IDENTIFICATION AND CHARACTERIZATION OF MANGANESE TOLERANCE GENES IN *Beta vulgaris subsp. maritima*

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF MANGANESE TOLERANCE GENES IN *Beta vulgaris subsp. maritima*

Manganese is an essential element for higher organisms however uptake of excess amount of manganese causes toxicity. *Beta vulgaris subsp. maritima*, the member of *Chenopodiaceae* family, is known to tolerate high concentration of sodium. Due to its living conditions, *Beta vulgaris subsp. maritima* adapted many different stress conditions. Therefore it is an ideal plant for studying plant tolerance mechanisms. In this study, we aimed to identify the genes which are responsible for manganese tolerance in *Beta vulgaris subsp. maritima* by screening its cDNA library in *Saccharomyces cerevisiae* cells. After initial screening in the presence of toxic manganese concentration; 2,7mM $MnCl_2$, three resistant yeast colonies were selected. After the sequence and similarity analysis, two genes which might involve in manganese tolerance were identified and named as *BmMn1* and *BmMn2*. The results of solid media tests with different yeast strains which transformed with the genes revealed that *BmMn1* provides a remarkable manganese tolerance like *BmMn2* and slightly nickel tolerance. They do not show tolerance to other metals such as zinc, cadmium, boron and cobalt. Identified manganese concentrations in Δ*pmr1* yeast strains transformed with *BmMn1*, *BmMn2* or empty vector pointed that BmMn1 and BmMn2 transport excess manganese out of the cell. In addition, GFP localization in the yeast cell proved that the BmMn1 and BmMn2 are located in Golgi apparatus. qRT-PCR analyses of *Beta vulgaris subsp. maritima* which was exposed to 2mM Mn^{2+} suggested a dynamic regulation for the expression of these two genes. The results indicate that *BmMn1* and *BmMn2* have a role in detoxification of excess amount of manganese in *Beta vulgaris subsp. maritima*.

ÖZET

Beta vulgaris subsp. maritima' daki MANGAN TOLERANS GENLERİNİN BELİRLENMESİ VE KARAKTERİZASYONU

Mangan gelişmiş organizmalar için gerekli elementlerden biridir. Ancak yüksek miktarlarda alınan mangan toksik etki göstermektedir. *Chenopodiaceae* ailesinin bir üyesi olan *Beta vulgaris subsp. maritima* yüksek tuz toleransına sahip bir bitki olarak bilinir. Yaşam koşullarına bağlı olarak, *Beta vulgaris subsp. maritima* farklı stres koşullarına adapte olduğundan, bitki tolerans mekanizmalarını incelemek için ideal bir bitkidir. Bu çalışmada, *Beta vulgaris subsp. maritima*'nın cDNA kütüphanesinin, *Saccharomyces cerevisiae* hücreleri içerisinde taranması yoluyla, *Beta vulgaris subsp. maritima*' daki mangan tolerans mekanizmalarında görev alan genlerin tanımlanması amaçlanmıştır. 2,7 mM toksik mangan konsantrasyonu varlığında yapılan ilk taramada üç dayanıklı maya kolonisi seçilmiştir. Sekans ve benzerlik analizleri sonucu, *BmMn1* ve *BmMn2* olarak adlandırılan iki genin mangan toleransında rol oynayabileceğini göstermiştir. Bulunan bu iki genin aktarıldığı farklı maya hatlarıyla yapılan büyüme testleri sonucuna göre *BmMn1*, *BmMn2* gibi kaydedeğer bir mangan toleransı ve az miktarda nikel toleransı göstermiştir. Ancak bu iki gen, çinko, kadmiyum, bor ve kobalt gibi diğer metaller için tolerans göstermemiştir. *BmMn1*, *BmMn2* veya boş vektörün aktarıldığı Δ*pmr1* maya hatlarında ki belirlenmiş mangan konsantrasyonları, bu genlerin fazla manganı hücre dışına taşıdığını göstermiştir. 2mM $MnCl_2$ 'e maruz bırakılan *Beta vulgaris subsp. maritima*' ların gerçek zamanlı PCR sonuçlarına göre bu iki genin dinamik bir regülasyon gösterdiği tespit edilmiştir. Bu sonuçlar, yüksek miktardaki mangan varlığında *BmMn1*, *BmMn2* genlerinin *Beta vulgaris subsp. maritima*' nın detoksifikasyonunda rol oynayabileceğini göstermiştir.

