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Functional Homologs of Fungal Metallothionein Genes from Arabidopsis

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Metallothioneins (MTs) are cysteine-rich proteins required for heavy metal tolerance in animals and fungi. Two cDNAs encoding proteins with homology to animal and fungal MTs have been isolated from Arabidopsis. The genes represented by these cDNAs are referred to as *MT1* and *MT2*. When expressed in an MT-deficient (*cup1*$^{\Delta}$) mutant of yeast, both *MT1* and *MT2* complemented the *cup1*$^{\Delta}$ mutation, providing a high level of resistance to $CuSO_4$ and moderate resistance to $CdSO_4$. Although the MT-deficient yeast was not viable in the presence of either 300 μM $CuSO_4$ or 5 μM $CdSO_4$, cells expressing MT1 were able to grow in medium supplemented with 3 mM $CuSO_4$ and 10 μM $CdSO_4$, and those expressing MT2 grew in the presence of 3 mM $CuSO_4$ and 100 μM $CdSO_4$. In plants, MT1 mRNA was more abundant in roots and dark-grown seedlings than in leaves. In contrast, MT2 mRNA accumulated more in leaves than in either roots or dark-grown seedlings. MT2 mRNA was strongly induced in seedlings by $CuSO_4$, but only slightly by $CdSO_4$ or $ZnSO_4$. However, MT1 mRNA was induced by $CuSO_4$ in excised leaves that were submerged in medium. These results indicated that Arabidopsis MT genes are involved in copper tolerance. Plants also synthesized metal binding phytochelatins (poly[γ-glutamylcysteine]glycine) when exposed to heavy metals. The results presented here argue against the hypothesis that phytochelatins are the sole molecules involved in heavy metal tolerance in plants. We conclude that Arabidopsis MT1 and MT2 are functional homologs of yeast MT.

INTRODUCTION

Heavy metals, such as copper and zinc, pose an interesting dilemma for all organisms. These metals are essential micronutrient elements required for a variety of processes in cellular metabolism. However, these metals are toxic at supraoptimal concentrations. All organisms must therefore critically balance the cellular concentrations of these potentially toxic metals. Although this can be accomplished in part by modulating uptake, organisms have developed cellular mechanisms to adapt to changes in the concentrations of metals. Such mechanisms may also be involved in dealing with other nonessential heavy metals, such as cadmium and mercury. In animals and fungi, a class of small cysteine-rich, metal binding proteins named metallothioneins (MTs) are of primary importance (reviewed by Hamer, 1986). MTs form complexes with heavy metals, and transcription of MT genes is typically regulated by metals (Thiele, 1992), providing a direct activation of this protection mechanism. The role of MTs in metal tolerance has been verified by analysis of deletion mutants that are unable to express MTs. In both yeast and mouse, such MT gene deletion strains grow under normal conditions but are hypersensitive to copper and cadmium, respectively (Hamer et al., 1985; Michalska and Choo, 1993). Whereas there is also evidence for the involvement of MTs in metal tolerance in some prokaryotes (Olafson et al., 1988; Turner et al., 1993), there is comparatively little information about MTs in plants.

The only MT protein from plants to be unequivocally characterized is a zinc binding protein from wheat, termed E_c (Lane et al., 1987). This has been confirmed by DNA sequence analysis of genes encoding E_c (Kawashima et al., 1992). E_c represents a class II MT (Kagi and Schaffer, 1988). However, E_c accumulates during embryogenesis, and its synthesis is regulated by abscisic acid rather than by metals. Although E_c is indeed the only MT protein to be purified and characterized from plants, genes encoding putative MTs have been isolated from a variety of plants, including monocots and dicots (de Miranda et al., 1990; Evans et al., 1990; de Framond, 1991; Kawashima et al., 1991; Snowden and Gardner, 1993). The structure and expression of MT genes in plants have been reviewed recently by Robinson et al. (1993). The proteins encoded by these genes contain two cysteine-rich domains, with the majority of cysteine residues present in a Cys-Xaa-Cys arrangement typical of MTs. However, unlike animal MTs, proteins predicted from plant MT genes, with the exception of E_c, possess a central domain containing aromatic and hydrophobic amino acids. The cysteine residues are responsible for most of the similarity between the plant and animal MTs. For example, *Mimulus guttatus* MT and equine MT1 share 18 identical amino acid residues of which 11 are cysteines. The proteins predicted from most plant MT genes share more than 50%

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identity between each other at the amino acid sequence level. However, the wheat E_c protein and the protein encoded by the barley B22E gene (Klemsdal et al., 1991) are more distantly related, having between 20 and 40% identity with other plant MTs. When the pea MT gene, *PsMT*$_A$, is expressed in *Escherichia coli*, this protein is able to bind copper, cadmium, and zinc (Kille et al., 1991; Tommey et al., 1991). Robinson et al. (1993) have shown recently that, under conditions of iron sufficiency, the abundance of pea MT mRNA is correlated with the concentration of copper in the growth medium. However, heavy metals have been shown to have no stimulatory effect on the expression of *Mimulus* and soybean MT genes (de Miranda et al., 1990; Kawashima et al., 1991). Consequently, the function of MT genes in plants remains uncertain.

