

Molecular characterization of a rice *metal tolerance protein*, *OsMTP1*

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Abstract Rice (*Oryza sativa* L. ‘Nipponbare’) cDNA subtractive suppression hybridization (SSH) libraries constructed using cadmium (Cd)-treated seedling roots were screened to isolate Cd-responsive genes. A cDNA clone, encoding the rice homolog of *Metal Tolerance Protein (OsMTP1)*, was induced by Cd treatment. Plant MTPs belong to cation diffusion facilitator (CDF) protein family, which are widespread in bacteria, fungi, plants, and animals. *OsMTP1* heterologous expression in yeast mutants showed that *OsMTP1* was able to complement the mutant strains’ hypersensitivity to Ni, Cd, and Zn, but not other metals including Co and Mn. *OsMTP1* expression increased tolerance to Zn, Cd, and Ni in wild-type yeast BY4741 during the exponential growth phase. *OsMTP1* fused to green fluorescent protein was localized in onion epidermal cell plasma membranes, consistent with an *OsMTP1* function in heavy metal transporting. *OsMTP1* dsRNAi mediated by transgenic assay in rice seedlings resulted in heavy metal sensitivity and changed the heavy metal accumulation in different organs of mature rice under

low-concentration heavy metal stress. Taken together, our results show that *OsMTP1* is a bivalent cation transporter localized in the cell membrane, which is necessary for efficient translocation of Zn, Cd and other heavy metals, and maintain ion homeostasis in plant.

Keywords Metal tolerance protein · Heavy metal · Rice

Abbreviations

MTP	Metal tolerance protein
CDF	Cation diffusion facilitator
NRAMP	Natural resistance-associated macrophage protein
ZIP	Zn-regulated transporter-/Fe-regulated transporter-like protein
TMD	Transmembrane domain
ZAT	Zinc transporter of <i>Arabidopsis thaliana</i>
AAS	Atomic absorption spectrometer
dsRNAi	Double-stranded RNA interference
SD medium	Synthetic defined medium
RT-PCR	Reverse transcription PCR
DIG	Digoxigenin
ORF	Open reading frame
EGFP	Enhanced green fluorescent protein
SSH	Subtractive suppression hybridization
ISH	In situ hybridization

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Introduction

Heavy metals such as Zn, Mn, Ni, Fe, Co, and Cu are essential trace elements for numerous physical processes in plants. In trace amounts, they can act as important

cofactors for many enzymes and function in cellular physical processes (Williams and Mills 2005). In high quantities, they are toxic through biomolecular inactivation by blocking essential functional groups or displacement of essential metal ions (Goyer 1997). Other heavy metals such as Cd, Pb, and Hg are non-essential elements in plants and are generally considered toxic, because they can displace some essential elements or react with sulfur and nitrogen in amino acid side chains (Clemens 2001). Plants have developed a series of uptake, extrusion, chelation, trafficking, and storage mechanisms to maintain essential trace element homeostasis, exclude non-essential trace elements, and to alleviate heavy metal toxicity (Hall and Williams 2003).

Some characterized plant transporter proteins responsible for metal homeostasis are membrane proteins that mediate heavy metal movement through membranes. These transporters can be classified into channels, carriers, and pumps (Williams et al. 2000). Among these transporters, cation diffusion facilitator (CDF) family proteins are crucial for metal ion homeostasis. Members of the diverse CDF family have been cloned from bacteria, fungi, plants, and animals (Mäser et al. 2001). Most CDF proteins possess four to six putative transmembrane domains (TMDs) with cytoplasmic N and C termini, and the most conserved amphipathic TMDs I, II, V, and VI regions that are likely involved in metal transfer (Haney et al. 2005). According to protein sequence alignment and substrate specificities of some characterized transporters, CDF members from three kingdoms (Archaea, Eubacteria, Eukaryotes) were classified into three subgroups: Zn-CDF, Fe/Zn-CDF, and Mn-CDF (Montanini et al. 2007).

In plants, CDF proteins were also designated as metal tolerance proteins (MTPs). The first plant CDF protein identified was ZAT (zinc transporter of *Arabidopsis thaliana*). Because of its involvement in *Arabidopsis* heavy metal tolerance, ZAT was later renamed as *AtMTP1* (*Metal Tolerance Protein 1*) (Van-Der-Zaal et al. 1999). *AtMTP1* over-expression in *Arabidopsis* confers improved growth with supra-optimal Zn concentrations in the medium, and an *AtMTP1* T-DNA insertion mutant line displays enhanced sensitivity to high Zn concentrations (Kobae et al. 2004), suggesting that *AtMTP1* is involved in Zn tolerance. Heterologous expression of *AtMTP3* in yeast double mutant *zrc1Δ cot1Δ* restores Zn and Co tolerance, and *AtMTP3* ectopic over-expression increases Zn accumulation in *Arabidopsis* leaves, and enhances Zn tolerance, while *AtMTP3* RNAi causes Zn hypersensitivity. Hence, *AtMTP1* and *AtMTP3* function similarly in responding to Zn. Unlike *AtMTP1*, however, *AtMTP3* seems to be involved in metal transportation under Fe deficiency (Arrivault et al. 2006). Sequence analyses showed that *AtMTP1* and *AtMTP3* belonged to Zn-CDF, while

AtMTP11 belonged to Mn-CDF (Montanini et al. 2007). Functional analysis showed that *AtMTP11* can specifically complement the Mn-hypersensitive yeast mutant *pmr1Δ* (Delhaize et al. 2007), and might be involved in both Mn tolerance and homeostasis mechanisms in *Arabidopsis*. MTP genes have also been cloned from other plants, especially from some heavy metal hyperaccumulator plants such as *Arabidopsis halleri* (*AhMTP1*) (Dräger et al. 2004), *Thlaspi goesingense* (*TgMTP1*) (Kim et al. 2004), and *Stylosanthes hamata* L. (*ShMTP1*) (Delhaize et al. 2003). These genes can all response to Zn, Cd, or other bivalence heavy metal ions.

Rice is the most important cereal crop in China. It is also a model monocotyledon plant. Heavy metal contamination has become a serious environmental problem, and has greatly affected rice growth and quality. Heavy metal accumulation in water, soil, and air increasingly accompanies industrial pollution. Heavy metals accumulated in rice are toxic to growth and metabolism. Rice *Metal Tolerance Protein1* (*OsMTP1*) gene expression is induced by Cd treatment, as indicated by our previous SSH study. Analysis of the rice genome has identified ten MTP genes (Montanini et al. 2007), and *OsMTP1* belongs to the Zn-CDF subgroup. In this study, we mainly focus on *OsMTP1* functional identification. Our results showed that *OsMTP1* heterologous expression in a yeast mutant restored Ni, Zn, and Cd tolerance. *OsMTP1* dsRNA interference can decrease markedly the rice seedling tolerance to heavy metals. Furthermore, down-regulation of *OsMTP1* by double-stranded RNA interference (dsRNAi) can also change the heavy metal accumulation in different organs of rice. These results suggest that *OsMTP1* is necessary for efficient translocation of Zn, Cd and other heavy metals as well as ion homeostasis in plant.

Materials and methods

Plant materials

Wild-type rice japonica variety Zhonghua 11 was used. Transgenic and wild-type plants were grown under a routine management regime in an experimental field at the South China Botanical Garden (Guangzhou, China). Rice seedlings were grown on one-half-strength MS medium with or without appropriate selection and heavy metals.