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CHAPTER 1

INTRODUCTION

1.1. *Beta vulgaris subsp. maritima*

Beta vulgaris subsp. maritima which is known as the sea beet or the wild beet is a short-lived perennial and sometimes a self incompatible plant (Boutin-Stadler et al. 1989; Letschert 1993). Wild beet an ancestor of sugar beet (Stevanato et al. 2001) is a relative of spinach and swiss chard. Subspecies of the sea beets with different phenotypes were recorded from different geographical regions. The Mediterranean sea beets are generally annual unlike the British Isles sea beets which are perennial. In addition, the leaves of Mediterranean sea beets are darker green, waxy and more hairy in contrast to the British Isles sea beet (Doney et al. 1990).

Although all plants have a special ability in order to adapt different environments due to having no powers of locomotion, some plant species have a greater ability themselves to adapt stressful conditions such as heavy metal containing soils, drought, salinity, high and low temperature. The sea beet grows in the coastal regions of all Mediterranean Sea, many Middle East countries and the Atlantic coasts of northern Europe (Doney et al. 1990). The living ability of *Beta vulgaris subsp. maritima* in large niches is provided by adaptation of many challenging environments via accumulation of many stress resistance genes (Doney 1993). Sea beets are able to grow under high salinity which is normally considered toxic for other plant species. It can also resist for many serious diseases such as cercospora and rhizomania (Stevanato et al. 2001). The relatives of wild beet; spinach and swiss chard are also known as cadmium accumulators (Bingham 1979). In addition, it is known that the highest amounts of manganese are found in beet roots at the concentration between 36 and 113 $mg.kg^{-1}$ DW (Sillanpää 1982). Studies showed that *Beta vulgaris subsp. maritima* survives at many challenging environments however its tolerance mechanisms are poorly understood at molecular level.

1.2. The Element Manganese

Manganese element was discovered by the chemist Johan Gottlieb Gahn (1745 – 1818) in 1774. Manganese is very abundant in earth's crust therefore it is found in soils remarkably high as the concentrations between 500 and 1,000 $mg.kg^{-1}$ (Aubert and Pinta 1977). In general, there are two forms of manganese in soils; as divalent cation (Mn^{2+}) a soluble form and tetravalent cation (Mn^{4+}) an insoluble form for plants (Aubert and Pinta 1977).

Manganese is found in very small amounts in plants and if its concentration in soil increases, manganese causes toxicity (Maze 1936). Mn^{2+} is absorbed by roots from soil as divalent cation form (Mn^{2+}). The mobility of Mn^{2+} along phloem is negligible (Riesen and Feller 2005) therefore Mn^{2+} is generally transported by xylem from roots to leaves (Page and Feller 2005; Marschner 1995).

Acidity has a direct impact on Mn^{2+} absorption from soil by roots. It increases at pH 6 or less on the other hand, it significantly decreases at pH 7 and more (Maas, Moore, and Mason 1968). The optimum absorption for Mn^{2+} is between pH 4.5 and pH 6 (Bowen 1969).

1.3. The Essential Micronutrient Manganese

Manganese is an indispensible element in plants. It can catalyze a reaction such as catalyzing MnSOD (Manganese-containing Superoxide Dismutase) or activate an enzyme such as activating PEP carboxykinase (Hänsch and Mendel 2009).

Manganese plays a vital role in oxygen evolution from H_2O in photosystem II (Gonzalez and Lynch 1997). In this reaction, manganese which is a binding site of two molecules of water stores energy to oxidize these molecules (Marschner 1995). In addition, manganese is used in redox reactions in that Mn (II) oxidation state form is oxidized to Mn (III) and Mn (IV) in plants (Marschner 1995).

Mn^{2+} participates in the structure of MnSOD which is found in mitochondria and peroxisomes in plants (Marschner 1995). This enzyme behaving as an antioxidant and reduces concentration of superoxide via oxidating itself (Kenten and Mann 1949; Bowler et al. 1991). Moreover, manganese is included in the tricarboxylic acid cycle (TCA) and it catalyzes many enzymes (Marschner 1995) such as PEP carboxykinase,

malic enzyme, isocitrate dehydrogenase. One of the examples is the activation of PEPCK (Phosphoenolpyruvate carboxykinase) by Mn^{2+}. PEPCK (Phosphoenolpyruvate carboxykinase) is an enzyme in carbon fixation and it catalyzes decarboxylation of oxaloacetate into phosphoenolpyruvate (PEP) and carbon dioxide (Walker and Leegood 1996). PEP catalyzed by PEPC (PEP carboxylase) is an indispensible substrate in carbon fixation occurred in C-4 plants (Hatch and Slack 1970) and in CAM plants (Osmond 1978).

