Poplar Metal Tolerance Protein 1 Confers Zinc Tolerance and Is an Oligomeric Vacuolar Zinc Transporter with an Essential Leucine Zipper Motif

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Cation diffusion facilitator (CDF) proteins are a recently discovered family of cation efflux transporters that might play an essential role in metal homeostasis and tolerance. Here, we describe the identification, characterization, and localization of PtdMTP1, a member of the CDF family from the hybrid poplar Populus trichocarpa × Populus deltoides. PtdMTP1 is expressed constitutively and ubiquitously, although at low levels. Heterologous expression in yeast showed that PtdMTP1 was able to complement the hypersensitivity of mutant strains to Zn but not to other metals, including Cd, Co, Mn, and Ni. PtdMTP1 fused to green fluorescent protein localized to the vacuolar membrane both in yeast and in plant cells, consistent with a function of PtdMTP1 in zinc sequestration. Overexpression of PtdMTP1 in Arabidopsis confers Zn tolerance. We show that PtdMTP1, when expressed in yeast and Arabidopsis, forms homooligomers, a novel feature of CDF members. Oligomer formation is disrupted by reducing agents, indicating possible disulfide bridge formation. PtdMTP1 also contains a conserved Leu zipper motif. Although not necessary for oligomer formation, Leu residues within this motif are required for PtdMTP1 functional activity.

INTRODUCTION

Transition elements such as Fe, Co, Ni, Cu, and Zn play a wide variety of roles in biology as enzyme cofactors and must be absorbed from the soil by plants. However, either naturally or as a result of human activity, these metals can be present at potentially toxic concentrations. A number of other metal ions with no known biological function—including Cd, Pb, and Hg—also are potentially highly toxic for plants. Two basic strategies for decreasing the toxicity of metals are apparent: chelation and efflux from the cytosol, either into the apoplast or by intracellular sequestration. A number of cation transporter families have emerged recently and been classified in plants (Mäser et al., 2001), and although it is apparent that some (e.g., the Zrt- and Irt-like protein [ZIP] family) are involved in the high-affinity uptake of metals for nutritional purposes, overexpression of a member of another family (the cation diffusion facilitator [CDF] family) can lead to decreased metal toxicity and enhanced accumulation (van der Zaal et al., 1999).

Interest regarding transporters that facilitate the accumulation of potentially toxic metals has centered on their potential exploitation in phytoremediation. Phytoremediation is an emerging technology potentially effective and applicable to a number of different contaminants and site conditions (Lasat, 2002; Pilon-Smits and Pilon, 2002). The practical use of many well-known hyperaccumulators, such as Thlaspi caerulescens, for metal phytoremediation might be limited because they are slow growing and produce little biomass. The use of larger plants that are not currently classified as hyperaccumulators can compensate for somewhat lower accumulation factors in aboveground organs with greater biomass production and high transpiration rates. Poplar, which has emerged as a model system for genomic approaches to wood formation and tree physiology (Sterky et al., 1998; Taylor, 2002; Kohler et al., 2003), also is a good candidate for phytoremediation purposes (Gordon et al., 1998; Rugh et al., 1998; Pilon-Smits and Pilon, 2002; Stanton et al., 2002; Di Baccio et al., 2003). Thus, an ideal plant for phytoremediation purposes would combine high biomass production and superior capacity for pollutant accumulation and tolerance.

Among essential metals, zinc plays critical roles in a wide variety of biochemical processes; therefore, intracellular zinc concentrations must be maintained at adequate levels to support cell growth (Gaither and Eide, 2001). Recent reviews have discussed the basis for the mechanisms of Zn tolerance in plants and proposed that transport-mediated sequestration can contribute greatly to Zn tolerance (Williams et al., 2000; Clemens, 2001; Clemens et al., 2002b; Lasat, 2002). Gaither and Eide (2001) reported the major advances that have been made in the last decade through the discovery of two families of zinc transporters and their regulators in eukaryotes: the ZIP and CDF families. Members of the CDF family have been implicated in the metal tolerance mechanisms of a range of organisms and
are found in all biological kingdoms (Paulsen and Saier, 1997). ZRC1 and COT1 in Saccharomyces cerevisiae localize to the vacuole membrane and are thought to contribute to the storage of Zn and Co ions, respectively (Li and Kaplan, 1998; MacDiamid et al., 2000, 2002; Miyabe et al., 2001). MSC2, a third CDF member from S. cerevisiae, was shown to affect the cellular distribution of zinc, particularly the zinc content of nuclei (Li and Kaplan, 2001). ZHF in Schizosaccharomyces pombe is localized in the endoplasmic reticulum/nuclear envelope and plays an important role in cellular zinc homeostasis by mediating the transport of zinc into the endoplasmic reticulum (Clemens et al., 2002a). In yeast, overexpression of metal-tolerance proteins (MTPs; which are members of the CDF family) from Thlaspi goesingense was shown to confer resistance to Cd, Co, Ni, and Zn, possibly as a result of transport into the vacuole (Persans et al., 2001). Arabidopsis ZAT (also known as AtMTP1; Mäser et al., 2001) is expressed in all organs of the plant, and overexpression of ZAT can lead to enhanced zinc resistance and accumulation in roots (van der Zaal et al., 1999). Despite this recent progress, no localization has been assigned to zinc transporters of the CDF family in plants. Recently, a Mn transporter belonging to this family was shown to be localized to the vacuolar membrane of yeast, on the epidermal, and Arabidopsis root cells, consistent with a function for PtdMTP1 in Zn sequestration. Moreover, overexpression of PtdMTP1 in Arabidopsis confers Zn tolerance. We found that PtdMTP1 possesses key biochemical features: the expected CDF signature and Leu zipper motifs, both of which are necessary for its functional activity. We also demonstrate that PtdMTP1 forms oligomers that are disrupted by reducing agents.

RESULTS

Molecular Analysis of a Poplar Zinc Transporter

To examine the molecular basis of Zn homeostasis in poplar, we searched the EST database of Populus trichocarpa × Populus deltoides roots (Kohler et al., 2003) for sequence homology with known Zn transporters of the ZAT/MTP/CDF family. We identified a cDNA homologous with plant and yeast zinc transporters of the ZAT/MTP/CDF family. Recently, a Mn transporter belonging to this family was shown to be localized to the vacuolar membrane of yeast, on the epidermal, and Arabidopsis root cells, consistent with a function for PtdMTP1 in Zn sequestration. Moreover, overexpression of PtdMTP1 in Arabidopsis confers Zn tolerance. We found that PtdMTP1 possesses key biochemical features: the expected CDF signature and Leu zipper motifs, both of which are necessary for its functional activity. We also demonstrate that PtdMTP1 forms oligomers that are disrupted by reducing agents.