For heavy metal resistance assays of rice, homozygous T3 seeds of lines over-expressing *OsMTP1* or transformed with dsRNAi *OsMTP1* were sown on plates containing half-strength MS medium supplied with various concentrations of heavy metals. Wild-type plants were used as a control. After 2 weeks growth, rice seedlings were examined.

For measurements of Zn, Cd, and Ni contents in rice, wild-type rice and homozygous dsRNAi T3 rice seeds were grown hydroponically according to Yoshida et al. (1976). ZnSO₄ (0.2 mM), CdCl₂ (0.01 mM), and NiSO₄ (0.05 mM) were added to the liquid culture, respectively.

Plasmid constructs and plant transformation

To study *OsMTP1* function in plants, we performed transgenic over-expression and dsRNAi assays in rice. For *OsMTP1* over-expression (*OsMTP1*-OE) in rice, the *OsMTP1* open reading frame (ORF) was PCR amplified to obtain full-length cDNA fragments with AK100735 cDNA clone (KOME, <http://cdna01.dna.affrc.go.jp/cDNA/>) as a template using the following primers: pMTP1OEL: 5'-CGGGATCCATGGACAGCCATAACTCAGC-3' and pMTP1OER: 5'-CGGGATCCCTACTCGCGCTCAATCTGAA-3' (*Bam*HI sites are underlined). PCR fragments were cloned into the *Bam*HI site of pCU1301 (Chen et al. 2007) between the maize Ubi1 promoter and the nos site. For *OsMTP1* dsRNAi in rice, the 5' fragments of *OsMTP1* (500 bp) were PCR amplified using the following primers: pMTP1RIL: 5'-CGGGATCC GAGCTCATGGACAGCCA TAACTCAGC-3' (*Bam*HI and *Sac*I sites are underlined), and pMTP1RIR: 5'-GGGGTACCCTAGT GCTGAGAC AGCAAACATGAG-3' (*Kpn*I and *Spe*I sites are underlined). PCR products were first cloned into pMD18-T vector (TaKaRa, Japan) and confirmed by sequencing. *OsMTP1* dsRNAi fragments were released by digesting with *Sac*I and *Spe*I and subcloned into pTCK303 (*Sac*I–*Spe*I sites) to generate the forward insertion. Further dsRNAi fragments were released by digesting with *Bam*HI and *Kpn*I and subcloned into the same pTCK303 (*Bam*HI–*Kpn*I sites) to generate the reverse insertion (Wang et al. 2004). The over-expression construct (*OsMTP1*/pCU1301) and dsRNAi construct (*OsMTP1*IRI/pTCK303) were introduced into *Agrobacterium* strain EHA105 and transformed into wild-type Zhonghua 11 following a standard procedure (Hiei et al. 1994).

Bacterial and yeast strains, media, and transformation

Escherichia coli strain JM109 was used for molecular cloning. The metal tolerance assay was performed on wild-type *Saccharomyces cerevisiae* strain BY4741, the Zn-sensitive *zrc1Δ* (Y00829) strain, the Zn and Co sensitive *cot1Δ* (Y01613) strain, the Cd-sensitive *ycf1Δ* (Y04069) strain, the Mn-sensitive *pmr1Δ* (Y04534) strain, the Ni-sensitive *smf1Δ* (Y06272) strain, and vacuole acidification mutant strains *vph2Δ* (Y04969). Yeast mutants were derived from the parental strain BY4741 and were all obtained from Euroscarf (<http://www.uni-frankfurt.de/>

[fb15/mikro/euroscarf/](http://www.uni-frankfurt.de/)). Growth was in yeast potato dextrose or in synthetic defined (SD) medium with 2% (w/v) galactose, supplemented as necessary.

The *OsMTP1*-coding region was inserted into the *Nco*I sites of pYES260 (from Euroscarf) behind the GAL1 promoter, yielding pYES260-*OsMTP1*. The plasmid was introduced into the yeast according to the method of Gietz and Schiestl (2007). Transformed cells were selected on solid SD medium (plus histidine, leucine, and methionine, without uracil) overnight and cultured in liquid SD medium overnight. The overnight cultures of transformed cells were each diluted to OD 1.0 at 600 nm. Diluted transformed cells (2 μl) were then spotted onto SD media plates with different heavy metals. For yeast growth curves, growth rates were measured according to OD 600 values.

RT-PCR and Northern blot analysis

Gene expression was first analyzed by semi-quantitative RT-PCR. Total RNAs were extracted from tissues using Trizol reagent (Invitrogen, USA) according to the instructions. Briefly, 5 μg total RNA was denatured at 65°C for 5 min followed by quick chilling on ice in a 14 μl reaction containing 1 μl oligo (dT)12–18 (50 μM) primer, and 1 μl of 40 μM dNTP mixture (10 mM each dATP, dGTP, dCTP, and dTTP at neutral pH). After addition of 4 μl 5× reaction buffer, the reaction was incubated at 37°C for 2 min, 1 μl (200 U) of M-MLV reverse transcriptase (Promega, Shanghai, China) was added to the reaction and incubated at 42°C for another 50 min. After terminating, the reaction was heated at 70°C for 15 min for inactivating. Thermocycling conditions were 94°C for 5 min followed by 30–35 cycles of 94°C for 1 min, 55–60°C for 30 s, and 72°C for 30 s. Due to the use of the *OsMTP1* 5'-terminal fragment (500 bp) to perform the dsRNAi, we detected the *OsMTP1* 3'-terminal (250 bp) to determine the efficiency of RNA-interfere with RT-PCR. The constitutively expressed *OsActin7* gene was used as internal control. The primers used were as follows: 5'-GGTCCTGATGGA GAGCACGC-3' and 5'-CTACTCGCGCTCAATCTGAA-3' for *OsMTP1*, 5'-CGTGACCTTACCGACAACCT-3' and 5'-GCACCTGAACCTTTCTGCTC-3' for *OsActin7*.

Northern blotting was performed to identify *OsMTP1* transcripts in transgenic rice and to test Cd-inducible *OsMTP1* expression in rice roots and shoots. Total RNA from transgenic plant leaves and 2-week-old Cd-treated rice roots and shoots were isolated with Trizol as described by the manufacturer (Invitrogen, USA) and blotted onto a nylon membrane (Millipore, USA). Digoxigenin (DIG)-labeled specific probes of the *OsMTP1* ORF were made using a PCR DIG probe synthesis kit (Roche, Germany) with primers pMTP1OEL and pMTP1OER. The membrane

was hybridized with the DIG-labeled probe for 16 h at 65°C in DIG Easy Hyb (Roche, Germany), then were washed twice at 37°C in 2× SSC and 0.1% (w/v) SDS for 10 min, followed by washing twice at 65°C in 0.2× SSC and 0.1% (w/v) SDS for 30 min. The membranes were then subjected to immunological detection following the manufacturer's instructions (Roche, Germany).