1.4. Manganese Toxicity

The symptoms of manganese toxicity include immature leaf chlorosis (caused by decreasing of chlorophyll amount) and leaf necrosis, reduction of CO_2 assimilation in immature leaves and brown speckles on mature leaves (Clairmont, Hagar, and Davis 1986; Gonzalez and Lynch 1997). In addition, manganese toxicity diminishes carotenoid content providing photosynthesis in the deprivation of chlorophyll (Clairmont, Hagar, and Davis 1986).

Moreover, acute manganese toxicity results changes in structure of enzymes with antioxidant activity in plants leading an ROS increase and in turn cell membrane damage and reduce shoot and root growth (Xue et al. 1995).

The adaptation of excess manganese can develop under harsh environmental conditions including low solubility, cation mobility and ion antagonism (Lidon 2002). However this adaptation develops only when genotypes of plant species allow.

1.5. Manganese Transporter Proteins

Many proteins involve in uptake and transportation of manganese from soil to leaves (Fig. 1.1). Such as AtECA, AtIRT1 or AtNRAMP3 regulate manganese uptake from soils to roots. AtFRD3 transports manganese from root cells to xylem similar to OsYSL, a role in manganese transport from root cells to phloem. Manganese homeostasis is regulated by AtNRAMP3, AtNRAMP4 and AtECA3 in shoots and by many proteins such as OsNRAMP2, AtMTP11 and AtCCX3 in leaves.

