Functional activity and role of cation-efflux family members in Ni hyperaccumulation in *Thlaspi goesingense*

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The ability of *Thlaspi goesingense* to hyperaccumulate Ni seems to be governed in part by enhanced accumulation of Ni within leaf vacuoles. We have characterized genes from *T. goesingense* encoding putative vacuolar metal ion transport proteins, termed *m*etal *t*olerance *p*roteins (TgMTPs). These proteins contain all of the features of cation-efflux family members, and evidence indicates they are derived from a single genomic sequence (*TgMTP1*) that gives rise to an unspliced (*TgMTP1t1*) and a spliced (*TgMTP1t2*) transcript. Heterologous expression of these transcripts in yeast lacking the TgMTP1 orthologues COT1 and ZRC1 complements the metal sensitivity of these yeast strains, suggesting that TgMTP1s are able to transport metal ions into the yeast vacuole in a manner similar to COT1 and ZRC1. The unspliced and spliced TgMTP1 variants differ within a histidine-rich putative metal-binding domain, and these sequence differences are reflected as alterations in the metal specificities of these metal ion transporters. When expressed in yeast, *TgMTP1t1* confers the highest level of tolerance to Cd, Co, and Zn, whereas *TgMTP1t2* confers the highest tolerance to Ni. *TgMTP1* transcripts are highly expressed in *T. goesingense* compared with orthologues in the nonaccumulators *Arabidopsis thaliana*, *Thlaspi arvense*, and *Brassica juncea*. We propose that the high-level expression of *TgMTP1* in *T. goesingense* accounts for the enhanced ability of this hyperaccumulator to accumulate metal ions within shoot vacuoles.

The genus *Thlaspi* contains numerous species that hyperaccumulate Ni. For example, field-collected specimens of *Thlaspi goesingense* Hálácsy from an ultramafic site in Redschlag, Austria, have been recorded with shoot Ni concentrations as high as 12,400 μg/g shoot dry biomass (1.2%) (1, 2). In the laboratory, we analyzed individuals from this *T. goesingense* population and confirmed their hyperaccumulator status (3). To determine the physiological basis of this Ni hyperaccumulation phenotype, we have investigated a number of physiological parameters in both the hyperaccumulator and the related nonaccumulator *Thlaspi arvense*. Production of Ni chelates in root exudates in the hyperaccumulator and nonaccumulator were found to be equivalent (4), as well as rates of Ni translocation to the shoots (3). However, the hyperaccumulator was found to be more Ni tolerant when compared with the nonaccumulator (3). Ni tolerance in the hyperaccumulator is related to its enhanced ability to compartmentalize Ni in shoot vacuoles (5) with 75% of the intracellular leaf Ni in the hyperaccumulator being localized to the vacuole (5). Furthermore, vacuoles from the hyperaccumulator contain approximately double the Ni of the nonaccumulator, even though protoplasts from each species contained equal amounts of Ni (5). We conclude that vacuolar compartmentalization of Ni in the hyperaccumulator plays a major role in Ni tolerance and hyperaccumulation, although little is known about its molecular mechanism (6). Here we present data characterizing the functional properties and role of *T. goesingense* metal tolerance protein (TgMTP)-1 in the mechanism of Ni hyperaccumulation, and propose a role for TgMTP1 in enhanced vacuolar compartmentalization of Ni in the hyperaccumulator *T. goesingense*.

Materials and Methods

Plant Material and Culture. Seeds of the Ni hyperaccumulator *T. goesingense* were collected from an ultramafic site in Redschlag, Austria (3). Seeds of *Arabidopsis thaliana* (Col-3) were purchased from Lehle Seeds (Round Rock, TX), and seeds of *Thlaspi arvense* and *Brassica juncea* (426308) were obtained from the Crucifer Genetics Cooperative (Univ. of Wisconsin, Madison, WI). Plants were grown and harvested as described (7).

Plasmids, Bacterial, and Yeast Strains. The *Escherichia coli* strain TOP10 F′ [F′ [*lacI*^{q} Tn*10*(TetR)] *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*-Δ-M15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (StrR) *endA1 nupG*] was used for PCR and reverse transcription (RT)-PCR product cloning and plasmid DNA production. The *TgMTP1* genes were cloned into the pYES plasmid (Invitrogen) and subcloned into p425 GAL1 (ATCC no. 87331). For yeast expression, the following strains were transformed by means of a chemical method (8): *cot1* (CYP514), (*MAT*α *ura3–52 leu2–3,112 trp1-Δ1 cot1-Δ1*::*URA3*); *zrc1* (CYP502), (*MAT*α *ura3–52 leu2–3,112 trp1-Δ1 zrc1-Δ100*::*LEU2*; ref. 9); and Δ*pep5* (BJ7964), (*MAT*α *ura3–52 leu2Δ1 his3Δ200 trp1Δ101* Δ*pep5*::*TRP1*; ref. 10).

