A mutagenic study identifying critical residues for the structure and function of rice manganese transporter OsMTP8.1

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Rice (Oryza sativa) MTP8.1 (OsMTP8.1) is a tonoplast-localized manganese transporter of the cation diffusion facilitator family. Here we present a structure-function analysis of OsMTP8.1 based on the site-directed and random mutagenesis and complementation assays in manganese hypersensitive yeast, in combination with three-dimensional (3D) structure modeling based on the crystal structure of the Escherichia coli CDF family member, EcYiiP. Two metal-binding sites are conserved in OsMTP8.1 with EcYiiP, one is between transmembrane helices TM2 and TM5, the other is the cytoplasmic C-terminus. In addition to these two metal-binding sites, there may exist other Mn-binding sites such as that at the very end of the CTD. Two residues (R167 and L296) may play an important role for the hinge-like movement of CTDs. Several mutations such as E357A and V374D may affect dimer formation, and S132A may induce a conformational change, resulting in a loss of transport function or modification in metal selectivity. The N-terminus of OsMTP8.1 was not functional for Mn transport activity, and the real function of NTD remains to be investigated in the future. The findings of the present study illustrate the structure-function relationship of OsMTP8.1 in Mn transport activity, which may also be applied to other plant Mn-CDF proteins.

Manganese (Mn) is an essential micronutrient for plant growth and development. In addition to being a cofactor in many enzymatic processes involved in glucose metabolism and energy production, it also acts as a cofactor of MnSOD in the mitochondrion and as part of the water-splitting complex in PSII in the chloroplast1,2. Despite its importance, Mn toxicity is common in acidic soils because the amount of exchangeable Mn increases in the soil solution3.

Recently, some of the genes responsible for Mn uptake and homeostasis in rice (Oryza sativa L.) have been identified. Members of the transporter gene family include natural resistance-associated macrophage protein (NRAMP)4–7, yellowstripe-like (YSL)8,9, metal tolerance protein (MTP)10,11, and vacuolar iron transporter (VIT)12. Mn uptake in rice roots is mediated by two polarized plasma membrane transporters, OsNramp5 and OsMTP913. OsNramp5 is an influx transporter that has been localized to the distal side of the exodermis and endodermis6. In contrast, OsMTP9 is an efflux transporter that has been localized to the proximal side of both cell layers11. OsYSL2 and OsYSL6 have been implicated in Mn homeostasis. OsYSL2 is a metal-nicotianamine transporter that is required for long-distance transport of iron and Mn8. OsYSL6 translocates Mn from the apoplasm to the symplast when plants are exposed to high levels of Mn4. OsMTP8.1 that was isolated from a rice shoot cDNA library conferred Mn tolerance in Saccharomyces cerevisiae, and its expression was found to enhance tolerance and accumulation of Mn, but not other heavy metals. OsMTP8.1 has been localized to the tonoplast and transports cytosolic Mn2+ into the vacuole. Disruption of OsMTP8.1 results in decreased chlorophyll content in the youngest leaf blades. Rice mtp8.1 Tos-17 insertion mutants and transgenic OsMTP8.1 RNAi knockdown lines are hypersensitive to Mn and accumulate less Mn in shoots and roots than wild-type plants10. However, its structure-function relationship remains elusive to date.

MTPs belong to the cation diffusion facilitator (CDF) transporter family and are important for the maintenance of cation homeostasis in bacteria, yeast, plants, and mammals14–16. MTPs play a critical role in removing...
transition metals from the cytosol either by intracellular sequestration or by cellular export. Phylogenetic analysis has indicated that the CDF family members can be divided into three major groups based on metal ion specificity: Zn-CDF, Fe/Zn-CDF, and Mn-CDF. To date, Ec YiiP is the only full-length CDF member that has been crystallized. It was initially characterized at a 3.8\(\text{Å}\) resolution and subsequently improved to 2.9\(\text{Å}\). Ec YiiP is a Y-shaped homodimer and each protomer contains three zinc-binding sites. Site A is located at the center of TM2 and TM5 and acts as an active site for zinc transport. This active site is tetrahedrally coordinated by D45 and D49 of TM2 and H153 and D157 of TM5. Site B is located in the intracellular loop that connects TM2 and TM3. Site C hosts four Zn\(^{2+}\) ions that mediate an interaction between CTDs and stabilize dimers. The six helices of the transmembrane domain (TMD) are grouped into two bundles with four (TM1–TM2–TM4–TM5) and two (TM3–TM6) helices. When Zn\(^{2+}\) binds to site C, the C-terminus moves the TM3-TM6 pair, causing reorientation of TM5, which allosterically changes the geometry of the active Zn\(^{2+}\)-binding site (site A), and thereby accomplishes transmembrane transport of Zn\(^{2+}\).

