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Characterization of two genes encoding metal tolerance proteins from *Beta vulgaris* subspecies *maritima* that confers manganese tolerance in yeast

Isil Erbasol · Gonensin Ozan Bozdag · Ahmet Koc · Pai Pedas · Huseyin Caglar Karakaya

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Abstract Manganese (Mn^{2+}) is an essential micronutrient in plants. However increased Mn^{2+} levels are toxic to plant cells. Metal tolerance proteins (MTPs), member of cation diffusion facilitator protein (CDF) family, have important roles in metal homeostatis in different plant species and catalyse efflux of excess metal ions. In this study, we identified and characterized two *MTP* genes from *Beta vulgaris* spp. *maritima* (*B. v.* ssp. *maritima*). Overexpression of these two genes provided Mn tolerance in yeast cells. Sequence analyses displayed BmMTP10 and BmMTP11as members of the Mn-CDF family. Functional analyses of these proteins indicated that they are specific to Mn^{2+} with a role in reducing excess cellular Mn^{2+} levels when expressed in yeast. GFP-fusion constructs of both proteins localized to the Golgi apparatus as a punctuated pattern. Finally, Q-RT-PCR results showed that BmMTP10 expression was induced threefold in response to the excess Mn^{2+} treatment. On the other hand BmMTP11 expression was not affected in response to excess Mn^{2+} levels. Thus, our results suggest that the BmMTP10 and BmMTP11 proteins from *B. v.* ssp. *maritima* have non-redundant functions in terms of Mn^{2+} detoxification with a similar *in planta* localization and function as the *Arabidopsis* Mn-CDF homolog AtMTP11 and this conservation shows the evolutionary importance of these vesicular proteins in heavy metal homeostatis among plant species.

Keywords MTP · Heavy metal tolerance · Manganese · Yeast · Golgi · Plant

Abbreviations

B. v. ssp. *maritima*	*Beta vulgaris* subspecies *maritima*
MTP	Metal tolerance protein
Mn-CDF	Manganese cation diffusion facilitator
GFP	Green fluorescent protein
PMR1	Ca^{2+}/Mn^{2+} P-type ATPase

Introduction

As an essential micronutrient, manganese (Mn^{2+}) has important roles in many cellular events in plant cells by either activating enzymes such as phosphoenolpyruvate carboxykinase, malic enzyme, phenylalanine ammonia-lyase and isocitrate dehydrogenase, or as an essential constituent of protein complexes like manganese superoxide dismutase or the oxygen-evolving

I. Erbasol · G. O. Bozdag · A. Koc · H. C. Karakaya (✉)
Department of Molecular Biology and Genetics, Izmir Institute of Technology, 35430 Urla, Izmir, Turkey
e-mail: caglarkarakaya@iyte.edu.tr

P. Pedas
Plant and Soil Science Section, Department of Plant and Environmental Sciences, Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, Frederiksberg C, 1871 Copenhagen, Denmark

complex in photosystem II (Marschner 1995; Hänsch and Mendel 2009; Bowler et al. 1991; Kenten and Mann 1949).

Specific soil conditions such as acidity increases the Mn^{2+} availability and thereby stimulate Mn^{2+} absorption from roots (Maas et al. 1968) leading to accumulation of an excess level of Mn^{2+} that causes Mn^{2+} toxicity. Mn^{2+} toxicity diminishes carotenoid and chlorophyll contents (Clairmont et al. 1986) and reduces CO_2 assimilation in leaves (Gonzalez and Lynch 1997). Moreover, acute Mn^{2+} toxicity changes the structure of antioxidant enzymes and leads to increased reactive oxygen species levels that in turn damages cell membrane and reduces shoot and root growth (Xue et al. 1995). Thus free Mn^{2+} in plant cells should be kept in homeostatic control.

The cation diffusion facilitator (CDF) protein family is one of the heavy metal transporter families in both prokaryotic and eukaryotic cells (Guffanti et al. 2002; Li and Kaplan 2001; Peiter et al. 2007; Kambe et al. 2002). CDF proteins are characterized as divalent metal cation antiporters Me^{2+}/H^{+} (K^{+}) that provide Me^{2+} transport from cytosol to outside of the cell or to subcellular compartments, therefore they have a crucial role in heavy metal homeostasis (Guffanti et al. 2002; Kawachi et al. 2008). In plants CDF members have so far been localized to the Golgi apparatus and the vacuolar membrane (Delhaize et al. 2003; Peiter et al. 2007). They generally have six transmembrane (TM) domains and each of them carry a highly conserved signature sequences in their TMD II and TMD V that provides the metal selectivity (Montanini et al. 2007). Mn-MTP family is one of the subgroups of the CDF family that selectively transport Mn^{2+} (Montanini et al. 2007). Two studies with overexpression of Mn-MTP proteins resulted in increased tolerance toward excess Mn^{2+} levels (Peiter et al. 2007; Delhaize et al. 2003, 2007). ShMTP8 from *Stylosanthes hamata* provides Mn^{2+} vacuolar sequestration (Delhaize et al. 2003), whereas AtMTP11 and PtMTP11 from *Arabidopsis thalinana* and *Populus trichocarpa* catalyse efflux of excess Mn^{2+} via exocytosis of secretory vesicles from the Golgi apparatus (Peiter et al. 2007).

