Examining the Specific Contributions of Individual Arabidopsis Metallothioneins to Copper Distribution and Metal Tolerance\textsuperscript{1}[OA]

Woei-Jiun Guo\textsuperscript{2}, Metha Meetam, and Peter B. Goldsbrough\textsuperscript{*}
Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907

Metallothioneins (MTs) are small cysteine-rich proteins found in various eukaryotes. Plant MTs are classified into four types based on the arrangement of cysteine residues. To determine whether all four types of plant MTs function as metal chelators, six Arabidopsis (\textit{Arabidopsis thaliana}) MTs (MT1a, MT2a, MT2b, MT3, MT4a, and MT4b) were expressed in the copper (Cu)- and zinc (Zn)-sensitive yeast mutants, \textit{Δcup1} and \textit{Δzrc1 Δcot1}, respectively. All four types of Arabidopsis MTs provided similar levels of Cu tolerance and accumulation to the \textit{Δcup1} mutant. The type-4 MTs (MT4a and MT4b) conferred greater Zn tolerance and higher accumulation of Zn than other MTs to the \textit{Δzrc1 Δcot1} mutant. To examine the functions of MTs in plants, we studied Arabidopsis plants that lack MT1a and MT2b, two MTs that are expressed in phloem. The lack of MT1a, but not MT2b, led to a 30\% decrease in Cu accumulation in roots of plants exposed to 30\μM CuSO\textsubscript{4}. Ectopic expression of \textit{MT1a} RNA in the \textit{mt1a-2 mt2b-1} mutant restored Cu accumulation in roots. The \textit{mt1a-2 mt2b-1} mutant had normal metal tolerance. However, when MT deficiency was combined with phytochelatin deficiency, growth of the \textit{mt1a-2 mt2b-1 cad1-3} triple mutant was more sensitive to Cu and cadmium compared to the \textit{cad1-3} mutant. Together these results provide direct evidence for functional contributions of MTs to plant metal homeostasis. MT1a, in particular, plays a role in Cu homeostasis in the roots under elevated Cu. Moreover, MTs and phytochelatins function cooperatively to protect plants from Cu and cadmium toxicity.

Metal ions, including those of iron, zinc (Zn), and copper (Cu), are required for catalytic and structural properties of many proteins and are therefore essential for growth and development of all organisms. However, excessive amounts of these metals, or of nonessential metals such as cadmium (Cd) and lead, are toxic and inhibit plant growth. To maintain proper metal homeostasis, organisms are equipped with a repertoire of mechanisms to regulate the uptake and distribution of specific metal ions. Nonessential metals and excessive amounts of essential metals can be detoxified by a variety of mechanisms including secretion, compartmentalization, or chelation by metal ligands (Hall, 2002).

Metallothioneins (MTs) and phytochelatins (PCs) comprise two major classes of metal binding peptides found in many eukaryotic organisms. MTs are low molecular mass (4–14 kD) proteins with high Cys content (Hamer, 1986; Coyle et al., 2002). Nearly all Cys residues in MTs are arranged in metal-binding motifs (Cys-Cys, Cys-Xaa-Cys, or Cys-Xaa-Xaa-Cys) that provide sulphhydryl ligands for coordination of bivalent metal ions (Cobbett and Goldsbrough, 2002). MTs were first isolated as Cd-binding proteins from horse kidney and have subsequently been identified in animals, plants, fungi, and some bacteria (Cobbett and Goldsbrough, 2002). In \textit{Saccharomyces cerevisiae}, MTs play a primary role in detoxification of excess Cu (Ecker et al., 1989). In mammals, MTs provide protection against Cd toxicity and maintain Zn homeostasis (Palmiter, 1998; Suhy et al., 1999; Coyle et al., 2002). Mammalian MTs also play crucial roles in controlling reactive oxygen species and in signal transduction (Tamai et al., 1993; Palmiter, 1998).

Unlike MT proteins, PCs are enzymatically synthesized from glutathione by PC synthase (PCS) and have a general structure of (γ-Glu-Cys)\textsubscript{2,11}-Gly (Rauser, 1999). PCs are an essential component of the Cd detoxification mechanism in plants, some fungi, and a small number of invertebrates (Cobbett, 2000). The role of PCs in plants has been further defined through analysis of Cd-sensitive mutants of Arabidopsis (\textit{Arabidopsis thaliana}). In particular, the \textit{cad1-3} mutant was shown to be PC deficient and hypersensitive to Cd toxicity due to a mutation in the \textit{AtPCS1} gene (Howden and Cobbett, 1992; Howden et al., 1995; Ha et al., 1999). Recent evidence also shows that PCs participate in transport of Cd from roots to shoots as a mechanism to alleviate Cd accumulation in Arabidopsis root cells (Gong et al., 2003).