PtdMTP1 Is Expressed Constitutively

Reverse transcription (RT)–PCR analyses demonstrated that PtdMTP1 was expressed constitutively in most poplar tissues, as shown in Figure 2A. The highest level of PtdMTP1 transcript was detected in mature leaves and roots. We further measured the expression of PtdMTP1 by RT-PCR in root and leaf from poplar seedlings grown axenically and found that transcript level was not affected by exposure to low or high concentrations of Zn (0.038, 0.38, and 3.8 mM) for short or extended periods (3 to 5 days) (data not shown). The additional metals Mn, Cd, and Ni also were checked, and we found no response to these either. Transcript profiling experiments to study poplar adventitious root development revealed that PtdMTP1 was expressed constitutively, although at low levels, as shown in Figure 2B. Expression was independent of root development stage, in contrast to that of the extensin gene EXT1. The low expression level also was reflected by the low abundance of the PtdMTP1 clone in a Populus trichocarpa × deltoides root cDNA library (just 1 in >7000 ESTs [Kohler et al., 2003]; http://mycor.nancy.inra.fr/poplardb/index.html) and in the Populus spp. cDNA libraries (8 in >95,151 ESTs from various tissues; http://poppel.fysbot.umu.se). Fortuitously, in other cDNA array projects performed to study drought stress, root colonization
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by the ectomycorrhizal fungus *Paxillus involutus*, and leaf infection by the rust fungus *Melampsora larici-populina*, we found that PtdMTP1 expression was not affected significantly (data not shown).

Expression of PtdMTP1 in Yeast Confers Tolerance to Zn but Not to Cd, Co, Mn, or Ni

To characterize PtdMTP1 further, PtdMTP1 cDNA was expressed in *S. cerevisiae* mutant strains that are unable to grow in high concentrations of various metals, and growth was monitored on either control or metal-supplemented medium. Figure 3 shows that the wild-type strain was able to grow at high Zn concentrations (Figure 3B), whereas deletion of the ZRC1 gene, which encodes a transporter that sequesters Zn into the vacuole, rendered the mutant highly sensitive to Zn (Kamizono et al., 1989; Li and Kaplan, 1998). The Zn-sensitive phenotype of the *zrc1Δ* mutant was fully complemented by PtdMTP1. *cot1Δ* mutants are hypersensitive to both Zn and Co, because COT1 mediates the efflux of both ions into the vacuole (Conklin et al., 1992; Li and Kaplan, 1998; Lyons et al., 2000). Interestingly, although PtdMTP1 complemented the *cot1Δ* mutant phenotype when grown on Zn (Figures 3C and 3D), PtdMTP1 failed to complement when grown on Co (Figures 3E and 3F). We further

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**Figure 1.** An Unrooted, Parsimony-Based Tree of the CDF Gene Family.

The tree was generated using PAUP 4.0b10 (D. Swofford, Smithsonian Institution, Washington, DC) after sequence alignment with CLUSTAL X (Thompson et al., 1997). Bootstrap values are indicated (1000 replicates, full heuristic search option). Accession numbers are given at the end of Methods. Arabidopsis gene names follow the new nomenclature used by Delhaize et al. (2003).
transformed the ycf1Δ, pmr1Δ, and smf1Δ mutant strains, which are unable to grow on Cd, Mn, and Ni, respectively. YCF1 is an ABC transporter that confers Cd tolerance through the transport of Cd conjugates into the vacuole (Szczypta et al., 1994; Li et al., 1997). PMR1 is the yeast secretory pathway pump responsible for high-affinity transport of Mn²⁺ and Ca²⁺ into the Golgi and confers Mn tolerance by effectively removing Mn from the cytoplasm (Ton et al., 2002). SMF1 functions in the cellular accumulation of Mn, and the smf1Δ mutant was shown to be Ni sensitive (Supek et al., 1996). Transformation with PtdMTP1 did not restore the growth of ycf1Δ on 0.15 mM Cd (Figures 3G and 3H), of pmr1Δ on 3 mM Mn (Figures 3I and 3J), or of smf1Δ on 1.5 mM Ni (Figures 3K and 3L). The data obtained with the five mutant strains suggest that PtdMTP1 specifically transports Zn but not Co, Cd, Mn, or Ni.

**PtdMTP1 Partially Rescues the Growth of Yeast Vacuolar Acidification Mutants under Zn Stress**

Yeast vacuolar acidification mutants are unable to acidify their vacuoles and have increased sensitivity to some heavy metals, possibly because proton-driven antiport on the vacuolar membrane is required for the sequestration of metals into the lumen.
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(MTP1, a Poplar Zinc Transporter 2915) Therefore, to gain insight into the dependence of PtdMTP1 on a proton gradient, two vacuole acidification mutants (vma8Δ and vph2Δ) were transformed with PtdMTP1 and colonies were assayed subsequently for growth at different Zn concentrations. The VMA8 gene encodes a subunit of the catalytic domain of the vacuolar-type H⁺-ATPase, and the VPH2 protein is required for the biogenesis of a functional vacuolar ATPase (Bachhawat et al., 1993; Graham et al., 1995). Figure 4 shows that vma8Δ and vph2Δ are highly sensitive to Zn (no growth at 0.25 mM Zn) compared with the wild type (growth up to 15 mM; Figure 3). Interestingly, the growth of both acidification mutants was partially rescued when cells were transformed with PtdMTP1. These PtdMTP1-expressing transformants were able to grow at 0.25 mM Zn (Figure 4) and also partially at 0.5 mM Zn (data not shown).

Figure 3. Complementation of Yeast Mutants on Selective Media.

S. cerevisiae mutant strains were transformed with the empty vector pYES2 or with pYES2-PtdMTP1. Wild-type (wt) cells (strain BY4741) also were transformed with pYES2 as a control. Yeast cultures were adjusted to OD = 1.0, and 2 μL of serial dilutions (from left to right in each panel) were spotted on SD medium without extra metal ([A], [C], [E], [G], [I], and [K]) or supplemented with 15 mM Zn ([B] and [D]), 1 mM Co ([F]), 150 μM Cd ([H]), 3 mM Mn ([J]), or 1.5 mM Ni ([L]). Plates were incubated for 6 days at 30°C.