This situation is further confounded by the presence of another class of metal binding ligands in plants. Phytochelatins (PCs) are peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where n = 2 to 10 (reviewed by Rauser, 1990; Steffens, 1990). PCs are not restricted to plants, but are also found in yeasts, including *Schizosaccharomyces pombe* and *Candida glabrata*. Like MTs, PCs use cysteine-SH groups to form complexes with metals. In contrast to MTs, PCs are synthesized enzymatically using glutathione as the substrate, and synthesis is induced in vivo and in vitro by several metals. The formation of PC–metal complexes and increased accumulation of PCs in cadmium-tolerant cell lines (Steffens et al., 1986) suggest that PCs are involved in the tolerance of plants to cadmium. This is supported by analysis of cadmium-sensitive mutants of *S. pombe* (Mutoh and Hayashi, 1988; Ortiz et al., 1992). Some of these mutants are defective in either synthesis of PCs or assembly of cadmium–PC complexes. As found in many plants, *C. glabrata* is able to synthesize PCs and has genes for MTs. Information on the specific functions of these metal ligands in *C. glabrata* has been obtained. Transcriptional activation of MT genes occurs when yeast is exposed to copper but not to cadmium (Mehra et al., 1988). This response, leading to synthesis of MT proteins, is necessary for the expression of copper tolerance (Zhou et al., 1992). In contrast, PCs are synthesized when cells are exposed to cadmium (Mehra et al., 1988).

We are interested in understanding the roles of both MTs and PCs in plants and how plants adapt to increases in the concentrations of heavy metals. To examine the functional properties of plant MT genes, we cloned two cDNAs encoding MTs from Arabidopsis. Expression of these genes in an MT-deficient yeast mutant restored metal tolerance, demonstrating that Arabidopsis MTs are both structural and functional homologs of the yeast MT. Furthermore, we demonstrated that expression of MT genes in Arabidopsis is regulated by heavy metals.

RESULTS

Presence of Two MT Genes in Arabidopsis

In an experiment aimed at identifying ethylene-regulated genes (Zhou and Goldsbrough, 1993), we isolated and determined the nucleotide sequences of a number of cDNAs. A gene represented by one of these, designated *MT1*, encodes a 45–amino acid protein that shares 45% identity with trout MT (Bonham et al., 1987). Figure 1 shows the nucleotide and deduced amino acid sequences of the MT1 cDNA. The length of the transcript detected by the MT1 cDNA probe in RNA hybridization experiments (0.5 kb) indicates that this is a full-length cDNA. The 45–amino acid protein is the only possible open reading frame of the MT1 cDNA. The DNA sequence of another Arabidopsis cDNA encoding an MT protein has been entered in the DNA data base (K. Takahashi, Laboratory of Plant Nutrition, Department of Agricultural Chemistry Faculty of Agriculture, University of Tokyo, Tokyo, Japan; GenBank, EMBL, DDBJ accession number X62818). The gene corresponding to this cDNA will be referred to as *MT2* in this report. We isolated a cDNA for *MT2* by polymerase chain reaction (PCR) using oligonucleotide primers flanking the open reading frame and RNA isolated from leaves as the initial template. The MT2 cDNA encodes an 81–amino acid protein containing 14 cysteine residues. Compared to the cDNA sequence submitted by K. Takahashi, there is a single nucleotide substitution (AGC to AAC) at codon 64 of the PCR-amplified cDNA that results in a Ser-to-Asn conversion. This substitution was observed in three independent subclones of the PCR product and has been confirmed in the sequence of a cloned genomic DNA fragment containing *MT2* (data not shown). Another Arabidopsis MT2 cDNA has been sequenced independently and also contains AAC rather than AGC at codon 64 (G. Philipps and C. Gigot, Laboratoire de Biologie Moleculaire des Plantes-CNRS, Strasbourg, France; GenBank, EMBL, DDBJ accession number Z26416).

As shown in Figure 2, MT1 and MT2 cDNAs predict proteins that are more than 50% identical in their N-terminal and

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  1   GAAGAAACTACAATAGTTAATCAATCAAAG
 31   AGAAGTAAGAGAAATGGCAGATTCTAACTG
                    M  A  D  S  N  C    6
 61   TGGATGTGGCTCCTCCTGCAAATGTGGTGA
       G  C  G  S  S  C  K  C  G  D   16
 91   CTCTTGCAGTTGCGAGAAGAACTACAACAA
       S  C  S  C  E  K  N  Y  N  K   26
121   GGAGTGCGACAACTGTAGCTGTGGATCAAA
       E  C  D  N  C  S  C  G  S  N   36
151   CTGCAGCTGTGGGTCAAACTGTAACTGTTG
       C  S  C  G  S  N  C  N  C  *   45
181   ATGAAATTATTATGGTCTAAAATCATATAT
211   ATGGCAGAAAAATTGGGGAAAATATGTGTT
241   TTATGCTAAGAGATGTGTGTGTGTTGTTGG
271   AATAAAGACGTGACCGTTGTGTTGCGTATC
301   AACTCTCTTAAGCTTTGACTTTTCCCAGCT
331   TTGTATTTTCCTATGTATGGTAATGGTGTG
361   ATTGTGTAATGTTTTCATATGTAACGTAAA
391   AAAAATATTTATGTGACATTGACTTTTGTG
421   ACTACTAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide Sequence and Deduced Amino Acid Sequence of the Arabidopsis MT1 cDNA.

The numbers at left and right refer to the numbering of nucleotides and amino acid residues, respectively.