Genes encoding MTs and MT-like proteins have been found in various plant species (Cobbett and Goldsbrough, 2002). Compared to MTs in mammals, plant MTs have more diverse amino acid sequences. In angiosperms, MTs can be classified into four types

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\textsuperscript{2} Present address: Institute of Plant and Microbial Biology, Academia Sinica, Taipei 115, Taiwan.

\textsuperscript{*} Corresponding author; e-mail goldsbrough@purdue.edu.

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based on the conserved positions of Cys residues (Cobbett and Goldsbrough, 2002). There are some general trends in the expression of these four types of MTs, with type-1 MTs expressed predominantly in roots, type-2 MTs in leaves, type-3 MTs in fruits, and type-4 MTs in seeds (Zhou and Goldsbrough, 1994; Hsieh et al., 1995; Zhou and Goldsbrough, 1995; Hsieh et al., 1996; Guo et al., 2003). We have previously shown that the genome of Arabidopsis contains seven genes encoding MTs (Cobbett and Goldsbrough, 2002). Expression of the Arabidopsis MT genes closely follows the patterns found in other plant species, with the exception of MT3 whose expression is prominent in leaf mesophyll cells (Guo et al., 2003). More detailed analysis has shown that expression of the Arabidopsis MT1a and MT2b genes is particularly high in phloem tissues (Guo et al., 2003).

Despite the widespread occurrence of MTs and the relatively high level of RNA expression of many MT genes (Matsumura et al., 1999; Bausher et al., 2003; Moyle et al., 2005), the functions of MTs in plants remain poorly understood. Some evidence points to a role of plant MTs in Cu detoxification. In many plant species, including Arabidopsis, rice (Oryza sativa), and the metal hyperaccumulator Thlaspi caerulescens, MT gene expression is strongly induced by Cu treatment and, to a lesser degree, by Cd and Zn (Zhou and

Figure 1. Arabidopsis MTs provide Cu tolerance in S. cerevisiae. A, Cu tolerance of yeast strains expressing Arabidopsis MTs. Yeast cells were grown on media containing 50 μM CuSO₄. Numbers at the bottom indicate serial dilutions of the yeast cultures. Wild type (DTY3), Δcup1, and the mutant complemented with CUP1 were used as controls. B, Cu accumulation in yeast strains. Yeast cells were grown to log phase in SC-Trp medium, treated with 10 μM CuSO₄ for 10 h, and the Cu content of the cells was analyzed. The data represent the means ± se of three independent experiments.

Figure 2. Analysis of Zn tolerance and accumulation of yeast strains expressing Arabidopsis MTs. A, Zn tolerance of yeast strains expressing Arabidopsis MTs. Yeast cells were grown on medium containing 0.1 mM of ZnSO₄. Numbers at the bottom indicate serial dilutions of the yeast cultures. Wild type (CM100), Δzrc1Δcot1, and the mutant complemented with a Zn transporter from T. goesingense (MTP1) were used as controls. B, Zn content in yeast strains. Yeast cells were grown in SC-Trp medium with or without 50 μM ZnSO₄ for 24 h and the Zn content of the cells was analyzed. The data represent the means ± se of three independent experiments.
Expression of MT genes has also been shown to correlate closely with Cu tolerance among Arabidopsis ecotypes and between populations of *Silene vulgaris* and *Silene paradoxa* (Murphy and Taiz, 1995; van Hoof et al., 2001; Mengoni et al., 2003). However, the involvement of MTs in Cu detoxification in planta has not been conclusively demonstrated. In addition, the divergence of plant MT protein sequences and the complex expression patterns of MT genes suggest that the functions of MTs may not be limited to Cu detoxification. This is supported by studies showing that a type-2 MT in rice, OsMT2b, is involved in reactive oxygen species scavenging and signaling (Wong et al., 2004). In response to pathogen attack, this rice MT must be down-regulated to allow an oxidative burst that signals activation of host responses leading to disease resistance.

In this study, we demonstrate that all Arabidopsis MTs can function as metal chelators in vivo. To further understand the physiological roles of MTs in plants, we have studied Arabidopsis mutants that do not express specific MT genes. Characterization of these mutants indicates that MT1a, but not MT2b, plays an important role in the accumulation of Cu in roots. We have also shown that both MTs and PCs are involved in tolerance to Cu and Cd.

