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# Genes Encoding Proteins of the Cation Diffusion Facilitator Family That Confer Manganese Tolerance

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**The yeast *Saccharomyces cerevisiae* expressing a cDNA library prepared from *Stylosanthes hamata* was screened for enhanced $Mn^{2+}$ tolerance. From this screen, we identified four related cDNAs that encode membrane-bound proteins of the cation diffusion facilitator (CDF) family. One of these cDNAs (*ShMTP1*) was investigated in detail and found to confer $Mn^{2+}$ tolerance to yeast by internal sequestration rather than by efflux of $Mn^{2+}$. Expression of *ShMTP1* in a range of yeast mutants suggested that it functions as a proton:$Mn^{2+}$ antiporter on the membrane of an internal organelle. Similarly, when expressed in Arabidopsis, *ShMTP1* conferred $Mn^{2+}$ tolerance through internal sequestration. The ShMTP1 protein fused to green fluorescent protein was localized to the tonoplast of Arabidopsis cells but appeared to localize to the endoplasmic reticulum of yeast. We suggest that the ShMTP1 proteins are members of the CDF family involved in conferring $Mn^{2+}$ tolerance and that at least one of these proteins (ShMTP1) confers tolerance by sequestering $Mn^{2+}$ into internal organelles.**

## INTRODUCTION

Proteins belonging to the cation diffusion facilitator (CDF) family (also known as the cation efflux family) have been implicated in the metal tolerance mechanisms of a range of organisms. Indirect evidence suggests that these proteins are transporters that either sequester metal ions within cells or export metal ions out of cells (Paulsen and Saier, 1997). The CDF proteins described to date confer $Zn^{2+}$, $Cd^{2+}$, $Co^{2+}$, or $Ni^{2+}$ tolerance to a range of organisms, and many are located on internal membranes. In at least one instance, a protein of the CDF family was shown to be involved in $Zn^{2+}$ uptake rather than $Zn^{2+}$ efflux across the plasma membrane, indicating diverse functional roles for this class of transporters (Cragg et al., 2002). The mechanism of transport and the driving force that allows the metal ions to be transported are not well understood for most of the CDF proteins.

Plants possess a range of genes that encode CDF proteins, and there is evidence that some of these are involved in metal tolerance. For example, the *ZAT* gene of Arabidopsis confers increased $Zn^{2+}$ tolerance when overexpressed in plants (van der Zaal et al., 1999). The increased tolerance is thought to be attributable to internal sequestration of $Zn^{2+}$; however, the subcellular location of the protein and the site where $Zn^{2+}$ is sequestered are unknown. Bloß et al. (2002) expressed *ZAT* in *Escherichia coli* and studied the purified protein in reconstituted proteoliposomes. The protein transported $Zn^{2+}$ into proteoliposomes by a mechanism that relied on the $Zn^{2+}$ gradient across the membrane and not on a proton gradient. Persans et al. (2001) isolated genes (*TgMTP*s) that encode CDF proteins from the nickel-hyperaccumulating species *Thlaspi goesingense*, which conferred metal tolerance to *Saccharomyces cerevisiae* mutants defective in COT1 and ZRC1. Both COT1 and ZRC1 are yeast proteins of the CDF family that confer $Zn^{2+}$ and $Co^{2+}$ tolerance and are located on the vacuolar membrane (Li and Kaplan, 1998). Recently, MacDiarmid et al. (2002) provided evidence that ZRC1 acts as a proton antiporter to transport $Zn^{2+}$ into the vacuole. Persans et al. (2001) also suggested that the TgMTP1 proteins are involved in transporting metals to the vacuole, although the location of the proteins in vivo and their transport activities have yet to be demonstrated in either yeast or plants.

Proteins of the CDF family from diverse sources have the following features in common: (1) they share an N-terminal signature sequence that appears to be specific to the family; (2) the proteins possess six transmembrane-spanning regions; (3) they share a cation efflux domain; and (4) most of the eukaryotic members possess an intracellular His-rich domain that is absent from the prokaryotic members (Paulsen and Saier, 1997). Initial analysis of the Arabidopsis genome identified eight genes that encode proteins belonging to the CDF family (Mäser et al., 2001). Four of the putative proteins encoded by these genes possess all of the features described above that are common to the CDF family, whereas the other, more distantly related proteins (previously named AtMTPc1 to AtMTPc4 for metal tolerance protein) possess only a subset of these features (Mäser et al., 2001). The AtMTPc proteins have four to five predicted transmembrane domains; three of them possess only part of the N-terminal signal sequence, and all lack the His-rich domain.

*Stylosanthes hamata* is a tropical legume tolerant of acid soils in which high concentrations of soil $Mn^{2+}$ can occur and represent a potential source of genes that confer $Mn^{2+}$ tolerance (de Carvalho et al., 1980). Here, we describe the isolation of cDNAs from Stylosanthes that encode proteins of the CDF

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family with features similar to those of the previously named AtMTPc3 of Arabidopsis (Mäser et al., 2001). One of these cDNAs was characterized in detail and shown to confer $Mn^{2+}$ tolerance to yeast and plants by a mechanism that is likely to involve the sequestration of $Mn^{2+}$ into internal organelles.

## RESULTS

### Isolation of Stylosanthes cDNAs That Confer Mn Tolerance to Yeast

A yeast expression library was screened on agar medium that contained $Mn^{2+}$ at a concentration that was toxic to wild-type Saccharomyces. From this screen, colonies were selected that grew vigorously on the $Mn^{2+}$-toxic agar. To confirm that expression of the plant cDNAs was responsible for the $Mn^{2+}$-tolerance phenotype, the plasmids were isolated and transformed into the wild-type parental strain and rescreened on $Mn^{2+}$-toxic agar. Sequencing showed that the cDNAs encoded four related proteins with features similar to those of the CDF family of transporters.

A search of the databases showed a strong similarity of the Stylosanthes proteins to four proteins encoded by putative genes in the Arabidopsis genome (Figure 1). One of the Arabidopsis proteins, previously named AtMTPc3 (Mäser et al., 2001), was identified by homology as a member of the CDF family of transporters. The other three Arabidopsis proteins were not recognized previously as CDF proteins, and it is now apparent that they, along with AtMTPc3, form a distinct subgroup within the Arabidopsis CDF family (Figure 2). To simplify the nomenclature and to avoid the potential difficulties of defining subgroups by the inclusion of lowercase letters, the Arabidopsis genes that encode the CDF proteins have been renamed *AtMTP1* to *AtMTP12* (David Salt, personal communication). In keeping with this nomenclature, we named the Stylosanthes genes identified in the $Mn^{2+}$-tolerance screen *ShMTP1* to *ShMTP4*. As a result of their phylogenetic relationship to AtMTP1, three other proteins from the Arabidopsis CDF family, AtMTP2, AtMTP3, and AtMTP4, are thought to be $Zn^{2+}$ transporters (Mäser et al., 2001) (Figure 2); AtMTP1 also is known as ZAT.

The proteins AtMTP8 to AtMTP11 along with ShMTP1 to ShMTP4, although related to this clade, are clustered as a major group, and evidence presented here suggests that they are CDF proteins involved primarily in $Mn^{2+}$ transport. The translation products of rice ESTs and *Caenorhabditis elegans* sequences also showed strong similarity to the ShMTP family of proteins, indicating that the distribution of these types of proteins is not restricted to plants. The molecular masses of the ShMTP proteins are predicted to be 46.7 kD (ShMTP1; 416 amino acid residues), 46.4 kD (ShMTP2; 407 amino acid residues), 47.0 kD (ShMTP3; 414 amino acid residues), and 47.4 kD (ShMTP4; 416 amino acid residues). The amino acid sequences of the ShMTP proteins did not show significant similarity to other proteins that are known to transport $Mn^{2+}$, such as CAX2 (Hirschi et al., 2000), members of the NRAMP family (Mäser et al., 2001), $Ca^{2+}/Mn^{2+}$-ATPases (Lapinskas et al., 1995), and ABC-type ATPases (Bartsevich and Pakrasi, 1996).