RNA and DNA Isolation. Total RNA and genomic DNA were isolated as described (7).

PCR and RT-PCR Cloning of *TgMTP1* Genes. For isolation of the *TgMTP1* genomic clone, 5 μg of genomic DNA from *T. goesingense* was PCR-amplified, using 100 pmol of 5′ MTP primer (5′-GCAAGCTTATGGAGTCTTCAAGTCCCCATCATGAGGTTAATG-3′) and 100 pmol of 3′ MTP primer (5′-ATGAATTCTTAGCGC TCGATTTG TAT-3′) with 35 cycles of 95°C denaturation for 1 min, 50°C annealing for 2 min, 72°C extension for 2 min, and a final 5 min 72°C extension step. PCR products were cloned into pYES by using standard techniques (11). Putative clones were sequenced (Univ. of Arizona, Tucson, AZ) and identified by comparison with *ZAT* (12). For isolation of *TgMTP1* transcript cDNA, 100 ng of shoot mRNA isolated from *T. goesingense* plants treated with 50 μM Ni for 48 h were reverse transcribed by using 4 units of avian myeloblastosis virus (AMV) reverse transcriptase (Promega), 100 pmol of 5′ MTP primer, and 100 pmol of 3′ MTP for 30 min at 42°C. First-strand cDNA

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Abbreviations: CE, cation efflux; RT, reverse transcription; SNP, single-nucleotide polymorphism.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AY044452 (TgMTP1), AY044453 (TgMTP1t1), and AY044454 (TgMTP1t2)].

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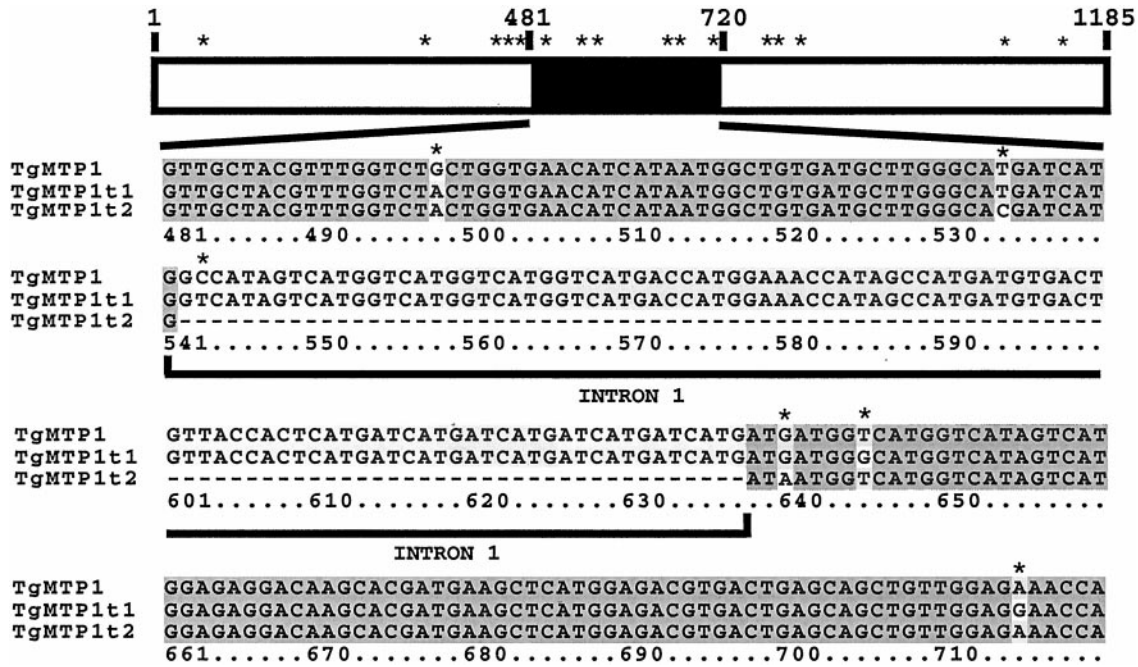

Fig. 1. Alignment of *TgMTP1* DNA sequences. The open box graphically illustrates the full-length alignment. The filled box (bp 481–720) is expanded to detail the alternatively spliced sequence. Nucleotides shaded in dark gray are conserved in all three sequences, and nucleotides in light gray are conserved only in two of the sequences. Intron 1 is illustrated with a dark line below the detailed sequence alignment. SNP positions are shown as asterisks.