By using site-directed mutagenesis, Kawachi et al. studied the structural basis of Zn\(^{2+}\) selectivity and transport activity in AtMTP1. They found that two Zn-binding sites (sites A and C) are conserved in AtMTP1 with Ec YiiP. They also found that the N-terminus of AtMTP1 is very important for metal selectivity. They suggested that the N-terminal domain, the His-rich loop, and the leucine zipper motif in TM6 may contribute to the high Zn\(^{2+}\) selectivity in AtMTP1. The His-rich loop of AtMTP1 acts as a sensor of cytosolic Zn to maintain an essential level of Zn in the cytosol. The sequence characteristics of OsMTP8.1 differs from that of AtMTP1; for example, OsMTP8.1 does not have the His-rich loop between TM4 and TM5, and no leucine zipper motif exists within TM6. The sequence of plant Mn-CDF members are highly similar and differ from Ec YiiP and AtMTP1 only in terms of structure-function relationships.

In the present study, we are used site-directed and random mutagenesis to investigate the structural basis of Mn transport activity in a plant Mn-CDF member, OsMTP8.1.

**Results**

**Subcellular Localization and Metal Selectivity of OsMTP8.1.** The full-length cDNA fragment of OsMTP8.1 (Os03g0226400) was RT-PCR amplified using sequence information derived from the Rice Annotation Project of NCBI (http://www.ncbi.nlm.nih.gov). OsMTP8.1 encodes a putative protein of 396 amino acids in length. Phylogenetic analysis of OsMTP8.1 indicated that it belongs to the Mn clade of the CDF family. Transport activity of OsMTP8.1 was assessed by complementation assay using a number of yeast mutants that were deficient in various metal transporters. OsMTP8.1 was expressed in *S. cerevisiae* yeast mutants lacking the endogenous metal transporters for Zn (*zrc1*), Co (*cot1*), Cu (*cup2*), Cd (*ycf1*), Ni (*smf1*), and Mn (*pmr1*). The results showed that a pmr1 strain but not other mutants were restored after transformation with OsMTP8.1 (Fig. 1A), thereby suggesting that OsMTP8.1 may be a Mn-specific transporter. OsMTP8.1 was localized to the vacuolar membrane when it is expressed heterologously in yeast (Fig. 1B). To further confirm the localization of OsMTP8.1 in rice, the cDNA of OsMTP8.1 was fused in frame to GFP in the expression vector *pZX1008*. Transient expression of OsMTP8.1-GFP in rice mesophyll protoplasts showed that green fluorescence was localized to the tonoplast (Fig. 1C). The tonoplast localization of OsMTP8.1 was consistent with the results of a previous study involving onion epidermal cells.

**Site-directed and Random Mutant OsMTP8.1 Expression in Yeast from TM1 to TM6.** We aligned OsMTP8.1 and some other reported Mn-CDF, which included AtMTP8, AtMTP11, CmMTP8, HvMTP8.1, PmMTP11.1, PmMTP11.2, and OsMTP9 with Ec YiiP (Fig. 2). Amino acids that were conserved within Mn-CDF were selected for replacement with aliphatic amino acid Ala. Random mutagenesis was performed on pFL61-OsMTP8.1 by using GeneMorph II random mutagenesis kits (Stratagene) according to the manufacturer’s instructions. The mutation products were transformed into *E. coli* more than 200 plasmids were extracted. Each plasmid was transformed into the Mn-sensitive mutant *S. cerevisiae* and more than 200 plasmids were selected for further study (Fig. S1). Primers for the mutations from random mutagenesis are shown in Table S1. Yeast strain pmr1 transformed with an empty vector pFL61 was unable to grow on medium supplemented with 3 mM or higher Mn concentrations, but yeast cells expressing pFL61-OsMTP8.1 could grow on medium supplemented with up to 8 mM Mn.