Construction of an *OsMTP1*–EGFP fusion expression vector and fluorescence microscopy

DNA fragments containing *OsMTP1* ORFs were obtained by PCR with forward primer pM1GL (5'-CGGGATCCA TGGACAGCCATAACTCAGC-3') and reverse primer pM1GR (5'-CGGGATCCTCGCGCTCAATCTGAATGG T-3') (*Bam*HI sites are underlined). PCR products were cloned into pMD18 T-vector, digested, and further cloned into pUC/EGFP made by ligating the 35S promoter from pB1m (Hua et al. 2004) and GFP from pB1101-EGFP into pUC18 in frame to produce the C-terminal EGFP-tagged fusion protein. After sequence confirmation, these vectors were bombarded into onion epidermal cells. After incubation at 22°C for 24 h, GFP fluorescence was visualized using confocal laser scanning microscope (LSM, 510 META, Zeiss, Germany). GFP excitation was 488 nm, and emission was detected between 500 and 575 nm.

mRNA in situ hybridization

Tissue sectioning was as described previously (Zhang et al. 2005). Briefly, rice vegetative and reproductive tissues were fixed in 4% (w/v) paraformaldehyde, dehydrated in an ethanol series, cleared with xylene, and embedded in paraffin. Paraffin-embedded tissues were cut into 8 µm-thick sections and then attached to glass microscope slides coated with polylysine hydrobromide. The 3' untranslated region (431 bp) of *OsMTP1* full-length cDNA was chosen as a template to generate DIG-labeled probe, which was PCR-amplified with full-length cDNA as template, and the primers were as follows: pM1RHL: 5'-CATCAGCCAT GTGACCATTC-3' and pM1RHR: 5'-CCATGCCAGTG CAGATTTAG-3'. PCR products were then cloned into pGEM-T vector (Promega, Shanghai, China) to generate recombinant plasmid pGOsM1.

DIG-labeled antisense probe was transcribed from *Sph*I-digested pGOsM1 using Sp6 RNA polymerase, and the sense RNA probe controls were transcribed from *Sac*I-digested pGOsM1 using T7 RNA polymerase. The hybridization mixture consisted of 50% (v/v) deionized formamide, 10% (w/v) dextran sulfate, 0.3 M NaCl, 10 mM Tris–HCl (pH 6.8), 10 mM NaH₂PO₄ (pH 6.8), 5 mM EDTA, 2.2 mM dithiothreitol, 0.6 µg/µl yeast

tRNA, 0.5 µg/µl yeast RNA, and about 500 ng denatured DIG-labeled RNA probe. About 100 µl mixture was mounted onto each slide and the slide was then covered with a clear coverslip. Hybridization was done at 65°C overnight. The slides were then washed twice in 2× SSC at room temperature, then in 1× SSC and 0.1× SSC at 55°C for 15 min each.

The DIG-labeled RNA was detected using anti-DIG alkaline phosphatase conjugate (anti-DIG-AP) according to the manufacturer (Roche, Germany). After the enzyme-catalyzed color reaction was done as previously described (Zhang et al. 2005), the slides were visualized with a microscope (AxioPlan 2 imaging, Zeiss). The hybridization signal was visible as an insoluble blue precipitate.

Measurement of heavy metal content in plants

Plant tissues were harvested, dried and desorbed in 10 ml HNO₃:H₂O₂ (4:1) at 100°C for 8 h. Then the HNO₃ in mixture was subsequently released through volatilization at 100°C for 24 h. The rest extraction was settled to the volume of 20 ml with 0.2% HNO₃. Zn, Cd, and Ni contents were analyzed by atomic absorption spectrophotometer (AAS, GBC3000).

Results

OsMTP1 is a member of rice CDF family

To isolate rice Cd-responsive genes, we constructed SSH libraries with Cd-treated or untreated rice roots. A cDNA fragment, designated as *OsMTP1*, was one of the Cd-induced genes in the SSH assay. *OsMTP1* cDNA (Os05g03780; Accession number AK100735) encodes a protein of 419 amino acids (45.57 kD). Analysis of the cDNA predicted sequence showed 69 and 78% identity with *AtMTP1* (Loci At2g46800; Mäser et al. 2001) at the nucleotide sequence and amino acid levels, respectively. On comparing with the rice genome sequence, it was found that *OsMTP1* was located on chromosome 5.

Previously, Montanini et al. (2007) identified 10 *MTP* genes from rice and 12 from *Arabidopsis* (Supplement data 1). The phylogenetic analysis of 22 *MTPs* in rice and *Arabidopsis* indicates that *MTPs* are grouped into three branches according to their amino acid sequence and possible ion-transporting specificity (Montanini et al. 2007). In rice, there are five Mn-CDFs, three Zn-CDFs, one Fe/Zn-CDF, and one unclassified CDF. Among these, *OsMTP1* has the highest homology with *AtMTP1*. *OsMTP1* functional domains including six putative TM domains and His-rich regions are conserved in all of these three members.

OsMTP1 is constitutively expressed and up-regulated by Cd

To understand the possible function of *OsMTP1* in rice, we examined its expression in different rice tissues by RT-PCR analysis (Fig. 1a). *OsMTP1* was expressed in various organs, including roots, leaves, stems and flowers. The highest level of *OsMTP1* transcript was detected in mature leaves and stems, which coincided with mRNA in situ hybridization results (Fig. 1b).

mRNA in situ hybridization of *OsMTP1* was performed using a DIG-labeled RNA antisense probe against *OsMTP1* cDNA 3'-untranslational region with control sense RNA. In rice leaves, *OsMTP1* mRNA accumulated specifically near vascular tissue (Fig. 1b, A–D), and longitudinal leaf sections showed that the signals were distributed in specific sieve vessel cell files (Fig. 1b, E). No positive reaction above background levels was detected on sections hybridized with sense RNA (Fig. 1b, F).

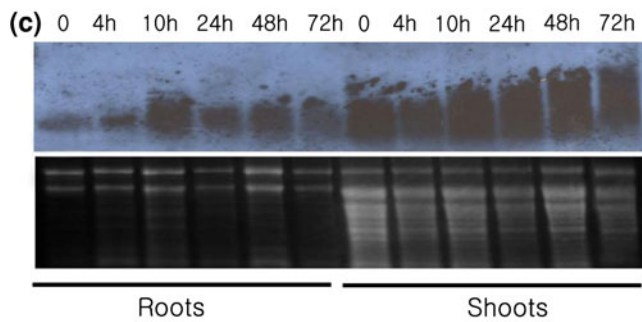
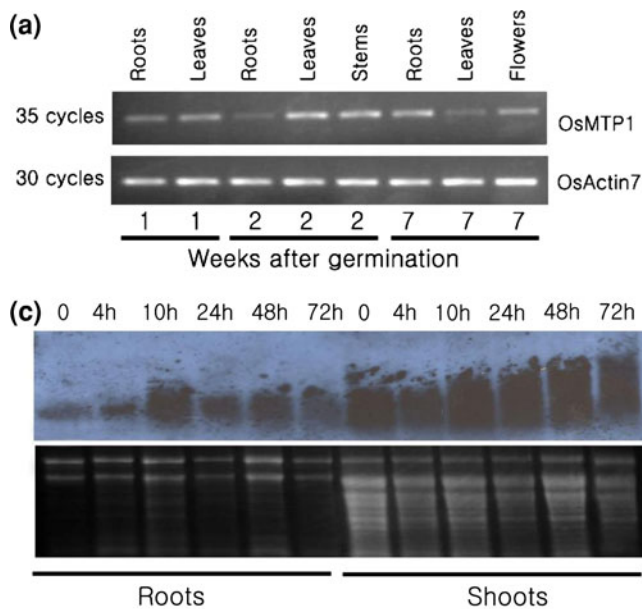
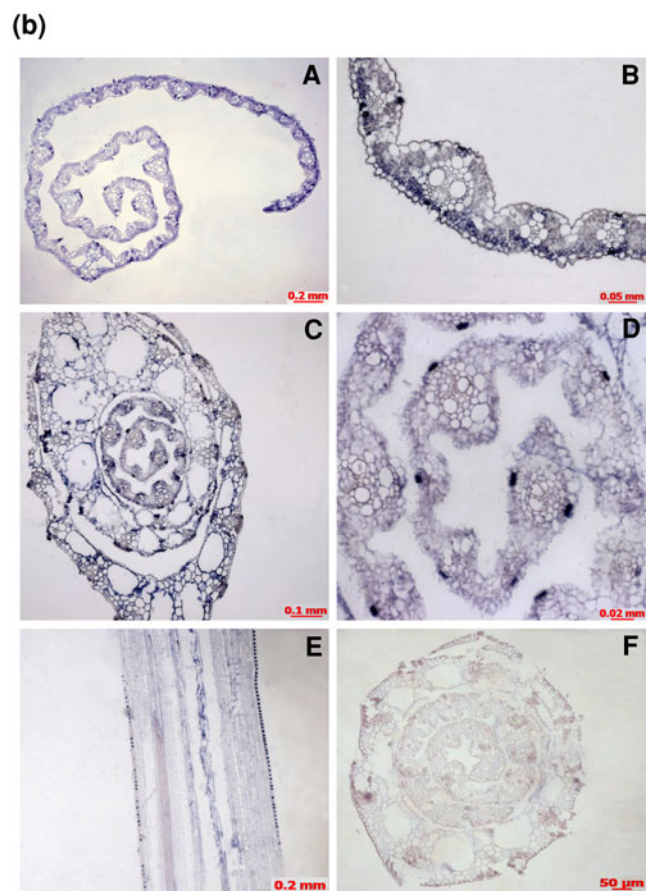