Analysis of the Stylosanthes sequences showed that, like the related Arabidopsis proteins (AtMTP8 to AtMTP11), they lack the complete N-terminal signature sequence for the CDF family and lack a His-rich region that is found commonly in eukaryotic members of the CDF family. Furthermore, transmembrane prediction programs identified models for the ShMTP family consisting of either four (http://www.cbs.dtu.dk/services/TMHMM/) or five (http://www.ch.embnet.org/software/TMPRED_form.html) membrane-spanning regions. Although the ShMTP and related Arabidopsis proteins lack the complete N-terminal signature sequence for the CDF family, all possess Ser and Asp residues (Figure 1) that are found to be fully conserved in other members of the CDF family (Paulsen and Saier 1997). All of these sequences possess a cation efflux domain, which is indicative of the CDF family as identified by the Pfam protein domain database (http://pfam.wustl.edu). Although the ShMTP1 protein lacks the complete N-terminal sequence suggested by Paulsen and Saier (1997) to be a signature sequence for the CDF class of proteins, we show here that the expression of *ShMTP1* in yeast and Arabidopsis confers phenotypes consistent with a role in $Mn^{2+}$ transport (see below). This finding indicates that the N-terminal signature sequence in itself is insufficient to identify all members of the CDF family, whereas the cation efflux domain is a more reliable indicator for this class of proteins.

### Expression of *ShMTP1* in Yeast Confers $Mn^{2+}$ Tolerance

Expression of *ShMTP1* in yeast conferred a high level of $Mn^{2+}$ tolerance on both solid (data not shown) and liquid media (Figure 3A). At $Mn^{2+}$ concentrations of $<$7.5 mM, no difference in growth rate was observed between cells expressing *ShMTP1* and control cells for the INVSc2 strain (data not shown). Analysis of cells growing in liquid medium with 5 mM $Mn^{2+}$, a concentration that did not restrict cell growth, showed that the yeast expressing *ShMTP1* accumulated $\sim$20 to 50% more $Mn^{2+}$ than control cells at each growth stage sampled over 16 h (Figure 3B). Because growth rates over the 16 h were similar for both genotypes, differences in $Mn^{2+}$ accumulation could not be explained either by differences in the dilution of cellular $Mn^{2+}$ caused by differing growth rates or by differences in cell viability. Similarly, when cells were exposed to 40 mM $Mn^{2+}$, a concentration that eventually was toxic to control cells, ShMTP-expressing cells accumulated more $Mn^{2+}$ than control cells over the initial 8 h of exposure (data not shown). These patterns of accumulation are consistent with an internal sequestration of $Mn^{2+}$ rather than with an efflux of $Mn^{2+}$ to the external medium. We would have expected less $Mn^{2+}$ to be accumulated in the $Mn^{2+}$-tolerant yeast compared with the control had the mechanism relied on $Mn^{2+}$ efflux from cells.

Expression of *ShMTP1* in yeast specifically conferred tolerance to $Mn^{2+}$, and no increased tolerance or sensitivity was observed to a range of other metals ($Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$, $Hg^{2+}$, $Zn^{2+}$, $Al^{3+}$, and $Cd^{2+}$ [data not shown]). The ionic radius of $Mn^{2+}$ is similar to that of $Ca^{2+}$, and the two ions can substitute for one another in many biological functions (Loukin and Kung, 1995). For instance, the Arabidopsis CAX1 protein is a specific $Ca^{2+}$ transporter, but the related protein CAX2 is able to transport

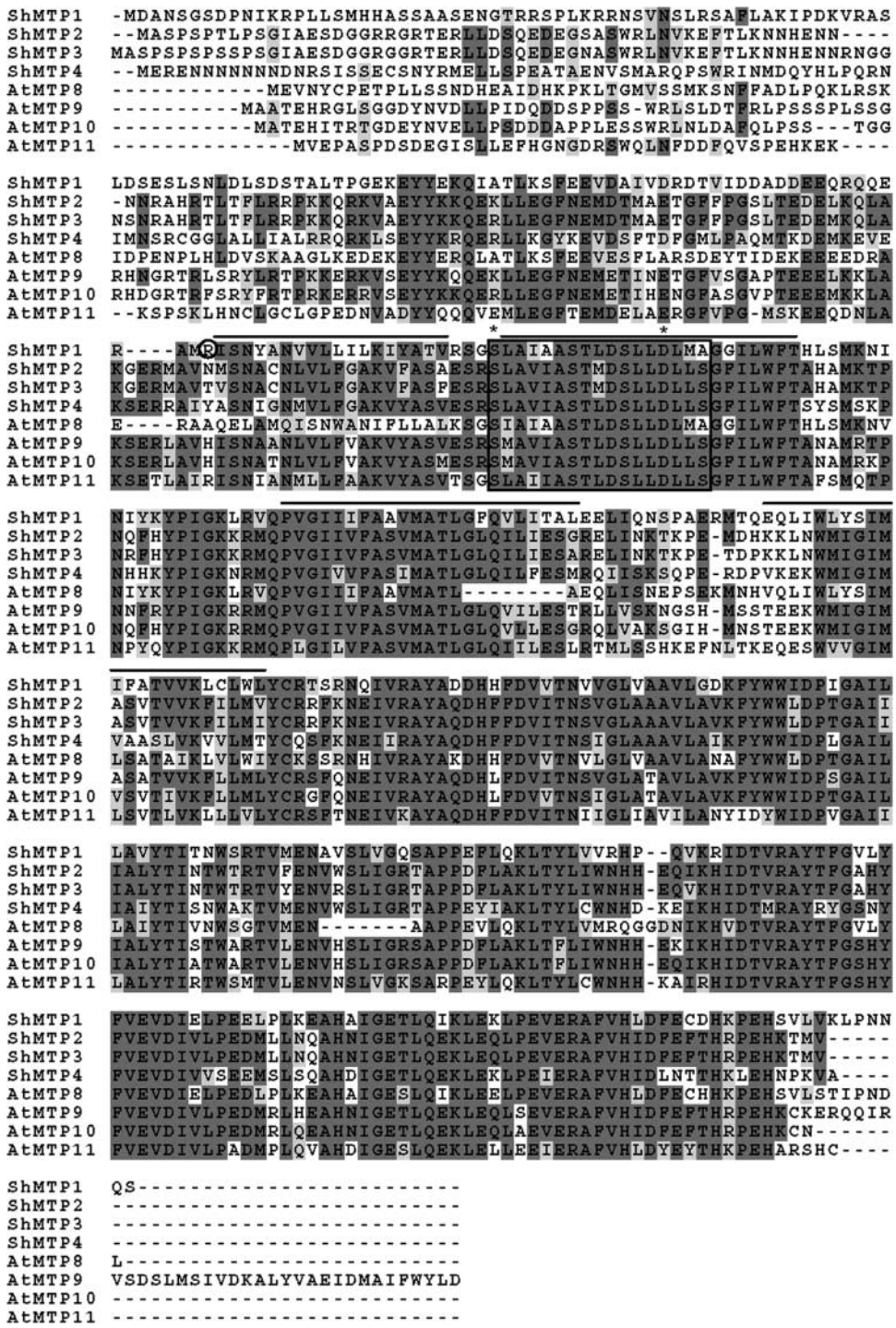


**Figure 1.** Analysis of the MTP Family of Proteins from Stylosanthes and Related Proteins from Arabidopsis.

CLUSTAL W alignment of the MTP proteins from Stylosanthes and Arabidopsis. Identical amino acids are indicated with dark shading, and similar amino acids are indicated with light shading. The membrane-spanning domains for ShMTP1 identified by TMHMM (version 2.0; http://www.cbs.dtu.dk/services/TMHMM/) are shown as lines above the sequence. The Arg residue at position 123, which when mutated to Ile abolishes the ability of ShMTP1 to confer $Mn^{2+}$ tolerance, is circled. The partially conserved N-terminal signature sequence for CDF proteins identified by Paulsen and Saier (1997) is boxed. The fully conserved Ser and Asp residues within this sequence are indicated by asterisks. The Arabidopsis genes are denoted by the names provided by the PlantsT World Wide Web site (http://plantst.sdsc.edu/). AtMTP8 originally was named AtMTPc3 (Mäser et al., 2001).

other metal ions, including $Mn^{2+}$, and its overexpression in plants confers a degree of $Mn^{2+}$ tolerance (Hirschi et al., 2000). Because *CAX1* and *CAX2* were isolated initially by their ability to complement a $Ca^{2+}$-sensitive yeast mutant (Hirschi et al., 1996), the ability of *ShMTP1* to complement a similar mutant also was assessed. However, *ShMTP1* was unable to complement the K667 yeast mutant with defects in $Ca^{2+}$ transporters at the vacuole (Cunningham and Fink, 1996), suggesting that it is unable to sequester $Ca^{2+}$ within internal organelles or to export $Ca^{2+}$ out of yeast cells (data not shown).