In this study, we show that two genes, *BmMTP10* and *BmMTP11* [*Beta vulgaris* spp. *maritima* (*B. v.* ssp. *maritima*) metal tolerance proteins (MTPs) 10 and 11], isolated from a cDNA library of *B. v.* ssp. *maritima* confer Mn^{2+} tolerance when expressed in yeast (*Saccharomyces cerevisiae*), putatively via vesicular trafficking and exocytosis from trans-Golgi. However, the transcript expression pattern indicated that the main role of these two *BmMTP* genes are related to supplying Mn^{2+} to Mn-dependent enzymes in the Golgi apparatus. In addition, this study shows remarkable similarities with other members of Mn-CDF family proteins (Delhaize et al. 2003, 2007; Peiter et al. 2007).

Materials and methods

Yeast strains, growth conditions, and cDNA library screening

Isogenic haploid deletion mutants: *YGL167C* (Δ*pmr1*), *YOR316C* (Δ*cot1*), *YDR135C* (Δ*ycf1*), and *YMR243C* (Δzrc1) of the wild type yeast (*Saccharomyces cerevisiae*) strain BY4741 (*MATa; his3; leu2; met15; ura3*) were obtained from the yeast deletion library (Invitrogen). YPD media (2 % glucose, 2 % peptone, 1 % yeast extract, and 2 % agar for solid media) and SD media (2 % glucose, 0.7 % yeast nitrogen base w/o amino acids, and 0.3 % of appropriate amino acids except the uracil marker) were used for yeast growth.

We constructed *B. v.* ssp. *maritima* cDNA library, inserted in the pAG426GPD vector, from Mn^{2+} treated plants. The yeast mutant, Δ*pmr1*, transformed with this cDNA library, was initially screened using SD plates supplied with 2.7 mM $MnCl_2$. Two Mn tolerant colonies were recovered from the initial screen, transformed cDNA were sequenced using vector based primers, and named as *BmMTP10* and *BmMTP11*. Then metal tolerance assays were performed using the individual yeast mutants transformed with either *BmMTP10* or *BmMTP11* cDNA or with its corresponding empty vector (pAG426GPD) control. The lithium acetate method was used for all yeast transformations (Burke et al. 1994).

For complementation assays, overnight cultures (180 rpm, at 30 °C) were diluted to the optical density (OD) of 0.2; 0.02; 0.002; 0.0002 at 600 nm using distilled sterile water and 5 µl of these cultures were spotted on solid SD-media with metal concentrations as 8 mM Mn^{2+}, 1 mM Co^{2+}, 150 µM Cd^{2+}, 1.8 mM Ni^{2+}, 15 mM Zn^{2+}, or without metals as controls. Yeast cells were incubated 5 days at 30 °C and photographed.

Sequence and phylogenetic analyses

BmMTP10 and *BmMTP11* homologous sequences were searched on BLASTP service of NCBI. Deduced amino acid sequences homologous to BmMTP10 and BmMTP11 were retrieved from GenBank and PopGenIE. These amino sequences were from dicotyledonous plants: *A. thaliana*, *Populus trichocarpa*, *Stylosanthes hamata*. Multiple sequence alignment was performed in ClustalW2, and conserved amino acids were highlighted and represented with logo with respect to Blosum 62 score by JALVIEW 2.7. BmMTP10 and BmMTP11 TM domains were identified using SOSUI web server. Molecular evolutionary analysis was performed in MEGA 5. Neighbour-joining (NJ) method was used for phylogenetic analysis and bootstrap calculations (1,000 replicatives). Sequence distances were calculated with Poisson correction model with uniform substitution rates among sites.

Determination of intracellular Mn^{2+} levels

Intracellular Mn^{2+} concentrations of *Δpmr1* yeast cells overexpressing *BmMTP10* and *BmMTP11* or containing empty vector (*pAG426GPD*) were measured using the method explained by (Mizuno et al. 2005). Overnight yeast cultures were diluted as 1/1,000 and 1 mM final concentration of $MnCl_2$ were added into the cultures. The cells expressing *BmMTP10* and *BmMTP11* genes were grown for 48 h, and the control cells (with empty vector) were grown for 60 h. Cell pellet were washed 3 times with 10 mM EDTA, dried for overnight at 70 °C. 5 mg of yeast cells were extracted by 65 % HNO_3 and filtrated. Intracellular Mn^{2+} contents (four replicates each) were quantified at ug/L (ppb) level using Agilent 7500ce inductively coupled plasma mass spectrometry (Agilent Technologies).

BmMTP10 and BmMTP11 localization

Construction of GFP fusions for expression in yeast cells

A PCR-based cloning strategy was used to generate *BmMTP10* and *BmMTP11* DNA with a mutated stop codon for C-terminal fusion to the *GFP* gene. A construct of the *pYES2-GFP* vector was kindly provided by Dr. Josefine Nymark Hegelund (University of Copenhagen) (Hegelund et al. 2010). To obtain *pYES2-BmMTP10-GFP* and *pYES2-BmMTP11-GFP* the coding sequences of *BmMTP10* and *BmMTP11* w/o stop codon were inserted in *Kpn1*/*XbaI* sites in the pYES2-GFP vector. The primers used were *BmMTP 10* (*BmMTP10F5′*-GGAGGTACCATGGAAAACA TAACTGAAAC-3′ and *BmMTP10R5′*-GGCTCTA GACCAAACCTTTGCTTTGTGCTC-3′) and *BmMTP 11* (*BmMTP11F5′*-CCGGGTACCATGCTGGAGGG TTTCAGTG-3′ and *BmMTP11R5′*-GGCTCTAGAC CATAGATGGGCTTGCGCATGC-3′). The PCR products were amplified using LA Taq (Takara Bio Inc.) and digested overnight with *Kpn1* and *Xba1*. The digested PCR products were ligated into the *pYES2-GFP* vector followed by sequencing.