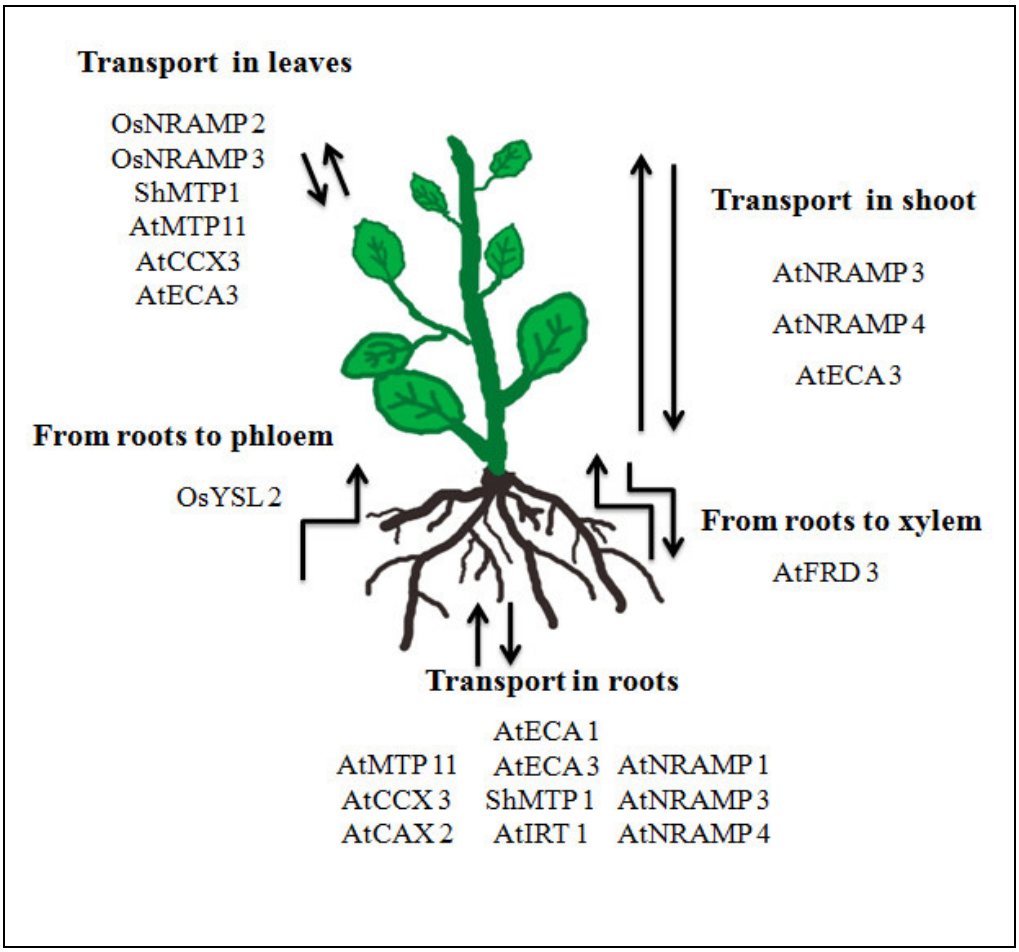

Figure 1.1. Expression regions of manganese transporter genes in plants. *Arabidopsis thaliana;* AtECA1 (Mills et al. 2008), AtMTP11(Delhaize et al. 2007), AtCCX3 (Morris et al. 2008), AtCAX2 (Hirschi et al.2000), AtECA3(Mills et al.2008), AtIRT1(Korshunova et al.1999),AtNRAMP1 (Cailliatte et al. 2010), AtNRAMP3 (Thomine et al. 2003),AtNRAMP4(Lanquar et al. 2010), AtFRD3 (Durrett, Gassmann, and Rogers 2007). *Stylosanthes hamate*; ShMTP1(Delhaize et al.2003). *Oryza sativa;* OsYSL2(Ishimaru et al.2010), OsNRAMP2,OsNRAMP3(Belouchi Kwan, and Gros 1997).

Although there have been still unknown proteins in manganese transport pathways, the vast majority of them have already been identified for plant cells (Fig.1.2). AtIRT1 (Iron Regulated Metal Transporter) is an iron transporter, localizes in plasma membrane, transports manganese, zinc and cobalt into cell as a cation transporter (Korshunova et al. 1999). OsYSL2 (yellow stripe 1 (YS1) - like protein) rice metal-NA transporter in plasma membrane provides phloem transport of iron and manganese (Koike et al. 2004; Ishimaru et al. 2010). The other transport protein localizing in plasma membrane is AtNRAMP1 (Natural Resistance-Associated Macrophage Protein 1), role in Mn^{2+} uptake into cell. AtNRAMP1 is stimulated especially by Mn^{2+} deficiency in roots (Cailliatte et al. 2010).

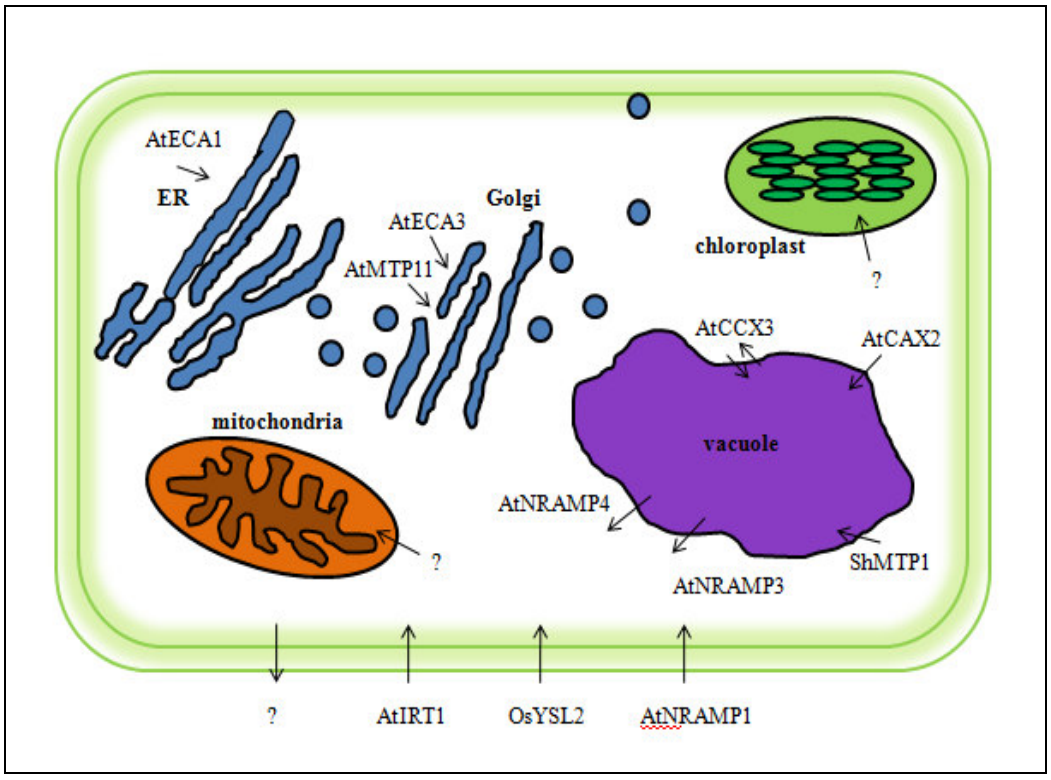

Figure 1.2.The manganese transport pathway in plant cells. AtIRT1(Korshunova et al.1999),OsYSL2 (Koikeet al. 2004), AtNRAMP1(Cailliatte et al. 2010), AtCAX2(Hirschi et al.2000),ShMTP1 (Delhaize et al.2003),AtCCX3(Morris et al.2008), AtNRAMP3 and AtNRAMP4 (Thomine et al.2003; Lanquar et al. 2010), AtECA1 (Wu et al. 2002), AtMTP11(Delhaize et al. 2007; Peiter et al. 2007), AtECA3 (Li et al. 2008; Mills et al. 2008)