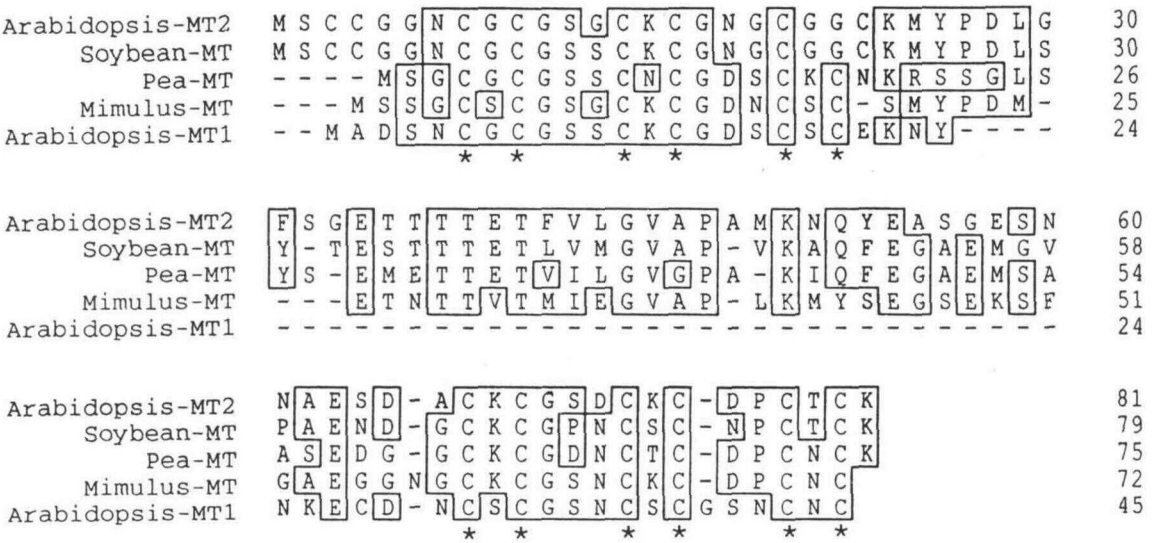

Figure 2. Alignment of Amino Acid Sequences of Arabidopsis MT1, MT2, and Other Plant MTs.

Asterisks indicate positions where cysteine residues are organized as Cys-Xaa-Cys. Numbers represent positions of amino acids in each MT. Residues that are identical or similar in at least three sequences are boxed. Dashes are included for optimal alignment.

C-terminal cysteine-rich domains. Twelve of 13 cysteine residues in MT1 and 10 of 14 in MT2 are organized as Cys-Xaa-Cys, which is typical of MTs. Unlike animal and fungal MTs, the amino acid sequences deduced from almost all previously reported plant MT genes contain a third domain of ~40 residues separating the two cysteine-rich domains. The central domains of plant MTs contain aromatic amino acid residues but no cysteine. Arabidopsis *MT2* also encodes this central domain and is therefore similar to the majority of MT genes isolated from other plant species. In contrast, Arabidopsis *MT1* encodes a smaller protein lacking the central domain and may represent another class of MT genes in plants. Although there is 53% overall identity between the amino acid sequences of MT1 and MT2, there is no significant homology between the cDNA nucleotide sequences. When MT1 and MT2 cDNAs were hybridized to genomic DNA, as shown in Figure 3, each cDNA detected distinct DNA fragments in Arabidopsis genomic DNA, indicating that Arabidopsis contains at least two MT genes.

Complementation of the Yeast *cup1*$^{\Delta}$ Mutation with Arabidopsis MT1 and MT2

Yeast contains a single MT gene (*CUP1*) that is required for copper tolerance. The *cup1*$^{\Delta}$ mutant is highly sensitive to heavy metals because of the lack of endogenous MT, and this mutant has been widely used to test the function of MT genes from several organisms (Ecker et al., 1986; Thiele et al., 1986; Chernaik and Huang, 1991). To test whether the expression of Arabidopsis MT genes protects yeast against copper and cadmium toxicity, we expressed Arabidopsis MTs in the *cup1*$^{\Delta}$ yeast mutant. cDNAs containing complete open reading frames of Arabidopsis *MT1* and *MT2* were inserted into the yeast expression vector pYCHE, as shown in Figure 4A, and introduced into yeast strain ABDE1, a *cup1*$^{\Delta}$ mutant (Ecker et al., 1986). Plasmids lacking any MT gene or carrying the hamster MT gene were used as negative and positive controls,

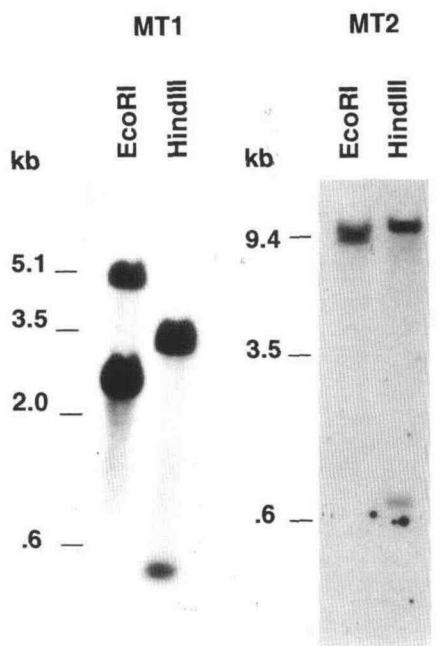

Figure 3. Genomic DNA Gel Blot Analysis of *MT1* and *MT2*.

Five micrograms of Arabidopsis DNA was digested with either EcoRI or HindIII, and blots were hybridized with the ^{32}P-labeled MT1 or MT2 cDNA probe as indicated. Molecular length markers are given at left in kilobases.

respectively. Figure 4B shows that the mRNAs for *MT1* and *MT2* are correctly transcribed in the yeast transformants. The yeast strains carrying these plasmids were streaked on agar plates containing copper or cadmium, as shown in Figure 5. Cells carrying the vector alone grew normally in the absence of these metals but were not viable on medium containing either 300 μM $CuSO_4$ or 5 μM $CdSO_4$ as a result of deletion of the *CUP1* locus. However, cells expressing Arabidopsis *MT1* or *MT2* or a hamster MT gene were resistant to 3 mM $CuSO_4$. When cadmium tolerance was tested, expression of Arabidopsis *MT1* or *MT2* or a hamster MT gene enabled cells to grow in the presence of 10, 100, and 500 μM $CdSO_4$, respectively. The difference in cadmium tolerance between yeast cells expressing *MT1* and *MT2* is not understood but suggests that the central domain of the MT2 protein may be involved in increasing cadmium tolerance. Alternatively, the differences in number and arrangement of cysteine residues between MT1 and MT2 may affect binding of cadmium to these proteins.