Preparation of yeast cells for confocal microscopy

The yeast strain InvSc1 (Invitrogen) was used for expression of the GFP-fusion constructs. Yeast transformants were grown on selective SD medium (2 % glucose, 0.7 % yeast nitrogen base without amino acids, and 0.2 % yeast synthetic drop-out supplement without uracil). BmMTP10-GFP and BmMTP11-GFP expression were induced for 5 h in 2 % galactose based medium. The cells were fixated on polysine slides (Thermo Scientific), and the transformed cells were visualized using a Leica TCS SP2/MP confocal laser scanning microscope (Leica Microsystems). Excitation for GFP was 488 nm, and emission was detected between 500 and 540 nm.

Plant material, growth conditions, and sampling

The species of sea beet *B. v.* ssp. *maritima TR 51196*, kindly provided by Dr. Ayfer Tan, Aegean Agricultural Research Institute Menemen Izmir (AARI), were used in this study. Seeds were germinated in autoclaved peat medium. After 7 days germination, seedlings were taken into aerated half-strength Hoagland solution at 25 °C with 12/12 h light/dark cycle (50 % relative humidity and 400 μmol m^{-2} s^{-1} light intensity). 2 mM Mn^{2+} (in Hoagland solution) was applied to the plants for 12 h. Root and leaf tissue of the control group were harvested just before manganese treatment. For Mn^{2+} treated plants, root and leaf tissue were harvested at every third hour for 12 h of total

treatment period to quantify the gene expression pattern.

BmMTP10 and *BmMTP11* expression analysis

Total RNA was isolated using RNA Isolation Kit (Invitrogen). Traces of genomic DNA were removed by DNase treatment (Fermentas). cDNAs were synthesized following instructions of cDNA Synthesis Kit manual (Fermentas). Three independent quantitative gene expression analyses were performed using MaximaSYBR Green qPCR Master Mix (Fermentas) with the IQ5 real-time PCR cycler system (Bio-Rad). *Beta actin* was used as a reference gene. Primers for *Beta vulgaris actin* (*BvACT1RTF*: 5′-AGACCT TCAATGTGCCTGCT-3′ and *BvACT1RTR* 5′-TCAG TGAGATCACGACCAGC-3′; product 187 bp), *B. v.* ssp. *maritima MTP 10* (*BmMTP10*RTF 5′-ACGAG CATACACTTTCGGTTCCCA-3′and *BmMTP10*RTR 5′-ACTTCCTGGAGTTGCTCGAGCTTT-3′; product 129 bp) and *B. v.* ssp. *maritima MTP 11* (*BmMTP 11*RTF 5′-AAGGCTGTACAAGTGTGGGATTCG-3′ and *BmMTP11*RTR 5′-AGTGGATCCAGACCA CAGCTCAAA-3′; product 140 bp). *Beta actin* primers were designed on *Beta vulgaris actin-1* (*BvACT1*) cDNA sequence that is homologous to *Arabidopsis actin-11* gene (*AtACT11*). Gene expressions levels were normalized with respect to *BvACT1* expression levels. PCR conditions were as follows: Initial denaturation at 95 °C for 10 min, and then 95 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s for 40 cycles. Q-RT-PCR data were calculated according to the Pfaffl's model. Gene expression levels of Mn^{2+} treated samples were calculated relative to the non-treated control.

Accession numbers

The GenBank accession numbers of the mentioned sequences are: *A. thaliana: AtMTP10*, NP_173081.2; *AtMTP11*, NP_181477.1; Arabidopsis actin-11 (*AtACT11*), NP_187818; *B. v.* spp. *maritima: BmMTP10*, AEP40484.1; *BmMTP11*, AEP40483.1; *Beta vulgaris*: Beta vulgaris actin (*BvACT1*), DQ866829.1; *Stylosanthes hamata*: ShMTP8, AY181256; ShMTP9, AY 181257; ShMTP10, AY181258; *ShMTP11*, AY181259. The PopGenIE accession numbers of the genes mentioned in this article are: *Populus trichocarpa*:PtrMTP 10, FGENESH4_PM.C_LG_X000667; PtrMTP11.1, ESTEXT_FGENESH4_PM.C_LG_X0839; PtrMTP11.2, EUGENE3.00080435.