Among these, there are five manganese transporters which localize in vacuolar membrane. AtCAX2 known as calcium ion transporter is capable to transport manganese and cadmium (Hirschi et al. 2000). ShMTP1 one of the members of CDF (cation diffusion facilitator) protein family provides sequestration of Mn^{2+} to vacuole in *Stylosanthes hamata* (Delhaize et al. 2003). AtCCX3 (Cation/calcium exchanger 3) is also a vacuolar membrane transporter which is able to transport both Na^{+} and Mn^{2+} and it balances the cation contents in Arabidopsis cells (Morris et al. 2008). There are two vacuolar membrane transporters which are the members of NRAMP family; AtNRAMP3 and AtNRAMP4, provide Mn^{2+} homeostasis by release of Mn^{2+} from vacuoles. AtNRAMP3 also transports of Fe^{2+} and Cd^{2+} from vacuole to cytoplasm (Thomine et al. 2003; Lanquar et al. 2010).

Some manganese transporter proteins play a role in the secretory pathway. AtECA1 (ER Ca^{2+} transporting ATPase) transports Mn^{2+} and Ca^{2+} into ER in order to maintain of both Ca^{2+} and Mn^{2+} homeostasis in cell (Wu et al. 2002). In addition,

AtMTP11, a member of CDF protein family, transports excess Mn^{2+} out of cell by exocytosis from Golgi to plasma membrane (Delhaize et al. 2007; Peiter et al. 2007). AtECA3 is also a Ca-ATPase in Golgi. However, it is distinct from other ATPases; AtECA1, AtECA2 and AtECA4. There are two functions of AtECA3. First, it accumulates Mn^{2+} into a Golgi-related compartment; second, it provides detoxification of high Mn^{2+} by transporting it to vacuole or out of cell via vesicular transport (Li et al. 2008; Mills et al. 2008).

CHAPTER 2

MATERIAL AND METHODS

2.1. Yeast Growth and Media

A haploid wild type (WT) *Saccharomyces cerevisiae* strain (BY4741 (*MATa; his3; leu2; met15; ura3*)), its isogenic haploid deletion mutants (YGL167C, YOR316C, YDR135C) obtained from the yeast deletion library (Invitrogen, Karlsruhe, Germany) and one haploid double mutant (Y04622 (ΔYLR130C) crossed with Y14087 (ΔYLR130C)) provided by Pai Pedas (Pedas et al. 2008) were used in this study.

YPD medium (with 2 % glucose, 2 % peptone, 1 % yeast extract and 2 % agar) and selective YNB medium (yeast nitrogen base) having required amino acids and bases were used.

2.2. Construction of *Beta vulgaris subsp. maritima* cDNA Library

2.2.1. Plant Growth and RNA Isolation

Beta vulgaris subsp. maritima seeds were firstly germinated in autoclaved peat and then seedlings needed to be grown enough for toxic Mn^{2+} application were taken into semi-strong Hoagland solution (Bozdag and Karakaya 2009) and grown under controlled environmental conditions (25 °C with 12h light/ 12h dark photoperiods at a light intensity of 40 µmol $m^{-2}.s^{-1}$ and 50% relative humidity). Then $MnCl_2$ solution was applied as the final concentration containing 2 mM Mn^{2+} for 7 days. At the end of the application period, the control and treated *Beta vulgaris subsp. maritima* roots and leaves were harvested, immediately frozen in liquid nitrogen and stored at -80 °C until total RNA isolation steps. Total RNA isolation was performed by Invitrogen RNA Isolation Kit according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

2.2.2. Synthesis of Gateway® – Compatible cDNA Library

Gateway®-compatible cDNA library were synthesized according to Ni et al. 2007. First-strand cDNA synthesis was performed by using following steps. The mix of 4 µl total RNA and oligo(d)T primer attB2-(d)T_{25} (15 µM) with the sequence 5´-ACCACTTTGTACAAGAAAGCTGGG T_{25}TVN-3´ was incubated at 72 °C for 2 minutes then at 0 °C for 2 minutes. 2 µl (5X) First Strand Buffer, 1 µl DTT (20 mM), 1 µl dNTP mix (10 mM) and 1 µl Power Reverse Transcriptase were added to the initial mixture and incubated at 42 °C for 1 hour. After the incubation, 1 µl oligo attB1-(r) G_3 (15 µM) with the sequence 5´-ACAAGTTTGTACAAAAAAGCAGGC TrGrGrG-3´ was added lastly and the final mixture was incubated at 42 °C for 1 hour.