Fig. 1 *OsMTP1* expression patterns in wild-type Zhonghua 11 rice plants. **a** RT-PCR analysis of *OsMTP1* expression. The amplification of the rice *Actin7* gene was used as a control to show the approximately equal total RNA amounts used. **b** In situ hybridization of *OsMTP1* mRNA in rice leaves. Blue precipitates indicate positive *OsMTP1* signals. Transverse sections of a mature leaf (A; B shows

We investigated *OsMTP1* expression patterns following Cd treatment using RNA Northern blots (Fig. 1c). The 2-week-old rice seedlings were exposed to 100 μ M CdCl₂ and *OsMTP1* gene expression was assayed with the *OsMTP1* ORF DIG-labeled probe. *OsMTP1* expression was induced in roots and shoots after a 10-h CdCl₂ treatment.

Identification of *OsMTP1* over-expressing and *OsMTP1*-dsRNAi transgenic plants

To further elucidate the *OsMTP1* role in rice, we generated *OsMTP1* over-expression and dsRNAi (*OsMTP1*-RI) transgenic plants. The *OsMTP1* over-expression and dsRNAi transgenes were under the control of the maize (*Zea mays*) ubiquitin 1 (Ubi1) promoter (Fig. 2a). Transgenic plants were generated by introducing the constructs into the japonica rice variety Zhonghua 11 by *Agrobacterium tumefaciens*-mediated transformation (Hiei et al. 1994).



enlargement of A); seedling leaf bud (C; D shows enlargement of C); and longitudinal sections of a seedling leaf bud (E); control shows seedling leaf bud (F). **c** *OsMTP1* expression under Cd stress analyzed by Northern blots. *OsMTP1* expression increased in rice seedlings treated with 100 μ M CdCl₂ for different periods

Positive T1 lines segregating at or near 3:1 under hygromycin selection and GUS staining were selected for further identification.

To examine *OsMTP1* expression in these transgenic lines, total RNA was isolated and RT-PCR was performed using specific primers. Northern blot results showed that in over-expression transgenic rice, the *OsMTP1* transcript was highly expressed (Fig. 2b). In dsRNAi transgenic rice plants, the dsRNA (1.4 kb) could be detected (Fig. 2b) and

was smaller than *OsMTP1* transcripts. Endogenous *OsMTP1* expression was relatively low in wild-type rice plants and cannot be detected at the same exposure time. *OsMTP1* transcripts were further analyzed with semi-quantitative RT-PCR. As shown in Fig. 1a, *OsMTP1* transcript was barely detected in the wild-type seedlings, but was greatly increased in the over-expression lines. In comparison, *OsMTP1* transcript was not detectable in the dsRNAi lines, respectively (Fig. 2c).