Results

Identification of *BmMTP10* and *BmMTP11*as manganese tolerance genes

In order to identify Mn^{2+} tolerance genes from *B. v.* ssp. *maritima*, we screened a *B. v.* ssp. *maritima* cDNA library in the Mn^{2+}sensitive yeast strain Δ*pmr1*. PMR1 is a member of the P-type ATPase family shown to be involved in loading of Mn^{2+} and Ca^{2+} into the Golgi apparatus (Lapinskas et al. 1995; Mandal et al. 2000). Yeast cells lacking PMR1 are therefore sensitive to high Mn^{2+} concentrations in the growth medium. From the yeast screen only two colonies were able grow in toxic levels of Mn^{2+} and the corresponding plasmids were isolated and used to confirm the Mn^{2+} tolerance phenotype in second transformation assays. Confirmation assays showed that these two genes could confer Mn^{2+} tolerance to the yeast cells up to 8 mM Mn^{2+} levels (Fig. 1).

After sequencing and evolutionary analyses, we named the genes of isolated two cDNAs as as *BmMTP10* and *BmMTP11* with respect to their homologous genes; *AtMTP10* and *AtMTP11* from *A. thaliana*.

Sequencing analysis indicated that open reading frame of *BmMTP10* is comprised of 948 nucleotides and the translated protein sequence contains 316 amino acids. The corresponding numbers for *BmMTP10* are 972 nucleotides and 324 amino acids, respectively.

We utilized BLASTP service for the BmMTP10 and BmMTP11 proteins to search for similar functional relatives. Close relatives of BmMTP10 and BmMTP11 were compared in terms of an amino acid alignment. According to the multiple alignment analysis, BmMTP10 and BmMTP11 have conversed amino acid domains those are also found in the members of CDFs family proteins (Fig. 2). In addition, the amino acid sequences of both BmMTP10 and BmMTP11 include the DXXXD signature sequence that is only found in the members of Mn-CDF family (Montanini et al. 2007). BmMTP10 showed high sequence homology with PtrMTP10, ShMTP9 and

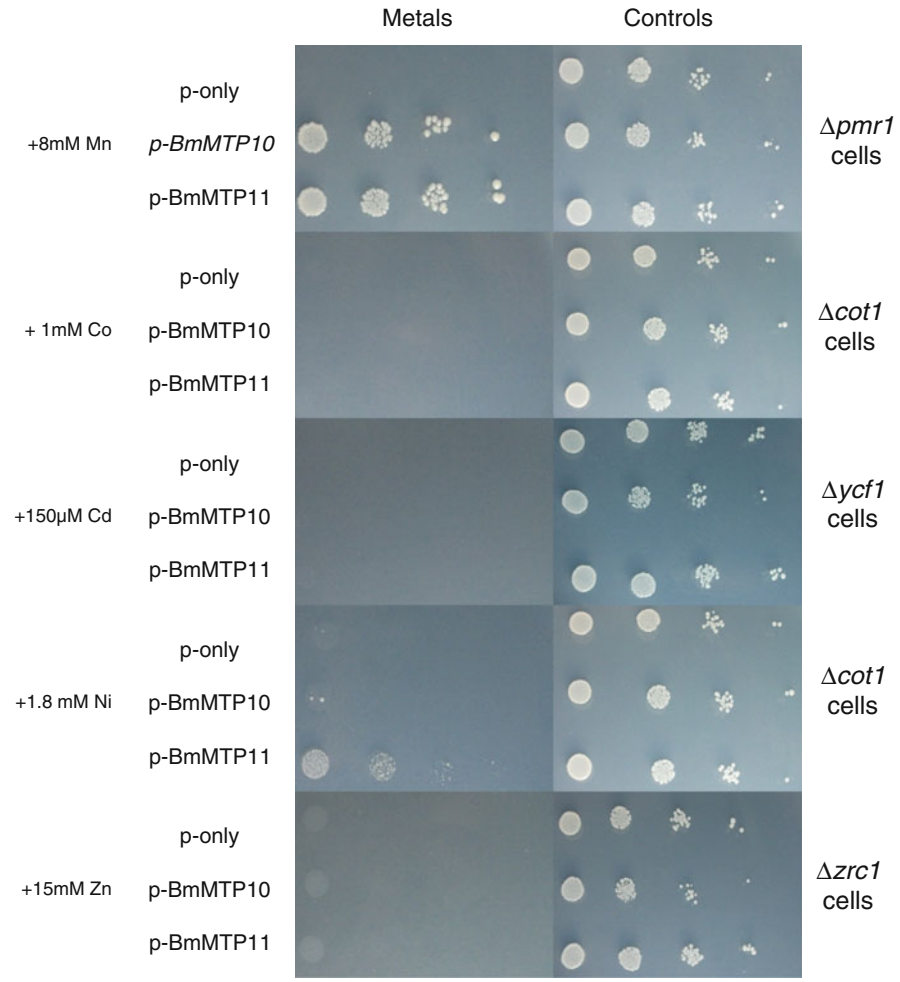

Fig. 1 Overexpression of *BmMTP10* and *BmMTP11* confer manganese resistance to *Δpmr1* cells. Four serial dilutions (0.2, 0.02, 0.002, and 0.0002 OD_{600}) of yeast cells transformed with an empty vector (p-only) or p-*BmMTP10* or p-*BmMTP11* were spotted on SD media supplemented with excess metal ions as specified. The plates were incubated at 30 °C for 5 days and photographed

AtMTP10. In addition, BmMTP11 revealed high sequence homology with PtrMTP11.1 and AtMTP11. There is 67 % similarity between BmMTP10 and BmMTP11. A multiple sequence alignment including 12 different amino acid sequences was used for NJ phylogenetic analysis (Fig. 3). Based on the evolutionary distances from BmMTP10 and BmMTP11, two major clusters were identified.