PtdMTP1:GFP Is Targeted to the Vacuolar Membrane in Yeast Cells

Phenotypic suppression by PtdMTP1 of the zrc1Δ phenotype indicates a putative vacuolar membrane localization of the transporter in yeast cells. We constructed the PtdMTP1:GFP fusion protein to determine the intracellular localization of PtdMTP1 more precisely. Because the PtdMTP1:GFP fusion gene complemented the zinc sensitivity of the zrc1Δ mutant (data not shown), the fusion protein was confirmed to retain its original function. The bright-field image in Figure 5A shows the clear presence of vacuoles in zrc1Δ yeast cells. At the standard image acquisition settings used for GFP visualization, autofluorescence from cells transformed with the untagged PtdMTP1 was absent (data not shown), so all detectable fluorescence in...
the transformants was GFP specific. Figure 5B shows that, when exponentially growing cells were analyzed by confocal laser scanning microscopy, green fluorescence resulting from PtdMTP1:GFP showed a ring-like pattern in the cells. We speculated that this ring-like pattern might reflect vacuolar membrane location. We then stained the vacuolar membrane with the lipophilic dye FM4-64 to identify the location of this organelle (Vida and Emr, 1995). After 60 min of internalization, FM4-64 was found exclusively at the vacuolar membrane in transformed S. cerevisiae cells (Figure 5C). Figure 5D shows that superposition of the GFP and FM4-64 images yields a coincident staining pattern, demonstrating that PtdMTP1 was localized to the vacuolar membrane. When the \textit{zrc1/H9004} mutant was transformed with the vector carrying only the GFP, the fluorescence was found throughout the cytosol (Figure 5F) and was not coincident with the staining of the vacuoles (Figures 5G and 5H).

**PtdMTP1 Localizes to the Vacuolar Membrane of Plant Cells**

Prediction by TargetP (Emanuelsson et al., 2000) indicated no preferred location of PtdMTP1. Therefore, we tested experimentally whether the vacuolar location of the transporter in yeast cells reflects the location in the plant. The PtdMTP1:GFP fusion protein was expressed transiently in living onion epidermal cells (Scott et al., 1999), which then were subjected to analysis by confocal laser scanning microscopy. The bright-field image in Figure 6A shows that, although onion epidermal cells have a large vacuole that effectively contours the cell, a clear separation from the cell periphery was seen in the region of the nucleus (inset). The corresponding fluorescence image in Figure 6B shows a clear localization of GFP to the indented region, rather than the cell periphery, unambiguously demonstrating a vacuolar location. Membrane-lined transvacuolar strands of cytoplasm spanning the cell also produced fluorescence, another typical feature of a vacuolar membrane location (data not shown).

Transgenic lines of Arabidopsis expressing PtdMTP1:GFP were used to confirm the vacuolar location of PtdMTP1 found by transient expression in onion cells. In elongated root cells, the large central vacuole usually occupies most of the cell volume, and the vacuolar and plasma membranes are closely juxtaposed. Conversely, cells in the root tip region contain several small immature vacuoles, so we focused on the examination of GFP fluorescence in root tips. Figure 6C shows the presence of numerous intracellular globular localization patterns in those cells, strongly suggesting a vacuolar location. The defined peripheral location of the AtMGT1:GFP fusion protein, as described previously by Li et al. (2001), is shown in Figure 6D as a control for plasma membrane localization.

**PtdMTP1 Confers Zn$^{2+}$ Tolerance in Planta**

The vacuolar membrane location of PtdMTP1 and its complementation of the Zn sensitivity phenotype of the yeast \textit{zrc1/H9004} mutants suggest that PtdMTP1 might provide Zn tolerance in planta. To address this possibility, we overexpressed PtdMTP1 in transgenic Arabidopsis plants under the control of the 35S promoter. All lines were phenotypically normal when grown on plates or in soil. Figure 7A shows that five representative homozygous lines performed significantly better at high Zn concentrations than did the controls. Two independently transformed lines that overexpressed PtdMTP1 were selected for further study. Figure 7B illustrates the increased Zn tolerance of these two lines when grown on plates in medium supplemented with excess Zn$^{2+}$ ions, compared with plants transformed with an empty vector grown under the same conditions. At 0.5 mM Zn, root development was nearly nonexistent in wild-type plants and in plants transformed with the empty vector, whereas it was only partially inhibited in lines expressing PtdMTP1 (data not shown). The two transgenic lines expressing PtdMTP1 produced more biomass than the control lines (untransformed plants and plants transformed with the empty vector), which grew poorly and finally died at 1.25 mM Zn (Figure 7B). These findings demonstrate that PtdMTP1 is capable of conferring Zn tolerance in planta.

**PtdMTP1 Forms Oligomers That Are Sensitive to Reducing Agents**

Size estimation by SDS-PAGE using metal blots indicated that AtZAT might form dimers when present in membranes (Bloss et
The discovery that PtdMTP1 forms multimers possibly stabilized by S-S bridges encouraged us to examine the possibility that other motifs are involved in functional aspects of protein–protein interaction. The Scanprosite interface (www.expasy.ch/cgi-bin/scanprosite) enabled the identification of a Leu zipper (LZ) motif in the C-terminal PtdMTP1 sequence from residues Leu-291 to Leu-314. Figure 10 shows that this LZ domain is conserved among plant CDF proteins. The most notable feature of the LZ is the presence of a repeating pattern of Leu residues at every seventh position \((LX_6)n\). More generally, however, other motifs are involved in functional aspects of protein–protein interactions. The overall architecture of coiled coils is defined by a 4,3 hydrophobic repeat in the primary amino acid sequence. Thus, the hydrophobic positions \(a\) and \(d\) of the sequence \((abcd)(e)(fg)\), fall on the same face of an \(\alpha\)-helix. In some cases, the Leu residues are replaced by Ile or Val and can include “skips” and “hiscs.”

**Figure 5.** Vacuolar Membrane Localization of the PtdMTP1:GFP Fusion Protein in Yeast, Viewed by Confocal Laser Scanning Microscopy.

Cells were visualized after 24 h of induction. Four images from the same cells are shown: bright-field image (A), GFP fluorescence from a PtdMTP1:GFP-expressing strain (B), FM4-64 staining of vacuolar membranes (C), and a pseudocolored merged image with GFP in green and FM4-64 in red (D). (E) to (H) are as for (A) to (D) except for (F) and (H), which show GFP fluorescence from nonfused GFP. Bars = 5 \(\mu\)m.

PtdMTP1 Contains a Leu Zipper Motif That Is Necessary for Function

The presence of both monomeric and dimeric forms and consequently refuting the heterodimeric formation of PtdMTP1:GFP with another unknown protein of \(~80\) kD. A third band running at \(~150\) kD also was observed (Figure 8B). Figure 8C demonstrates that oligomer formation also occurred in planta. Therefore, whether expressed in planta or in yeast, PtdMTP1 proteins are likely to be present at least as dimers; nevertheless, the presence of higher molecular mass complexes cannot be excluded and are indicated in Figure 8.