products were PCR amplified with 5 units of *Pfu* polymerase (Promega) and 25 cycles (see above). PCR products were cloned into pYES (11) and *TgMTP1* transcripts identified by sequencing. *TgMTP1t1* was identified and used to screen the remaining RT-PCR clones by means of colony hybridization after the Northern blot hybridization protocol. Resulting positive clones were sequenced and identified as a homolog of *TgMTP1t1* and named *TgMTP1t2.*

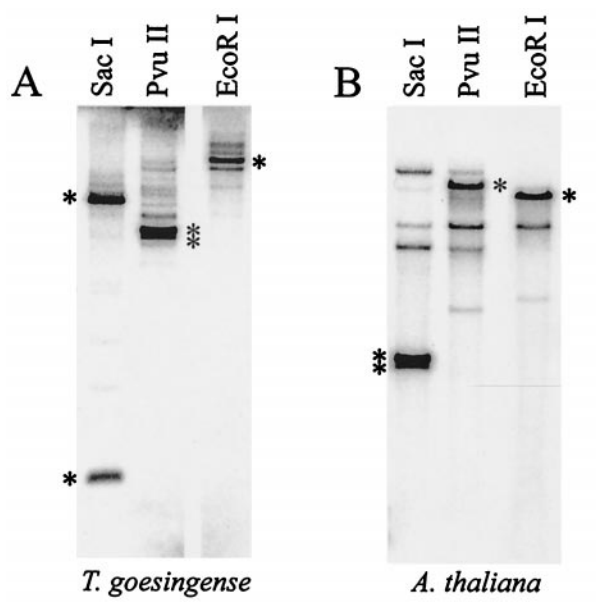

Fig. 2. Genomic Southern blot analysis of *TgMTP1* and *AtZAT*. (*A*) *T. goesingense* genomic DNA was cut with *Sac*I and *Pvu*II (internal cutters) and *Eco*RI (external cutter) and probed with *TgMTP1*. (*B*) *A. thaliana* genomic DNA was cut with *Sac*I (internal cutter) and *Pvu*II and *Eco*RI (external cutters) and probed with *AtZAT*. Major DNA fragments are marked with asterisks.

Alignments were performed by using CLUSTAL W (13). Analysis of conserved domains was performed by using reversed position-specific BLAST (14), and analysis of transmembrane domains was performed by using TMPRED (http://www.ch.embnet.org/software/TMPRED_form.html). Sequences were organized and analyzed at the Biology Workbench (http://workbench.sdsc.edu).

Yeast Metal Tolerance Assay. Yeast strains were grown to 1.5 OD_{600} in 10 ml of yeast nitrogen base (YNB) minimal medium SC amino acid supplement + adenine (SCA)/2% glucose − uracil − leucine for the *cot1* and *zrc1* mutants or YNB SCA/2% glucose − uracil for the Δ*pep5* mutant (YNB SCA = 6.7 g/liter yeast nitrogen base/2 g/liter SC amino acid supplement/100 mg/liter adenine). One milliliter of yeast culture was used to inoculate 50 ml of YNB SCA/4% galactose-uracil or -leucine (*cot1* and *zrc1*) or -uracil only (Δ*pep5*), containing 1.5% top agar (plant tissue-culture grade) and mixed well. This solution then was poured into 150 × 15-mm Petri dishes and allowed to cool. Sterile paper filter disks (6 mm) were placed at regular intervals

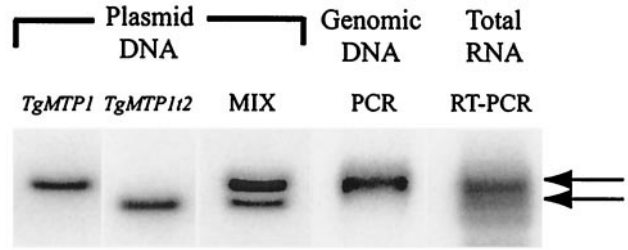

Fig. 3. Southern blot analysis of *TgMTP1* PCR and RT-PCR products. *TgMTP1* and *TgMTP1t2* cDNAs and a mixture of both (MIX) were prepared by digestion of pYES containing these clones. Full-length *TgMTP1* cDNA products were also amplified from both genomic DNA (PCR) and RNA (RT-PCR). All cDNAs were separated on an agarose gel, blotted, and probed with *TgMTP1*. Two major cDNA products were amplified from RNA (marked with arrows).