**RESULTS**

**Arabidopsis MTs Are Functional Chelators of Cu and Zn in Vivo**

It has been shown that MT1 and MT2 proteins from Arabidopsis can be purified by Cu affinity chromatography and are able to bind Cu ions in vitro (Murphy et al., 1997). When expressed in the yeast Δcup1 mutant, the MT1a and MT2a genes were also able to confer Cu and Cd tolerance (Zhou and Goldsbrough, 1994). To test the ability of the other Arabidopsis MT genes to function as metal chelators in vivo, six Arabidopsis MTs were expressed in the Cu-sensitive Δcup1 *S. cerevisiae* mutant, which lacks the yeast CUP1 MT (Hamer et al., 1985). Expression of these MTs was driven by a constitutive promoter derived from the glyceraldehyde-3-P dehydrogenase gene in a high copy number (2µm origin) yeast expression vector (Mumberg et al., 1995). MT1c was omitted from this experiment because it exhibits 94% sequence identity to MT1a and the MT1c mRNA is expressed at a very low level in Arabidopsis compared to MT1a. Growth of the Δcup1 mutant was completely inhibited by 50 µM CuSO4 (Hamer et al., 1985; Fig. 1A). Expression of the six Arabidopsis MT genes, as well as the yeast CUP1 gene, was able to restore Cu tolerance of the mutant to nearly the wild-type level (Fig. 1A). Similar results were also observed in the presence of 100 µM CuSO4 (data not shown).

To determine whether expression of the Arabidopsis MTs imparted tolerance by increasing the capacity of yeast to sequester Cu ions, cells were grown in liquid medium containing a noninhibitory concentration of CuSO4 (10 µM) for 10 h and the Cu concentration in cells was measured. Deletion of the CUP1 gene significantly reduced Cu content in the mutant, and complementation with the CUP1 gene restored Cu accumulation to 70% of the wild-type level (Fig. 1B). Expression of the Arabidopsis MT proteins also increased Cu accumulation to varying degrees in the Δcup1 mutant (Fig. 1B).

To determine if Arabidopsis MTs could also function as Zn ligands in vivo, we examined the ability of these proteins to restore tolerance to a Zn-sensitive yeast mutant. The Δzrc1 Δcot1 yeast mutant lacks two vacuolar Zn transporters, ZRC1 and COT1, and is hypersensitive to Zn due to accumulation of Zn in the cytoplasm (MacDiarmid et al., 2000). Growth of the Δzrc1 Δcot1 mutant was inhibited by 0.1 mM ZnSO4. Expression of MTP1, a plasma-membrane Zn efflux transporter from the nickel/Zn hyperaccumulator *Thlaspi goesingense* (Kim et al., 2004), restored Zn tolerance almost to that of the wild type (Fig. 2A). All Arabidopsis MTs were also able to restore some growth to the Δzrc1 Δcot1 mutant in the presence of 0.1 mM ZnSO4. However, the ability of different MTs to impart tolerance to Zn was more variable than with Cu. MT4a was the most effective of the Arabidopsis MTs in conferring Zn tolerance to the yeast mutant. Although MT4a and MT4b share 84% amino acid sequence identity, including nearly all
Cys residues, MT4a was reproducibly better than MT4b in imparting Zn tolerance in yeast. Accumulation of Zn was analyzed in yeast cells grown in medium containing 50 μM ZnSO₄. Cells of the Δzrc1 Δcot1 mutant had a lower level of Zn because of their inability to accumulate Zn in the vacuole (MacDiarmid et al., 2000). Expression of the T. goesingense efflux transporter MTP1 in Δzrc1 Δcot1 further decreased Zn accumulation in yeast cells (Kim et al., 2004; Fig. 2B). Although all Arabidopsis MTs provided some degree of Zn tolerance, only expression of MT4a, and to a lesser degree MT4b, resulted in the accumulation of significantly higher levels of Zn in the yeast mutant (Fig. 2B).

Because the Δzrc1 Δcot1 mutant is also hypersensitive to Cd and cobalt (Co; D. Salt, personal communication), the ability of Arabidopsis MTs to provide protection against these metals was also tested (Fig. 3). Interestingly, MT4a provided a high level of tolerance to Co, but was ineffective for Cd. The other MTs had little effect on Co tolerance but were able to provide a high level of Cd tolerance, superior to the wild-type strain. However, expression of these MTs had no effect on Cd accumulation in yeast (data not shown).