Double-stranded cDNA synthesis was performed by PCR amplification on Aplied Biosystems Gen Amp® PCR System 9700. The reaction was prepared via the mixture of 80 µl dH_2O, 10 µl (10X) Advantage 2 PCR buffer, 2 µl (10 mM) dNTP mix, 2 µl (15 µM) attB1 primer, 2 µl (15 µM) attB2 primer, 2 µl Adv. 2 Polymerase Mix and the 2 µl first- strand cDNA template prepared. The conditions of PCR reaction were 1 minute initial denaturation at 95 °C, 20 cycles of three temperatures as 15 seconds at 95 °C, 30 seconds at 64 °C, 6 minutes at 68 °C and finally one cycle 10 minutes at 68 °C. The quality of the synthesized cDNA library was controlled by performing agarose gel (1 %) electrophoresis.

2.2.3. Cloning of the cDNA Library with Gateway Technology®

The cDNA library was run on agarose gel (1%) with 100 voltages for 20 minutes. The agarose gel was excised from >500 bp length of cDNA bands and extracted by Fermentas DNA Extraction Kit (Fermentas, St. Leon-Rot, Germany).

Extracted cDNAs with the concentration of 160 ng were introduced to *pDONR221* (150 ng) entry vector (Invitrogen, Karlsruhe, Germany) by addition of 2 µl BP Clonase™ II enzyme with an overnight incubation at 25 °C. The reaction was terminated by addition of 2 µl protease K (Invitrogen, Karlsruhe, Germany) and the reaction mixture was incubated at 37 °C for 10 minutes and then at 70 °C for 10 minutes. After BP clonase reaction, the mixture was transformed to the OmniMAX™ 2 T1 Phage-Resistant (T1R) *Escherichia coli* strain (Fermentas, St. Leon-Rot, Germany)

by heat shock method. First, *Escherichia coli* competent cells were incubated on ice for 5 minutes. Then, 2 µl of BP clonase reaction mixture were added onto the cells and the transformation mixture was incubated on ice for 25 minutes. The competent cells heat shocked at 42 °C for 30 seconds. 2 minutes on ice, then 250 µl SOC medium was added into the transformation mixture and latest incubation was applied at 37 °C 200 rpm for 1 hour. Then the transformation mixture was plated on LB medium having kanamycin (50µg/ml). Grown colonies were collected in 50 ml falcon tubes and the plasmids were isolated using PureLink™ Quick Plasmid Maxiprep Kit (Invitrogen, Karlsruhe, Germany).

LR Clonase reaction was performed with the mixture of 160 ng/µl of plasmids which were obtained from the BP reaction, *pAG426GPD* (150ng) destination vector (Invitrogen, Karlsruhe, Germany), 3 µl TE buffer and 2 µl LR Clonase™ II enzyme mixture. The reaction was carried out overnight at 25 °C and 2 µl of the reaction mixture were transformed to the same cell line by the heat shock method described above. The transformation mixture was plated on LB medium including ampicillin (100 µg/ml). Plasmids were isolated by PureLink™ Quick Plasmid Maxiprep Kit (Invitrogen, Karlsruhe, Germany).

2.3. Yeast Transformation of Cloned cDNA Library

Yeast transformation was performed by Lithium Acetate (LiAc) method by reference to (Burke, Dawson, and Stearns 1994). Yeast cells were grown until mid-log phase at 5 ml YPD medium and washed with dH_2O. The cell pellets were resuspended with 0.1 M LiAc, transferred into 1.5 ml micro centrifuge tubes, centrifuged for 5 seconds at the top speed centrifuge and washed with 500 µl 0.1 M LiAc. After the removal of the LiAc, 240 µl PEG (50 % w/v), 36 µl (1 M) LiAc, 5 µl ssDNA, 10 µl DTT (0.3 mM), 20 µl plasmid DNAs, 10 µl dH_2O were added onto the cell pellets and was vortexed for 1 minute. Then the mixture was incubated at 30 °C (160 rpm) for 30 minutes and finally incubated for 30 minutes at 42 °C. Then it was plated on YNB-ura medium including 2700 µM Mn^{2+}. Plasmid isolation was performed from resistant colonies by using PureLink™ Quick Plasmid Miniprep Kit (Invitrogen, Karlsruhe, Germany). The isolated plasmids were sent to the Biotechnology Center in IYTE to identify the sequence of genes.