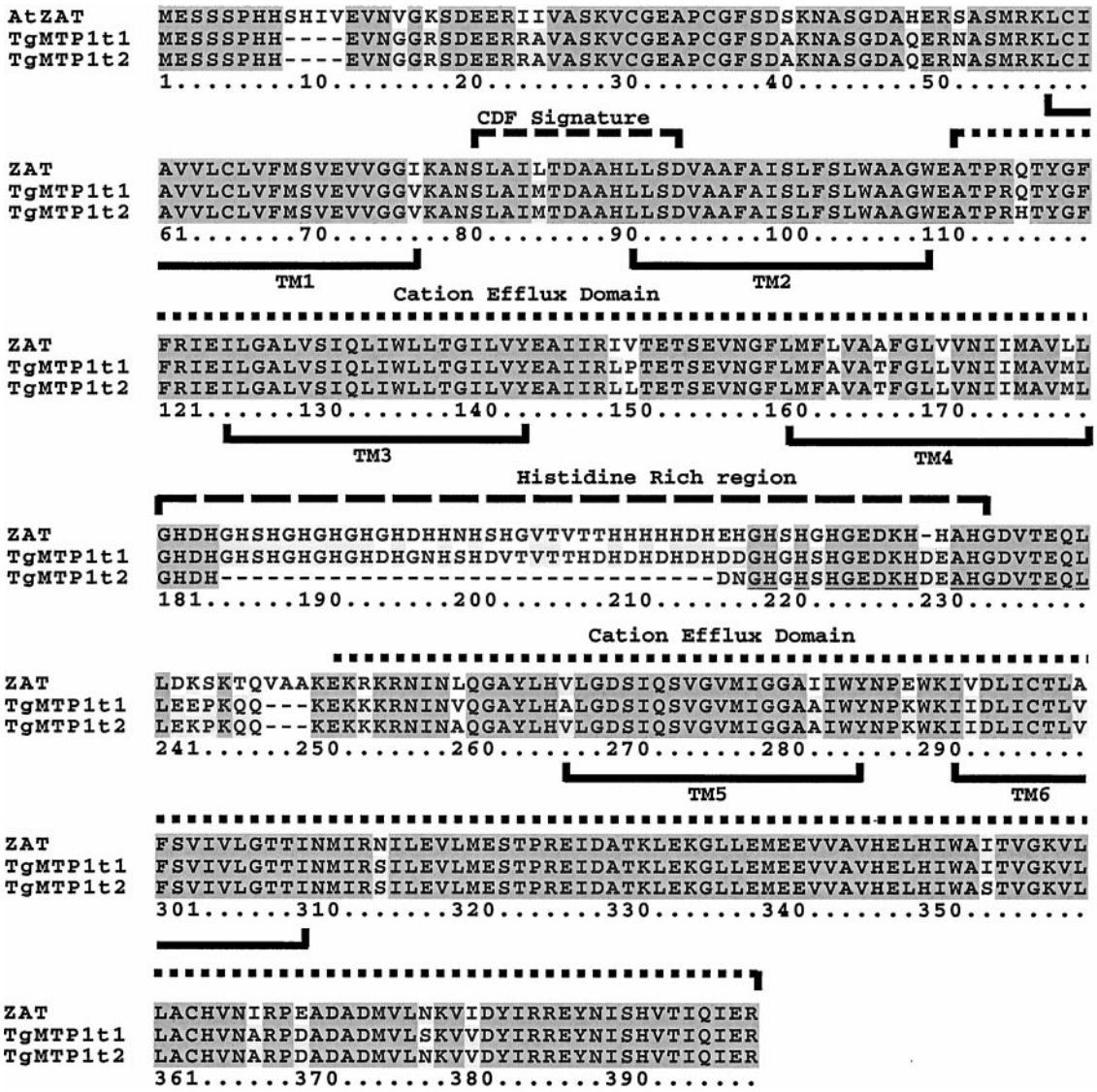

Fig. 4. Alignment of TgMTP1 and ZAT predicted amino acid sequences. The cation diffusion facilitator (CDF) signature sequence, CE domains, histidine-variable region, and transmembrane (TM) domains are shown. Amino acid residues shaded in dark gray are conserved; residues in light gray are present in two of the three protein sequences.

on the surface of the top agar, and solutions containing various metal ions were spotted on the disks. Plates were incubated at 30°C for 4–7 days, and the area of the zone of inhibition was measured.

RNA and DNA Blots. For Northern analysis, 30 μg of total shoot RNA per sample was analyzed by using described protocols (7). For genomic Southern analysis, 20 μg of genomic DNA per sample was digested with 60 units of each of the restriction enzymes (*Sac*I, *Pvu*II, and *Eco*RI) for 6–8 h at 37°C and was analyzed by using protocols as described (7). For probe preparation, cDNAs were digested with the appropriate restriction enzyme for 1 h at 37°C. Digested DNA was separated on a 1.5% agarose gel, and the appropriate size fragments were excised, recovered by electroelution, and labeled with ^{32}P following standard protocols (7).