Analysis of Arabidopsis Plants That Lack MT1a and MT2b

The capacity of MT1a and MT2b proteins to provide Cu tolerance in yeast (Fig. 1) together with phloem-localized and Cu-inducible expression of MT1a and MT2b (Guo et al., 2003) suggest that MTs may function in Cu homeostasis and distribution in plants. To examine this possibility, plants with insertional inactivation or reduced expression of the MT1a and MT2b genes were studied. Arabidopsis mutants with T-DNAs inserted in the MT1a and MT2b genes were obtained from the Salk Institute Genomic Analysis Laboratory. The positions of the T-DNA left border insertion sites were confirmed by DNA sequencing and are located 356 bp, 19 bp, and 18 bp downstream of the translational start codon in the mt1a-1, mt1a-2, and mt2b-1 mutants, respectively (Fig. 4A). The insertions in mt1a-1 and mt2b-1 are located in the first exon whereas the insertion in mt1a-1 is in the second exon. A double mutant (mt1a-2 mt2b-1) with insertions in both MT1a and MT2b was obtained from a cross between the mt1a-2 and mt2b-1 mutants. Expression of MT genes in the mutants was analyzed by RNA-blot hybridization (Fig. 4B) and reverse transcription (RT)-PCR (Fig. 4C). No hybridization signals or PCR products were detected for MT1a or MT2b RNA in the single or double mutants that were

Figure 4. Analysis of Arabidopsis deficient mutations in MT1a and MT2b. A, T-DNA insertions in Arabidopsis MT1a and MT2b genes. Black boxes represent exon sequences and the numbers indicate the positions of the insertions relative to translational start codons. The numbered arrowheads indicate locations and orientations of primers used for PCR analysis (see “Materials and Methods”). B, RNA expres-
homozygous for the corresponding T-DNA insertion alleles, indicating that the insertions resulted in complete loss of gene expression in these mutants. Expression of MT2a and MT3 RNA was not altered in the single or double mutants (Fig. 4B).

A number of independent transgenic Arabidopsis plants were also produced that express RNA interference (RNAi) for MT1a. Some of these plants had substantially reduced expression of the target MT1a RNA (Fig. 4D) and normal expression of other MT RNAs (data not shown). These plants were used in some experiments to verify that phenotypes observed in insertional knockouts of MT1a were indeed due to the lack of this MT.

The mt1a and mt2b-1 single mutants, the mt1a-2 mt2b-1 double mutant, and the RNAi transgenic plants showed no visible phenotypes when grown under normal conditions in either soil or hydroponic culture. Analysis of leaves also showed no significant differences in elemental composition between the mutant and wild-type plants grown under these conditions (data not shown). These results indicate that MT1a and MT2b are not essential for normal growth and development under these conditions.

**MT1a Deficiency Decreases Cu Accumulations in Roots**

To examine the role of MTs in plants exposed to excess Cu, seeds of the insertion mutants and one MT1a RNAi line (Fig. 4D, MT1a-RNAi-7) were germinated on normal Murashige and Skoog (MS) medium for 4 d and then transferred either to fresh MS medium or to medium containing 30 μM CuSO₄ for 10 d. Root growth and dry weight of the mutants and the RNAi line were indistinguishable from the wild type regardless of the Cu concentration in the medium (data not shown). No significant differences were observed in the Cu contents of mutant and wild-type seedlings grown in normal MS medium (Fig. 5, A and B). Compared to plants grown on normal medium, the seedlings transferred to medium containing 30 μM CuSO₄ accumulated much more Cu in both roots and shoots. However, the Cu content in roots of both the mt1a mutants and the MT1a-RNAi-7 line was approximately 30% lower than in the wild type or the mt2b-1 mutant (Fig. 5B). The mt1a-2 mt2b-1 double mutant did not show any further decrease in Cu accumulation in roots compared with the mt1a mutants. The Cu contents in shoots of the mutants and MT1a-RNAi-7 line were not significantly different from the wild-type level (Fig. 5A). The concentrations of iron, Zn, magnesium, and manganese in mutant and wild-type plants were also measured but showed no significant differences (data not shown).

Lower accumulation of Cu was also observed in roots of the mt1a-1 mutant, compared to the wild type, when plants were grown for 5 weeks in a hydroponic solution containing 30 μM CuSO₄ (data not shown). These results indicate that MT1a, but not MT2b, is important for Cu accumulation in Arabidopsis roots.

To confirm that the decreased Cu content in roots of mt1a mutants was indeed the result of deficiency in...
MT1a, the MT1a complementary DNA (cDNA) driven by the cauliflower mosaic virus 35S promoter was transformed into the mt1a-2 mt2b-1 double mutant. Two independent transgenic lines (35S-MT1a-1 and 35S-MT1a-9) were analyzed for Cu content as described above. Expression of this transgene was able to restore Cu accumulation in roots of the mt1a-2 mt2b-1 double mutants to the level seen in wild-type plants (Fig. 5C).