To confirm the presence of oligomers, cotransformation of yeast cells with both PtdMTP1:V5 and PtdMTP1:GFP constructs was performed. Because the two tagged species have significantly different molecular masses, heterodimeric and homodimeric chimeric proteins are expected to migrate differentially on gels of low acrylamide concentration. In one approach, anti-GFP antibodies were used to detect PtdMTP1 proteins. Figure 9A shows that in addition to the homodimeric species running with an apparent molecular mass of \(~140\) kD, a band with a lower mass of \(~120\) kD also was detected, indicating the presence of heterodimeric PtdMTP1:V5/PtdMTP1:GFP complexes. Complementary data were obtained when the converse experiment was performed; that is, anti-V5 antibodies revealed the presence of a heterodimeric complex, which although appearing fainter, had a higher molecular mass compared with that of the homodimeric complex. We also considered the possibility that PtdMTP1 complexes could be formed during the extraction procedure. To test this possibility, we mixed cells that expressed either PtdMTP1:GFP alone or PtdMTP1:V5 alone and then processed the mixture for protein gel blot analysis as described above. Heterodimers were not observed when the blot was probed with anti-GFP antibodies or with anti-V5 antibodies (Figure 9A). To determine whether PtdMTP1 dimerization occurred through disulfide bridges, extracts containing the PtdMTP1:GFP dimers were treated with the reducing agent DTT before gel loading. As shown in Figure 9B, increasing DTT concentration in the sample led to increasing dissociation of the dimers. At 20 mM DTT, almost complete dissociation was observed. Similar results were found when extracts containing PtdMTP1:V5 were used as well as with the reducing agents \(\beta\)-mercaptoethanol and tributylphosphine (data not shown).
cups” (Leung and Lassam, 1998). This explains the unsuccessful searches for LZ motifs in some other CDF proteins (e.g., AtMTP1) with search programs that are designed to detect only the conventional (LX6)n motif, even though the motifs are effectively present (Figure 10).

To determine the role of the conserved LZ in the oligomeric structure and biological activity of PtdMTP1:GFP, the heptadic Leu residues 293, 300, 307, and 314 were changed individually or in pairwise combination to Ala residues. Ala was chosen as a substituting residue for Leu because (1) Ala has a strong propensity to form an α-helix and its substitution has minimal disruptive effects on the α-helical structure characteristic of a LZ domain in proteins, and (2) Ala lacks the bulky hydrophobic side chain of Leu, which is believed to be the major hydrophobic force for a LZ domain to facilitate protein interaction (Luo et al., 1999). Figure 11 (bottom gel) shows that all of the mutant proteins were expressed at levels identical to that of the wild-type protein. Substitution of one or two of the four Leu residues did not alter the size of the fusion protein oligomer, as determined by gel electrophoresis, nor was the subcellular localization of the mutant PtdMTP1:GFP proteins changed. However, as shown in Figure 11, although the L293A substitution had no apparent effect on function, the L314A substitution completely abolished the functional complementation of the zrc1/Δ mutant by PtdMTP1 on 15 mM Zn. Other single substitutions (L300A and L307A) showed intermediate patterns: the reduction in yeast growth at high Zn concentration was marked but not fully abolished. Double Leu substitution mutants confirmed the patterns observed with the single Leu mutants, with a slight additional effect in growth inhibition, especially for the L300A-L307A mutant (Figure 11).

**Residue Substitutions in the CDF Motif Abolish Yeast Complementation**

PtdMTP1 contains the CDF signature, which begins with Ser-80 and continues just past the fully conserved Asp-93. We created four mutants (D86A, H89A, H89K, and D93A) by site-directed

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**Figure 6. Vacuolar Membrane Localization of PtdMTP1:GFP in Planta.**

(A) and (B) Transient expression of PtdMTP1:GFP in onion epidermal cells. Bar = 50 μm.

(A) Bright-field image along the middle plane of a cell showing the nucleus (n) and invagination of the vacuolar membrane (inset).

(B) GFP fluorescence in the same cell concentrated to the vacuolar membrane, which follows the cell contour except in the perinuclear region (inset).

(C) and (D) Vacuolar localization of PtdMTP1:GFP in Arabidopsis. The red fluorescence is caused by cell walls and nuclei stained with propidium iodide. Bars = 10 μm.

(C) Root tips of transgenic Arabidopsis expressing PtdMTP1:GFP.

(D) Control cells expressing AtMGT1:GFP, a transporter located in the plasma membrane.
mutagenesis of either semi (D86 and H89) or fully (D93) conserved residues. All of the mutant proteins were expressed at levels identical to that of the wild-type protein (Figure 11, bottom gel), still formed dimers, and were localized on the vacuolar membrane (data not shown). However, as shown in Figure 11, all four mutants failed to complement the Zn hypersensitivity of Zrc1Δ cells, as shown by the complete abolition of yeast growth on 15 mM Zn.

DISCUSSION

The novel transporter from the hybrid poplar *Populus trichocarpa* × *deltaoides*, PtdMTP1, is a member of the CDF family. The CDF family can be subdivided into three (van der Zaaij et al., 1999; Gaither and Eide, 2001) or four (Mäser et al., 2001) groups. Following the classification given by Gaither and Eide (2001), PtdMTP1 is most closely related to group-III CDF members (Figure 1). Heterologous expression of PtdMTP1 in yeast lacking either of the vacuolar cytoplasmic efflux carriers ZRC1 or COT1 complements only the zinc sensitivity of these yeast strains, strongly suggesting that PtdMTP1 is able to selectively transport zinc ions into the yeast vacuole. These properties reflect those of AtZAT (AtMTP1), which when reconstituted in proteoliposomes was shown to transport Zn but not Co (Bloss et al., 2002). However, the AtZAT gene did not rescue the zinc sensitivity of a zrc1Δ single mutant or a zrc1Δ cot1Δ double mutant strain of *S. cerevisiae*, but it did rescue that of a zrc1Δ mutant strain of *S. pombe* (Bloss et al., 2002).
A critical question in establishing the physiological function of CDF transporters relates to their cellular location. Table 1 shows for a number of CDF family members a range of disparate locations, including on most intracellular membranes and the plasma membrane. Thus, ShMTP1, a *S. hamata* CDF member that specifically transports Mn, was found recently to be targeted to the vacuolar membrane in planta but localized to endoplasmic reticulum membranes when expressed in yeast (Delhaize et al., 2003). One of the homologous mammalian zinc resistance transporters, ZNT1, has been localized to the plasma membrane and reported to function in Zn efflux (Palmiter and Findley, 1995). A second, related mammalian zinc resistance transporter, ZNT2, is located on intracellular membranes and appears to facilitate the vesicular sequestration of Zn (Palmiter et al., 1996a). The reported localization of MSC2, a CDF family member from *S. cerevisiae*, in the endoplasmic reticulum/nucleus and the higher nuclear zinc content of msc2Δ cells suggested a requirement for transporter-facilitated exchange of zinc between the cytosol and the nucleus (Li and Kaplan, 2001). ZHF, a CDF member from *S. pombe*, is hypothesized to mediate the transport of zinc into the endoplasmic reticulum and the nuclear envelope in fission yeast (Clemens et al., 2002a). By contrast, the *S. cerevisiae* CDF transporter ZRC1 has been localized to the yeast vacuole membrane (Li and Kaplan, 1998; MacDiarmid et al., 2000, 2002; Miyabe et al., 2001). The present study demonstrates the targeting of PtdMTP1::GFP fusion proteins to the vacuolar membranes of yeast, living onion epidermal cells, and Arabidopsis cells, effectively confirming the physiological role of the transporter in vacuolar Zn sequestration.