Results and Discussion

Members of the cation-efflux (CE) family (15), also known as the cation diffusion facilitator (CDF) family, are known to be involved in Zn efflux across the plasma membrane and into intracellular vacuoles in mammals (16). Recent evidence suggests ZAT, an *A. thaliana* CE family member, also possibly effluxes Zn into the vacuole, although direct evidence is lacking (12). We initiated an investigation of the role of CE family members in the mechanism of vacuolar metal ion accumulation in plants by cloning cDNAs encoding putative vacuolar metal ion transporter from the Ni hyperaccumulator *T. goesingense*. This species is known to have an enhanced capacity to accumulate Ni within vacuoles in the shoot as part of its hyperaccumulation mechanism (5).

By using PCR, we isolated an ortholog of the *A. thaliana ZAT* gene from the hyperaccumulator *T. goesingense* and named it *TgMTP1* (*T. goesingense m*etal *t*olerance *p*rotein; Fig. 1). Although ZAT holds prior authority, to allow for expansion of the CE plant family, we propose that MTP would be a better base name for ZAT-related proteins (see ref. 15 for discussion).

To establish the presence, and estimate the copy number of *TgMTP1* in the *T. goesingense* genome, we performed genomic Southern blot analyses by using three restriction enzymes, *Sac*I,