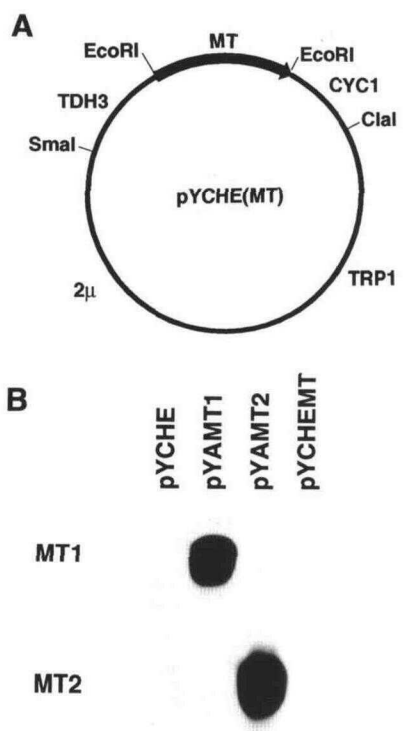

Figure 4. Expression of Arabidopsis MT1 and MT2 mRNAs in Yeast.

(A) Diagram of the pYCHE vector used to express MTs in yeast. DNA fragments of MT genes were inserted into the EcoRI site between the glyceraldehyde-3-phosphate dehydrogenase (*TDH*) promoter and the cytochrome *c* oxidase (*CYC*) terminator as described in Methods. Plasmids were introduced into yeast strain ABDE1.

(B) MT mRNA expression in yeast. Total RNA was isolated from yeast cells carrying pYCHE (no MT), pYAMT1 (Arabidopsis MT1), pYAMT2 (Arabidopsis MT2), or pYCHEMT (hamster MT). After electrophoresis and transfer to nylon membranes, RNAs were hybridized to the ^{32}P-labeled MT1 or MT2 cDNA.

Differential Expression of MT1 and MT2 mRNAs in Arabidopsis

Accumulation of MT1 and MT2 mRNAs in Arabidopsis is regulated in a tissue-specific manner. Figure 6 shows the RNA gel blot analysis of *MT1* and *MT2* expression in different tissues. MT1 mRNA was highly expressed in roots and etiolated seedlings, but the level of MT1 mRNA in leaves was only one-third that in roots. In contrast, MT2 mRNA was approximately eight times more abundant in leaves than in roots of 2-week-old plants.

Metal Induction of MT mRNA Expression

Metal-regulated expression is a common feature of MT genes from animals and fungi. Experiments with *Mimulus* and soybean have failed to demonstrate any increase in MT gene expression in response to either copper or cadmium (de Miranda et al., 1990; Kawashima et al., 1991). This may indicate that plant MT genes are not regulated by heavy metals. However, experimental conditions may not have been used where increased MT gene expression in response to metals could be observed. Experiments described by Robinson et al. (1993) support this latter hypothesis. The abundance of $PsMT_A$ mRNA increased when copper was supplied together with an iron chelate to pea roots.

The differential expression of *MT1* and *MT2* in Arabidopsis tissues prompted us to examine the effects of metal treatment on MT gene expression in a variety of tissues. To obtain uniform exposure, plant material was submerged in liquid medium containing heavy metals and shaken continuously to provide aeration. Figure 7A shows the levels of MT1 and MT2 mRNAs in 7-day-old seedlings that had been exposed to various concentrations of $CuSO_4$, $ZnSO_4$, or $CdSO_4$ for 30 hr. MT2 mRNA was present at a low level in control seedlings but increased in seedlings treated with heavy metals. Among the metals tested, copper was the most efficient inducer of MT2 mRNA. Treatment of seedlings with 50 μM $CuSO_4$ for 30 hr produced a 5.5-fold increase in the level of MT2 mRNA, as determined by densitometry. The time course of MT2 mRNA expression in copper-treated seedlings is shown in Figure 7B. Treatment of seedlings with 50 μM $CuSO_4$ caused a steady increase in MT2 mRNA for up to 72 hr. The effects of zinc or cadmium on MT2 mRNA expression were less pronounced. In the experiment shown in Figure 7A, the level of MT2 mRNA increased to twice and 2.5-times that of the control after exposure of seedlings to 1 mM $ZnSO_4$ and 150 μM $CdSO_4$, respectively. In repeated experiments, copper was consistently the most effective inducer of MT2 mRNA. Results with zinc and cadmium have been more variable, ranging from almost no induction in one experiment to the response seen in Figure 7A.