To obtain clues regarding the mechanism of $Mn^{2+}$ tolerance conferred by the ShMTP1 protein, *ShMTP1* was expressed in a range of previously described $Mn^{2+}$-sensitive yeast mutants (Figure 4). The *cnb1* mutant is defective in Ca homeostasis as a result of inactive calcineurin, and its sensitivity to $Mn^{2+}$ is thought to be the result of CNB1 also being involved in $Mn^{2+}$ homeostasis (Farcasanu et al., 1995). The increased sensitivity of *pmr1* mutants to $Mn^{2+}$ has been attributed to the absence of the $Ca^{2+}/Mn^{2+}$-ATPase activity located at the Golgi complex, which normally acts to reduce cytosolic $Mn^{2+}$ concentrations, with subsequent export of $Mn^{2+}$ to the external medium by exocytosis (Lapinskas et al., 1995). The *VMA8* gene encodes the D subunit of the catalytic domain of the V-type $H^+$-ATPase, and mutations in this gene prevent yeast from effectively acidifying their vacuoles (Xu and Forgac, 2000). Yeast mutants that are unable to acidify their vacuoles have increased sensitivity to a range of metals, including $Mn^{2+}$ (Ramsay and Gadd, 1997). One explanation for this sensitivity is that the sequestration of $Mn^{2+}$ into the vacuole relies on a proton-driven antiporter on the tonoplast.

We confirmed that the mutants *cnb1* and *pmr1* were more sensitive to $Mn^{2+}$ on both solid and liquid media (Figure 4), but the *vma8* mutant showed a weaker $Mn^{2+}$-sensitive phenotype that was apparent only on solid medium (data not shown). Expression of *ShMTP1* conferred $Mn^{2+}$ tolerance to the control strain, the *pmr1* mutant, and the *cnb1* mutant but not to the *vma8* mutant (Figure 4). Indeed, in contrast to all other strains, *ShMTP1* expression made the *vma8* mutant more sensitive to 10 mM $Mn^{2+}$ (Figure 4D). Expression of *ShMTP1* also was unable to confer increased $Mn^{2+}$ tolerance to a *vph2* mutant (data not shown), another vacuolar acidification mutant with defects

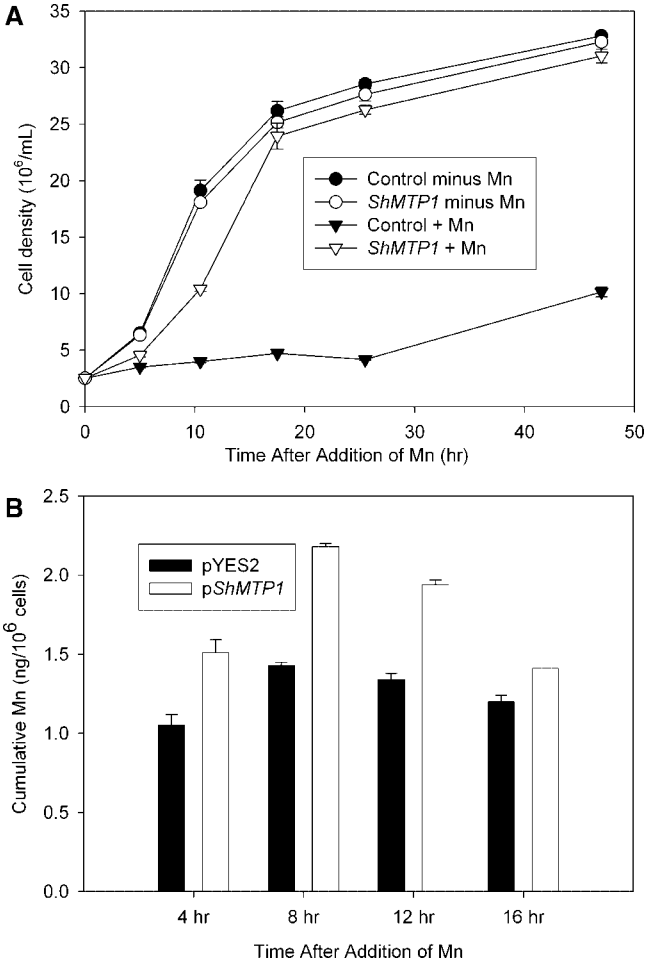


**Figure 3.** Effect of *ShMTP1* Expression on $Mn^{2+}$ Tolerance and $Mn^{2+}$ Accumulation of Saccharomyces Strain INVSc2.

**(A)** Liquid medium inoculated with Saccharomyces to an initial density of $2.5 \times 10^6$ cells per mL was subsampled periodically to monitor growth at basal $Mn^{2+}$ concentration (circles) or in the presence of 40 mM $Mn^{2+}$ (inverted triangles). Closed symbols represent the growth of yeast transformed with the empty vector, and empty symbols represent the growth of yeast expressing *ShMTP1*.

**(B)** Mn accumulation by Saccharomyces transformed with *ShMTP1* (open columns) or empty vector (control; closed columns). $Mn^{2+}$ (5 mM) was added to yeast cultures at an initial density of $2.5 \times 10^6$ cells per mL, and subsamples were collected at the time intervals shown. The growth of both genotypes was unrestricted by 5 mM $Mn^{2+}$ and was similar to the growth of cells in basal Mn (as shown in **[A]**) over 16 h. The yeast depleted $Mn^{2+}$ in the medium by <1% during the experiment. Data shown are means and standard errors ($n = 3$).

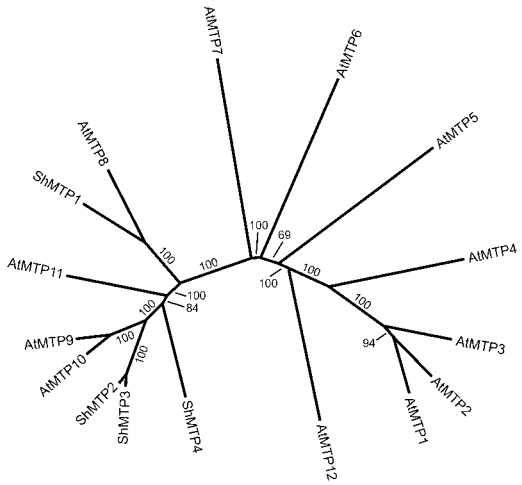


**Figure 2.** Phylogenetic Relationships of the CDF Proteins from Stylosanthes and Arabidopsis.

The unrooted tree was drawn using PHYLIP (Felsenstein, 1989) after the sequences were aligned with CLUSTAL W (Thompson et al., 1994). The branch values indicate the number of times (%) that each branch topology was found during bootstrap analysis.

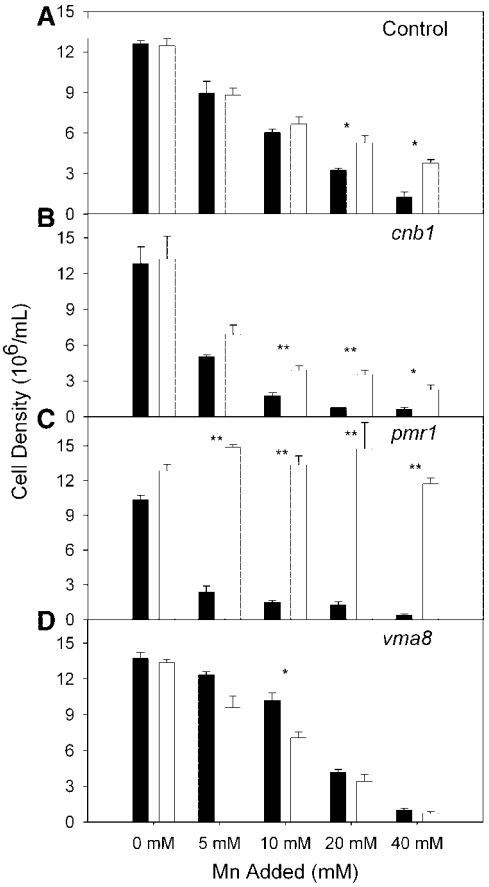


**Figure 4.** ShMTP1 Confers $Mn^{2+}$ Tolerance to a Range of $Mn^{2+}$-Sensitive Yeast Mutants.

Mutants of Saccharomyces strain BY4743 were transformed with the empty pYES2 vector (black bars) or with *ShMTP1* in pYES2 (white bars). The *ade1* strain was used as the control with a wild-type level of $Mn^{2+}$ tolerance **(A)**, whereas *cnb* **(B)**, *pmr1* **(C)**, and *vma8* **(D)** are $Mn^{2+}$-sensitive mutants as described in the text. Cell growth was measured when cell density had reached $\sim 12 \times 10^6$ cells per mL after medium was inoculated to an initial density of $1.3 \times 10^6$ cells per mL. Data shown are means and standard errors ($n = 3$). Statistically significant differences in growth between yeast expressing *ShMTP1* and yeast harboring the empty vector at a particular $Mn^{2+}$ concentration are shown by single asterisks ($P < 0.05$) or double asterisks ($P < 0.01$) as determined by *t* test on data normalized to their respective 0-$Mn^{2+}$ controls.