2.4. Sequence Analyses of the Identified *Beta vulgaris subsp. maritima* Genes

The identified genes from the *Beta vulgaris subsp. maritima* cDNA library were searched on BLAST service of NCBI. Multiple sequence alignment analysis was performed by ClustalW2. Phylogenetic tree of the identified genes was created by JALVIEW 2.6.1 multiple alignment editor and further changes were performed via Fig Tree java console application.

2.5. Metal Tolerance Assay

Identified genes were tested for their resistance activities by exposing with different metal stresses; Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} or B^{2+}. The wild type and haploid deletion mutants were transformed with empty vector *pAG426GPD*, *BmMn1* or *BmMn2* containing *pAG426GPD* vector. Overnight cultures at 30 °C were diluted with dH_2O to an OD_{600} of 0.2, 0.02, 0.002, 0.0002 and spotted as 5 µl on solid YNB plates (–ura; 2 % glucose) with Mn^{2+} (8mM), Ni^{2+} (1.8 mM), Co^{2+} (1 mM), Zn^{2+}(15 mM), Cd^{2+}(150 µM) or B^{2+}(110 mM) and the YNB plates (–ura; 2 % glucose) without Mn^{2+}, Ni^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+} or B^{2+}. The yeast colonies were grown continuously for 5 days at 30 °C.

2.6. Identification of Manganese Concentration in Yeast Cells

Manganese concentrations of haploid Δ*pmr1* yeast strains including *BmMn1*, *BmMn2* or empty vector (*pAG426GPD*) were detected by reference to Mizuno et al. 2005. Pre-cultured yeast cells were grown overnight in YNB (–ura) medium. The cells were diluted with the same medium as the final concentration having 1/1000 diluted yeast cells and Mn^{2+} were added into the cell cultures as the final concentration containing 1 mM Mn^{2+}. The cells transformed with the identified genes were grown for 48 hours and the cells transformed with empty vector were grown for 60 hours then all cells were washed three times with 10 mM EDTA and one time with ultrapure water and dried overnight at 70 °C. 5 mg of yeast cells were extracted by 65% HNO_3 and filtrated with 0.20 µm Minisart® filters (Gottingen, Germany). Mn^{2+} contents of the

extracts were identified by Induced Coupled Plasma-Mass Spectrometry (ICP-MS) in the Environmental Development, Implementation and Research Center, IYTE.

2.7. Identification of BmMn1 and BmMn2 Localization

2.7.1. Construction of GFP Fusions for Expression in Yeast Cells

A PCR-based cloning strategy was used to generate *BmMn1* and *BmMn2* 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-BmMn1-GFP* and *pYES2-BmMn2-GFP* the coding sequences of *BmMn1* and *BmMn2* w/o stop codon were inserted in *Kpn1*/*XbaI* sites in the pYES2-GFP vector. The primers used were *BmMn1* forward (5´-CCGGGTACCATGCTGGAGGGTTTCAGTG-3´) and reverse (5´-GGCTCTAGACCATAGATGGGCTTGCGCATGC-3´) and *BmMn2* forward (5´-GGAGGTACCATGGAAAACATAACTGAAAC-3´) and reverse (5´-GGCTCTAGACCAAACCTTTGCTTTGTGCTC-3´). The PCR products were amplified using LA Taq (Takara Bio Inc., Shiga, Japan) and digested o/n with *Kpn1* and *Xba1*. The digested PCR products were ligated into the *pYES2-GFP* vector followed by sequencing.

2.7.2. Preparation of Yeast Cells for Confocal Microscopy

The yeast strain InvSc1 (Invitrogen, Karlsruhe, Germany) was used for expression of the GFP-fusion constructs. Yeast transformants were grown on selective glucose containing (SD) medium with the required supplements; 2% glucose, 0.7% yeast nitrogen base w/o amino acids and 0.2% yeast synthetic drop-out medium supplement without uracil (Sigma-Aldrich, Taufkirchen, Germany). The expression of BmMn1-GFP and BmMn2-GFP were induced for five hours in 2% galactose based medium. The cells were fixated on polysine slides (Thermo Scientific, Waltham USA) and the transformed cells were visualized using a Leica TCS SP2/MP confocal

laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Excitation for GFP was 488 nm, and emission was detected between 500 and 540 nm.

