Jiang, 1998; Jiang, 2000). Thus favoring incorporation of zinc and copper into enzymes rather than cadmium.

To extend the observation that zinc best stabilizes class MT3, and to determine if excess of this MT might affect tolerance and accumulation of cadmium and zinc, plants overexpressing MT3 were generated and challenged with these metals. MT3 lines did not accumulate significantly more cadmium and zinc, as compared to wildtype, when grown on media spiked with those metals. MT3 overexpression lines did, however, show a significant increase in the

zinc to cadmium ratio, when challenged with equimolar amounts of both metals as compared to wildtype plants. Most interesting was the phenotype that MT3 overexpression lines exhibited when germinated on excess zinc. These plants were highly chlorotic and smaller in stature than wildtype. Further, they did not segregate as expected in a 3:1 ratio despite the fact that a single transgene insert had already been verified in these lines. Because T_2 seeds contain genetic information from both the single insert female parent and the male pollen, a dominant effect of a transgene can be observed. One likely explanation of this maternally segregating phenotype is that MT3 is inhibiting mitochondrial function necessary for proper embryogenesis and germination. Mitochondria are maternally inherited. It has been shown that mitochondria are inhibited by the presence of excess MT and excess zinc, in combination, *in vitro*. This work represents *in vivo* support for MT importing excess zinc into the mitochondria, thus poisoning electron transport.

Additional biological questions are currently being addressed in our lab to further characterize the ancient classes of plant MTs. To determine if MT classes can compensate for each other in plants, endogenous levels of all four classes of MTs in MT1 RNAi plant lines and MT3 overexpression plant lines challenged with excess or limiting amounts of metals are being monitored using HPLC. Nutrient depletion experiments are being performed on MT3 overexpression lines to determine if excess MT will allow plants to survive on soils from areas with levels of nutrition, where zinc and copper rich fertilizers are necessary.

Plants already play a natural role in phytoremediation, which is the ability to uptake, sequester and detoxify pollutants. However, most plants that have inherent abilities to hyperaccumulate metals in a biologically significant manner are typically of low biomass. High biomass plants engineered to express metal binding proteins in above ground tissues may act as

sinks for toxic metals. This work has demonstrated that class 1 metallothioneins bind Cd in plants, and that plants cannot accumulate the same amount of cadmium when these genes are suppressed, suggesting MT1's candidacy for a role in phytoremediation strategies.

A primary root function is to extract appropriate levels of 16 nutrient metals, like copper and zinc, from soil, while excluding toxic metals such as cadmium, arsenic and mercury. Nutrient enhancement can be achieved by increasing zinc levels in edible portions of plants, like seeds. The importance of finding metal-binding molecules in plants that preferentially bind zinc has become of key importance because of the positive impact it can have on human health. MT3, which we have shown to be best stabilized by zinc, over all metals tested, and to be biologically active when overexpressed in the seed, may be a candidate molecule for such a role.

Certainly, phytoremediation strategies include multiple genes and approaches to solve environmental problems. More often than not, genes must be modified for codon bias, and subcellular targeting before use in these strategies. MTs are excellent candidates for these modifications. They are small enough to diffuse through membranes if small targeting sequences were to be added, and perhaps stabilization may be increased by modifying some of the cysteine spacing patterns in the metal binding domains.

In conclusion, the activities shown in plants altered in expression for MTs, either suppressed by RNA interference or overexpressed by a constitutive promoter, suggest potential roles for both enhancing nutritional qualities of crops and for use in phytoremediation of toxic sites. Towards these ends, future work with MTs will include the pairing of plants overexpressing MTs with metal ion pumps to enhance import into the plant root system and transporter molecules that direct metal-bound MTs to aboveground tissues and/or vacuoles.

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