Lack of MTs Increases Metal Sensitivity in PC-Deficient Plants

MTs and PCs constitute two classes of Cys-rich metal chelators in plants. PCs are required for Cd tolerance in plants. In Arabidopsis, the PC-deficient cad1-3 mutant exhibited a 10-fold decrease in Cd tolerance and a 2-fold decrease in Cu tolerance (Howden et al., 1995; Ha et al., 1999). Although both PCs and MTs can bind a variety of metals in vitro, the interaction between these ligands in metal tolerance has not been investigated. The Arabidopsis mt1a-2 mt2b-1 double mutant has normal tolerance to Cu and Cd (data not shown). However, the impact of MT deficiency on metal tolerance may have been masked by the presence of PCs. To examine the potential redundancy between MTs and PCs, the mt1a-2 mt2b-1 double mutant was crossed with the cad1-3 mutant to obtain the mt1a-2 cad1-3, mt2b-1 cad1-3, and mt1a-2 mt2b-1 cad1-3 mutants. These plants were then tested for Cd and Cu tolerance relative to the cad1-3 mutant. In the presence of 30 or 40 μM CdSO₄, root growth of the mt1a-2 cad1-3 and mt2b-1 cad1-3 mutants was similar to that of cad1-3 plants (Fig. 6A). However, root growth of the mt1a-2 mt2b-1 cad1-3 triple mutant was significantly more sensitive to Cd after transfer of seedlings to Cd-containing medium (Fig. 6A). When the mt1a-2 mt2b-1 cad1-3 triple mutant was germinated and grown on MS medium containing 30 μM CdSO₄, root growth was more severely inhibited than observed in cad1-3 seedlings (Fig. 6B).

Similar results were observed when Cu sensitivity was analyzed. Using root length as an assay for Cu tolerance was complicated by the extensive development of lateral roots in seedlings grown for prolonged periods on agar plates containing additional Cu. Therefore, biomass was used as an alternative measure of Cu tolerance. Seedlings were grown on normal MS medium for 4 d and then transferred to medium containing various concentrations of CuSO₄. Dry weight of seedlings was measured after 10 d. The dry weight of the mt1a-2 mt2b-1 cad1-3 plants was similar to or slightly higher than that of the cad1-3 mutant under normal conditions. However, 30 μM CuSO₄ inhibited growth of the mt1a-2 mt2b-1 cad1-3 mutant, and to a lesser degree the mt2b-1 cad1-3 mutant, more than in the cad1-3 plants (Fig. 6C).

DISCUSSION

Arabidopsis MTs Can Impart Metal Tolerance in Vivo

MTs are Cys-rich metal-chelating proteins that have important roles in metal homeostasis in yeast and
Native MT proteins have proven to be very difficult to isolate from plants. Therefore, heterologous expression has often been used to provide evidence for the metal-binding properties of MT proteins. Here we have demonstrated that six Arabidopsis MTs have metal-binding properties. This diversity among plant MTs suggests that the four MT types may have different metal binding properties and distinct functions.

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Table 1. Genotypes of yeast strains used in this study

<table>
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<tr>
<th>Name</th>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CuWT</td>
<td>DTY3</td>
<td>MATa; trp1-1; leu2-3; leu2-112; gal1; His−; ura3-50; cup1s (single copy)</td>
<td>Hamer et al. (1985)</td>
</tr>
<tr>
<td>Δcup1</td>
<td>DTY4</td>
<td>MATa; trp1-1; leu2-3; leu2-112; gal1; His−; ura3-50; cup1::URA3+</td>
<td>Hamer et al. (1985)</td>
</tr>
<tr>
<td>Δzrc1Δcot1</td>
<td>CM137</td>
<td>MATa; can1-100; his3-11; his3-15; leu2-3; leu2-112; trp1-1; ura3-52; Δzrc1::URA3; Δcot1::KanR</td>
<td>MacDiarmid et al. (2000)</td>
</tr>
<tr>
<td>ZnWT</td>
<td>CM100</td>
<td>MATa; can1-100; his3-11; his3-15; leu2-3; leu2-112; trp1-1; ura3-52</td>
<td>MacDiarmid et al. (2000)</td>
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MT1a Is Important for Cu Accumulation in Roots

Arabidopsis MT1a is expressed abundantly in roots, and both MT1a and MT2b are expressed in phloem of several organs. These observations suggest that MT1a may have an important role in roots, and that MT1a and MT2b may be involved in distribution of Cu via the phloem (Guo et al., 2003). However, Arabidopsis plants that lack MT1a and MT2b did not exhibit any unusual phenotypes when grown under normal conditions. This may be explained by functional compensation by other MTs (MT2a and MT3) or by other metal homeostasis mechanisms. It is worth noting that MT-deficient mammals and fungi are also able to grow and develop normally under standard conditions (Hamer, 1986; Coyle et al., 2002).