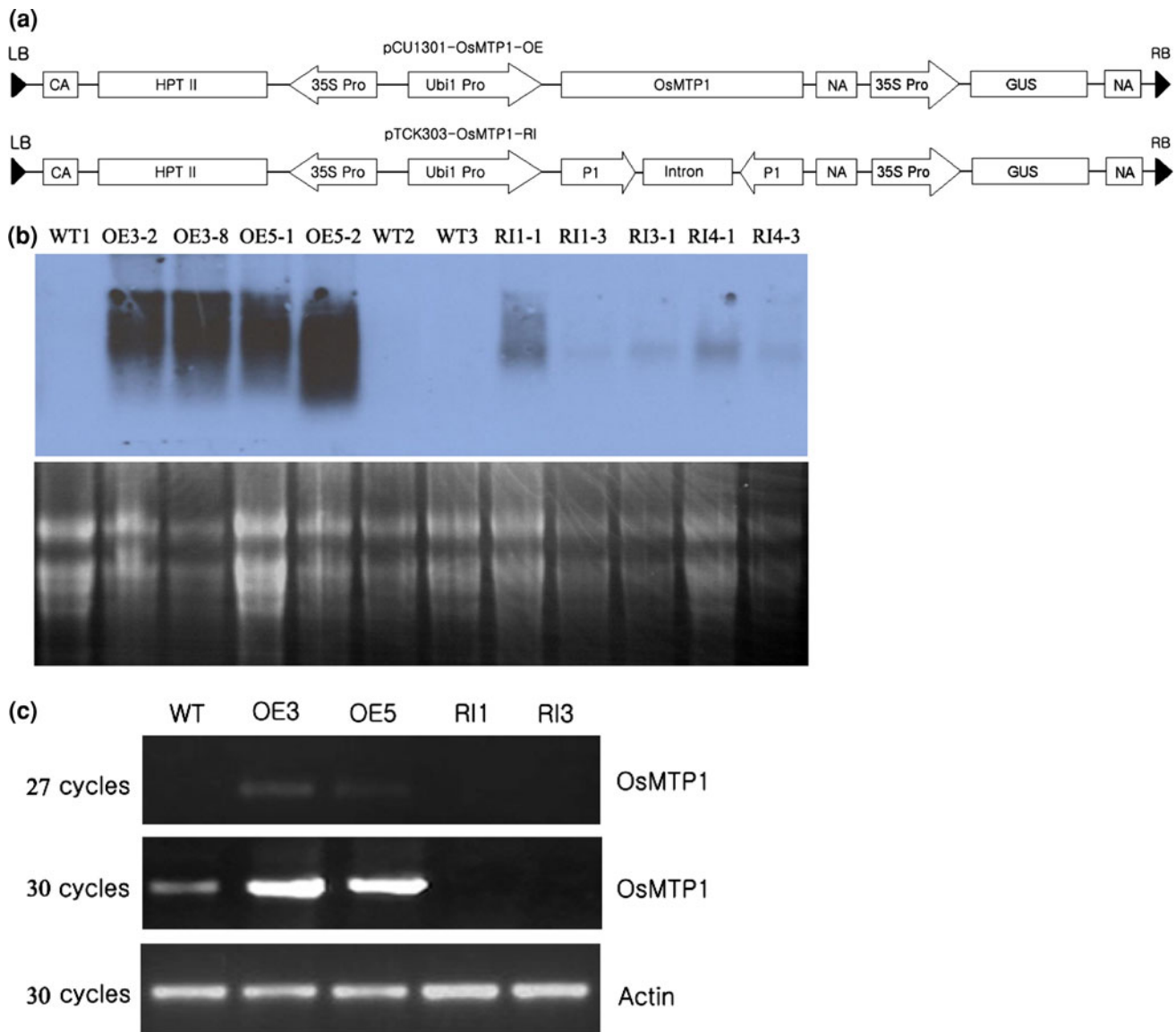


Fig. 2 *OsMTP1* expression patterns in transgenic rice. **a** Constructs used for rice transformation. The full-length cDNA encoding the *OsMTP1* gene (1,257 bp) was inserted into the *Bam*HI site of pCU1301 with sense orientation to construct the over-expression vector in rice. The 3' terminal partial *OsMTP1* cDNA (500 bp) was inserted into the *Sac*I/*Spe*I site (behind the intron) in antisense orientation and *Bam*HI/*Kpn*I site (before the intron) in sense orientation to construct the dsRNAi vector in rice. **b** Northern blot

analysis of *OsMTP1* transcript levels in transgenic rice. Total RNA extracted from rice leaves at the heading stage was probed with full-length *OsMTP1* cDNA. Loading of approximate amounts of RNA was confirmed by ethidium bromide staining. **c** RT-PCR analysis of *OsMTP1* expression in wild type, over-expression, and dsRNAi rice plants. Amplification of the rice *Actin7* gene was used as a control to show the approximately equal amounts of total RNA used in the RT-PCR analysis

OsMTP1 dsRNAi transgenic plants exhibit obviously decreased Zn, Cd, and Ni tolerance

We performed the transgenic assay in rice to identify *OsMTP1* biological roles and elucidate the relevance between *OsMTP1* transcripts and heavy metal resistance. Wild-type and transgenic T3 homologous seeds were chosen to detect Zn, Cd, and Ni tolerance. Over-expression or dsRNAi of *OsMTP1* both decreased Zn, Cd, and Ni tolerance in rice, particularly when grown for 2 weeks in solid MS medium supplemented with different concentrations to determine at what levels the aberrant phenotypes became apparent (Fig. 3a). Seedling length (including root and shoot) was measured to monitor heavy metal tolerance (Fig. 3b). Under heavy metal (Zn, Cd, and Ni) stress at different concentrations, down-expression of *OsMTP1* transgenic rice grew less than wild type, while the growth of over-expression of *OsMTP1* rice was slightly suppressed by metal stress. This result indicated that the abnormal expression level of *OsMTP1* may cause ionic disorder, and then affect the growth condition of rice transgenic plants.

OsMTP1 dsRNAi transgenic plants have changed Zn, Cd, and Ni content

We measured the Zn, Cd and Ni levels in mature dsRNAi transgenic rice plants and wild-type rice plants that had been exposed to low-concentration of heavy metals during the whole growth period. We found that the metal content in rice roots, leaves and seeds showed obvious disparities between dsRNAi transgenic rice plants and wild-type rice plants (Fig. 4a–c). In rice roots, the part of a crop which is immersed in nutrient fluid supplied with heavy metal, dsRNAi transgenic plants had higher level accumulation of Zn, Cd and Ni contents than wild-type plants, while in rice aerial parts (leaves, stems, and seeds), the heavy metal concentrations were different from root parts. As a whole, in leaves and stems, the Zn and Cd accumulation levels were higher in wild type than in dsRNAi transgenic rice plants, while Ni accumulation level was similar between wild type and dsRNAi transgenic rice plants. The same results were also detected in the rice seeds. Therefore, these results support that *OsMTP1* may be important in rice cation homeostasis mechanisms and down-regulation of *OsMTP1* by dsRNAi can affect the heavy metal accumulation in different rice organs.

Expression of *OsMTP1* in yeast confers tolerance to Ni, Zn, and Cd but not Mn

To characterize OsMTP1 specificity as a metal transporter, we performed the complementation assays in a series of yeast mutants, including *zrc1Δ*, *cot1Δ*, *smf1Δ*, *ycf1Δ*,

pmr1Δ, and *vph2Δ* (Fig. 5a). *OsMTP1* heterologous expression can recover some of these mutants' metal tolerance. *zrc1Δ* yeast mutant is a Zn-sensitive strain, in which *ZRC1* gene encoding transporter that sequesters Zn into the vacuole was deleted (Li and Kaplan 1998). *OsMTP1* did not complement the *zrc1Δ* Zn-sensitive phenotype. *cot1Δ* is 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, *OsMTP1* complemented the *cot1Δ* phenotype when grown on Zn but not Co. We further 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 Mn and Ca transport of Cd conjugates into the vacuole (Szczyпка et al. 1994; Li et al. 1997). PMR1 is a yeast secretory pathway pump responsible for high-affinity transport into the golgi and confers Mn tolerance by effectively removing Mn from the cytoplasm (Ton et al. 2002). SMF1 functions in Ni and Mn cellular accumulation, and the *smf1Δ* mutant is Ni sensitive (Supek et al. 1996). To our surprise, *OsMTP1* heterologous expression in *smf1Δ* can complement the Ni-sensitive phenotype, indicating that OsMTP1 can transport Ni in yeast. *OsMTP1* can also partially complement the Cd-sensitive phenotype of *ycf1Δ* mutant, but did not increase Mn tolerance in *pmr1Δ*. *OsMTP1* could not complement the Zn-sensitive phenotype of *vph2Δ*, which is unable to acidify the vacuole (Fig. 5a).

To further confirm the *OsMTP1*'s contribution to heavy metal tolerance in yeast, *OsMTP1* was heterologously expressed in yeast mutant strains *smf1Δ*, *cot1Δ*, and *ycf1Δ* and detected their Ni, Zn, and Cd tolerance (Fig. 5b). Yeast mutant strains transformed with empty vector pYES260 or pYES260-*OsMTP1* had the same growth rate without heavy metal stresses, while *OsMTP1* heterogonous expression in *smf1Δ*, *cot1Δ*, and *ycf1Δ* elevated their growth rate under 0.5 mM Ni, 5 mM Zn, and 0.1 mM Cd stress (Fig. 5b).

OsMTP1 is localized to the plasma membrane

To examine OsMTP1 localization, we fused *OsMTP1* ORF to the enhanced green fluorescent protein (EGFP) gene under 35S promoter control. The recombinant and empty vectors were bombarded into onion cells and confocal microscopy showed that OsMTP1-EGFP was efficiently targeted to the cell periphery, suggesting OsMTP1-EGFP plasma membrane localization (Fig. 6a), while EGFP was distributed throughout the cell including the cytoplasm and nucleus (Fig. 6c). Moreover, we also performed the bioinformatics analysis of OsMTP1's subcellular localization with three different programs (Supplement data 2). WoLF