BmMTP10 and BmMTP11 proteins have high Mn^{2+} specificity

In order to understand the metal substrate selectivity of BmMTP10 and BmMTP11, *BmMTP10* and *BmMTP11* were tested whether they are able to provide metal tolerance for other divalent cations. For the analysis, the growth results of different yeast mutant strains, expressing *BmMTP10* and *BmMTP11* under the control of a constitutive promoter, were compared with the growth results of the corresponding empty-vector *pAG426GPD* transformed cells.

As seen in Fig. 1, while these two genes were able to rescue the Mn^{2+}-sensitive phenotype very efficiently, they were not able to tolerate toxic Co^{2+}, Cd^{2+}, and Zn^{2+}. Only the BmMTP11 protein showed a partial rescue for tolerating toxic nickel (Ni) (1.8 mM) (Fig. 1). These results indicate that both transporters have high specificity for Mn^{2+}.

Expression of *BmMTP10* and *BmMTP11* lower the yeast intracellular Mn^{2+} levels

In literature, there are examples of metal detoxifying membrane proteins functioning via reducing the intracellular metal levels (Mizuno et al. 2005). Since both BmMTP10 and BmMTP11 proteins contain

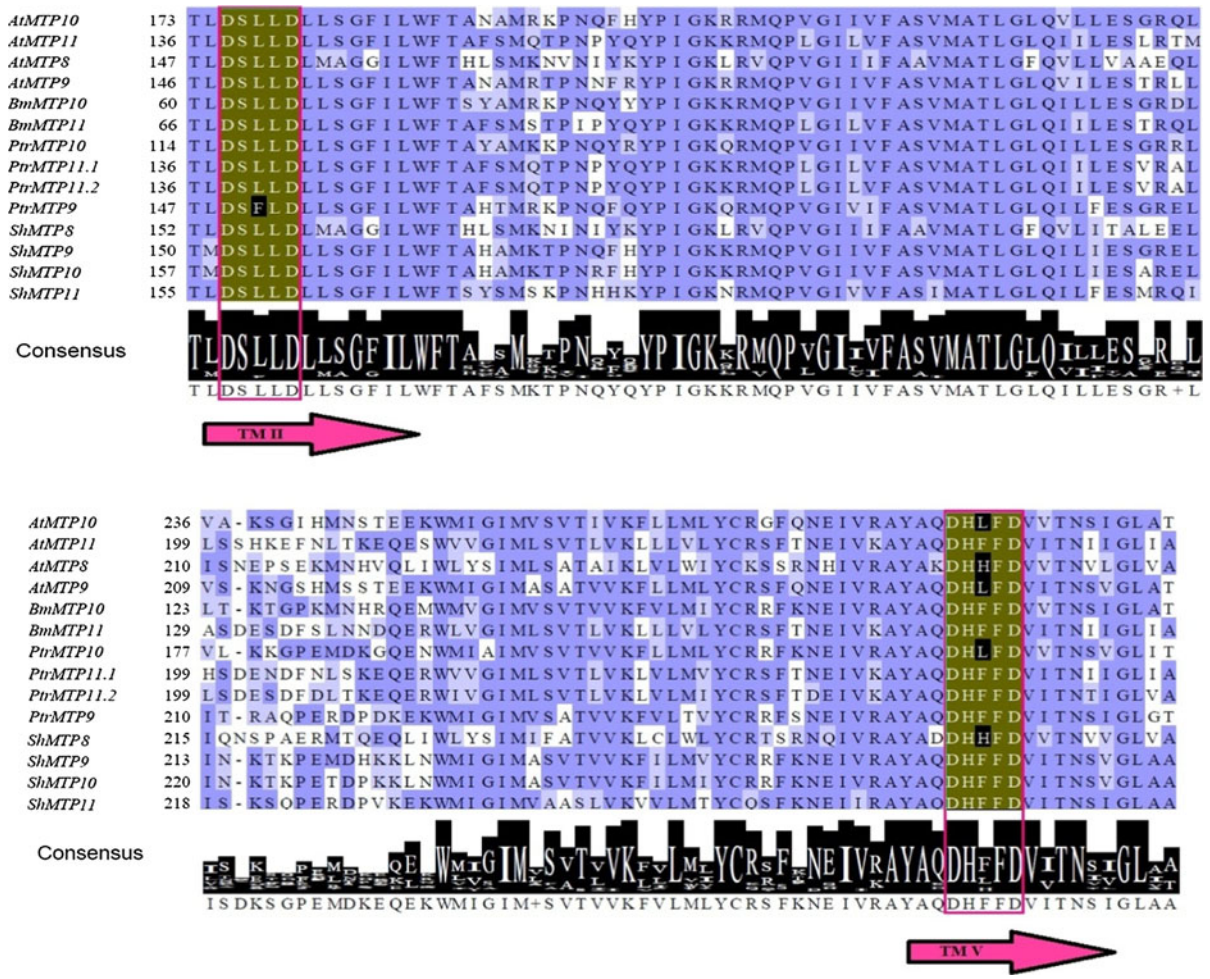

Fig. 2 Conserved signature sequence; DXXXD of Mn-MTP proteins (Montanini et al. 2007); including the deduced BmMTP10 and BmMTP11. *Same colors* on columns indicate conserved amino acids between BmMTP10, BmMTP11 and other MTP-proteins. Conserved amino acid sequences were *highlighted* and represented with *logo* with respect to Blosum 62 score with JALVIEW 2.7. TM II and TM V domains were marked, respectively. Predicted TM domains were identified using SOSUI web server. (Color figure online)

predicted TM domains, we investigated whether these two proteins function as efflux transporters. Therefore, we analyzed intracellular Mn^{2+} levels of cells expressing *BmMTP10*, *BmMTP11* or the empty vector by ICP-MS.