in the assembly of the V-type $H^+$-ATPase (Jackson and Stevens, 1997). The requirement for a functional V-type $H^+$-ATPase to obtain the $Mn^{2+}$-tolerance phenotype conferred by ShMTP1 suggests that some direct or indirect reliance on a proton gradient is required for the functioning of this protein.

It is unlikely that *ShMTP1* complements the functions defective in the *cnb1* mutant, because wild-type CNB1 is a protein phosphatase and not a membrane-bound protein (Farcasanu et al., 1995). Therefore, it is more likely that ShMTP1 acts independently of CNB1 to confer $Mn^{2+}$ tolerance by some other mechanism. Because *ShMTP1* was able to confer a relatively high level of $Mn^{2+}$ tolerance to the *pmr1* mutant (Figure 4B), the mutant most sensitive to $Mn^{2+}$ stress, and the PMR1 protein is able to transport $Mn^{2+}$, it was possible that *ShMTP1* complemented other functions defective in the *pmr1* mutant. In addition to being sensitive to high external $Mn^{2+}$ concentrations, *pmr1* mutants are susceptible to $Mn^{2+}$ and $Ca^{2+}$ deficiency caused by EGTA (Wei et al., 2000). Both $Ca^{2+}$ and $Mn^{2+}$ are essential elements for yeast, and PMR1 is the primary mechanism for transport of the ions into the Golgi, where $Mn^{2+}$ plays specific roles in the glycosylation of proteins and $Ca^{2+}$ is involved in accurate sorting of proteins to the vacuole (Dürr et al., 1998). EGTA is an effective chelator of both $Mn^{2+}$ and $Ca^{2+}$ and is able to reduce the concentration of these ions to the cell and ultimately the Golgi complex, where they are required. Therefore, we tested the hypothesis that ShMTP1 was able to complement other functions of PMR1 in addition to conferring $Mn^{2+}$ tolerance by assessing the EGTA sensitivity of *pmr1* cells expressing *ShMTP1*. If expression of *ShMTP1* were able to complement or partially complement the functions of the high-affinity $Ca^{2+}/Mn^{2+}$-ATPase, then the *pmr1* mutant would be expected to regain tolerance to EGTA. However, *ShMTP1* expression further increased the EGTA sensitivity of the *pmr1* mutant, indicating that *ShMTP1* did not complement the EGTA-sensitive phenotype of the *pmr1* mutant (Figure 5A). The EGTA sensitivity of *pmr1* cells was rescued by the external addition of $Mn^{2+}$ but required higher $Ca^{2+}$ concentrations to achieve a similar effect (Figure 5B).

### Expression of *ShMTP1* in Arabidopsis Confers $Mn^{2+}$ Tolerance

Expression of *ShMTP1* in Arabidopsis conferred increased $Mn^{2+}$ tolerance, as found for yeast. The increased tolerance in four independent homozygous lines expressing differing levels of ShMTP1 protein was shown by greater shoot weights compared with controls (Figures 6A and 6B). One line that had a relatively low shoot yield with basal $Mn^{2+}$ showed a growth stimulation when supplied with excess $Mn^{2+}$, suggesting that in this line, $Mn^{2+}$ homeostasis was perturbed by the high-level expression of *ShMTP1* (Figure 6A, line 2). The ability of ShMTP1 to protect seedlings from $Mn^{2+}$ toxicity is illustrated in Figure 6C. In this experiment, the control seedlings died or grew poorly after germination on toxic $Mn^{2+}$ concentrations, whereas the line expressing *ShMTP1* showed robust growth on the same substrate. Two Arabidopsis lines expressing *ShMTP1* were analyzed in greater detail for shoot yield and Mn accumulation over a range of $Mn^{2+}$ concentrations (Figure 7). The two lines showed greater $Mn^{2+}$ tolerance than the control at all $Mn^{2+}$ levels and accumulated Mn in their shoots at concentrations similar to that in the control line with up to 1 mM $Mn^{2+}$ in the substrate. At higher $Mn^{2+}$ concentrations, the $Mn^{2+}$-tolerant lines accumulated more Mn than the control line, and at $>1.5$ mM $Mn^{2+}$, the control line either produced insufficient shoots for analysis or died soon after germination (Figure 7).

In a different experiment to measure accumulation over a shorter term, seedlings initially grown hydroponically with a basal $Mn^{2+}$ concentration were exposed to 100 $\mu$M $Mn^{2+}$ and Mn concentrations were determined in roots and shoots over a time course. The low $Mn^{2+}$ concentration used in this experi-

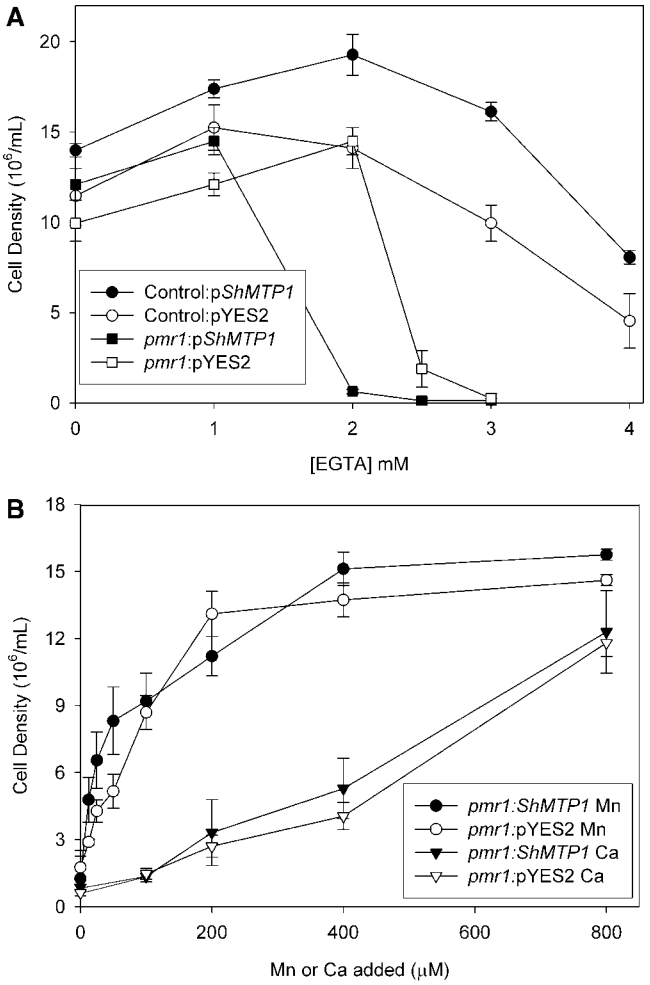


**Figure 5.** Expression of *ShMTP1* Exacerbates the EGTA Sensitivity of the Saccharomyces *pmr1* Mutant, Whereas Exogenously Added $Mn^{2+}$ and $Ca^{2+}$ Ameliorate the EGTA Sensitivity.

**(A)** Effect of EGTA on the growth of control (circles) and *pmr1* mutant (squares) cells of Saccharomyces strain BY4743 either expressing *ShMTP1* (closed symbols) or harboring the empty vector (open symbols). Cell growth was measured when the cell density had reached $\sim 12 \times 10^6$ cells per mL for the 0-EGTA controls after medium was inoculated to an initial density of $1.3 \times 10^6$ cells per mL. Data shown are means and standard errors ($n = 3$).