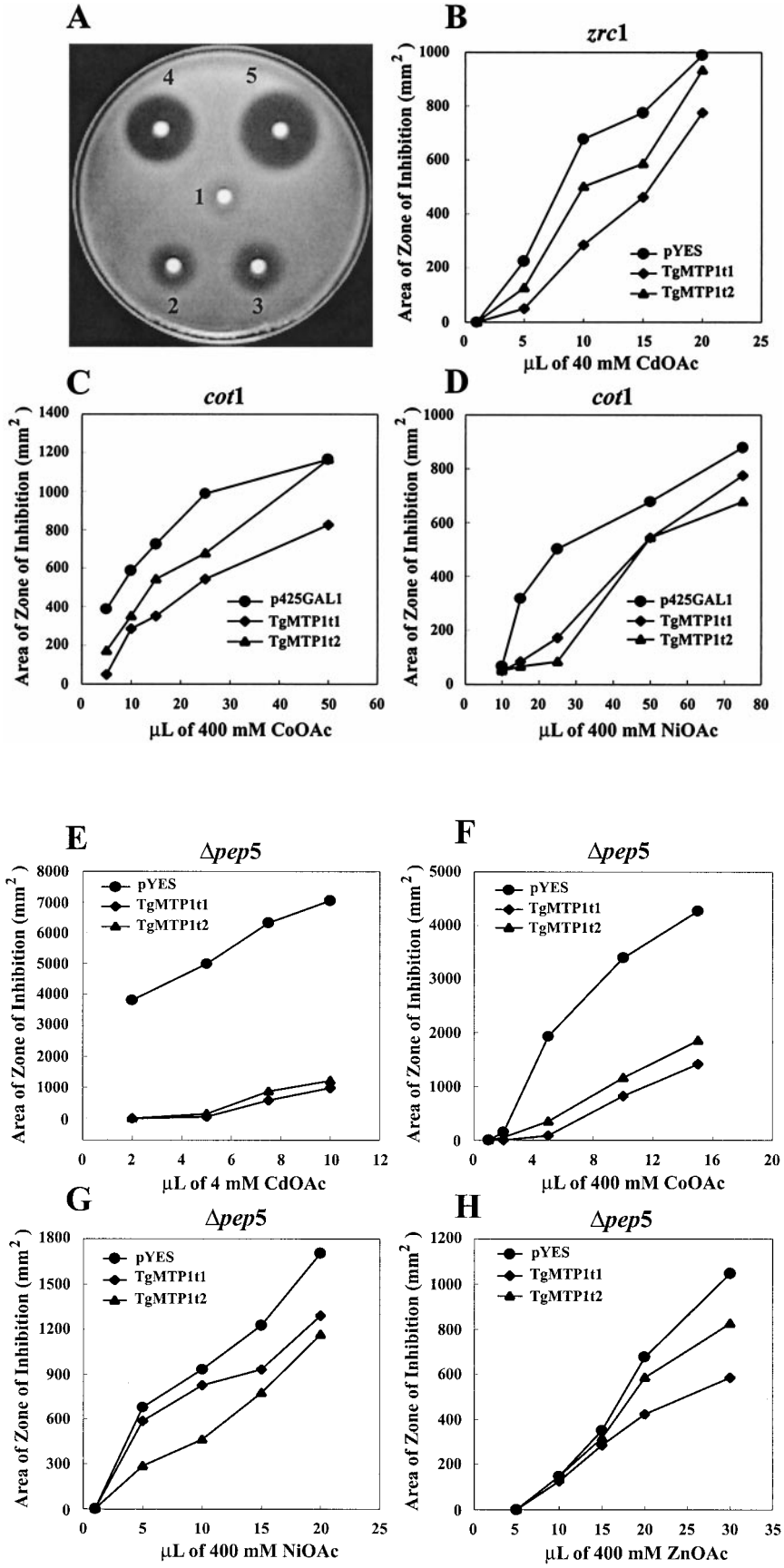

Fig. 5. Quantification of yeast metal tolerance. (*A*) An example of a yeast metal tolerance-assay plate showing the zones of *cot1* growth inhibition around Co-soaked filter discs, with discs 1–5 containing 2, 4, 6, 10, and 20 μmol of Co, respectively. (*B–H*) Quantification of Cd, Co, Ni, and Zn tolerance of *cot1*, *zrc1*, and Δ*pep5* yeast strains expressing *TgMTP1t1* and *TgMTP1t2*. Metal tolerance was quantified by using the assay system described in *A*.

*Pvu*II, and *Eco*RI (Fig. 2). As predicted for a single-gene copy, *Sac*I and *Pvu*II (internal cutters) produced two major bands, with *Eco*RI (external cutter) producing a single major band (Fig. 2*A*). As a control, we also performed an *A. thaliana* genomic Southern blot of *ZAT*, which is known to be a single-copy gene [The Arabidopsis Genome Initiative (AGI) http://www.arabidopsis.org/]. As predicted for the single-copy *ZAT*, *Sac*I (internal cutter) produced two major bands, and *Pvu*II and *Eco*RI (external cutters) produced a single major band each (Fig. 2*B*). The lighter bands observed on the *A. thaliana* Southern blot represent cross-hybridization of the *ZAT* probe with the known *ZAT* homologs *AtMTPa1* (AGI no. AT3 g61940), *AtMTPa2* (AGI no. AT3 g58810), and *AtMTPb1* (AGI no. AT2 g29410; ref. 15). The lighter bands observed in the *T. goesingense* Southern blot (Fig. 2*A*) also represent cross-hybridization of the *TgMTP1* probe with orthologs of the *A. thaliana AtMTPa1*, *AtMTPa2*, and *AtMTPb1* sequences. We confirmed the presence of such orthologs in *T. goesingense* by cloning and sequencing *TgMTPa1* and *TgMTPb1* (data not shown).

To determine the intronic gene structure of *TgMTP1*, we cloned transcript cDNA from total shoot RNA by using RT-PCR. Two transcript sequences, *TgMTP1t1* and *TgMTP1t2*, were cloned. Sequence alignments (Fig. 1) revealed 14 single-nucleotide polymorphisms (SNPs) between the sequences, and a 96-bp deletion between nucleotides 541 and 638 in *TgMTP1t2* (Fig. 1).

Because the hyperaccumulator seed used in our studies was collected from a wild *T. goesingense* population, the SNPs observed in the *TgMTP1* sequences most likely reflect different *TgMTP1* alleles in this population and not multiple *TgMTP1* genes within the *T. goesingense* genome. Such a conclusion is supported by the genomic Southern blot analysis that shows *TgMTP1* to be a single-copy gene (Fig. 2).

There are two alternative explanations for the origin of the 96-bp deletion in *TgMTP1t2*. Either it represents an intron that is spliced from *TgMTP1* or *TgMTP1t2* represents the transcript of an unidentified allele of *TgMTP1*. To distinguish between these two possibilities, we performed PCR and RT-PCR on genomic and total RNA by using primers that amplify full-length *TgMTP1*. The PCR products were run on an agarose gel, blotted, and probed with *TgMTP1* (Fig. 3). Amplification of *TgMTP1* from genomic DNA gave rise to a single product equal in size to full-length *TgMTP1*. Such a conclusion is consistent with the genome containing only a full-length *TgMTP1* sequence. However, amplification of *TgMTP1* from RNA produced two products that hybridized with *TgMTP1*. The largest and most abundant fragment was equal in size to *TgMTP1* (Fig. 3), whereas the second smaller product was equal in size to *TgMTP1t2* (Fig. 3). Amplification of this smaller second product exclusively from RNA is consistent with the 96-bp deletion in *TgMTP1t2* being an intron and confirms that *TgMTP1t2* represents an alternatively spliced transcript of *TgMTP1*.