Plate growth assays indicated that the mutants have different sensitivities to Mn (Fig. 3). A total of 63 single-substitution mutations conferred nearly the same level of Mn tolerance as that of wild-type OsMTP8.1 (Fig. S2). A reduced level of Mn tolerance was conferred by 23 mutations, which included N107A, N110A, L113A, S126A, I127A, L140A, I145A, S155A, I156A, V158A, K160A, I163A, K165A, V171A, G184A, V189A, L226A, W227A, N235A, V238A, L247A, A293T, and S295P. These mutants were able to grow in the presence of 3 mM Mn, but not with 8 mM Mn. These residues included S106A, I122A, S136A, G143A, F148A, G164A, G172A, I173A, F185A, I237A, Y241A, H245A, N252A, G255A, D270A, W285A, and L296A. Finally, 18 mutations that included K117A, T133I, D135A, D139A, L146A, Y161A, P162A, R167A, A177R, M180A, M216A, K223A, D244A, F247A, D248A, V260A, W285A, and L296A resulted in a complete loss of ability to confer Mn tolerance, which indicated that these amino acids are critical for Mn transport activity of OsMTP8.1.

**The function of N- and C-terminal domains of OsMTP8.1.** The OsMTP8.1 protein has six transmembrane (TM) domains, an N-terminal domain, and a C-terminal domain. To study the function of the N- and C-terminal domains of OsMTP8.1, we construct several deletion mutants. For the N-terminal domain, Mn...
Tolerance conferred by these deletion mutants was equivalent to that conferred by wild-type OsMTP8.1 (Fig. 4A). These results indicate that the N-terminal domain does not contribute to Mn transport activity in OsMTP8.1. For the C-terminal domain, we constructed 7 deletion mutants, and only the △392–396 mutant conferred Mn tolerance in the presence of 3 mM and 8 mM Mn, whereas other mutants did not confer any Mn tolerance even at a concentration of 3 mM Mn (Fig. 4B). To further determine which residues are required for Mn transport of OsMTP8.1, several substitute mutants were constructed. Mutations E304A, T325A, R327A, F336S, L359A, and F373L conferred reduced levels of Mn tolerance. The ability to confer Mn tolerance was significantly reduced in the H316A, D324A, L349A, E357A, Q360A, and E379A mutants. Approximately 15 mutants showed a complete loss in their ability to confer Mn tolerance; these mutants included T310A, G332A, V337D, E338A, D340N, H353A, G356R, E370A, R371A, V374D, H375Q, D377A, H382Y, E385A, and H386A (Fig. 4B). Mutations conferred nearly the same level of Mn tolerance as wild-type were shown in Fig. S2.

Zinc tolerance assays. The OsMTP8.1 mutants were also tested for Zn tolerance in the Zn-hypersensitive yeast strain zrc1. The ZRC1 gene encodes a multicopy suppressor of Zn toxicity that has been localized to the vacuolar membrane of S. cerevisiae. Two mutants, F75A and S132A, conferred tolerance to high levels of Zn (18 mM) but not other metals including Cd, Ni, Cu, Co or Fe (Figs 5A and S3). F75 was localized to the NTD, and S132 to EL1. Furthermore, to investigate the metal transport activity of these two mutants, the accumulation of Zn and other metal cations was compared in zrc1 that expressed OsMTP8.1 or the mutants. The results showed that two mutants accumulated 1.6-fold the amount of Zn of the wild type OsMTP8.1 (Fig. 5B). Significant differences were not detected in the concentrations of other metals (data not shown).

Cellular localization of the mutants within yeast cells showed a vacuolar localization similar to the wild-type proteins (Fig. S4), confirming that the mutant proteins are expressed and functional. A summary of effects of all mutations in OsMTP8.1 as determined by yeast complementation assays are presented in Fig. 6.
3D structural modeling of OsMTP8.1. To determine the function of mutated residues involved in the determination of metal transport activity, we constructed a 3D model of OsMTP8.1 by using the SWISSMODEL software (Fig. S5). The final models turned out to be strongly biased toward E. coli YgiP (3H90). The N-terminal domain of OsMTP8.1 was much longer than EcYgiP (Fig. 2) and was not reflected in the 3D model.