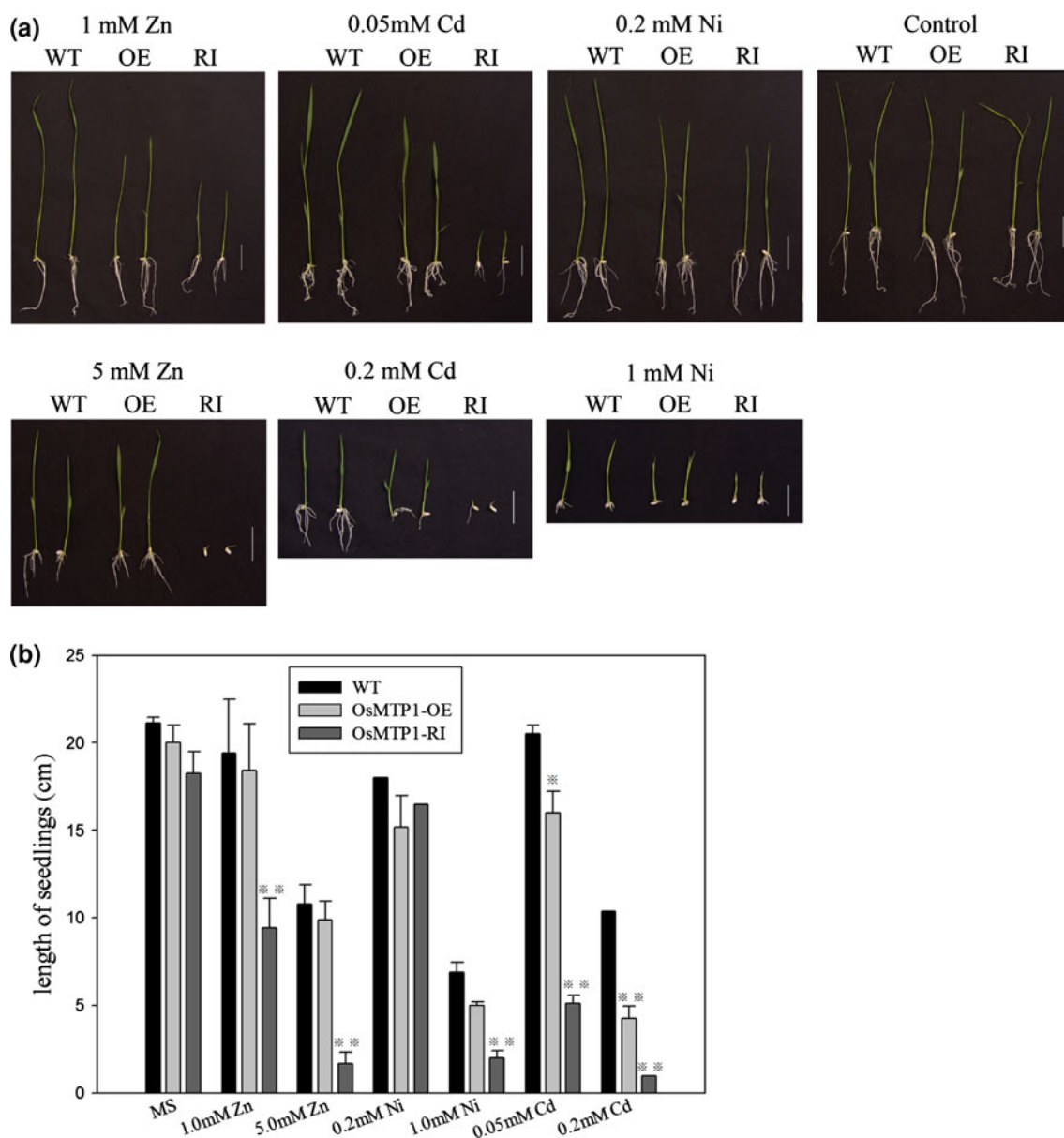


Fig. 3 Heavy metal tolerance test of *OsMTP1* over-expression and dsRNAi rice plants. **a** Phenotype comparison of wild type, over-expression, and dsRNAi rice plants grown under different heavy metal ion levels. Rice seedlings were grown for 12 days on solid phyto gel containing one-half-strength MS medium (control) or medium supplied with different Zn, Cd, and Ni concentrations.

Images show two plants each of wild-type control (left), *OsMTP1* over-expression (middle), and dsRNAi (right). White bars 3 cm. **b** Average rice seedling shoot and root length, averaged for 7–10 independent strains. Error bars represent the SE. * $P < 0.05$, and ** $P < 0.01$

PSORT predictions showed that *OsMTP1* localization at the plasma membrane, vacuolar membrane and endoplasmic reticulum, scored, respectively, 8.0, 3.0 and 3.0. This analysis showed that *OsMTP1* may mainly distribute at the plasma membrane, which is coincided with our EGFP fusion protein assay results. The Target P predicted a relatively high *OsMTP1* score value for a chloroplast transit peptide (0.675, cTP), and ARAMEMNON predicted *OsMTP1* distributed primarily in chloroplast with a high absolute value (8.9). These two above predictions indicated

that *OsMTP1* might be involved in chloroplast ion metabolism, while the correlative experimental evidence needs further investigation. We also performed the transmembrane domain prediction by TMHMM2; and the result showed that *OsMTP1*, had six transmembrane regions (Supplement data 3), could be a membrane-integrated protein. These predictions, together with our experimental results of *OsMTP1*–EGFP fusion protein subcellular localization, suggest that *OsMTP1* may be mainly plasma membrane localized, and probably less distributed in