Further experimentation showed that MT1a plays an important role in Cu homeostasis and/or distribution. When exposed to 30 µM CuSO4, the roots of the mt1a-1 and mt1a-2 plants accumulated 30% less Cu than the wild type and the mt2b-1 mutant. These results suggest that MT1a, but not MT2b, may function in sequestration of excess Cu in root cells. In the absence of MT1a, Cu may bind to other ligands, including other MTs. However, the capacity of MT1a-deficient roots to accumulate Cu is significantly reduced. When this capacity is surpassed, plants may down-regulate Cu transporters (e.g., AICOP1; Sancenon et al., 2004) to limit further uptake of Cu or pump excess Cu into the rhizosphere. Excess Cu may also be transported to the shoots, but we did not observe an increase in Cu accumulation in...
shoots of the mutants compared to the wild type. The lower accumulation of Cu in roots of the mt1a mutants was not accompanied by a change in tolerance to the metal. This suggests that the MT-deficient plants are still able to cope with the elevated Cu concentration, perhaps through transport mechanisms or by another detoxification system. The destructive reactive oxygen species generated by free Cu ions can also be scavenged by various cellular antioxidants and enzyme systems. The lack of MT1a and MT2b did not significantly affect Cu accumulation in shoots, suggesting that MT1a and MT2b are not essential for transport or accumulation of Cu in shoots. However, we cannot rule out the possibility that MT1a plays a role in the transport of Cu from shoots to roots. In this scenario, Cu may be transported via the xylem to shoots where MT1a is then involved in remobilization of Cu to roots or other sink tissues via the phloem. The lack of MT1a in phloem could reduce the amount of Cu redirected to the root tissues. This possibility is supported by the observation that MT1a RNA is highly expressed in senescing leaves (Guo et al., 2003), when Cu is mobilized to sink tissues. We are currently examining the effect of MT deficiency on Cu distribution in young and senescing plants, and whether MT1a proteins can translocate between roots and shoots.

Functional Interaction between MTs and PCs

In addition to participating in Cu accumulation, the results presented here provide evidence that both PCs and MTs contribute to Cu and Cd tolerance and may overlap in their functions. Growth of the mt1a-2 mt2b-1 cad1-3 triple mutant was more sensitive to Cu and Cd than either the cad1-3 or the mt1a-2 mt2b-1 mutants (Fig. 6). The increase in metal sensitivity was observed only when PC deficiency was combined with lack of both MT1a and MT2b, but not with either MT alone. These results suggest that PCs can compensate for the loss of MTs in metal detoxification. Because MT1a and MT2b comprise the major MT isoforms that are expressed in phloem tissues (Guo et al., 2003), the effect of MT deficiency on Cd hypersensitivity in PC-deficient plants may be due to the lack of MTs in the phloem. In the absence of PCs, MT1a and MT2b may participate in transport or sequestration of Cd in the phloem. This hypothesis is supported by a recent report that Cd accumulates in the phloem of Arabidopsis plants and that translocation of Cd from mesophyll cells to phloem may constitute an important mechanism for Cd tolerance in Arabidopsis (Bellegem et al., 2007). PCs may also have a role in the transport of Cu in the xylem or phloem tissues. It has already been shown that PCs can translocate from roots to shoots and vice versa, and that PCs mediate root-to-shoot transport of Cd (Gong et al., 2003; Chen et al., 2006).

In conclusion, the results presented in this study provide direct evidence for a role of plant MTs in metal homeostasis and distribution. We have shown that (1) Arabidopsis MTs function in metal homeostasis in vivo and may differ in their metal chelating properties; (2) MT1a is involved in the sequestration of Cu in roots; (3) MTs and PCs have overlapping functions in Cu and Cd tolerance. Further and more detailed investigations of Arabidopsis MT-deficient mutants will be necessary to understand the specific functions of each MT and their interactions with other metal homeostatic factors. The expression of MT genes in plants is regulated by a wide variety of developmental and environmental conditions including senescence, pathogen infection, and oxidative stress. Characterization of MT-deficient mutants under these and other conditions may reveal additional roles for MTs in plants.