**(B)** Growth of the *pmr1* mutant in the presence of EGTA was measured when cell density had reached $\sim 12 \times 10^6$ cells per mL for 0-EGTA controls after medium was inoculated to an initial density of $1.3 \times 10^6$ cells per mL. The medium contained 2.0 mM EGTA (*pmr1*:*ShMTP1*; closed symbols) or 2.75 mM EGTA (*pmr1*:pYES2; open symbols) supplemented with various $Mn^{2+}$ (circles) or $Ca^{2+}$ (inverted triangles) concentrations. The different EGTA concentrations used for the strains reflect their relative sensitivities to EGTA as shown in **(A)**. Data shown are means and standard errors ($n = 5$ or 6).

ment relative to the agar experiments reflects the binding of $Mn^{2+}$ by components in the agar that effectively reduce the $Mn^{2+}$ activity in solution. However, prolonged exposure (7 days) to this $Mn^{2+}$ concentration proved toxic to the control plants. The accumulation of Mn in shoots and roots was similar for both control and ShMTP1-expressing plants over 7 days of Mn exposure (data not shown). These data indicate an internal tolerance of $Mn^{2+}$ by the *ShMTP1*-expressing plants that is consistent with the sequestration of $Mn^{2+}$ into an internal compartment.

### ShMTP1 Is Located at the Plant Tonoplast

The presence of up to five membrane-spanning regions in ShMTP1 indicates that the protein is membrane bound, and TargetP (www.cbs.dtu.dk/services/TargetP/) predicted the chloroplast as the most likely location for ShMTP1. To localize ShMTP1 in plant cells, we prepared a construct that fused the green fluorescent protein (GFP) to the C terminus of the full ShMTP1 protein. Expression of the chimeric gene in tobacco and Arabidopsis showed that instead of in the chloroplast, as predicted by TargetP, ShMTP1 was located at the tonoplast, although no obvious tonoplast-targeting signal was evident in the sequence. Figure 8A shows that the fluorescence of the ShMTP1:GFP fusion protein in tobacco trichomes was associated with a discrete cellular region that for the most part tracked the cell wall, but the clear bulge that coincided with the presence of the nucleus (Figure 8B) was consistent with a tonoplast location for ShMTP1. Because these cells were heavily vacuolated, the presence of a signal that largely tracked the cell walls was expected, because the vacuole occupies most of the cell volume and the tonoplast would be pushed up against the plasma membrane, with only a thin layer of cytoplasm remaining between the membranes. Figure 8C shows the GFP-only control localizing to the cytoplasm. The thin layer of cytoplasm is visible, as is the strong fluorescence of the nucleus, which was the pattern observed by others when GFP was expressed without specific targeting signals attached (Haseloff et al., 1997).

Similarly, in epidermal cells of tobacco leaves, the fluorescence showed bulges that were consistent with the presence of nuclei, although these were less obvious than those observed for the trichomes because of the irregular shape of the cells (Figure 8E). Chloroplasts present in the leaf cells provided additional evidence to support a tonoplast location for ShMTP1. In many cases, the green fluorescence from GFP was seen to skirt the red autofluorescence of the chloroplasts toward the inside of the cell and away from the cell wall (Figure 8F); based on reasoning similar to that described above for the nucleus, this finding is consistent with a tonoplast location for the protein. Furthermore, the simple structure of Arabidopsis roots allowed the meristematic tissues to be seen clearly. In young developing root cap cells, GFP-only expression showed relatively large cytoplasmic volume compared with that of older cells (Figure 8H). By contrast, the expression of ShMTP1:GFP showed young root cap cells with bubble-like structures that eventually occupied most of the volume of older cells (Figure 8G). These bubble-like structures likely represent the tonoplasts of developing vacuoles.

In contrast to its effect in plants, an ShMTP1:GFP fusion protein expressed in yeast did not show clear localization to the tonoplast. High-level expression under the control of the *GAL1* promoter resulted in the accumulation of the fusion protein to

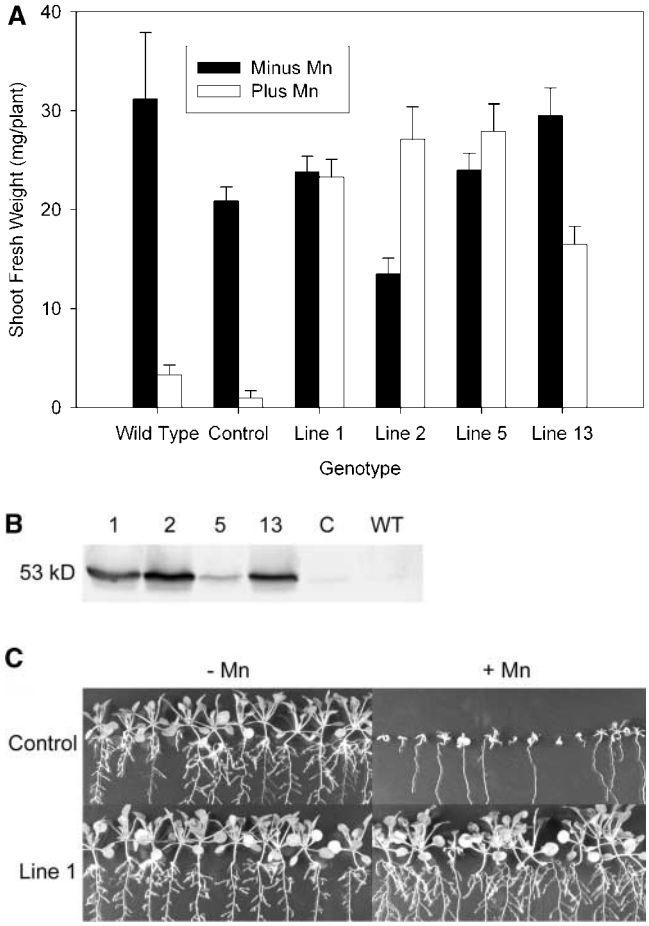


**Figure 6.** *ShMTP1* Expression Confers $Mn^{2+}$ Tolerance to Arabidopsis.

**(A)** Shoot yield of Arabidopsis lines grown on nutrient agar for 16 days with basal $Mn^{2+}$ (closed columns) or supplemented with 2.5 mM $Mn^{2+}$ (open columns). The control is a homozygous transgenic line transformed with the empty vector, wild type indicates untransformed Arabidopsis, and lines 1, 2, 5, and 13 are a range of independent homozygous lines expressing *ShMTP1*. Data shown are means and standard errors ($n = 25$ to 34).

**(B)** Level of ShMTP1 expression in the various lines as determined with a specific antibody against ShMTP1. The identities of the lines are as described in **(A)**; C indicates the control, and WT indicates the wild type. The molecular mass of ShMTP1 estimated from the immunoblot was marginally larger than that predicted from the sequence (47 kD).

**(C)** Growth of the control line compared with line 1 on agar containing basal or 2.5 mM $Mn^{2+}$.

internal membranes such as the endoplasmic reticulum that differed markedly from that of the GFP-only control (Figures 8I to 8L). The pattern was unlike those described for the plasma membrane (Panek et al., 2000), the tonoplast (Suriapranta et al., 2000), or the Golgi (Ton et al., 2002) but was similar to that shown for the endoplasmic reticulum (Benghezal et al., 2000). Although no discrete signal was apparent at the plasma membrane or the vacuolar membrane, the notion that a small proportion of the protein was incorporated into these membranes could not be discounted.

Expression of the ShMTP1:GFP fusion protein conferred Mn tolerance to both yeast and Arabidopsis, indicating that the attached GFP peptide did not interfere with ShMTP1 function (data not shown). During construction of the cDNA that encoded the fusion protein, one plasmid was prepared in which a PCR-generated error mutated the codon encoding an Arg residue at position 123 of ShMTP1 to an Ile residue. The protein mutated at this one residue did not confer $Mn^{2+}$ tolerance when expressed in either yeast or Arabidopsis, even though the subcellular location in both cases was the same as that of the wild-type protein fused to GFP (data not shown). Although this Arg residue is present in only two of the MTP proteins shown in the alignment in Figure 1, it immediately precedes the first membrane-spanning domain and appears to be critical for ShMTP1 function.