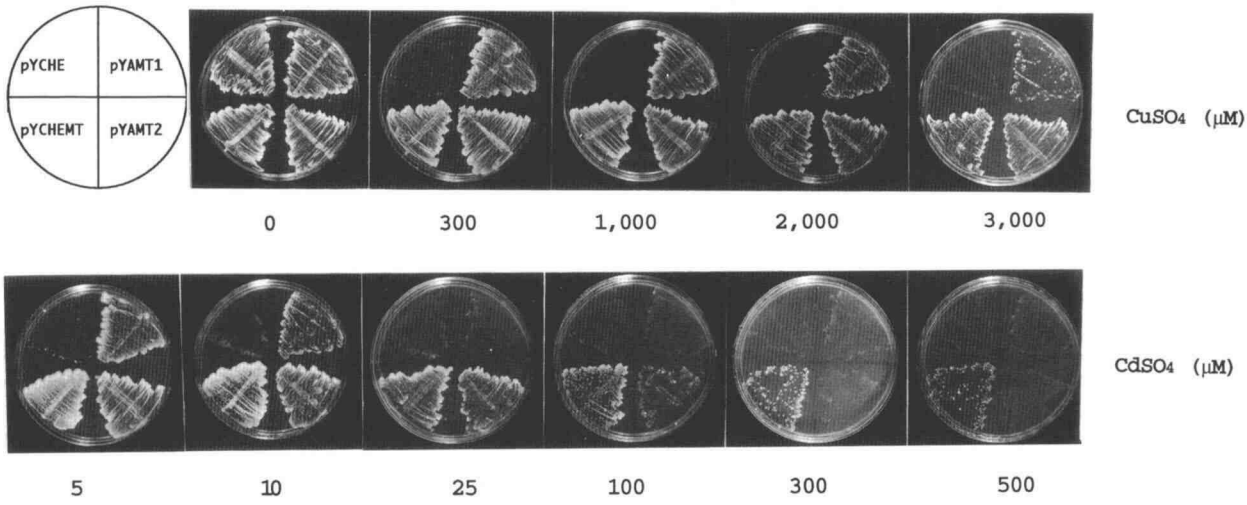

Figure 5. Complementation of Copper and Cadmium Sensitivity of the *cup1*$^{\Delta}$ Yeast Mutant by Arabidopsis MT1 and MT2.

The strains were streaked on plates as shown. The yeast strains carrying pYCHE (vector alone), pYAMT1 (Arabidopsis MT1), pYAMT2 (Arabidopsis MT2), or pYCHEMT (hamster MT) were grown on synthetic agar medium (Chernaik and Huang, 1991) containing various amounts of $CuSO_4$ or $CdSO_4$ as indicated below the plates and photographed after incubation at 30°C for 4 days.

The abundance and metal regulation of MT1 mRNA in seedlings did not parallel that of MT2 mRNA. *MT1* was expressed at a relatively high level in seedlings under control conditions. In repeated experiments, induction of MT1 mRNA by metals has not been observed. The small changes in MT1 mRNA abundance shown in Figure 7A (i.e., small declines in response to copper and zinc and a small increase in response to cadmium) have not been consistently observed. We further examined the effects of metals on the expression of *MT1* in leaves, where the mRNA is at a low level under normal conditions. Figure 8 shows that the abundance of MT1 mRNA in excised leaves increased after exposure to these metals. The level of MT1 mRNA increased to more than three times that of the control in response to copper. The effects of zinc and cadmium were less pronounced. These results indicated that both *MT1* and *MT2* have the ability to be induced by copper. The significance of smaller changes in MT mRNA abundance in response to zinc and cadmium is not yet understood.

DISCUSSION

Arabidopsis MT1 and MT2 Are Functional Homologs of Fungal MTs

Among the plant MT genes that have been identified, the metal binding properties of only two MTs, $PsMT_A$ from pea and E_c from wheat, have been studied (Lane et al., 1987; Kille et al., 1991; Tommey et al., 1991). When expressed in *E. coli* either as a glutathione *S*-transferase fusion or as a native protein, $PsMT_A$ binds copper, cadmium, and zinc, with the highest affinity for copper. Lane et al. (1987) found that E_c protein purified from wheat germ contains zinc. However, it has not been demonstrated if either $PsMT_A$ or E_c can provide heavy

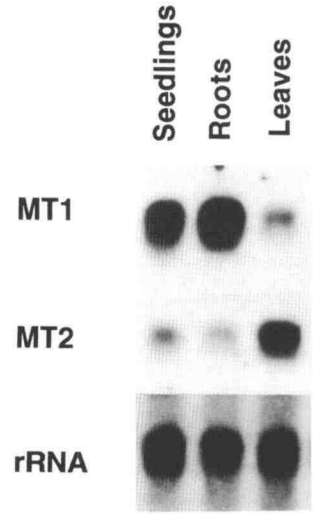

Figure 6. Differential Expression of MT1 and MT2 mRNA in Arabidopsis Plants.

RNA was isolated from dark-grown seedlings and from roots or leaves of 2-week-old light-grown plants. The RNA blots were hybridized with the ^{32}P-labeled MT1 or MT2 cDNA. The blots were then stripped and hybridized with an rDNA probe for 18S rRNA to confirm equal loading of RNA.