Biochemical studies and X-ray structural analysis of EcYgiP indicated that it has three Zn$^{2+}$-binding sites: site A is a tetrahedral Zn$^{2+}$-binding site situated in the transmembrane domain and is composed of two residues from TM2 (Asp45 and Asp49) and two residues from TM5 (His153 and Asp157), two cytoplasmic binding sites, sites C and D. Residues essential for Mn binding in site A and site C of OsMTP8.1 are shown in red. Point denotes a gap in the alignment.

Figure 2. Amino acid sequence alignment of OsMTP8.1 with EcYgiP and other CDF family proteins. TiCzr (GenBank Acc. No. AJ307316.1) from Thermus thermophilus, AtMTP1 (GenBank Acc. No. At2g46800), AtMTP8 (GenBank Acc. No. At3g58060) and AtMTP11 (GenBank Acc. No. At2g39450) from Arabidopsis thaliana, GMTP8 (GenBank Acc. No. AFJ42703) from Curcuma longa, HvMTP8.1 (GenBank Acc. No. AFF33387) from Hordeum vulgare, PmMTP1.1 (Phytozome: POPTR_0010s21810) and PmMTP11.2 (Phytozome: POPTR_0008s04940) from Populus trichocarpa, OsMTP8.1 (Phytozome: LOC_Os01g30050) and OsMTP8.1 (Phytozome: LOC_Os03g12530) from Oryza sativa, and E. coli K12 YgiP are aligned. The sequence of AtMTP1 lacks the histidine-rich loop (D185 to K250). The transmembrane helices (TM), external (vacuolar) loops (EL), intracellular (cytosolic) loops (IL), and C-terminal cytoplasmic α-helices and β-sheets of EcYgiP are shown above the alignment. Residues essential for Mn binding in site A and site C of OsMTP8.1 are shown in red. Point denotes a gap in the alignment.

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B and C. Site B is localized to the intracellular loop that connects TM2 and TM3 (IL1), in which Zn\(^{2+}\) is coordinated with one water molecule and three residues Asp68, His71 and His75, and site C in the C-terminal domain that hosts two Zn\(^{2+}\) ions bound in a binuclear cluster by Asp285, His232, His248, His283, and His261 from the neighboring subunit of \(Ec\)YiiP. This site hosts four Zn\(^{2+}\) ions that mediate a tight interaction between CTDs and play an important role in stabilizing the dimer\(^{19,20}\).

According to the 3D model, the active Mn-binding site (site A) of \(Os\)MTP8.1 consists of D135 and D139 from TM2 and D244 and D248 from TM5 (Fig. 7A). The sequence DxxxD (x = any amino acid) in TM2 and TM5 are conserved in Mn-CDF. Mutations in any of these four residues appeared to abolish Mn transport activity of \(Os\)MTP8.1. These results suggested functional conservation of site A between \(Ec\)YiiP and \(Os\)MTP8.1.

Sequence alignment indicated no conserved regions between \(Ec\)YiiP and \(Os\)MTP8.1 in site B (Figs 2 and 7B). However, mutations Y161A and P162A in the intracellular loop between TM2 and TM3 appeared to abolish Mn transport function, and mutation G164A apparently strongly reduced the transport function of \(Os\)MTP8.1. These three residues are conserved among Mn-CDFs. These results indicated that IL1 is essential for \(Os\)MTP8.1 function, but may not likely form a metal-binding site as \(Ec\)YiiP.

A number of highly conserved residues were detected at site C of \(Os\)MTP8.1, including D324, D340, H353, H375 and D377, thereby implicating its role in metal binding (Fig. 7C). Mutations D340A, H353A, H375A, and D377A showed a complete loss in the ability to confer Mn tolerance, and D324A presented a significant reduction in function. In addition to the conserved residues, 9 mutations also abolished Mn tolerance, namely, T310A, G332A, V337A, E338A, G356A, E370A, H382A, E385A, and H386A. Sequence alignment indicated that these residues are highly conserved in Mn-CDF but less conserved with \(Ec\)YiiP, indicating that the structure of CTD for Mn binding in Mn-CDF may be different from that of \(Ec\)YiiP.