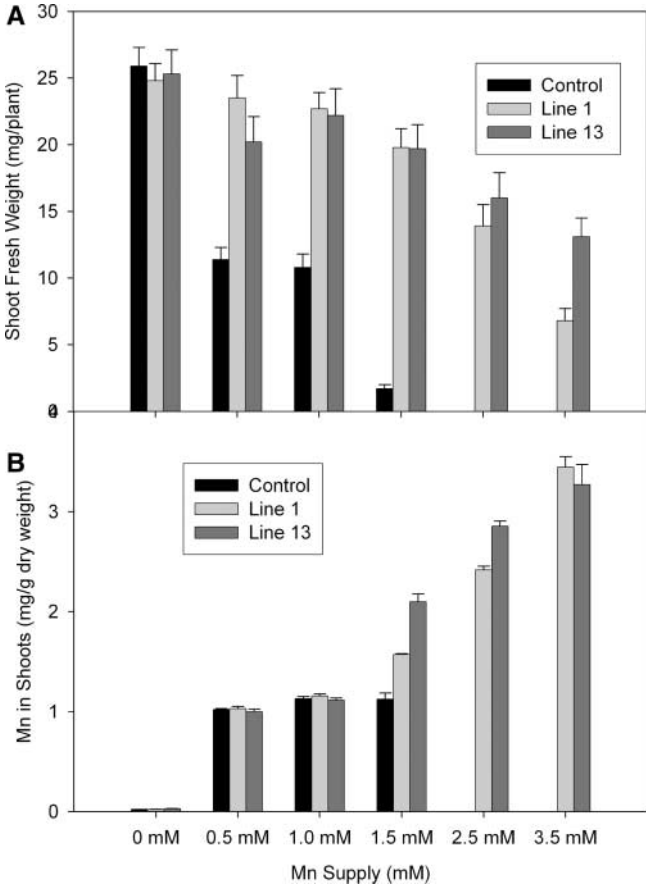


**Figure 7.** Effect of *ShMTP1* Expression on the Accumulation of $Mn^{2+}$ in Arabidopsis Shoots.

**(A)** Shoot yield of the control and lines expressing *ShMTP1* (lines 1 and 13) grown with a range of $Mn^{2+}$ concentrations for 21 days. Data shown are means and standard errors ($n = 50$).

**(B)** $Mn^{2+}$ concentration in shoots of the control and lines expressing *ShMTP1* (lines 1 and 13) grown with a range of $Mn^{2+}$ concentrations for 21 days. Data shown are means and standard errors for bulked samples from three Petri dishes ($n = 3$).

No data are shown for the control line at concentrations of >1.5 mM because of insufficient shoot material for analysis.

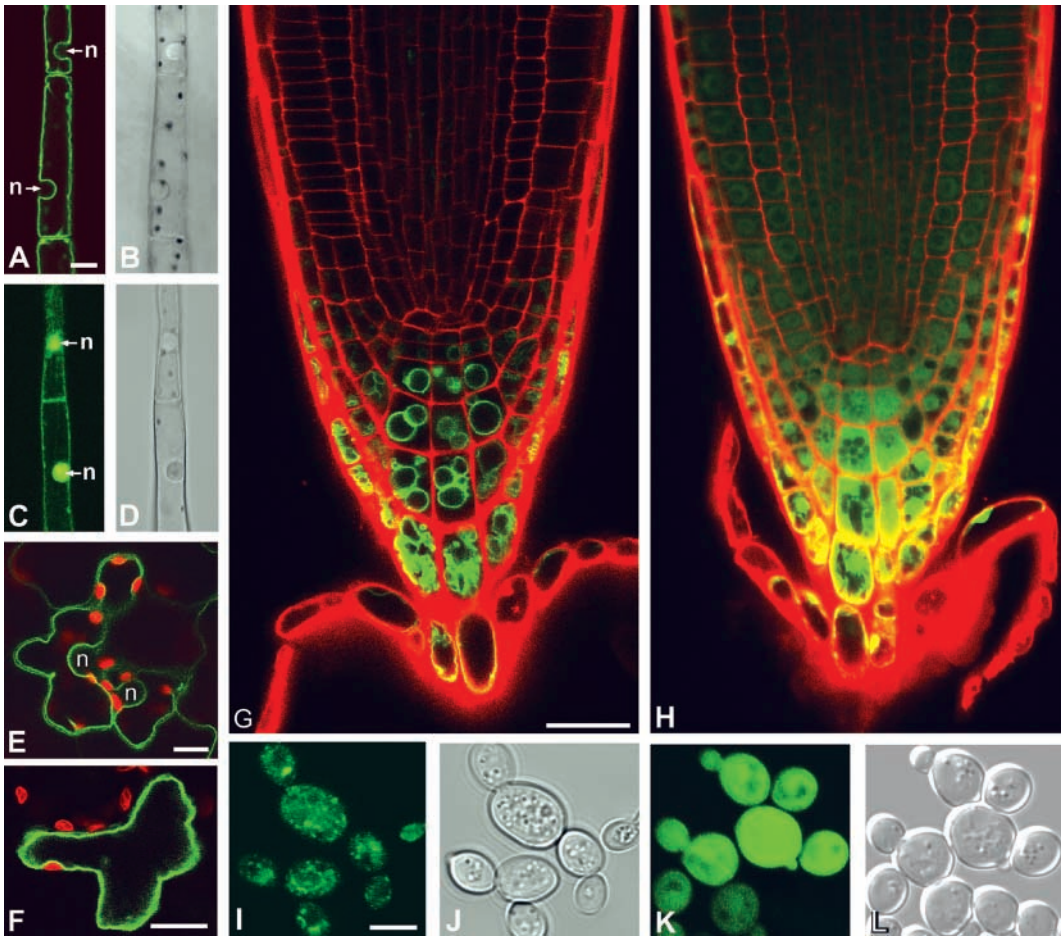


**Figure 8.** Confocal Microscopy Showing Fluorescence in Transgenic Tobacco, Arabidopsis, and Yeast Expressing an ShMTP1:GFP Fusion Protein.

**(A)** to **(D)** Tobacco trichomes expressing the ShMTP1:GFP fusion protein **(A)** or GFP alone **(C)** with the positions of the nuclei (n) shown. The corresponding light-field images are shown for trichromes expressing the ShMTP1:GFP fusion protein **(B)** or GFP alone **(D)**. Bar in **(A)** = 10 μm; the same magnification is used for **(B)** to **(D)**.

**(E)** and **(F)** Transient expression of ShMTP1:GFP in epidermal cells of tobacco leaves. Chloroplasts are indicated by the red autofluorescence, and the locations of nuclei (n) are indicated. Bars = 10 μm.

**(G)** and **(H)** Root tips of Arabidopsis expressing the ShMTP1:GFP fusion protein **(G)** or GFP alone **(H)**. The red fluorescence is caused by cell walls stained with propidium iodide. Bar in **(G)** = 20 μm; the same magnification is used for **(H)**.

**(I)** to **(L)** Fluorescence of yeast expressing ShMTP1:GFP **(I)** or GFP alone **(K)**. The same cells visualized by Normaski optics are shown in **(J)** and **(L)**, respectively. Bar in **(I)** = 4 μm; the same magnification is used for **(J)** to **(L)**.

## DISCUSSION

The *ShMTP1* gene encodes a protein of the CDF family that is likely to be an $Mn^{2+}$ transporter. The close similarity of three other cDNAs from Stylosanthes that also confer $Mn^{2+}$ tolerance to yeast as well as four putative proteins from Arabidopsis with a high degree of homology with these proteins suggests that members of this CDF subfamily have related functions. Expression of *ShMTP1* in several yeast strains provided indirect evidence that the ShMTP1 protein acts to sequester $Mn^{2+}$ to internal organelles. The enhanced Mn accumulation into cells expressing ShMTP1 at high external $Mn^{2+}$ concentrations and the increased EGTA sensitivity of the *pmr1* mutant expressing ShMTP1 both are consistent with internal $Mn^{2+}$ sequestration.