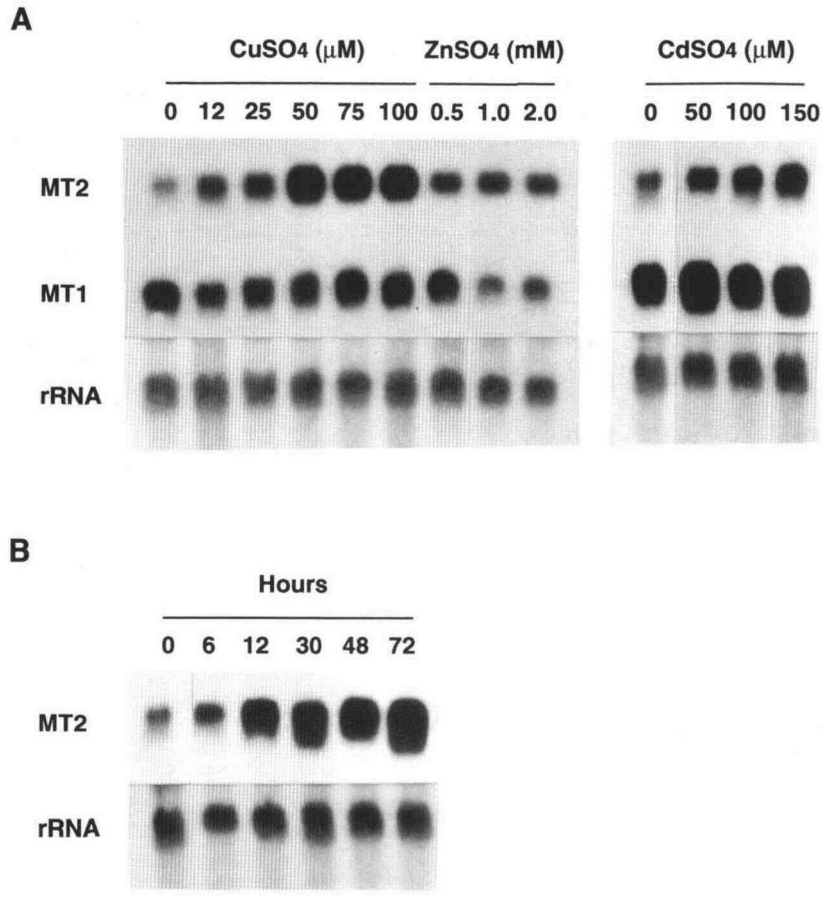

Figure 7. Metal-Regulated Expression of MT2 mRNA in Arabidopsis.

(A) Expression of MT2 and MT1 mRNA in seedlings. Seedlings were exposed to various concentrations of $CuSO_4$, $ZnSO_4$, or $CdSO_4$ for 30 hr. **(B)** Time course of $CuSO_4$-induced MT2 mRNA expression in seedlings. Seedlings were exposed to 50 μM $CuSO_4$ for the times indicated. RNA blots were hybridized with the ^{32}P-labeled MT2 cDNA or an rDNA probe as described in the legend to Figure 6.

metal tolerance in vivo. In this report, we show that Arabidopsis has at least two MT genes. The abundance of MT1 mRNA in leaves and MT2 mRNA in seedlings increased after treatment with copper. Furthermore, expression of *MT1* and *MT2* in the yeast *cup1*$^{\Delta}$ mutant restored metal tolerance. The ability of Arabidopsis MTs to complement the yeast MT-deficient mutation and the regulation of these MT genes by metals demonstrate that structural and functional homologs of the yeast MT gene are indeed present in plants. Mammalian MT genes similarly complement this yeast mutation (Thiele et al., 1986), indicating that at least some properties of MT genes are conserved between plants, animals, and fungi.

The Structure of MT1 and MT2

Similar to animal MTs, most plant MTs, including MT1 and MT2 from Arabidopsis, have two distinct cysteine-rich domains

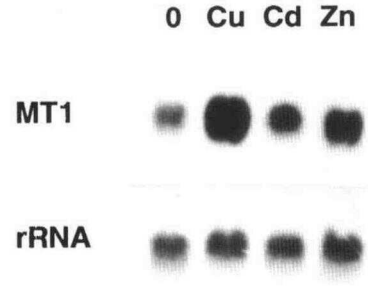

Figure 8. Induction of MT1 mRNA Expression by Metals.

Leaves were excised from plants and submerged in Murashige and Skoog liquid medium containing 50 μM $CuSO_4$ (Cu), 100 μM $CdSO_4$ (Cd), 1 mM $ZnSO_4$ (Zn), or no metal (0) and incubated with shaking (120 rpm) at 22°C for 17 hr. The RNA blot was hybridized with the ^{32}P-labeled MT1 cDNA or an rDNA probe as indicated.

(Figure 2). The exception to this is E_c from wheat, where cysteines are distributed throughout the 80-amino acid protein (Kawashima et al., 1992). These two domains are characteristic features of animal MTs, where the cysteines are organized in clusters and there is a lack of aromatic and hydrophobic amino acids. Most plant MTs also contain a large central domain separating the two cysteine-rich domains. This central domain contains aromatic and hydrophobic amino acids but no cysteine and does not fit the criteria for MTs described by Hamer (1986). However, Arabidopsis MT1 is unique among the characterized plant MTs with regard to its size and absence of this central domain. MT1 is more similar to animal MTs and shares 45% identity at the protein level with the MT from rainbow trout.

The nucleotide sequence divergence between MT1 and MT2 cDNAs allows them to be used as gene-specific probes. As shown by genomic DNA gel blot analysis, there are at least two MT genes in the Arabidopsis genome. Hybridization of each probe to two DNA fragments with each restriction enzyme indicates that there may be more MT genes in the Arabidopsis genome. MT gene families have been identified in *Mimulus* and pea (de Miranda et al., 1990; Evans et al., 1990), but within each species these genes are highly homologous. For example, three MT genes have been isolated from pea and are more than 95% identical to each other at the amino acid sequence level (Robinson et al., 1992). Plant MT genes have also been classified based on the arrangement of cysteine codons (Robinson et al., 1993). Arabidopsis *MT1* and *MT2* would be listed as type 1 and type 2, respectively, under this classification. The presence of divergent MT genes within a species, similar to that described here for Arabidopsis, has been reported in *C. glabrata* (Mehra et al., 1989) and *Caenorhabditis elegans* (Freedman et al., 1993). The significance of two distinct MT genes in Arabidopsis is not yet understood. Whereas the cysteine-rich domains are responsible for metal binding, as demonstrated in animal MTs (Otvos and Armitage, 1980; Chernaik and Huang, 1991), the central domain has no known function. Nevertheless, conservation of the central domain in plant MTs implies that it is functionally important. When the pea $PsMT_A$ gene was expressed in *E. coli*, the central domain of this protein was the target for extensive proteolysis (Kille et al., 1991). It is not known if similar proteolysis occurs in plants or if such processing is functionally important, but this might explain the failure to detect these proteins in plants. The structural differences between *MT1* and *MT2* suggest that these genes may play distinct roles in Arabidopsis. Further support for this hypothesis comes from the contrasting patterns of expression of MT1 and MT2 mRNAs in plants.