![Figure 8. Oligomerization of PtdMTP1 Expressed in Yeast and Arabidopsis.](image)

*S. cerevisiae* expressing either PtdMTP1::GFP (A) or PtdMTP1::V5 (B) were induced or not with 2% (w/v) galactose (gal). (C) shows the expression of PtdMTP1::GFP in Arabidopsis control plants (wt) or three transgenic lines (t1 to t3) overexpressing PtdMTP1::GFP. Proteins were extracted and separated on 5% (w/v) SDS-PAGE gels under nonreducing conditions and analyzed further using immunoblotting methods. The positions of the PtdMTP1::GFP and PtdMTP1::V5 monomers, dimers, and oligomers are indicated. The antibodies used for detection are noted below each gel. Numbers beside the gels indicate molecular masses in kilodaltons.

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![Figure 9. PtdMTP1 Forms Dimers.](image)

(A) Coexpression of PtdMTP1::GFP and PtdMTP1::V5 fusion proteins in yeast shows dimer formation. As indicated above lanes 1 to 3 (from left), proteins were extracted from cells expressing only PtdMTP1::V5, only PtdMTP1::GFP, or both (PtdMTP1::GFP/PtdMTP1::V5). The right lane was loaded with proteins from cells expressing only PtdMTP1::GFP mixed with cells expressing only PtdMTP1::V5 (PtdMTP1::GFP + PtdMTP1::V5). (B) Disruption of the PtdMTP1::GFP dimer by DTT. Samples were treated with increasing concentrations of DTT for 1 h before loading. Cells were processed for immunodetection as described in the legend to Figure 8. The positions of homodimers and heterodimers are indicated. The antibodies used for detection are noted below each gel. Numbers beside the gels indicate molecular masses in kilodaltons.
In eukaryotes, two families of transporters have been implicated in zinc transport. ZIP family members play prominent roles in cytosolic zinc import, transporting zinc from outside the cell (Pence et al., 2000) or from within an intracellular compartment (Gaither and Eide, 2001). The second group of transporters, the CDF family, transports zinc in a direction opposite that of the ZIP transporters, promoting cytosolic zinc efflux. Chardonnens et al. (1999) reported evidence that transport across the vacuolar membrane plays an essential role in naturally selected zinc-tolerant *Silene vulgaris*. van der Zaal et al. (1999) suggested that increased Zn transport into the vacuoles of Zn-tolerant species such as *S. vulgaris* might be attributable to a ZAT-like protein, although data on ZAT localization were lacking. The present study demonstrates that the ZAT homolog PtdMTP1 is located at the vacuolar membrane, which is consistent with a role for PtdMTP1 in zinc sequestration. Zinc transporters—either of the ZIP or CDF families—have not previously been identified plant vacuolar membranes.

PtdMTP1 mRNA was expressed constitutively throughout the hybrid poplar used in this study and was not expressed differentially by varying the Zn concentrations. Similarly, RNA gel blot experiments in Arabidopsis indicated that ZAT mRNA was expressed throughout the plant at uniform but low levels and was not induced by increasing the Zn concentrations (van der Zaal et al., 1999). Expression of the Ni transporter MTP1 from *T. goesingense* failed to increase during Ni exposure, although in this MTP1-related Ni hyperaccumulator, MTP1 is much more highly expressed than in nonaccumulator species, presumably conferring enhanced ability to accumulate Ni ions within vacuoles (Persans et al., 2001). Under metal stress, the regulation of CDF transporters might occur post-transcriptionally, or these transporters might simply perform a housekeeping function.

To date, no clear picture has emerged regarding whether plant CDF transporters might energize metal transport through coupling to antiport with H⁺. Bloss et al. (2002) showed that the rate and extent of Zn uptake in proteoliposomes containing purified and reconstituted ZAT1 (AtMTP1) were unaffected by the presence of a protonotive force, indicating the probable absence of H⁺ coupling. By contrast, the failure of the Mn transporter ShMTP1 to complement the Mn-sensitive phenotype of yeast mutants defective in vacuolar acidification led Delhaize et al. (2003) to conclude that this CDF transporter is H⁺/H⁺ coupled. We adopted a similar approach, expressing PtdMTP1 in yeast vacuolar ATPase mutants in which ATP-driven H⁺/H⁺ pumping is severely defective (vph2Δ; Bachhawat et al., 1993) or in which vacuolar ATPase activity is abolished completely (vma8Δ; Graham et al., 1995). Our results demonstrate the complementation of the Zn-sensitive mutant phenotype, albeit not to the extent to which complementation occurred when PtdMTP1 was expressed in a zrc1Δ background. It is possible, then, that PtdMTP1, like ZAT1, is not H⁺ coupled, although these complementation results should be interpreted with caution. The possible presence of PtdMTP1 on nonvacuolar endomembranes in yeast (Figure 5D) might confer a partial complementation phenotype if acidification of compartments bounded by such membranes is not vacuolar ATPase dependent. This conclusion raises the question of whether PtdMTP1 actively sequesters Zn in the vacuolar lumen through energized transport.

**Figure 10.** Conservation of a LZ Motif in Plant CDFs.

Numbers in parentheses correspond to the first residue in each sequence. Gray-shaded residues indicate conserved “d” positions of the Leu/Ile/Val heptad repeats. Sequences were aligned as described in the legend to Figure 1. Accession numbers are given at the end of Methods.
or whether the capacity to confer Zn tolerance arises through the formation of a passive Zn permeation pathway that provides access to the additional intracellular volume afforded by the vacuolar lumen. Nevertheless, a clear physiological role in conferring Zn tolerance in planta is indicated by the ability of plants that overexpress PtdMTP1 to survive and grow at high Zn concentrations that are lethal to control plants.