One interpretation of the EGTA sensitivity of *pmr1* mutants is that they are more prone than wild-type cells to Mn deficiency as a result of restricted $Mn^{2+}$ transport to the Golgi. ShMTP1 expression further exacerbated the susceptibility of *pmr1* cells to EGTA, suggesting that $Mn^{2+}$ supply to the Golgi was restricted in these cells. Consistent with this hypothesis is the observation that an increase in external $Mn^{2+}$ concentration was able to overcome the growth inhibition by EGTA (Figure 5B). The ability of external $Ca^{2+}$ to ameliorate EGTA sensitivity, albeit less effectively than $Mn^{2+}$, suggests that $Ca^{2+}$ is able to substitute for $Mn^{2+}$ in critical processes or that at high concentrations it frees up $Mn^{2+}$ that otherwise would be chelated by EGTA. The enhanced $Mn^{2+}$ tolerance and the increased EGTA sensitivity of the *pmr1* mutant expressing *ShMTP1* indicate that

ShMTP1 may act as a transporter to sequester $Mn^{2+}$ from the cytosol to an organelle or organelles at the expense of the Golgi complex. Increased EGTA sensitivity might not be apparent in wild-type yeast expressing *ShMTP1* if the high affinity of PMR1 for $Mn^{2+}$ ($K_m$ of ~20 nM [Mandal et al., 2000]) effectively outcompetes ShMTP1 for $Mn^{2+}$.

ShMTP1 in yeast appeared to be associated primarily with the endoplasmic reticulum, suggesting that this, or some other internal organelle, is the site of $Mn^{2+}$ sequestration. The endoplasmic reticulum can act as a store for metal ions, and recently, Clemens et al. (2002a) showed that a CDF protein of *Schizosaccharomyces pombe* located at the endoplasmic reticulum is involved in Zn accumulation to this organelle. The requirement of an active V-type $H^+$-ATPase for effective $Mn^{2+}$ tolerance to be conferred by ShMTP1 in yeast suggests that, as in plants, the tonoplast also is the site where a small proportion of the ShMTP1 functions as an $Mn^{2+}/H^+$ antiporter. Alternatively, because the V-type $H^+$-ATPase is not restricted to the tonoplast (Forgac, 1999), it also is possible that the loss of this activity in other organelles, such as the endoplasmic reticulum, disrupts the proton gradient across the membranes and prevents ShMTP1 from functioning as an $Mn^{2+}/H^+$ antiporter. It is known that some vacuolar proteins are mistargeted in mutants of the V-type $H^+$-ATPase (Robinson et al., 1988), which may explain the inability of ShMTP1 to confer $Mn^{2+}$ tolerance in the *vma8* and *vph2* mutants. However, analysis of the ShMTP1:GFP fusion in the acidification mutants and wild-type yeast cells showed a similar distribution of fluorescence, indicating that the mutations did not affect the gross distribution of ShMTP1 (data not shown). Similarly, the distribution of ZRC1 was unaffected when expressed in acidification mutants, indicating that mistargeting of proteins is not a general phenomenon in these mutants (MacDiarmid et al., 2002). Evidence that some CDF proteins require a proton gradient to transport metals comes from MacDiarmid et al. (2002) and Guffanti et al. (2002), who showed that CDF proteins from yeast and *Bacillus subtilis* can act as $Zn^{2+}/H^+$ antiporters.

In plants, ShMTP1 was localized specifically at the tonoplast, suggesting that the sequestration of $Mn^{2+}$ to the vacuole is the mechanism that confers $Mn^{2+}$ tolerance. This finding indicates that the signal that targets ShMTP1 to the tonoplast in plants is not recognized effectively by yeast and illustrates the notion that plant proteins are not necessarily localized to the same compartment in yeast even when the apparent phenotypes resulting from their expression, such as tolerance to a metal, are similar in both organisms. We attempted to directly measure a transport function for ShMTP1 in membrane vesicles derived from both yeast and plants using $^{54}Mn^{2+}$. However, we were unable to detect enhanced $Mn^{2+}$ transport conferred by ShMTP1 using either cation gradients ($Na^+$ and $K^+$) or proton gradients as driving forces (data not shown). The inability to detect a transport function in vitro could indicate either that ShMTP1 is not an $Mn^{2+}$ transporter or, more likely, that specific conditions in either the membrane preparations or the transport assays are required to maintain an active protein.

Overexpression of *ShMTP1* conferred a clear increase in $Mn^{2+}$ tolerance to Arabidopsis that was associated with an increased tolerance to internal $Mn^{2+}$. Whether *MTP*-like genes can explain some of the natural variation observed in $Mn^{2+}$ tolerance within plant species is not known. The observation that a range of species tolerate internal Mn rather than exclude $Mn^{2+}$ (Horst, 1988) is consistent with a role for these genes in the $Mn^{2+}$ tolerance of natural populations. Other genes that encode transporters capable of protecting plants from $Mn^{2+}$ toxicity include *CAX2* and *ECA1*. *CAX2* encodes a protein located at the tonoplast that transports several metals, including $Ca^{2+}$, $Mn^{2+}$, and $Cd^{2+}$ (Hirschi et al., 2000). Expression of *CAX2* in tobacco conferred some tolerance to $Mn^{2+}$, although this was not demonstrated as greater shoot weights. *ECA1* encodes a $Ca^{2+}$ pump that also is capable of transporting $Zn^{2+}$ and $Mn^{2+}$ to the endoplasmic reticulum (Wu et al., 2002). Arabidopsis seedlings mutated in *ECA1* are sensitive to $Mn^{2+}$, and although overexpression of *ECA1* complements this phenotype, $Mn^{2+}$ tolerance did not appear to be increased above that of wild-type seedlings.

The *ShMTP1* gene encodes a membrane protein that differs from both *CAX2* and *ECA1*, and in view of its ability to confer a strong $Mn^{2+}$-tolerance phenotype to Arabidopsis, it may have applications in increasing the $Mn^{2+}$ tolerance of selected plant species for agriculture by genetic manipulation. $Mn^{2+}$ toxicity is one of the factors that limit plant production on acid soils, and some plant species, such as alfalfa, are particularly sensitive to the high $Mn^{2+}$ concentrations that can occur in acidic soils (Foy, 1983). Furthermore, the increased tolerance of $Mn^{2+}$ by sequestration to internal organelles allowed the transgenic plants to accumulate $Mn^{2+}$ in shoots at concentrations that otherwise were toxic to nontransgenic plants. The ability to tolerate high internal concentrations of a heavy metal is one of the traits required for the genetic engineering of plants that are capable of hyperaccumulating metals (Clemens et al., 2002b). The development of fast-growing plants capable of hyperaccumulating heavy metals has applications in the bioremediation of sites contaminated with toxic metals.

## METHODS

### Yeast Strains and Culture

A *Stylosanthes hamata* cDNA library cloned into a modified pYES2 vector (Smith et al., 1995) was used to transform *Saccharomyces cerevisiae* strain INVSc2 (Invitrogen, Carlsbad, CA) (*MATα his3-Δ1 ura3-52*) using a method described by Gietz et al. (1995). Primary transformants were selected on medium that lacked uracil, washed off the plates with sterile water, and stored in 15% glycerol at −80°C until used. Putative $Mn^{2+}$-tolerance genes were isolated by screening ~$10^6$ cells on Petri plates that contained synthetic complete medium (Rose et al., 1990) supplemented with His, 40 mM $MnCl_2$, and galactose to induce expression of the cloned cDNAs inserted behind the *GAL1* promoter of pYES2. Plasmids from yeast colonies that grew on the medium were rescued in *Escherichia coli* (strain XL1-Blue; Stratagene, La Jolla, CA) and retransformed into the parental yeast strain to confirm that the $Mn^{2+}$ tolerance was conferred by the plasmid. Other yeast strains used included a $Ca^{2+}$-sensitive mutant (K667) (*vcx1*::*hisG cnb1*::*LEU2 pmc1*::*TRP1 ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1* [Cunningham and Fink, 1996]) and a range of $Mn^{2+}$-sensitive mutants in the diploid strain BY4743 (*MATa/a his3Δ1/his3Δ1 leu2Δ0 /leu2Δ0 lys2Δ0/LYS2 MET15/met15Δ0 ura3Δ 0/ura3Δ0*). These mutants were generated by the *Saccharomyces* Genome Deletion Project (http://www-sequence.stanford.

edu/group/yeast_deletion_project/) and included *ade1* (used as a control with wild-type levels of $Mn^{2+}$ tolerance; disrupted in YAR015W), *cnb1* (disrupted in YKL190W), *pmr1* (disrupted in YGL167C), *vma8* (disrupted in YEL051W), and *vph2* (disrupted in YKL119C). Detailed descriptions of these mutants can be obtained from the *Saccharomyces* Genome Database (http://genome-www.stanford.edu/Saccharomyces/). For growth experiments in liquid medium, the strains were cultured in synthetic complete medium supplemented with His, Lys, Leu, Trp, and adenine at the concentrations given by Rose et al. (1990).