Regulation of *MT1* and *MT2* Expression

Whereas the yeast complementation study demonstrated that both *MT1* and *MT2* are able to provide heavy metal tolerance in vivo, there is no direct evidence for their role in plants. An understanding of how these genes are regulated might provide some insight into their possible function in plants. We found that expression of MT genes in Arabidopsis is developmentally regulated. Whereas MT1 mRNA is predominantly expressed in roots, MT2 mRNA is present mainly in leaves. This suggests that different organs in Arabidopsis use different MT isoforms. Interestingly, tissue-specific expression of MT isoforms was also observed in mammals (reviewed by Hamer, 1986).

Arabidopsis MT gene expression is also regulated by copper. In animals and fungi, metal-induced expression of MT genes is responsible for the accumulation of MT proteins and hence for metal tolerance (Hamer et al., 1985; Ecker et al., 1986; Michalska and Choo, 1993; Zhou and Thiele, 1993). Animal MT genes are induced most efficiently by cadmium (Richards et al., 1984). In contrast, fungal MT genes are induced primarily by copper (reviewed by Butt and Ecker, 1987). The metal-regulated expression of Arabidopsis MT genes is more similar to that of fungal MT genes, with greatest induction by copper. This suggests that Arabidopsis MTs may be involved in tolerance to heavy metals, especially copper.

MT genes have been used as model systems to study metal-regulated gene expression (reviewed by Thiele, 1992). Metal-responsive elements have been identified and characterized in animal and yeast MT gene promoters. Genes encoding transcription factors that are required for MT gene expression have also been cloned from yeast (Thiele, 1988) and *C. glabrata* (Zhou and Thiele, 1991). The metal response element of the yeast *CUP1* gene has been used to drive expression of a reporter gene in transgenic plants (Mett et al., 1993). Only a low level of reporter gene activity was obtained and this increased only twofold after exposure of plants to copper. When ACE1, the transcription factor required for copper-regulated expression of *CUP1* in yeast, was also expressed in plants, the reporter gene was induced more than 10-fold after copper treatment. Therefore, there is divergence between plants and yeast in the mechanisms used to control MT gene expression in response to copper. This will be examined further by analyzing the promoter elements that are required for metal responsiveness of Arabidopsis MT genes.

Arabidopsis MT genes are regulated by copper, and increased expression of a pea MT gene in response to copper has been reported recently (Robinson et al., 1993). However, metal-regulated expression of MT genes was not reported in *Mimulus* and soybean (de Miranda et al., 1990; Kawashima et al., 1991). Metal regulation of MT genes in plants may only occur at certain developmental stages, in specific tissues, or under particular conditions of mineral nutrition. Expression of the *C. elegans* MT genes, *mtl-1* and *mtl-2*, is induced by cadmium exclusively in intestinal cells (Freedman et al., 1993). The metal-inducible expression of *mtl-1* is attenuated in adult animals compared to larvae. Relatively few experiments examining the metal induction of plant MT genes have been reported. Establishing systems in which plant MT genes are induced by heavy metals will facilitate the study of metal-regulated gene expression in plants.

Possible Roles of PCs and MTs in Plants

The discovery of metal binding PCs in plants led to the proposal that PCs are the functional analogs of fungal and animal MTs (Grill et al., 1987; Jackson et al., 1987). Moreover, it has been proposed that plants and animals use divergent heavy metal sequestration mechanisms, with PCs being the only class of molecules used by plants for metal binding in both tolerance to excess metals and normal metal ion homeostasis (Grill et al., 1987). Our results disagree with this hypothesis and indicate that the role of PCs in plants needs to be reevaluated.

Many plants are equipped to synthesize both MTs and PCs as metal binding ligands. A similar situation has been described in *C. glabrata*, where PCs are induced by cadmium and MTs are produced in response to copper (Mehra et al., 1988). *C. glabrata* mutants that are unable to transcribe MT genes are sensitive to copper but not cadmium (Zhou et al., 1992). Plants may have evolved similar mechanisms for tolerating different metals. In tobacco, inhibiting PC synthesis with buthionine sulfoximine reduced cadmium tolerance but had no effect on copper tolerance (Reese and Wagner, 1987). Similarly, cadmium-sensitive mutants of Arabidopsis show only a small reduction in copper tolerance (Howden and Cobbett, 1992). Expression of either *MT1* or *MT2* imparts high levels of copper tolerance to yeast but has only a limited effect on cadmium tolerance, relative to the hamster MT gene. In Arabidopsis plants, induction of MT genes was more effective with copper than cadmium. Taken together, these results suggest that PCs and MTs function with distinct metal specificities in Arabidopsis.

METHODS

Growth and Metal Treatment of Plants

Arabidopsis thaliana, ecotype Columbia, was obtained from M. Estelle (Indiana University, Bloomington). Seeds were surface-sterilized with 2% sodium hypochlorite, germinated, and grown on Murashige and Skoog (1962) agar medium (Sigma) for 4 days at 22°C in the dark to obtain etiolated seedlings. Leaf and root tissues were obtained from seedlings that were grown on agar medium for 14 days at 22°C under continuous light. For metal treatment, sterilized seeds were germinated in Murashige and Skoog liquid medium and grown for 7 days at 22°C with continuous shaking (120 rpm). Seedlings were treated with metals by adding solutions of metal salts to the liquid medium. To expose leaf tissue to heavy metals, leaves were excised from 2-week-old plants, submerged in Murashige and Skoog liquid medium containing 50 μM $CuSO_4$, 100 μM $CdSO_4$, 1 mM $ZnSO_4$, or no metal, and incubated with shaking (120 rpm) at 22°C for 17 hr.