Previous studies involving metal blots have suggested that AtZAT might form dimers (Bloss et al., 2002), although direct evidence for this contention has been lacking. The protein gel blots presented here show that the dominant extractable form of PtdMTP1, whether expressed in yeast or plants, is oligomeric. Further experiments with other CDF members and other species are needed to determine whether CDF oligomerization is a common feature of plant CDFs and is present in members of other kingdoms. Although it is clear that PtdMTP1 can form homooligomers, the possibility of heterooligomer formation with other CDF proteins cannot be excluded where CDF proteins are encoded by multigene families. Stable oligomer formation in PtdMTP1 might be dependent, at least in part, on the

![Figure 11. Evaluation of the Functionality of PtdMTP1 Mutants by Yeast Complementation Assay.](image)

Yeast zrc1Δ mutants were transformed with the empty vector pYES2, with pYES2-PtdMTP1, or with various mutant pYES2-PtdMTP1 vectors. Cultures were adjusted to OD = 1.0, and 2 μL of serial dilutions were spotted on SD medium without extra metal (top panels) or supplemented with 15 mM Zn (middle panels). Plates were incubated for 6 days at 30°C. Protein gel blot analysis of microsomes of transformed mutants with anti-GFP antibodies is shown in the bottom panel. Cells were processed for immunodetection as described in the legend to Figure 8. WT, wild type.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Substrate</th>
<th>Organism</th>
<th>Localization</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
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<td>Zn</td>
<td>Hybrid poplar</td>
<td>Vacuole</td>
<td>Present study</td>
</tr>
<tr>
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<td>Vacuole</td>
<td>Delhaize et al. (2003)</td>
</tr>
<tr>
<td>ZNT7</td>
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<td>H. sapiens</td>
<td>Golgi</td>
<td>Kirschke and Huang (2003)</td>
</tr>
<tr>
<td>ZHF1</td>
<td>Zn, Co, Cd</td>
<td>S. pombe</td>
<td>Endoplasmic reticulum</td>
<td>Clemens et al. (2002a)</td>
</tr>
<tr>
<td>ZNT6</td>
<td>Zn</td>
<td>H. sapiens</td>
<td>Golgi</td>
<td>Huang et al. (2002)</td>
</tr>
<tr>
<td>MCS2</td>
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<td>S. cerevisiae</td>
<td>Endoplasmic reticulum/nucleus</td>
<td>Li and Kaplan (2001)</td>
</tr>
<tr>
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<td>S. cerevisiae</td>
<td>Vacuole</td>
<td>Li and Kaplan (1998)</td>
</tr>
<tr>
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<td>S. cerevisiae</td>
<td>Vacuole</td>
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</tr>
<tr>
<td>ZNT1</td>
<td>Zn</td>
<td>H. sapiens</td>
<td>Plasma membrane</td>
<td>Palmiter and Findley (1995)</td>
</tr>
</tbody>
</table>
formation of one or more disulfide bonds, because the addition of DTT resulted in a shift toward the monomeric form.

Interestingly, we discovered a LZ motif in PtdMTP1. The LZ domain is a helical structure that forms coiled coils and was identified originally as a highly conserved motif mediating the binding of transcription factors to DNA (Landschulz et al., 1988). The role of LZ domains in promoting the homodimerization and heterodimerization of transcription factors has been well documented (Turner and Tjian, 1989). LZ domains also have been shown to mediate protein–protein interactions (Surks et al., 1999) and intraprotein oligomerization (Simmerman et al., 1996) and also were implicated in the formation of macromolecular complexes (Marx et al., 2001). The finding of a LZ motif in PtdMTP1 encouraged us to examine its functional importance. Our results indicate that the conserved Leu residues are not necessary for oligomer formation but are required for the functional activity of PtdMTP1. Analogous findings have been reported for other systems. Thus, triple Leu-to-Ala substitutions on the paramyxovirus fusion proteins demonstrated that the conserved Leu residues in the LZ motif were not necessary for oligomer formation but were required for the fusogenic ability of the protein (Reitter et al., 1995). Subunit assembly of animal Shaker K+ channels does not depend on the Leu heptad repeat, even though substitutions of the Leu residues in the repeat produced large effects on the voltage dependence of conductance and prepulse activation curves (McCormack et al., 1991). It seems likely that the LZ in PtdMTP1 mediates subtle interactions that are required for the function of the multimeric complex.

It appears that the fourth and last Leu residue (Leu-314) of the LZ motif is particularly critical for the function of PtdMTP1. Interestingly, this final Leu residue of the heptad repeat was found to be highly conserved among plant CDFs (Figure 10). Studies of the AIZAT protein have revealed that the C-terminal putative intracellular region is critical for activity, because truncation just after the last transmembrane domain was not able to confer Zn resistance or Zn accumulation in Arabidopsis (van der Zaal et al., 1999). This truncation removed the final two residues of the LZ domain (Val-318 and Leu-319) and, although not precluding an important role for other residues at the C terminus, it is intriguing to speculate that the removal of Leu-319 alone likely would have abolished function.

PtdMTP1 possesses the CDF family–specific signature sequence (Paulsen and Saier, 1997) that begins with Ser-80 and continues just past Asp-93. Substitution with Ala of any of the semi (Asp-86 or His-89) or fully (Asp-93) conserved residues within this sequence yielded a nonfunctional protein that was unable to rescue semi (Asp-86 or His-89) or fully (Asp-93) conserved residues within this sequence yielded a nonfunctional protein that was unable to rescue zrc1Δ mutant yeast. Because the mutated amino acids are charged residues, it remained possible that the mutation abolished a salt bridge. However, in the case of His-89, the H89K mutant also was nonfunctional; thus, disruption of a salt bridge seems unlikely. Therefore, this motif might be essential for in vivo transport activity of the protein but is not required for oligomerization or for tonoplast localization of PtdMTP1. The demonstration of oligomerization abilities and the identification of a LZ motif provide a new foundation for understanding the structure and function of the CDF family, proteins that could be important in phytoremediation technologies.

To breed plants with superior phytoremediation potential, one possible strategy is to enhance metal accumulation in high-biomass species (Pilon-Smits and Pilon, 2002). Indeed, the combination of fast-growing and high-biomass-producer characteristics together with the metal hyperaccumulation trait would be an invaluable tool in phytoremediation technology. Hybrid poplars have been grown primarily for raw material production, but the utility of poplar now extends to the treatment of wastewater, nutrient removal from agriculture runoff, and phytoremediation of industrial landfills (Stanton et al., 2002). It was demonstrated recently that poplar plants have the potential to be used for plantations in Zn-contaminated soils (Di Baccio et al., 2003). Because vacuolar sequestration is clearly a process to target to improve metal tolerance (Küpper et al., 1999; Persans et al., 2001; Clemens et al., 2002b), poplar species/clones exhibiting high zinc accumulation–like characteristics could be screened using MTP1 protein as a suitable marker with a view to improving the potential of this gene for Zn phytoremediation. Poplar clones overexpressing PtdMTP1 also could be used for phytoremediation technologies.

METHODS

Plant Growth Conditions

Hybrid poplar (Populus trichocarpa × Populus deltoides cv Beaupré) was grown in a greenhouse in a controlled environment as described previously (Kohler et al., 2003). Arabidopsis thaliana plants (ecotype Columbia) were grown in Klasman Substrate No. 1 compost (Klasman-Deilmann, Geest, Germany) in a greenhouse at 23°C with a 16-h photoperiod. Alternatively, seeds were sterilized and grown at 22°C with a 16-h photoperiod on agar plates containing sterile MS medium (Murashige and Skoog, 1962).