### Plant and Yeast Transformations

The *ShMTP1* cDNA was ligated into the SalI-SphI site of the pDH51 expression cassette, which uses the 35S promoter and terminator of *Cauliflower mosaic virus* (Pietrzak et al., 1986). Subsequently, the EcoRI fragment containing the Stylosanthes cDNA was introduced into the EcoRI site of the binary vector pPLEX502 (Schünmann et al., 2003). Plant transformation vectors were transferred into *Agrobacterium tumefaciens* strain AGL1 by triparental mating. To localize ShMTP1, GFP was fused to the C terminus of ShMTP1. An in-frame fusion of GFP attached to the C terminus of ShMTP1 was prepared by PCR amplification of the coding sequence of *ShMTP1* with the primers 5′-CTAAACGAATTCCCCGGGATGGACGCCAATTCGGG-3′ and 5′-CTCCACGAATTCTGACTGATTGTTGGGCAG-3′. The primers included EcoRI sites (underlined), and the reverse primer lacked a stop codon. The coding region of a GFP optimized for expression in plants was removed from pMng1004 (Upadhyaya et al., 1998) with NcoI and NotI, end-filled to yield a blunted fragment, and then inserted into the SmaI site of pART7 (Gleave, 1992) in the correct orientation with respect to the 35S promoter.

The *ShMTP1* PCR product was digested with EcoRI and inserted into the EcoRI site of the pART7 vector upstream of and in-frame with the GFP coding region. The resulting cassette was removed as a NotI fragment, inserted into the NotI site of pPLEX502, and transferred to Agrobacterium as described above. A similar construct was prepared for the expression of a ShMTP1:GFP fusion protein in yeast except that the GFP was derived from the plasmid pCBJ4 (Benghezal et al., 2000), because the GFP version optimized for plant expression showed poor expression in yeast. The *ShMTP1* cDNA that lacked a stop codon was inserted into the EcoRI site of pBC SK (Stratagene) with its 3′ end adjacent to the PstI site. The GFP coding region from pCBJ4 was amplified by PCR with the following primers that included PstI sites (underlined): 5′-AACTGCAGATGAGAGGAGAAGAACTTTTCAC-3′ and 5′-AACTGCAGTTATTTGTATAGTTCATCCATGC-3′. The resulting PCR product was digested with PstI and inserted into the PstI site with its 5′ end adjacent to the 3′ end of *ShMTP1* in pBC SK. A NotI-SalI fragment containing the construct then was introduced into a modified version of pYES2 in which the KpnI site was converted to SalI (Smith et al., 1995), and yeast was transformed as described above.

The plant *ShMTP1*:*GFP* construct yielded a protein with the additional peptide EFGTP inserted between the ShMTP1 and GFP coding regions, whereas the yeast construct included the peptide EFLQ between the coding regions. Transient expression of the *ShMTP1*:*GFP* fusion in tobacco leaves was undertaken using an Agrobacterium infiltration method as described by Yang et al. (2000). Stable tobacco transformants were obtained subsequently by incubating the infiltrated leaves on Murashige and Skoog (1962) agar medium that contained 100 μg/mL kanamycin. Arabidopsis was transformed using the floral-dip technique (Clough and Bent, 1998) or by root transformation (Valvekens et al., 1988). Primary transformants were selected on nutrient agar medium (Richardson et al., 2000) supplemented with 50 μg/mL kanamycin. Subsequent generations also were selected on kanamycin to identify lines homozygous for the introduced genes. GFP expression in yeast and plant tissues was detected using a Leica SP2 confocal laser scanning microscope (Wetzlar, Germany). Roots were stained with 10 μg/mL propidium iodide before observation.

### Protein Gel Blot Analysis

The region encoding most of the N-terminal hydrophilic region of ShMTP1 (amino acids 8 to 125) was amplified by PCR of the *ShMTP1* coding region with the forward primer 5′-TTCGGATCCAAACATCAAG-3′ (BamHI site underlined) and the reverse primer 5′-AAACTGCAGTGGAAATTCTCATTGGCTCTTTC-3′ (PstI site underlined). The PCR product was subcloned into the BamHI-PstI sites of the *E. coli* expression vector pQE-31 (Qiagen, Clifton Hill, Victoria, Australia) that incorporates a poly-His tag onto the N terminus of the expressed polypeptide. The growth of expression cultures included 2% (w/v) glycerol before induction to eliminate protein toxicity that can occur as a result of basal transcription. After overnight growth, a 1:10 dilution was made into broth that included 0.1% glycerol, and the culture was grown at 37°C until the $OD_{600}$ reached 0.6. Expression of the polypeptide was induced by isopropyl-thio-β-galactoside (1 mM), and the culture was grown at 30°C for another 4 to 6 h. The protein was purified under denaturing conditions on a nickel affinity column according to the manufacturer's procedures (Qiagen). The purified protein was used to generate a polyclonal antibody in a rabbit, and the antibody was purified by passage through an affinity column using ShMTP1 as the ligand.

Conditions for immunoaffinity purification with cyanogen bromide–activated Sepharose 4B (Sigma) were according to Caughey et al. (1999) except that the antibody was eluted with only 0.1 M Gly, pH 2.9. For protein gel blot analysis, plant tissues were homogenized in an equal weight-to-volume ratio with 0.1 M EGTA that contained 0.25% (v/v) Tween 20. The extracts were centrifuged at 13,000*g* for 5 min and assayed for protein content (Bradford, 1976). Denaturing buffer was added to a sample that contained 100 μg of protein, and the sample was heated at 95°C for 5 min and then separated by SDS–denaturing protein electrophoresis. The proteins were transferred to nitrocellulose, and antigens were detected with the purified anti-ShMTP1 and a secondary antibody conjugated to alkaline phosphatase using methods described by Rerie et al. (1991).

### Mn Assays

Yeast medium (250 mL) was inoculated with a saturated primary yeast culture to yield a cell density of ~$2.5 \times 10^6$ cells per mL ($OD_{600}$ of ~0.2). At various times after the addition of $MnCl_2$ to 5 mM, subsamples (40 mL) were removed and placed on ice. The cells were collected by centrifugation (3000*g* for 3 min), resuspended in 2 mL of ice-cold 10 mM $CaCl_2$ to desorb loosely bound Mn, and then transferred to small vials. After centrifugation (15,000*g* for 20 s), the cells were washed two more times with 2-mL aliquots of 10 mM $CaCl_2$ and finally once with deionized water. The cells were ashed at 550°C overnight, and the residue was taken up in a mixture of 75 μL of nitric acid and 75 μL of hydrogen peroxide. After the samples were heated to 100°C for ~15 min to clarify any residues, the volume was made up to 3 mL with deionized water. Plant shoots were collected, dried, and weighed before being ashed and prepared as described for the yeast. Roots were treated in the same manner after incubation in 10 mM $CaCl_2$ for 1 h to desorb any loosely bound Mn. The Mn content of the digested samples was determined by atomic absorption spectrophotometry.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

### Accession Numbers

The GenBank accession numbers for the sequences described in this article are as follows: ShMTP1, AY181256; ShMTP2, AY181257; ShMTP3,

AY181258; ShMTP4, AY181259; AtMTP1, NP_182203; AtMTP2, NP_191753; AtMTP3, NP_191440; AtMTP4, NP_180502; AtMTP5, NP_187817; AtMTP6, NP_182304; AtMTP7, NP_564594; AtMTP8, NP_191365; AtMTP9, NP_178070; AtMTP10, NP_173081; AtMTP11, NP_181477; AtMTP12, NP_178539; rice sequences, BAB67872, AAN52756.1, BAA993621.1; and *Caenorhabditis elegans* sequences, AAA81718.3, NP_509279.1, NP_504288.1, T16470, NP_498611.1, and T16640.

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