Yeast Strain and Vector

Saccharomyces cerevisiae ABDE-1 (*arg4-8, leu2-112, his7-2, trp1-289, ade5*, and *cup1*$^{\Delta}$) (Ecker et al., 1986) and pYCHEMT (Chernaik and Huang, 1991), which contains a Chinese hamster metallothionein (MT) gene, were kindly provided by P.-C. Huang (Johns Hopkins University, Baltimore). pYCHEMT (Figure 4A) carries the *N*-(5′-phosphoribosyl)anthranilate isomerase (*TRP1*) gene and a 2 μ replication origin conferring high copy number in yeast cells. The transcription cassette within the vector contains the promoter of the yeast glyceraldehyde-3-phosphate dehydrogenase (*TDH*) gene and the transcription terminator of the yeast cytochrome c oxidase (*CYC*) gene.

DNA and RNA Isolation and RNA and DNA Gel Blot Analyses

Total RNA was isolated from yeast and Arabidopsis as described previously (Ausubel et al., 1987; Goldsbrough et al., 1990). For RNA gel blot analysis, RNA was fractionated by formaldehyde–agarose gel electrophoresis. Gels were loaded with 10 μg of total RNA per lane. RNA was transferred to a nylon membrane (Schleicher & Schuell) and cross-linked with UV light. RNA blots were hybridized with ^{32}P-labeled MT1 or MT2 cDNAs. Blots were also hybridized with an rDNA probe for 18S rRNA to confirm equal loading of RNA. Hybridization was quantified by densitometry of autoradiograms with different exposure times by using a PD-120 densitometer (Molecular Dynamics, Sunnyvale, CA). Genomic DNA was isolated from Arabidopsis leaves as described by Goldsbrough et al. (1990). Five micrograms of DNA was digested with restriction enzymes, separated on an agarose gel, and transferred to a nylon membrane. Duplicate blots were hybridized with ^{32}P-labeled MT1 or MT2 cDNAs.

Cloning of the MT1 cDNA from Arabidopsis

An Arabidopsis cDNA library was originally screened for cDNA clones representing mRNAs induced by ethylene using a differential screening procedure (Zhou and Goldsbrough, 1993). One of the cDNAs identified by this method was sequenced using a Sequenase DNA sequencing kit (U.S. Biochemical Corp.); this cDNA has been shown to encode an MT-like protein and was named Arabidopsis MT1 cDNA. The DNA sequence of the MT1 cDNA has GenBank, EMBL, and DDBJ accession number L15389.

Cloning of the MT2 cDNA

The sequence of the MT2 cDNA was originally determined by K. Takahashi and entered into the GenBank, EMBL, and DDBJ data base (accession number X62818). A DNA fragment containing the MT2 open reading frame was obtained by polymerase chain reaction (PCR) amplification using upstream and downstream primers (5′-CCAGAATTCTCGAGAAAAATGTCTTGC-3′, 5′GTCGAATTCACTTGCAGGTGCAAG-3′) corresponding to the sequences flanking the open reading frame of this cDNA. EcoRI sites were included in the primers to aid in cloning the amplified fragment. Two micrograms of total RNA from leaves was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) and the downstream primer for *MT2*, and the resulting cDNA was used as template for amplification (Kawasaki, 1990). The amplified DNA fragment was digested with EcoRI and inserted into the EcoRI site of pBluescript SK– (Stratagene).

Construction of Yeast Expression Vectors

Because of the multiple EcoRI sites in pYCHEMT, direct insertion of Arabidopsis MT cDNAs into this plasmid was impossible. To construct

the desired expression vectors, the SmaI-ClaI fragment containing the MT expression cassette from pYCHEMT was subcloned into pBluescript SK−. The hamster MT sequence was deleted by EcoRI digestion. The MT1 cDNA in pBluescript SK− was flanked by an upstream EcoRI site and a downstream XhoI site. A DNA fragment containing the open reading frame of *MT1* with EcoRI sites at both ends was synthesized by PCR with the SK primer (Stratagene) and a downstream primer with an EcoRI site (5′-CAAGAATTCCATCAACAGTTACAGTTTGAC-3′). After EcoRI digestion, DNA fragments containing MT1 or MT2 were inserted in place of the hamster MT gene in the subcloned MT expression cassette. The new SmaI-ClaI fragments containing *MT1*, *MT2*, or no insert were then transferred from pBluescript SK− into pYCHEMT, replacing the original SmaI-ClaI fragment. The newly generated plasmids were named pYAMT1, pYAMT2, and pYCHE, respectively.

Yeast Transformation and Measurement of Metal Tolerance

Yeast (ABDE1) cells were transformed with different constructs using a lithium acetate procedure (Stearns et al., 1991), and transformants were selected by growth on tryptophan-deficient agar medium (1.7 g/L yeast nitrogen base, 5 g/L ammonium sulfate, 5 g/L casamino acids [autoclaved for 30 min to remove tryptophan], 20 g/L glucose, 30 mg/L adenine, 20 mg/L histidine, 30 mg/L arginine, 40 mg/L leucine, 40 mg/L lysine, and 2% bactoagar) (Chernaik and Huang, 1991). Transformed yeast strains were streaked on tryptophan-deficient plates containing various amounts of $CdSO_4$ or $CuSO_4$ and photographed after incubation for 4 days at 30°C.

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