As seen in Fig. 4, Δ*pmr1* cells transformed with the pAG426GPD empty plasmid resulted in a significantly higher Mn^{2+} accumulation compared with cells transformed with the *BmMTP10* and *BmMTP11* cDNA′s, respectively. Expression of *BmMTP10* and *BmMTP11* in Δ*pmr1* cells decreased the Mn^{2+} levels to 55 and 56 % of the level of their corresponding empty-vector transformed cells, respectively. These results suggest that expression of both *BmMTP10* and *BmMTP11* lower the intracellular Mn^{2+} level.

BmMTP10 and BmMTP11 localizations

BmMTP10 and BmMTP11 are predicted membrane proteins with five putative TM domains. We therefore wanted to determine their cellular membrane localizations. C-terminal regions of both genes were tagged with GFP for localization experiments. The microscopy analysis for C-terminal GFP-fusion constructs of *BmMTP10* and *BmMTP11* expressed in yeast cells resulted in a punctuated pattern, similar to localizations

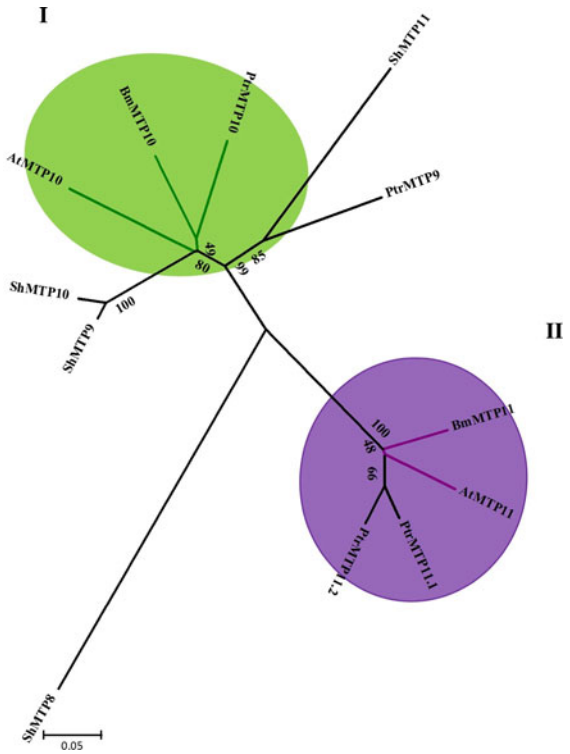

Fig. 3 Phylogenetic tree of the Mn-MTP proteins. The neighbor-joining phylogenetic tree based on the multiple amino acid sequence alignment was built with MEGA 5. Clusters are represented with the colours; *green* and *purple* with respect to nearest distance from BmMTP10 and BmMTP11 and proteins most closest to BmMTP10 or BmMTP11 are marked with *same colored branch line*. The *scale bar* indicates an evolutionary distance of 0.05 amino acid substitution per site. (Color figure online)

obtained with the yeast Golgi localized proteins Pmr1p (Mandal et al. 2003) and Gyp1p (Du and Novick 2001). These results showed that BmMTP10 and BmMTP11 have a localization pattern resembling the Golgi apparatus (Fig. 5).

Expression analysis of BmMTP10 and BmMTP11 in response to manganese stress

In some studies, abiotic stress tolerance genes were shown to be induced at the transcriptional level (Nakashima et al. 2007; Sunkar et al. 2003). Within this context we analyzed whether *BmMTP10* and *BmMTP11* transcript levels were induced when *B. v.* ssp. *maritima* plants were exposed to a 2 mM Mn^{2+} treatment. A Q-RT-PCR approach was used to analyze transcriptional responses in root and leaf tissues.

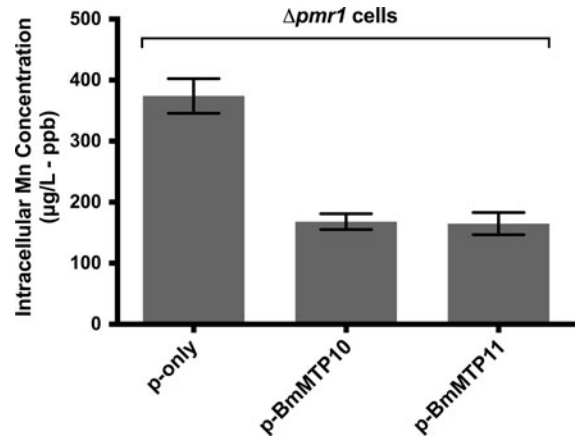

Fig. 4 Intracellular manganese concentrations in the Δ*pmr1* yeast cells that were grown in 1 mM Mn^{2+} supplemented SD media for 48 h (for p-*BmMTP10* or p-*BmMTP11* cells) or 60 h (for p-only cells) at 30 °C. Cells were transformed with either empty vector (p-only) as control, or p-*BmMTP10* or p-*BmMTP11* overexpression vectors. *Bars* represent mean ± SEM ($n = 4$) manganese concentrations