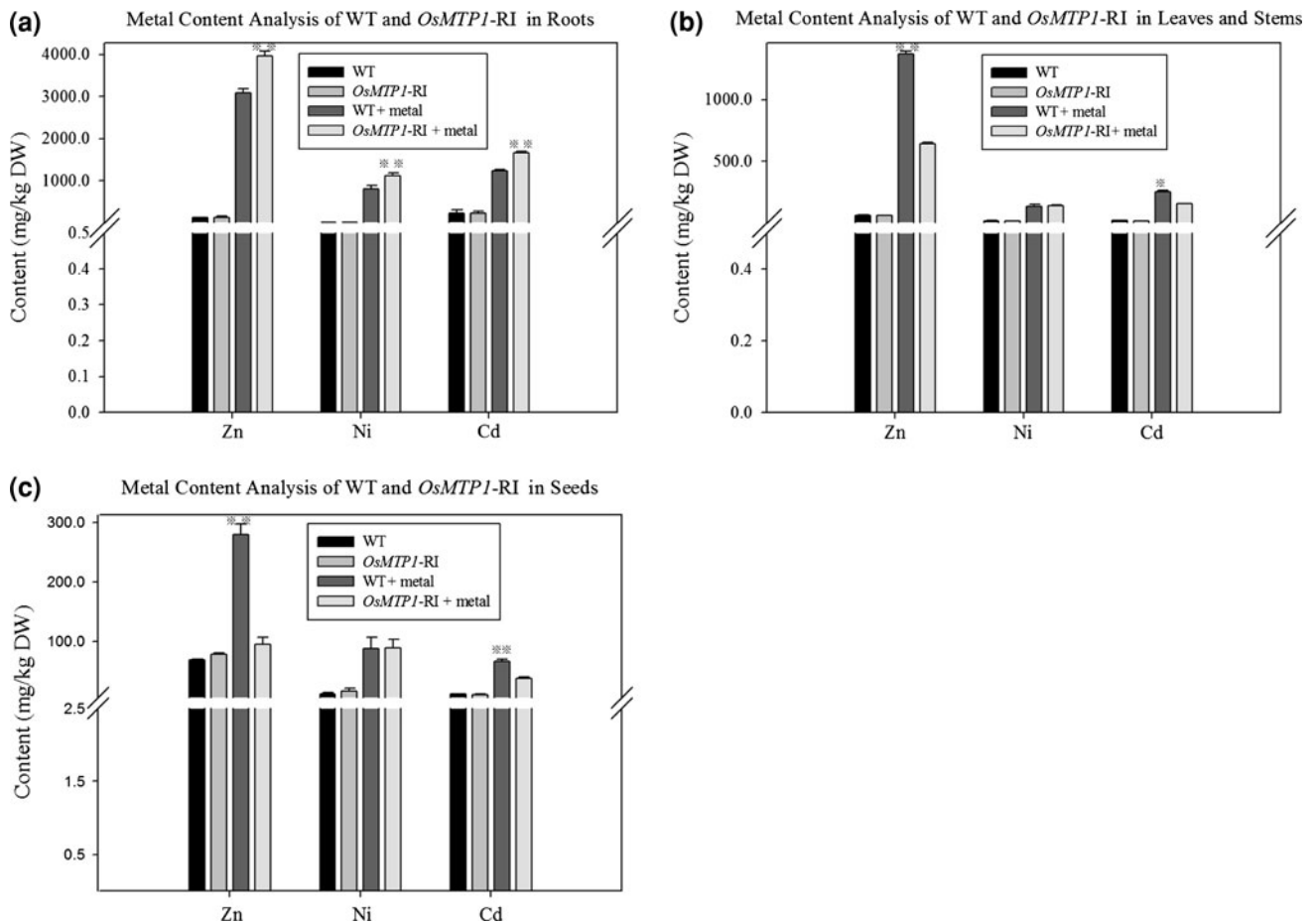


Fig. 4 Heavy metal content analyses of wild type and dsRNAi rice plants. Potted rice plants grown in hydroponics supplied with ZnSO_4 (0.2 mM), CdCl_2 (0.01 mM), and NiSO_4 (0.05 mM). After seed maturity, roots, leaves and stems, seeds were harvested for AAS (GBC3000) analyses. Mean values from four to six independent

experiments are shown. Error bars represent the SE. * $P < 0.05$, and ** $P < 0.01$. **a** Zn, Ni, and Cd accumulation in rice roots. **b** Zn, Ni, and Cd accumulation in rice leaves and stems. **c** Zn, Ni, and Cd accumulation in rice seeds

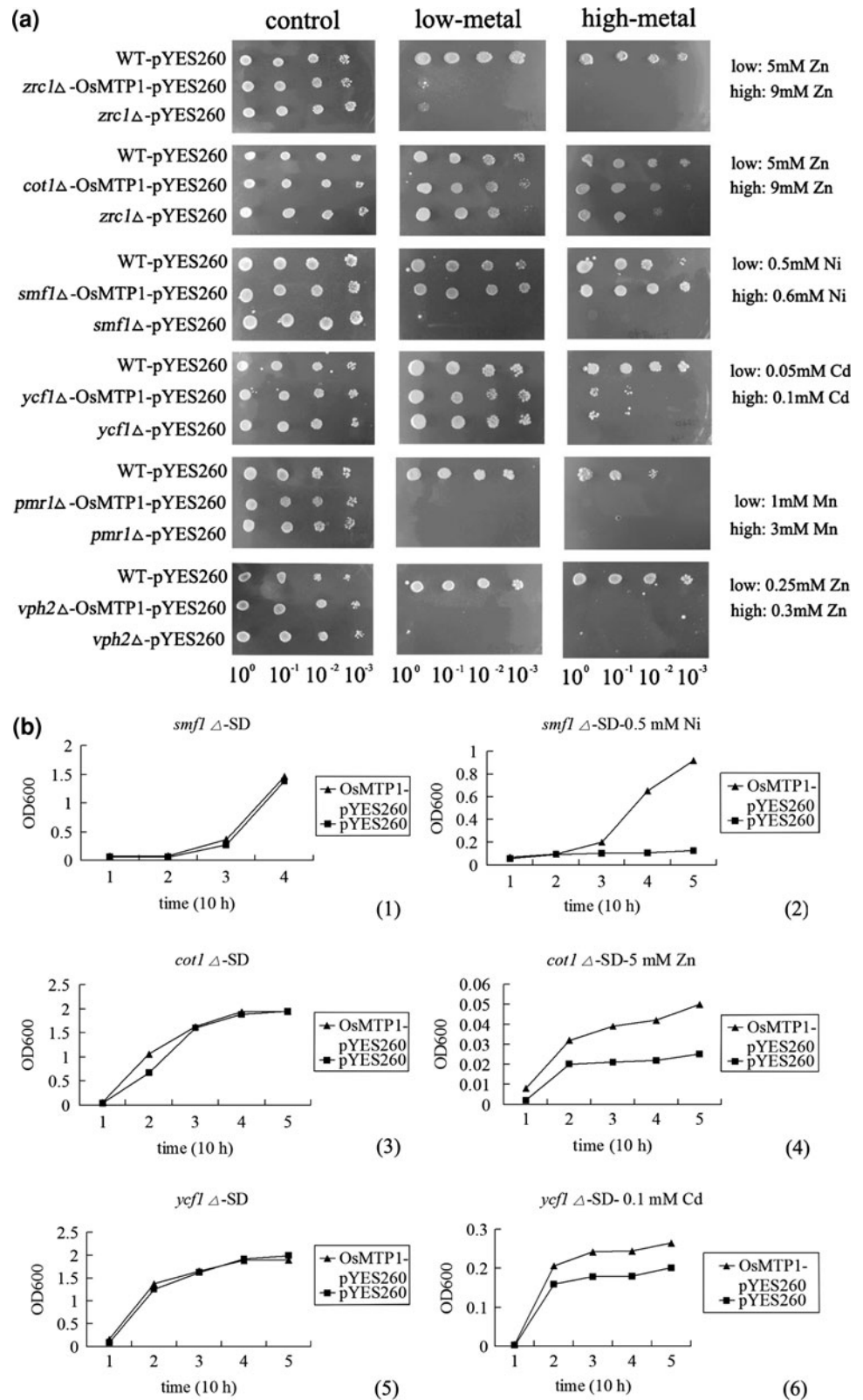
chloroplast and other organelle membrane, functioning in transmembrane transporting of metallic ions.

Discussion

Heavy metal pollution severely affected plant growth and development. As the most important cereal crop in China, rice and its growing space have been polluted by heavy metals, particularly Cd. Plants have developed a series of strategies to decrease metal ion toxicity, one of which is the cellular transport system. There are several groups of metal transport proteins identified in plants, including P_{IB} -type ATPases (Eren and Argüello 2004), CDF proteins, natural resistance-associated macrophage proteins (NRAMP), and Zn-regulated transporter-/Fe-regulated transporter-like proteins (ZIP, Mäser et al. 2001). CDF proteins were later renamed as MTPs in plants, and are now identified as a group of important metal transporting proteins.

Plant MTPs were first characterized in *Arabidopsis*, and are now cloned from a wide variety of plants species, including *Arabidopsis*, poplar, *T. goesingense*, *Brassica juncea*, and tobacco. Most MTPs have four to six putative transmembrane domains and a signature N-terminal amino acid sequence. A His-rich region between TMD four and five may be specifically involved in metal binding. Here, we described a Cd-induced gene, *OsMTP1* in rice. Bioinformatics analyses indicated that *OsMTP1* encodes a 45.57 kD membrane protein with six TMDs. Homologous analysis revealed that *OsMTP1* displayed many characteristic features of MTPs such as *AtMTP1* and *PtdMTP1* (Damien et al. 2003), implying a similar molecular mechanism and physiological function among them. In this study, RT-PCR analyses demonstrated that *OsMTP1* had relatively low expression, while Cd treatment slightly induced it in roots and shoots. These results suggest that *OsMTP1* responded to Cd stress.