The boundary sequences of plant nuclear mRNA introns are defined by a GU 5′ donor site and an AG 3′ acceptor site; although in rare instances, 5′ GC/AG 3′ or 5′ AU/AC 3′ also occur (17). The 5′ donor site in *TgMTP1* is GC (Fig. 1), a known but rare donor site. However, the 3′ acceptor site in *TgMTP1* seems to be UG, an unknown acceptor site. Our evidence (Figs. 1–3) strongly supports *TgMTP1t2* being produced by splicing of *TgMTP1*. However, because the *TgMTP1* sequence shown in Fig. 1 does not contain complete splice sites, we speculate that another as yet unidentified *TgMTP1* allele is present in the Redschlag *T. goesingense* population that contains the appropriate splice sites, and it is this allele that gives rise to the spliced *TgMTP1t2* transcript. In support of this, we have recently isolated two other genomic *TgMTP1* alleles that contain a GU 5′ donor site, created by a C to U polymorphism (data not shown). We note that the SNPs in *TgMTP1* cluster around both the 5′ and 3′ intron border sites (Fig. 1), making the generation of allelic splice variant likely. Instances of such SNPs creating new splice sites are well established in plants (18).

A search for conserved domains (http://workbench.sdsc.edu) classifies TgMTP1 as CE proteins, with both TgMTP1 splice variants possessing all features of CE family members (Fig. 4). These include the N-terminal signature sequence (19), CE domain (http://pfam.wustl.edu/), six predicted membrane-spanning domains (19), and a variable histidine-rich region near the middle of the protein sequence (20) that is predicted to project into the cytoplasm. It has been suggested that this type of histidine-rich domain may be involved in metal binding or transport (19). The CE family includes yeast COT1 and ZRC1 proteins (9, 21), mammalian ZNT1–4 proteins (20, 22), and bacterial CzD proteins (23). All these proteins are known to be involved in transport of metal ions including Ni, Zn, Co, and Cd, and there is strong evidence that COT1 and ZRC1 are responsible for transport of Co/Ni and Zn/Cd into the yeast vacuole (24).

Alignment of TgMTP1 with *A. thaliana* ZAT shows these proteins to be orthologs, with unspliced TgMTP1t1 showing 87% identity to ZAT (Fig. 4). Alignment of the predicted amino acid sequence of the unspliced and spliced variants of TgMTP1 shows that splicing differences are located exclusively in the central histidine-rich variable region of these proteins (Fig. 4). To investigate whether these sequence differences confer different functional properties to the proteins, we heterologously expressed them in yeast mutants deficient in the TgMTP1 evolutionary orthologs COT1 and ZRC1 (9, 21). Both COT1 and ZRC1 are members of the CE family, and yeast mutants lacking these genes are sensitive to Co, Ni, Cd, and Zn (9, 21). COT1 and ZRC1 are localized to the yeast vacuolar membrane (24) and are thought to be involved in transporting metal ions into the vacuole.

Yeast mutants *cot1* and *zrc1* heterologously expressing *TgMTP1t1* and *TgMTP1t2* were assayed for metal tolerance. Fig. 5*A* shows a representative metal-tolerance assay plate, with zones of yeast growth inhibition being clearly visible around the metal ion-soaked discs. Expression of *TgMTP1t1* and *TgMTP1t2* in *cot1* and *zrc1* yeast complemented the Cd, Co, and Ni sensitivity of these strains (Fig. 5 *B–D*), and we conclude that the *T. goesingense* TgMTP1 proteins act as functional orthologs of the yeast vacuolar metal ion transport proteins COT1 and ZRC1.

To further confirm the ability of *TgMTP1* to confer metal tolerance, we heterologously expressed both *TgMTP1t1* and *TgMTP1t2* in the metal-sensitive yeast mutant Δ*pep5*. This mutant is metal-sensitive because it forms numerous small immature vacuoles instead of properly enlarged mature vacuoles (10). Both variants of *TgMTP1* were able to suppress the Cd, Co, Ni, and Zn sensitivity of the Δ*pep5* mutant (Fig. 5 *E–H*). The suppression of metal sensitivity in Δ*pep5* may be explained in two ways. Either TgMTP1 proteins are targeted to the small immature vacuoles in this yeast mutant where they are involved in sequestering metal ions, or TgMTP1 proteins are targeted to the plasma membrane where they efflux metal ions from the yeast. Further studies are required to distinguish these mechanisms.