For Zn resistance assays of Arabidopsis lines expressing PtdMTP1 and lines transformed with the empty vector pART27, homozygous T3 seeds were sown on plates containing half-strength MS medium supplemented with various concentrations of ZnSO4. Wild-type plants were also used as a control. After 3 weeks of growth, plant fresh weights were determined.

Cloning of PtdMTP1, cDNA Array, and Reverse Transcriptase–PCR Analysis

Standard techniques were used for DNA preparation (Sambrook and Russell, 2001). A cDNA library was made from the adventitious root system of 2-month-old rooted cuttings as described by Kohler et al. (2003). The pTriplex2 phagemid clones in Escherichia coli were obtained using the mass excision protocol according to the manufacturer’s instructions (Clontech, Erembodegem, Belgium), and DNA sequencing on randomly chosen clones was performed as described by Kohler et al. (2003). Generation and analysis of cDNA arrays were performed as reported elsewhere (Lacourt et al., 2002; Kohler et al., 2003). Developing root tissues of cuttings were harvested at six different stages (dormant bark tissues, root primary, root calli, emerging roots, primary roots, and lateral root tips), frozen in liquid N2, and stored at ~−80°C for RNA isolation. Photographs of the different developmental stages are available at http://mycor.nancy.inra.fr/poplarlab/index.html. EST database searches were performed using Basic Local Alignment Search Tool (BLAST; Altschul et al., 1990).

Total RNA isolation was performed with the RNeasy Plant Mini kit (Qiagen, Courtabœuf, France) from ~100 mg of frozen material. An av-
The yeast strains used for the heterologous expression of PtdMTP1 were CAGCCGCTCCAAACAGT-3
UBQFP1 (5'- using an Eppendorf Mastercycler (Eppendorf, Le Pecq, France). The suit-
ite site at both ends (sites underlined), and cloned at the XbaI site in the
leu2 modules were excised and ligated back into the pYES2 vector. To generate
/H11032 primer MTP1f and the 3'-mutant
Site-Directed Mutagenesis
plasmids containing corresponding single Leu-to-Ala codon substitu-
te PCR. Double Leu-to-Ala substitution mutants were generated by the
primers MTP1 FP1 (5'-H11032/CCTCCTTCTGAGTAATGCTG-3') and MTPRP1 (5'-TCAAGCACAAGCAGATCTGAGT-3') in the following conditions: 95°C for 1
minute followed by 35 cycles at 95°C for 5 s, 65°C for 5 s, and 68°C for 6 min
using an Eppendorf Mastercycler (Eppendorf, Le Pecq, France). The suit-
ability of the extracted RNA for RT-PCR amplification was checked by performing
RT-PCR control experiments with ubiquitin using the primers
UBQFP1 (5'-GGACCTCTGCGAGCAGTACGAAA-3') and UBQR1 (5'-TAA-
CAGCGCTCAGAAGACTG-3') in the same amplification conditions.

**Yeast Strains, Media, and Transformation**

The yeast strains used for the heterologous expression of PtdMTP1 were
BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0, zrc1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 ZRC1:kanMX4), cot1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YCF1:kanMX4), pmr1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 PMR1::kanMX4), smf1Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SMF1:: kanMX4), yma8Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 VPH2::kanMX4), and
vph2Δ (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 VPH2::kanMX4). The seven mutants used derived from the parental strain BY4741 and were all obtained from Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/euroscarf/). Growth was in yeast potato dextrose or in synthetic defined
media obtained from Euroscarf (http://www.uni-frankfurt.de/fb15/mikro/

**Site-Directed Mutagenesis**

Mutant PtdMTP1 plasmid clones were generated by oligonucleotide-
directed PCR mutagenesis. The desired amino acid codon changes were incorporated into a PCR-amplified fragment using the 5’ flanking
primer MTP1f and the 3’ flanking primer MTP1r and a series of mu-
tagenic primers (Table 2). The resulting mutant HindIII-BamHI frag-
ments were excised and ligated back into the pYES2 vector. To generate
single amino acid mutants, pYES2-PtdMTP1 was used as a template for
the PCR. Double Leu-to-Ala substitution mutants were generated by the
same process as the single mutants except that the PCR templates were
plasmids containing corresponding single Leu-to-Ala codon substitu-
tions instead of pYES2-PtdMTP1. The presence of targeted mutations in
all plasmid constructs was verified by DNA sequencing.

**Construction of GFP and V5 Fusions for Expression in Yeast**

To construct PtdMTP1-GFP, the GFP (S65T) cDNA was amplified from the
plasmid PFA6a-GFP6S5-kanMX6 with the primers GFP1 (5'-CCCTCTGAGCTATACCTTTCCATGCT-3') and GFP2 (5'-CCCTCTGAGCTATACCTTTCCATGCT-3'), introducing a XbaI site at both ends (sites underlined), and cloned at the Xbal site in the

pYES2 plasmid to generate a new plasmid called pYES2-GFP. Then, a
PtdMTP1 fragment, lacking the stop codon of the PtdMTP1 cDNA, was
amplified with the primers MTP1f and MTP1rnostop (5'-CCTCCCAG-
ATCCCCAGCTCTAGTGGTACATCAT-3') (BanHi site underlined) and cloned into the HindIII-BamHI-digested pYES2-GFP plasmid. PtdMTP1:V5 was constructed by inserting the HindIII-BamHI fragment of PtdMTP1 into pYES6/CT (Invitrogen, Carlsbad, CA). Mutant PtdMTP1 clones also were also fused in frame with GFP or V5 into the pYES2-GFP or pYES6/CT plasmid, respectively. As a control, the expression of nontargeted GFP in yeast was achieved by transforming cells with pYES2 in
which only the full-length GFP cDNA was cloned.

**Transient Expression of PtdMTP1 in Onion Cells**

The intracellular localization of PtdMTP1 was determined by monitoring the transient expression of a PtdMTP1:GFP translational fusion product in onion epidermal cells after DNA particle bombardment. The coding region of mGFP5, a green fluorescent protein modified for plants (Haseloff et al., 1997), was fused in-frame to the 3’ terminus of full-length PtdMTP1 cDNA using a three-step PCR procedure. First, a mGFP5 fragment was amplified by PCR from the plasmid pCAMBIA1303 using the primers
GFP5f (5'-AAAGGAGAAAGCTTCTCAAGCTATTGTC-3') and GFP5r (5'-AAACTCTATTGTTAATGCTACATGC-3'), introducing a XbaI site at the 3’ end of the fragment (site underlined). In a second step, a fragment consisting of the full-length PtdMTP1 cDNA (stop codon removed) plus the first 21 nucleotides of mGFP5 was amplified by PCR from the plasmid pYES2-PtdMTP1 with the primer pair MTP1 gpf (5'-AAATCTGAGTGGAGCAGCAGACTCCTA-3') and MTP1 gpfr (5'-AGTGGAGGAGCTCTAGTGGTACATG-3'), introducing a Xhol site at the 5’ end of the fragment (site underlined). DNA fragment were gel purified, mixed in a PCR mix lacking primers, denatured at 94°C for 1 min, annealed at 55°C for 1 min, elongated at 68°C for 3 min, and subsequently subjected to 25 PCR cycles after the addition of the MTP1 gpf and GSP5r primers. The resulting Xhol-XbaI fragment was cloned between the 5SS promoter of Cauliflower mosaic virus and the octopine synthase terminator in the Xhol-XbaI-digested pART7 plasmid (Gleave, 1992). The construct was coated onto gold particles (0.6 μm) and delivered into onion cells with a Helios Gene Gun System (Bio-Rad Laboratories, Hercules, CA). The bombardment pa-
rameters were as follows: discharge pressure of 150 p.s.i., and distance to target tissue of 3.5 cm. Onion cells were placed onto MS agar plates before bombardment and incubated at 22°C for 24 h after particle delivery.