MATERIALS AND METHODS

Expression of MT Proteins in Saccharomyces cerevisiae

The genotypes of Saccharomyces cerevisiae strains used in this study are listed in Table I. To express Arabidopsis (Arabidopsis thaliana) MTs in yeast, the open reading frames encoding MT1a, MT2a, MT2b, MT3, MT4a, and MT4b proteins were amplified using Pfu polymerase from individual cDNA plasmids with gene-specific primers containing restriction enzyme sites. The resulting fragments were cloned into the p424-CUP1 expression vector (Mumberg et al., 1995). The MT-CDNA-p424 plasmids and the empty vector were transformed into the yeast Δcmt1 and Δcmt1 Δcot1 mutants using the LiAc method (Gietz et al., 1995). The empty vector was also transformed into the corresponding wild-type strain, DTY3 or CM100. A construct containing the yeast MT gene (CUP1) was also transformed into Δcmt1 to provide a positive control for Cu tolerance experiments. The yeast Δcmt1 Δcot1 mutant expressing MTP1, a Zn transporter from the nickel/Zn hyperaccumulator Thlaspi goetzeiense (Kim et al., 2004), was a gift from Dr. David Salt.

Transformants were selected on synthetic complete (SC) medium lacking Trp (SC-Trp) and verified by PCR. The SC-Trp medium contained 0.67% yeast nitrogen base (Difco); 2% Glc; 0.01% of adenine, Arg, Cys, Leu, Lys, Thr, and uracil; 0.005% of Asp, His, Ile, Met, Phe, Pro, Ser, Tyr, and Val; and 2% agar for solid media.

Metal Tolerance Assays in Yeast

For analysis on plates, overnight cultures were inoculated into liquid medium at 30°C at an optical density (OD)600 of 0.2 to 0.3 and grown at 30°C to early mid-log phase (OD600, 0.8–1.0). The culture was then diluted to an OD of 0.2, from which serial dilutions (1, 10−1, 50−1, 100−1, and 500−1) were prepared with fresh SC-Trp medium and 2 μL of each dilution was spotted on SC-Trp medium containing various concentrations of CuSO4, ZnSO4, CdSO4, or Co(NO3)2. The plates were incubated at 30°C for 2 to 3 d and photographed.

Analysis of Metal Accumulation in Yeast

To measure Cu content in yeast cells, an overnight culture was inoculated into 50 mL of fresh SC-Trp medium at an OD600 of 0.2 to 0.3 and grown at 30°C for 24 h to late-log phase. Four milliliters of this culture was added to 1 mL of fresh SC-Trp medium and an OD600 of 0.2 and ZnSO4 added to a final concentration of 10 μM. Three replicates were inoculated at the same time for each yeast strain. The yeast cultures were grown at 30°C for 10 h. For Zn content analysis, the overnight cultures were inoculated into 5 mL of fresh SC-Trp medium at an OD600 of 0.2 and ZnSO4 added to a final concentration of 50 μM. Three replicates were inoculated for each strain. The cultures were grown at 30°C for 24 h. For both Cu and Zn content analyses, cells from 4 mL of cultures were collected by filtration (0.45 μm nitrocellulose filter; Millipore) and washed with citrate buffer (20 mM sodium citrate, 1 μM EDTA, pH 4.2) as described previously (Kim et al., 2004). The filters with cells were dried at 92°C for 24 h and digested in 2 mL of concentrated nitric acid at 115°C for 6 h. Digested samples were diluted to 4 mL with water and analyzed by atomic absorption spectrometry (Perkin Analyst 400; Perkin Elmer). Three replicates for each yeast strain were analyzed at the same time.
Plant Materials and Growth Conditions

Arabidopsis plants and mutants used in this study were all in Columbia ecotype. Plants were grown in a soil mix in a growth room or greenhouse. For growth on MS medium, seeds were sterilized for 8 min with 15% (v/v) household bleach containing a few drops of Tween 20, followed by three washes with sterile water. Seeds were stratified at 4°C in the dark for 3 d, germinated on 100–× 100–× 15-mm square petri dishes containing MS medium, and grown at 22°C with a 16-h/8-h light/dark photoperiod. The MS medium contained half-strength MS salts, 0.05% (w/v) MES, and 1% (w/v) Suc, solidified with 0.8% (w/v) agar.

The PCR products were digested with EcoRI and cloned into pKANNIBAL. The resulting insertions and confirmed by RNA analysis.