BmMTP10 transcript levels in roots and leaves showed a dynamic regulatory pattern after exposing the plants to 2 mM Mn^{2+}. Expression levels of *BmMTP10* in root cells (Fig. 6a) were induced up to twofold after 9 h of treatment. In leaves, it was induced approximately threefold after three hours and remains stable (Fig. 6b). In contrast, there were no significant changes in the expression level of *BmMTP11* in both root or leaf tissues as shown in Fig. 6c, d.

Discussion

There are three identified metal tolerance mechanisms that depend on lowering of the high metal concentrations in cells. First and well known mechanism is to export excess metal from cell via transporter proteins that are embedded in plasma membrane (Kim et al. 2004; Persans et al. 2001). However, up to now no TM proteins playing a role in Mn^{2+} extrusion have been identified. The second mechanism is storing excess metal into vacuole by vacuolar localized transporters such as AtCCX3, ShMTP8 and AtCAX2 (Delhaize et al. 2003; Hirschi et al. 2000; Morris et al. 2008) previously shown to have a role in Mn^{2+} detoxification. Besides these, excess metals are also excluded via vesicle mediated exocytosis by transport into the Golgi

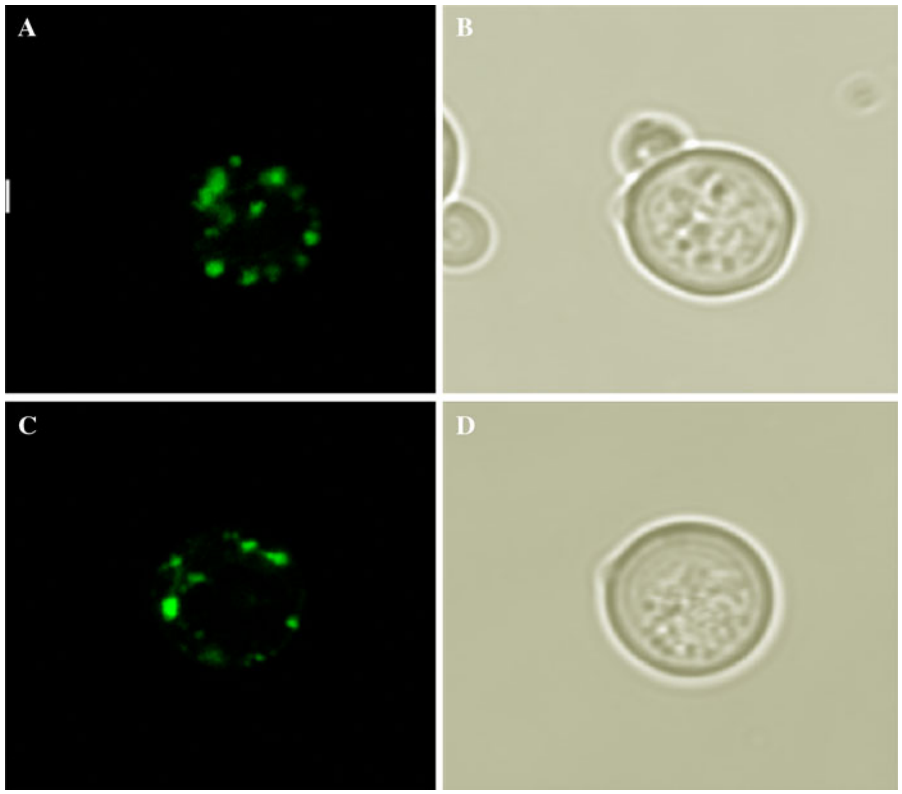

Fig. 5 BmMTP10 and BmMTP11 localization in yeast cells. BmMn10:GFP and BmMn11:GFP fusion proteins localize in a punctuated pattern resembling the Golgi apparatus. Confocal laser scanning microscopy of InvSc1 yeast cells expressing BmMTP10:GFP (**a**) and BmMTP11:GFP (**c**) were performed after 5 h of induction. Bright-field images (**b**, **d**) are shown for the cells shown in **a**, **c**, respectively. (Color figure online)

apparatus. Two proteins belonging to the CDF family; PtMTP11.1 and AtMTP11 have been shown to increase the tolerance against Mn^{2+} toxicity (Peiter et al. 2007).

In this study, we identified two MTP genes from *B. v.* ssp. *maritima* encoding Mn^{2+} transport proteins that are able to provide tolerance to excess Mn^{2+} levels when expressed in yeast (Fig. 1). Metal tolerance assay performed in various mutant yeast strains pointed out that these two genes provide significant Mn^{2+} tolerance in contrast to Co^{2+}, Cd^{2+}, and Zn^{2+}. Furthermore, Δ*cot1* yeast cells transformed with *BmMTP11* showed a relatively weak Ni^{2+} tolerance. Some MTPs have also shown to increase the tolerance to other metal toxicities than Mn^{2+}, such as AtMTP11 and ShMTP8 both being able to increase the Cu^{2+} tolerance in yeast (Delhaize et al. 2007).