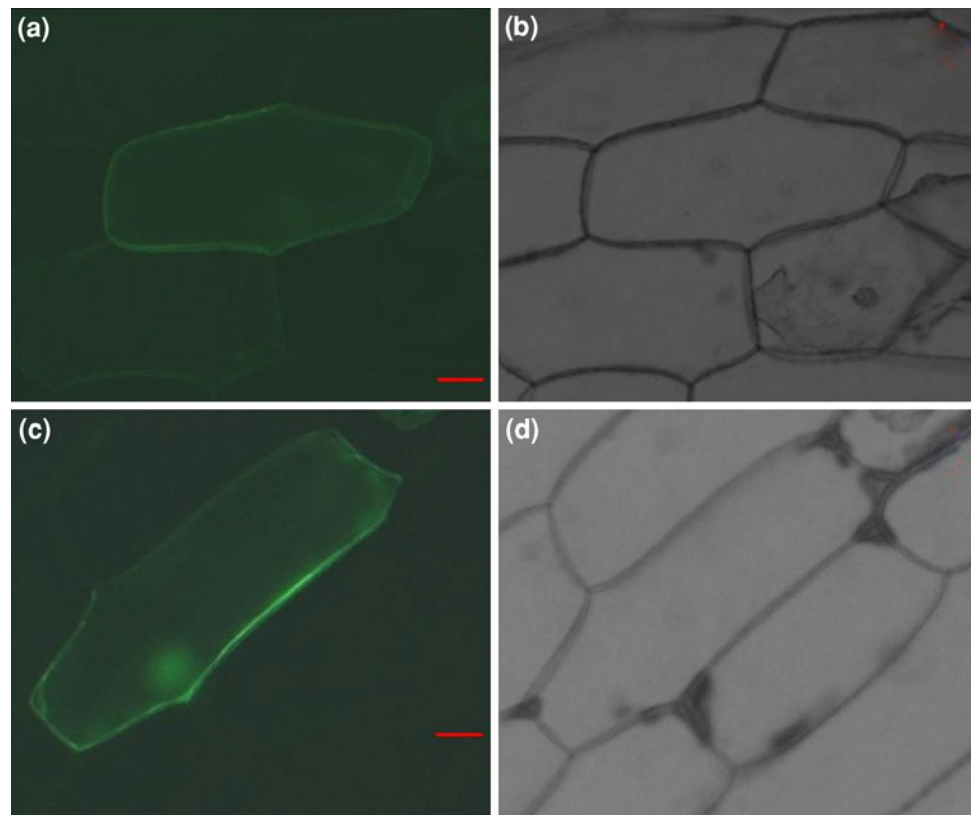
Fig. 5 Complementation of yeast mutants on selective media. **a** *S. cerevisiae* wild type (WT) and mutant strains were transformed with the empty vector pYES260 or with pYES260-OsMTP1. Yeast cultures were adjusted to OD₆₀₀ = 1.0, and 2 μ l serial dilutions (from left to right in each panel) were spotted on SD medium without (*control*) or with different heavy metal concentrations. Plates were incubated for 6 days to 2 weeks at 30°C. **b** *OsMTP1* confers Ni, Zn, and Cd tolerance when expressed in *smf1* Δ , *cot1* Δ , and *ycf1* Δ yeast mutants. Yeast mutant (*smf1* Δ , *cot1* Δ , and *ycf1* Δ), transformed with the empty vector pYES260 or pYES260-OsMTP1, were grown in liquid SD culture (*control*) or supplemented with Ni (0.5 mM), Zn (5 mM), and Cd (0.1 mM). The optical density at 600 nm (OD₆₀₀) of the cultures was determined after different periods



We also performed transgenic experiments in rice to analyze whether *OsMTP1* was involved in rice heavy metal tolerance. *OsMTP1* over-expression and dsRNAi transgenic rice

plants were generated. We found that *OsMTP1* dsRNAi transgenic rice seedlings had obviously decreased heavy metal tolerance, while the growth of *OsMTP1* over-expression

Fig. 6 Transient *OsMTP1*–EGFP expression in onion epidermal cells. **a** Confocal micrograph of *OsMTP1*–EGFP. **b** Light field image of **a**. **c** Confocal micrograph of EGFP. **d** Light field image of **c**. Scale bar 50 μ m



seedlings was suppressed slightly by heavy metal. These results indicated that both up- and down-regulated *OsMTP1* expression in rice may function in cation absorption and transferring balance, disrupting or unbalancing rice growth and development. The remaining challenge is to determine the specific contribution of *OsMTP1* in maintaining ionic equilibrium in rice plants.

In rice, *OsMTP* gene family includes ten *MTP* members. Among them, *OsMTP1* and other two *MTPs* (*OsMTP5* and *OsMTP12*) belong to the Zn-CDF subgroup (Montanini et al. 2007; Gustin et al. 2011). It is possible that these three Zn-CDF members are functionally redundant. At low-concentration Zn, Cd and Ni stresses, dsRNAi transgenic plants had higher heavy metal contents than wild type in rice roots, but had lower heavy metal contents than wild type in leaves and seeds. We speculated that in dsRNAi transgenic rice plants, due to the gene functional redundancy, down-regulation of *OsMTP1* could not affect the roots absorbing the heavy metals, but it could impair the translocation of heavy metal from rice roots to aerial parts. This indicated that *OsMTP1* might play crucial roles in heavy metal transcellular transport or translocation.

In the yeast mutant complementation assay, *OsMTP1* can totally suppress the Ni-sensitivity of the yeast *smf1* Δ mutant, and partially suppress the Zn, Cd sensitivity of the yeast *cot1* Δ and *ycf1* Δ . These data showed that *OsMTP1* demonstrated obvious metal-specificity, possibly preferentially

transporting Ni in yeast. While in rice plants, the metal-specificity was not obvious since the Zn, Cd and Ni content levels showed similar changed pattern in dsRNAi transgenic rice plants under low-concentration heavy metal stresses. Although the mechanism of metal selectivity has been discussed in previous reports (Damien et al. 2003; Montanini et al. 2007), and some *PtdMTP1* site-mutated assays were performed (Montanini et al. 2007), the mechanism remains unclear and needs further investigation.

In summary, *OsMTP1* contains all typical *MTP* domains and can complement the Ni-sensitive phenotype of yeast mutant *smf1* Δ . *OsMTP1* can also partially complement the Zn-sensitive phenotype of yeast mutant *cot1* Δ and the Cd-sensitive phenotype of yeast mutant *ycf1* Δ . These results indicate that *OsMTP1* can transport heavy metals and possessed cation-binding specificity. Although *OsMTP1* was expressed in leaves, stems, roots, and flowers, its expression was relatively low and regulated spatially and temporally during rice development and also showed differential response to Cd stress. Transgenic assays in rice showed that *OsMTP1* expression levels changed plant cation absorption and affected Zn, Ni, and Cd contents. Bioinformatic analyses indicated that *OsMTP1* is a membrane-integrated protein, and subcellular localization of *OsMTP1*/EGFP fusion protein showed that *OsMTP1* was mainly localized in plasmalemma. The identification and functional analysis of *OsMTP1* in rice should be helpful in

understanding its role in plant heavy metal transport. This is the first molecular identification of rice MTP and we propose that heavy metal (especially cadmium) transport through MTPs is indeed existent. Our observations may provide a key to the development of low-toxic heavy metal crops for food production, and are potentially of great importance for changed heavy metal remediation manners of other plants.

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