Previous biochemical and X-ray analyses have indicated that the \(Ec\)YiiP homodimer structure is stabilized by an interlocked salt bridge near the membrane surface that is formed by K77 and D207\(^{20}\). The corresponding residues for these two residues in \(Os\)MTP8.1 were R167 and G298, these two residues apparently did not form a salt bridge, and G298A conferred nearly the same level of Mn tolerance as that of wild-type \(Os\)MTP8.1. Interestingly, we found that R167 and L296 were critical for Mn transport activity of \(Os\)MTP8.1. These two residues were located near the membrane surface (Fig. 8B); we speculate that they play an important role in hinge movement of \(Os\)MTP8.1.

**Figure 3.** Metal tolerance complementation assays from TM1 to TM6 of \(Os\)MTP8.1 with random and site-directed mutagenesis. Complementation of \(S.\ cerevisiae\) mutant \(pmr1\) grown on selective media supplemented with 3 mM and 8 mM Mn\(^{2+}\). For complementation tests, transformants were pre-cultured in SD-Urea overnight. Pre-cultured cells were diluted to an OD\(_{600nm}\) of 1.0, 10 \(\mu\)L of cell suspensions were spotted on SD-Urea plates supplemented with the indicated concentrations of Mn\(^{2+}\). Plates were incubated for 72 h at 30°C.
Discussion

According to the phylogenetic study conducted by Montanini et al., Mn-CDF proteins can be differentiated from other CDF family members by the consensus sequence DXXXD in TM2 and TM5. The active Mn-binding site (site A) of OsMTP8.1 is formed by four Asp residues, of which D135 and D139 are located in TM2 and D244 and D248 in TM5 (Fig. 7A). These key residues of the Mn-binding site are highly conserved in other Mn-CDF family proteins (Fig. 2).

In contrast to EcYiiP, OsMTP8.1 does not contain the Mn-binding site B in the cytosolic loop (IL1) between TM2 and TM3. This site is also not conserved within other CDF family members such as MmCDF3 from *Mariculalis maris* MCS104 and the plant zinc transporter AtMTP121. IL1 is the interface for dimerization of EcYiiP protomers. In OsMTP8.1, IL1 contains critical residues (Y161, P162, and G164) for transport function (Fig. 7B); these residues may be involved in dimerization of OsMTP8.1. These results also suggest that there is an alternative access mechanism for Mn to reach the active transport site from CTD of OsMTP8.1 via the intracellular cavity.

The cytoplasmic binding site (site C) of OsMTP8.1 has been localized to the CTD-CTD interface and is composed of several highly conserved residues (D324, D340, H353, H375, and D377) (Fig. 7C). EcYiiP showed extensive outer-shell constraints around the binuclear zinc coordination in site C, which were composed of D233, E250, D265, I282, and Q284. These residues apparently establish an extensive network of outer-shell interactions at the CTD interface, thereby stabilizing the CTD-CTD association. In OsMTP8.1, these residues correspond to T325, E342, E357, V374, and L376 (Fig. 7C). Yeast complementation assays support the essential role of T325, E357, and V374 and may contribute to the function of stabilizing the CTD-CTD association in OsMTP8.1 (Fig. 7C). The sequence HKPEH from residue 382 to 386 in OsMTP8.1 is highly conserved among Mn-CDF and distinguishable from EcYiiP (Fig. 2), and mutations H382A, E385A, and H386A abolished Mn transport activity. We therefore propose that there may be another Mn-binding site at the end of the CTD of OsMTP8.1. The 3D model of OsMTP8.1 ended at 380 C, and lost the structural information of this site, and the binding information remains to be elucidated in future research investigations. Recently, the 3D structures of the CTDs of several CDF proteins were resolved, e.g., the CzxB of *T. thermophiles*22, the apo form of a CDF protein of *T. maritima*23, and MamM of *M. gryphiswaldense*24. These findings demonstrate that CTDs adopt a metallochaperone-like fold, and
the metallochaperones can carry metal ions to various protein targets. It has been suggested that the CTD plays a role in sensing the Mn ions and delivering these to the TMD region of OsMTP8.1.