60 μM CdSO4. After 2 d, seedlings with inhibited root growth and brown root were transferred to 

medium. Four-day-old seedlings were transferred to MS medium containing 30 μM CdSO4 and grown for another 10 d. Shoots and roots from 14-d-old seedlings were pooled from four to five MS plates (32–40 seedlings), washed in deionized water, then washed first in deionized water, followed by citrate buffer (20 mM sodium citrate, 1 mM EDTA, pH 4.2), and then 25 mM CaCl2 (pH 5), 5 min for each wash. All the washes were performed with ice-cold solutions. After a final rinse in deionized water, tissues were dried at 95°C for 24 h and the dry weight of each sample was determined. The dry tissues were digested in 1 mL of concentrated nitric acid for 12 h at 115°C. The digested samples were diluted to 3 mL with water and analyzed by atomic absorption spectrometry.

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LITERATURE CITED


Analysis of Arabidopsis Metallothioneins

RNA Expression Analysis

RNA was isolated from 8-d-old seedlings grown in liquid MS medium using TRIZOL Reagent (Invitrogen). For RNA blots, 5 μg of RNA was separated on a formaldehyde agarose gel, transferred to a nylon membrane, UV cross-linked, and hybridized with 32P-labeled cDNA probes as described previously (Guo et al., 2003). For RT-PCR analysis of MT1a and MT2b transcripts in mutants, cdna was synthesized in a 20-μL reaction containing 2 μg of RNA as described (Smith et al., 2004). The resulting cdna was diluted to 400 μL, and 1 μL was used as the template in a 15-μL reaction and subjected to 35 cycles of PCR (94°C for 3 min, 54°C for 30 s, and 72°C for 1 min) using Taq DNA polymerase. Amplification of a tubulin cDNA (TUB6, At5g12250) using the forward primer (5′-TGGGACTCTGCATTTACCT-3′) and the reverse primer (5′-GAAA-GCATGTCACC-3′) was used to normalize results from different samples.

Measurement of Cu Content in Plant Organs

To measure Cu content, seedlings from different lines were germinated vertically on MS medium. After 4 d, seedlings were transferred to MS plates with or without 30 μM CdSO4, and grown for another 10 d. Shoots and roots from 14-d-old seedlings were pooled from four to five MS plates (32–40 seedlings), washed in deionized water, then washed first in deionized water, followed by citrate buffer (20 mM sodium citrate, 1 mM EDTA, pH 4.2), and then 25 mM CaCl2 (pH 5), 5 min for each wash. All the washes were performed with ice-cold solutions. After a final rinse in deionized water, tissues were dried at 95°C for 24 h and the dry weight of each sample was determined. The dry tissues were digested in 1 mL of concentrated nitric acid for 12 h at 115°C. The digested samples were diluted to 3 mL with water and analyzed by atomic absorption spectrometry.

RNAi Construct to Reduce Expression of MT1a

An RNAi construct to silence MT1 expression was developed by inserting two identical fragments of MT1a cDNA in opposite orientations in the pKANNIBAL vector (Wesley et al., 2001). A 388-bp fragment of MT1a cDNA including 35 bp of 5′ untranslated region and 220 bp of 3′ untranslated region was amplified from an Arabidopsis cDNA plasmid using primers that added BamHI or XhoI flanks to the 5′- and 3′-end EcoRII sites to the 3′-end of the PPT product.

The PCR products were digested with XhoI and EcoRII on the pKANNIBAL vector. The PCR products were digested with XhoI and EcoRII on the binary vector pART. The final construct, MT1a-pART, as well as the empty vector, pART, were transferred into Agrobacterium tumefaciens strain GV3300 and used for transformation of Arabidopsis by the floral dip method (Clough and Bent, 1998). Transformants were identified by germinating seeds on MS medium containing 50 μg mL−1 kanamycin. Three of 24 transformants showed clear evidence of reduced MT1 RNA expression (Fig. 4D). The line with the lowest level of MT1 RNA expression (MT1a-RNAi-7) was used in experiments to examine the effect of MT1 deficiency on Cu accumulation.

Expression of MT1a in the mt1a-2 mt2b-1 Mutant

To express MT1a under the control of the 35S promoter the MT1a cDNA was cloned between the BamHI and NcoI sites of the binary vector pCK-

GFP-S65C in place of the GFP open reading frame. The resulting plasmid was transformed into the mt1a-2 mt2b-1 double mutant as described above. Transformants were identified on MS medium containing 7 μg mL−1 35Sufosinate (Basta). Six transformed T2 plants were obtained and two T2 plants heterozygous for a single copy transgene were used in this study.