Sequence similarity analyses between BmMTP10, BmMTP11 and other Mn-CDF proteins, showed that PtrMTP10 (83 % amino acid identity) and AtMTP10 (80 % amino acid identity) are significantly similar to BmMTP10. In addition, AtMTP11 (86 % amino acid identity) revealed high similarity with BmMTP11. The multiple alignment amino acid sequence analysis showed that BmMTP10 and BmMTP11 have highly conserved domains with the members of Mn-CDF family and their amino acid sequences carry the DXXXD signature sequence in their TM II and TM V that is only conserved for Mn-CDF sub-family (Fig. 2) (Montanini et al. 2007). The phylogenetic tree pointed out that group I includes close relatives of BmMTP10 and group II includes close relatives of BmMTP11 (Fig. 3).

Based on the decreased Mn^{2+} content in Δ*pmr1* yeast cells expressing either of the two BmMTP proteins, the putative membrane localization could be either at the plasma membrane or the Golgi apparatus resulting in either a direct efflux or efflux via exocytosis of secretory vesicles as previously shown for AtMTP11 (Peiter et al. 2007). However, C-termini tagged BmMTP-GFP fusion proteins expressed in yeast resulted for both proteins in a punctuated pattern

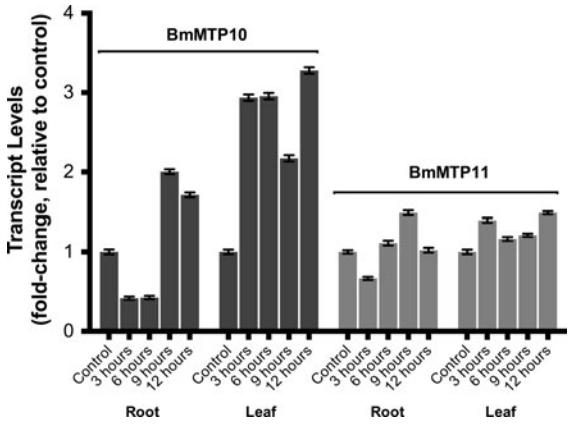

Fig. 6 *BmMTP10* and *BmMTP11* root and leaf specific expression patterns in *Beta vulgaris* spp. *maritima.* Plants were exposed to 2 mM Mn^{2+} for 12 h (samples were collected for every 3 h). The cDNA, isolated from the root and leaf tissues those harvested right before Mn^{2+} exposure, were taken as control group. All expression levels were normalized with respect to *BvACT1* housekeeping gene. Fold-change values of the transcript levels were calculated relative to the control groups in each four cases. Bars show the mean ± SEM ($n = 3$) fold-change in transcripts relative to the controls

resembling the Golgi apparatus. The yeast data do therefore suggest that BmMTP10 and BmMTP11 are involved in Mn^{2+} tolerance via an efflux mechanism by loading Mn^{2+} into the Golgi apparatus and subsequently exocytosis of secretory vesicles.

The gene expression levels of *BmMTP10* and *BmMTP11* were analyzed by qRT-PCR after addition of 2 mM Mn^{2+} to *B. v.* ssp. *maritima*. The expression levels of *BmMTP10* were induced in both root and shoot tissue. This induction is in contrast to the decreased and fluctuating transcript expression pattern of *AtMTP11*, previously shown to have a role in transport of excess Mn^{2+} out of the cell via vesicular mediated exocytosis, with *Arabidopsis* plants exposed to short term exposure of Mn toxicity (0–48 h and 2 mM Mn^{2+}). The expression level of *BmMTP11* did not show any regulation in relation to the toxic Mn^{2+} treatment. The differential regulation of the *BmMTP* genes indicates that the genes are not redundant. The in vivo study suggested that both BmMTP transporters are involved in Mn^{2+} export. However, neither of the transcript levels for the two transporters was induced to a level implicating them having a role in Mn^{2+} toxicity tolerance.

It has previously been shown that Mn^{2+} has an essential role in N-linked and O-linked protein glycosylation in the secretory pathway in yeast (Durr et al. 1998) and in mammalian cells (Kaufman et al. 1994). Moreover, the PMR1 pump has a vital role in supplying Mn^{2+} for these processes in addition to its main function as Mn^{2+} detoxification (Durr et al. 1998). *BmMTP10* and *BmMTP11* expressed in the *Δpmr1* yeast strain provided specifically Mn^{2+} tolerance and their protein localizations were observed in the Golgi apparatus but their *in planta* expression levels under toxic Mn^{2+} concentration did not show a marked regulation. Thus, BmMTP10 and BmMTP11 may be putatively expressed in Golgi membrane to both supply Mn^{2+} ions for glycosylation processes and detoxify of excess Mn^{2+} similar to the PMR1 transporter in yeast.

In summary we have identified and characterized two genes encoding the first identified Mn^{2+} tolerance proteins from *B. v.* spp. *maritima*. The proteins are suggested to have non-redundant functions, localized in the Golgi apparatus and involved in Mn^{2+} tolerance via an efflux mechanism through the secretory pathway.

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