The six transmembrane helices of the TMD in EcYuiP consist of two independent bundles; TM1, TM2, TM4, and TM5 form a four-helix bundle, whereas TM3 and TM6 compose another bundle. The orientation of the TM3–TM6 bundle is stabilized by four interlocking salt bridges formed by K77 of TM3 and D207, which are located in IL3. The charge interlock also plays an important role in stabilizing dimer associations and is essential for Zn transport. When Zn binds to site C, it triggers a hinge-like movement of two CTDs, inter-CTD movements alter the TM3-TM6 bundle orientation and causes reorientation of TM5. The orientation of TM5 with respect to TM2 affects the coordination geometry of the active Zn-binding site, thereby facilitating Zn transport. In OsMTP8.1, two residues (R167 from TM3 and L296 from TM6) near the membrane surface play critical roles in Mn transport. Mutation M180A in TM3 and W285A in TM6 abolished Mn transport activity of OsMTP8.1 (F75A and S132A). Two mutations of OsMTP8.1 can confer Zn tolerance phenotype in yeast sensitive mutant.

**Figure 5.** Two mutations of OsMTP8.1 can confer Zn tolerance phenotype in yeast sensitive mutant.
(A) Yeast cell suspensions of a zrc1 or pmr1 transformed with empty vector pFL61, or with pFL61 containing wild-type OsMTP8.1 or mutated OsMTP8.1 cDNAs were pre-cultured in SD-Urea overnight. Pre-cultured cells were diluted to OD600 nm of 1.0, 10 μL of cell suspensions were spotted on SD-Urea plates supplemented with the indicated concentrations of Mn2+ or Zn2+. Plates were incubated at 30°C for 72 h. (B) Concentration of Zn in yeast cells determined in the wild-type BY4741(WT) and mutant strain zrc1 transformed with empty vector pFL61, OsMTP8.1, and two mutants of OsMTP8.1 (F75A and S132A). Yeast cells were grown in liquid SD medium supplemented with 0.5 mM ZnCl2 at an initial OD600 = 0.1. Bars with different letters are significantly different (Tukey’s test, p < 0.01).
N258 of AtMTP1 (Fig. 8A), and mutant L146A completely lost its ability to transport Mn and I237A conferred reduced levels of Mn tolerance (Fig. 3). There is a long His-rich loop between TM4 and TM5 of AtMTP1, but the loop between TM4 and TM5 of OsMTP8.1 was very short (Fig. 2). Residues L146 and I237 of OsMTP8.1 may have different mechanisms for metal transport due to S101 and N258 of AtMTP1. We propose that mutations L146A and I237A affect the coordination geometry between TM2 and TM5 and abolish Mn transport activity in OsMTP8.1.

The N-terminal deletions showed no effect of Mn transport activity compared to that in wild-type OsMTP8.1 (Fig. 4A). In AtMTP1 and PtdMTP1, two Cys residues are required for Zn transport activity; these two residues are well conserved among plant Zn-CDF proteins21,28. The N-terminal deletion mutant, which lacks the Cys residues, also did not confer Zn tolerance. Compared to the Zn-CDF proteins, the function of the N-terminal domain of OsMTP8.1 requires further investigation.

Previous studies have shown that several sites influence metal specificity in CDF family proteins. In AtMTP1, the His-rich loop of IL2 and sequences within NTD are responsible for metal selectivity, and several residues within transmembrane domain were also found to influence metal selectivity21. In OsMTP1, residue L82, which is located in EL1, may also play a role in substrate specificity29. This also found in other CDF family proteins such as ScZRC130. Two mutants, F75A located in the NTD and S132A located in EL1, also conferred tolerance to Zn besides Mn.