**Generation of Transgenic Arabidopsis Expressing PtdMTP1 or PtdMTP1:GFP**

For PtdMTP1 and PtdMTP1:GFP overexpression in Arabidopsis, expres-
sion cassettes present in pART7 (see above) were isolated as Ncol frag-
ments and cloned in Ncol-digested pART7 (Gleave, 1992). The resulting
plasmids were used to transform Agrobacterium tumefaciens strain
GV3101. Transformed plants were selected for spectinomycin resistance and were used to transform Arabidopsis by the floral-dip method (Clough and Bent, 1998). Plant transformants were selected on MS agar plates containing 50 mg/L kanamycin. Subsequent generations also were selected on kanamycin to identify homozygous lines. Arabidopsis trans-
genic plants expressing AIMGT1:GFP (Li et al., 2001) were the kind gift of L. Li and S. Luan (University of California, Berkeley).

**Cell Labeling and Confocal Microscopy**

Yeast vacuolar membranes were selectively stained with the red fluores-
cent probe FM4-64 (Molecular Probes, Leiden, The Netherlands). This li-
pophilic styryl dye has been reported to stain yeast vacuolar membranes
selectively (Vida and Emr, 1995). Cells were incubated with FM4-64 for 30 min at 30°C, washed with SD medium, and incubated 1 h before observation by confocal microscopy. These incubation times are sufficient for the dye to reach the vacuolar membrane with no residual plasma membrane or endosomal staining (Humair et al., 2001). Staining of root cell walls and nuclei was achieved by incubating roots in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 10% (v/v) glycerol, 100 μM pepstatin A. Lysates were centrifuged at 3,000 g for 5 min to remove debris and subsequently at 13,000 g for 15 min to pellet the microsome fraction. Finally, the proteins were homogenized in the extraction buffer. Even if an equivalent number of cells or mass of plant tissue was processed in each extraction, protein quantitation was performed with the Bio-Rad protein assay to standardize the extracts for protein concentration.

**Table 2. Oligonucleotides for Amino Acid Substitution by PCR Mutagenesis**

<table>
<thead>
<tr>
<th>Name</th>
<th>Positions</th>
<th>Sequence (5’ to 3’)</th>
<th>Mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mut1f</td>
<td>241-270</td>
<td>CTGGCAATCTTGGTgcACTGCACCAGTGGT</td>
<td>D86A</td>
</tr>
<tr>
<td>Mut1r</td>
<td>270-241</td>
<td>CAATCGACTGACgcAGTCAGATTGCGCAG</td>
<td>H89A</td>
</tr>
<tr>
<td>Mut2f</td>
<td>250-279</td>
<td>TGGACTGATGGTgcCTGAGAGCCGAGTGG</td>
<td>H89K</td>
</tr>
<tr>
<td>Mut2r</td>
<td>279-250</td>
<td>ATCTGAAGAAGAcCGCAGATCATGCAA</td>
<td>L307A</td>
</tr>
<tr>
<td>Mut3f</td>
<td>282-291</td>
<td>CTGGTTATAGTgcGGGACCAAACTTGAA</td>
<td>L300A</td>
</tr>
<tr>
<td>Mut3r</td>
<td>291-262</td>
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<tr>
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<tr>
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<td>291-262</td>
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<td>L300A</td>
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<td>Mut6f</td>
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<td>291-262</td>
<td>TTAACAGAAAAATGgcGTTGACAGTCAA</td>
<td>L300A</td>
</tr>
</tbody>
</table>

a Two partially complementary primers (f and r) were used in each PCR mutagenesis for the introduction of amino acid codon substitutions.
b The region of the PtdMTP1 sequence in each oligonucleotide is marked by the positions of the 5’ oligonucleotide. All positions are numbered in reference to the first nucleotide of the PtdMTP1 coding sequence.
c Uppercase letters indicate that the sequence is exactly the same as the wild-type PtdMTP1 DNA sequence. Lowercase letters indicate the mismatched nucleotides designed for specific mutations.
d The designated amino acid substitutions resulting from each mutagenic primer pair.

**Protein Gel Blot Analysis**

Protein extracts were mixed with sample buffer (60 mM Tris-HCl, pH 6.8, 25% [v/v] glycerol, 2% [w/v] SDS, and 0.0125% [v/v] bromphenol blue) either with (1 to 200 mM) or without the reducing agent DTT and subjected to SDS-PAGE (Laemmli, 1970), and proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Blots were probed either with mouse anti-V5-HRP antibodies (Invitrogen) (1:5000) or with rabbit GFP antiserum (Invitrogen) (1:5000). In the latter case, goat anti-rabbit peroxidase-conjugated antibodies (Calbiochem-Novabiochem, Bad Soden, Germany) (1:10,000) were used as the secondary reagent. Proteins were revealed by enhanced chemiluminescence using the ECL protein gel blot detection kit (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions.

**Accession Numbers**

The GenBank accession numbers for the sequences described in this article are as follows: *Populus trichocarpa* × *deltoide*: PtdMTP1, AY450453;
The Plant Cell

Eucalyptus grandis: EgMTP1, AAL25646; Arabidopsis thaliana: 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; and AtMTP12, NP_178539.

Other accession numbers are AAK91869; NP_013649; NP_014961; ScMSC2, NP_010491; ScMMT2, NP_015100; and ScMFT1, NP_173081; AtMTP11, NP_181477; and AtMTP12, NP_178539.


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Poplar Metal Tolerance Protein 1 Confers Zinc Tolerance and Is an Oligomeric Vacuolar Zinc Transporter with an Essential Leucine Zipper Motif
Damien Blaudez, Annegret Kohler, Francis Martin, Dale Sanders and Michel Chalot
Plant Cell 2003;15;2911-2928; originally published online November 20, 2003;
DOI 10.1105/tpc.017541

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