In all of the yeast strains tested, the variant proteins TgMTP1t1 and TgMTP1t2 showed consistent differences in their ability to confer metal tolerance (Fig. 5). TgMTP1t1 derived from the unspliced transcript had the highest capacity to confer tolerance to Cd, Co, and Zn (Fig. 5 *B*, *C*, *E*, *F*, and *H*), whereas TgMTP1t2 derived from the spliced transcript had the highest capacity to confer tolerance to Ni (Fig. 5 *D* and *G*). The amino acid sequences of the two variant TgMTP1s differ exclusively in the central histidine-rich domain of the proteins, with TgMTP1t2 lacking this region almost entirely (Fig. 4). Loss of this histidine-rich domain in TgMTP1t2 seems to confer increased Ni specificity to this protein.

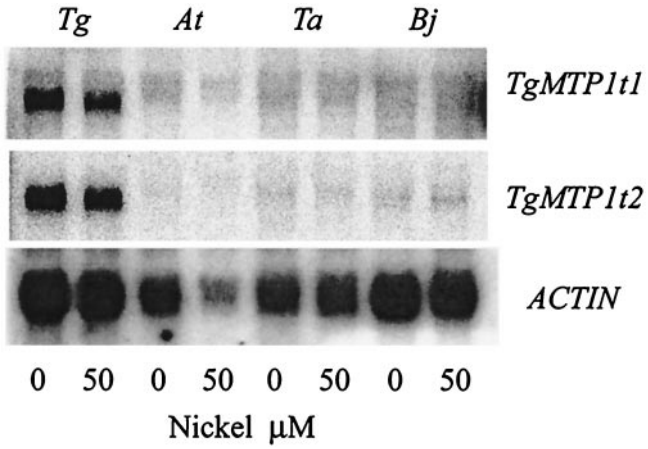

Fig. 6. Northern blot of *TgMTP1* expression in hyperaccumulator and nonaccumulator species. Total RNA was isolated from shoots of the hyperaccumulator *T. goesingense* and the nonaccumulators *A. thaliana*, *T. arvense*, and *B. juncea* after exposure to Ni for 48 h. Northern blots equally loaded with 30 μg of total RNA were probed with *TgMTP1t1*, stripped, and reprobed with *TgMTP1t2*, and finally stripped and reprobed with an *A. thaliana* actin probe as an RNA loading control.

To determine whether the steady-state levels of *TgMTP1* mRNAs are induced by Ni or are constitutively higher in the hyperaccumulator, we performed Northern analysis on both the hyperaccumulator *T. goesingense* and the nonaccumulators *T. arvense*, *A. thaliana*, and *B. juncea*. This analysis showed that *TgMTP1* transcripts are more abundantly expressed in shoots of the hyperaccumulator compared with the nonaccumulators (Fig. 6). Probing of the Northern blot with a full-length *T. arvense* ortholog of *TgMTP1* confirmed that these results were not caused by differential hybridization of the *TgMTP1* probe (data not shown) but genuinely reflect increased levels of the *TgMTP1* transcript in *T. goesingense*. Increased expression of *TgMTP1* transcripts in the hyperaccumulator is constitutive and not effected by exposure to either high concentration of Ni for short periods (100, 250, or 500 μM for 24 or 48 h) or lower concentrations for extended periods (100 μM for 1–4 weeks; data not shown). Ni hyperaccumulation in *T. goesingense* is a known constitutive trait (25), which supports the observation that *TgMTP1* is constitutively expressed. Increased expression of TgMTP1 in *T. goesingense* is consistent with this protein playing an important role in detoxification of Ni by vacuolar compartmentalization, a known mechanism of Ni tolerance in this hyperaccumulator (5).

In summary, we present an analysis of the functional activity and role of the TgMTP1 proteins in Ni hyperaccumulation in *T. goesingense*. Compared with nonaccumulator species, these proteins are constitutively highly expressed in the hyperaccumulator as two variant forms, TgMTP1t1 and TgMTP1t2, that are derived from the alternative splicing of the single-copy genomic clone *TgMTP1*. Heterologous yeast expression of these proteins complemented the metal sensitivity of the yeast mutants *cot1* and *zrc1* that lack functional vacuolar metal ion transport proteins. The complementation of metal sensitivity in these yeast mutants confirms that the TgMTP1 proteins can act as function orthologs of the yeast COT1 and ZRC1 vacuolar metal ion transporters. Sequence differences between the two splice variants of TgMTP1 cause altered metal ion selectivity in the proteins, with *TgMTP1t1* conferring the highest tolerance to Cd, Co, and Zn, whereas *TgMTP1t2* confers the highest tolerance to Ni. We propose that the overexpression and alternate splicing of these TgMTP1 metal ion transport proteins in *T. goesingense* are involved in the enhanced ability of this hyperaccumulator to compartmentalize Ni in its shoot vacuoles; a process critical to the ability of this plant to hyperaccumulate Ni (3, 5).

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