In conclusion, by using site-directed and random mutagenesis and complementation assays in yeast, and in combination with 3D structure modeling based on the E. coli Zn transporter YiiP, we have identified residues that are essential for Mn transport in the plant Mn-CDF member, OsMTP8.1. We also presented a structure-function relationship involving OsMTP8.1 for Mn transport activity that may also be applied to other plant Mn-CDF proteins. Two metal binding sites are conserved in OsMTP8.1 with E. coli YiiP, one is the active Mn-binding site between TM2 and TM5, and the other is the CTD-binding site. Several mutations may affect dimer formation or cause a conformational change in the protein, thereby resulting in a loss of transport function or modification in metal selectivity. The N-terminus of OsMTP8.1 is not functional for Mn transport activity, and the real function of NTD remains to be investigated in future studies.

Methods
Site-directed and random mutagenesis of OsMTP8.1. OsMTP8.1 was inserted into the NotI sites of pFL6114 to obtain the expression plasmid. Site-directed mutagenesis and random mutagenesis were performed on the pFL61-OsMTP8.1 vector using QuickChange II XL site-directed mutagenesis and GeneMorph II random mutagenesis kits (Stratagene) according to the manufacturer’s instructions32. Sequences encoding N- or C-terminally truncated mutants of OsMTP8.1 were amplified by PCR, and the DNA fragments obtained were
inserted into the Nol site of the pFL61 vector. The primers used and the mutations induced are listed in Table S2. All constructs generated in the present study were verified by DNA sequencing.

**Heterologous expression of mutated OsMTP8.1 in yeast.** The mutants and the wild-type yeast strains used in this research were obtained from the Euroscarf collection. Plasmids were transformed into the Mn-sensitive mutant Y04534 (pmr1) or Zn-sensitive mutant Y00829 (zrc1) and its parental strain BY4741 for Mn and Zn complementation analyses. Yeast transformation was performed by using the lithium acetate/PEG transformation method. Positive colonies were selected on synthetic dropout (SD) plates containing the appropriate selective markers. Yeast strains expressing empty vector, wild-type OsMTP8.1, or mutated OsMTP8.1 variants were pre-cultured in SD-Ura liquid medium at 30 °C for 16 h. Pre-cultured cells were diluted to an OD600 nm of 1.0, and 10-μL aliquots were spotted onto SD-Ura plates containing various concentrations of metals as indicated elsewhere. Plates were incubated at 30 °C for 3 days.

**Subcellular localization in rice protoplast and yeast.** For the subcellular localization in rice protoplasts, the OsMTP8.1 cDNA fragment without a stop codon was amplified and was cloned into the BamHI and KpnI sites of pXZP008. The construct was transformed into rice mesophyll protoplasts and GFP fluorescence was observed using a confocal laser scanning microscopy (Confocal System-UitraView VOX, Perkin Elmer). For the subcellular localization in yeast, the ORFs were amplified by using primers containing the MluI site, and cloned into the yeast expression vector pFL61-GFP. The construct was transformed into pmr1 and GFP signal was observed by confocal laser scanning microscopy.

**Bioinformatics analysis of OsMTP8.1.** Multiple sequence alignments of TtCzrB (GenBank Acc. No. AJ307316.1) from Thermus thermophilus, Arabidopsis thaliana AtMTP1 (GenBank Acc. No. At2g46800), AtMTP8 (GenBank Accession Number At3g58060) and AtMTP11 (GenBank Acc. No. At2g39450), Cucumis sativus Cs
MTP8 (GenBank Acc. No. AF124703), Hordeum vulgare HsMTP8.1 (GenBank Acc. No. AF833387), PtMTP11.1 (Phytome: POPTR_0010s21810) and PtMTP11.2 (Phytome: POPTR_0008s04940) from Populus trichocarpa, OsMTP9 (Phytome: LOC_Os01g03914) and OsMTP8.1 (Phytome: LOC_Os03g12530) from Oryza sativa, and E. coli K12 YiiP (P69380) were performed using multalin. The 3D model of OsMTP8.1 was generated by homology modeling using the SWISSMODEL software (http://swissmodel.expasy.org/) based on the structure of EcYiiP (PDB ID 3H90). Images were generated by using PyMOL 1.6.x.

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X.C., J.L. and W.Z. planned the experiments, X.C., J.L., L.W. and G.M. performed the experiments, and X.C., J.L. and W.Z. analyzed the data and wrote the paper.

**Additional Information**
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