2.8. Gene Expression Analysis

Beta vulgaris subsp. maritima plants were exposed to 2 mM Mn^{2+} for 12 hours. Leave and root samples were harvested in every 3 hours and immediately frozen in liquid nitrogen. Then these samples were stored at -80 °C until total RNA isolation steps. Total RNA isolation was performed as described above. cDNAs in order to use in qRT-PCR were synthesized by using Fermentas cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). IQ5 real-time PCR cycler system (Bio-rad, München, Germany) were used for determining quantitive gene expression analyses. The PCR reaction mixture was prepared as 12.5 µl of Fermentas Maxima^{TM} SYBR Green qPCR Master Mix (2X) (Fermentas, St. Leon-Rot, Germany), 0.5 µl reverse and forward primers (10 pmol/ µl) and 2 µl cDNA template. PCR amplification conditions were: Initial denaturation at 95 °C for 10 min. as one cycle, and then 95 °C for 20 s, 55 °C for 30 s, 72 °C for 30 s as 40 cycles.

Beta actin was used as a positive control. The forward primer was *BmAct*RTF 5´-AGACCTTCAATGTGCCTGCT-3´and the reverse primer was *BmAct*RTR 5´-TCAGTGAGATCACGACCAGC-3´. The primers were used to amplify 187 bp of *Beta vulgaris subsp. maritima* beta actin cDNA.

The forward primer of one of the identified gene in this study was *BmMn1*RTF 5´-AAGGCTGTACAAGTGTGGGATTCG-3´ and the reverse was *BmMn1*RTR 5´-AGTGGATCCAGACCACAGCTCAAA-3´ and these primers were used to amplify 140 bp of the *BmMn1* cDNA. The forward primer of the second identified gene in this study was *BmMn2*RTF 5´-ACGAGCATACACTTTCGGTTCCCA-3´ and the reverse was *BmMn2*RTR 5´-ACTTCCTGGAGTTGCTCGAGCTTT-3´ and these primers were used to amplify 129 bp of the *BmMn2* cDNA.

CHAPTER 3

RESULTS

3.1. Identification of *BmMn1* and *BmMn2*

After transformation of cDNA library of *Beta vulgaris subsp. maritima* to Δ*pmr1* yeast strain, three yeast colonies were able to grow under 2700 µM Mn^{2+} toxic stress within 5 days. Plasmids (three samples for each colony) including the genes (named as *BmMn1a*, *BmMn1b* and *BmMn1c*, *BmMn2a*, *BmMn2b* and *BmMn2c*, *BmMn3a*, *BmMn3b* and *BmMn3c*) that may play a role in Mn^{2+} tolerance were isolated from these three colonies. After restriction of plasmids, the fragments were run on agarose gel (1%) to see size of the insert (Figure 3.1)

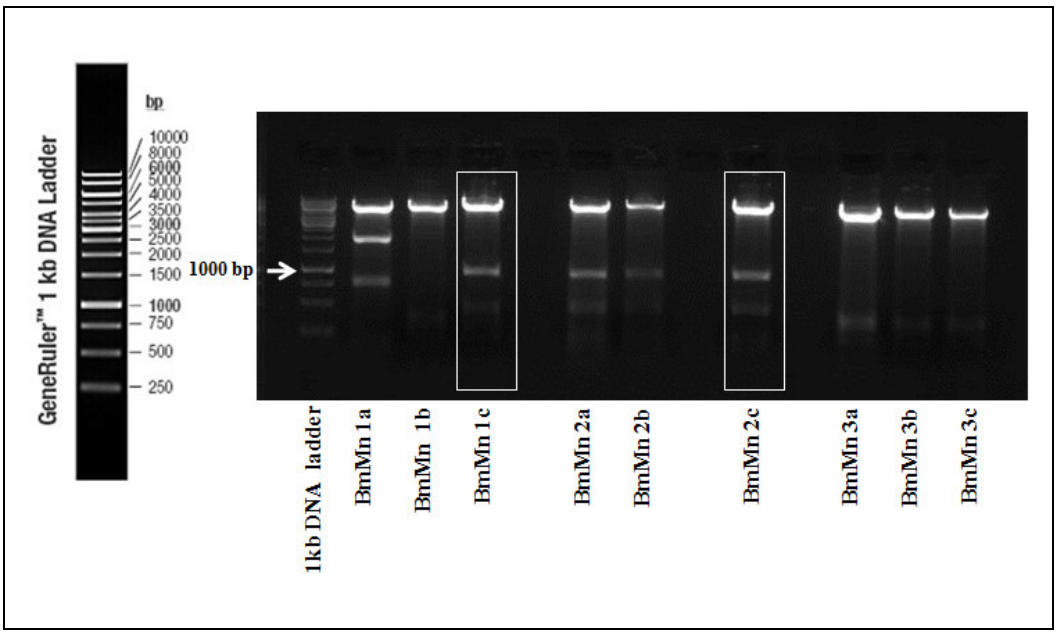

Figure 3.1. Agarose gel display for the plasmids isolated from the three resistant colonies.

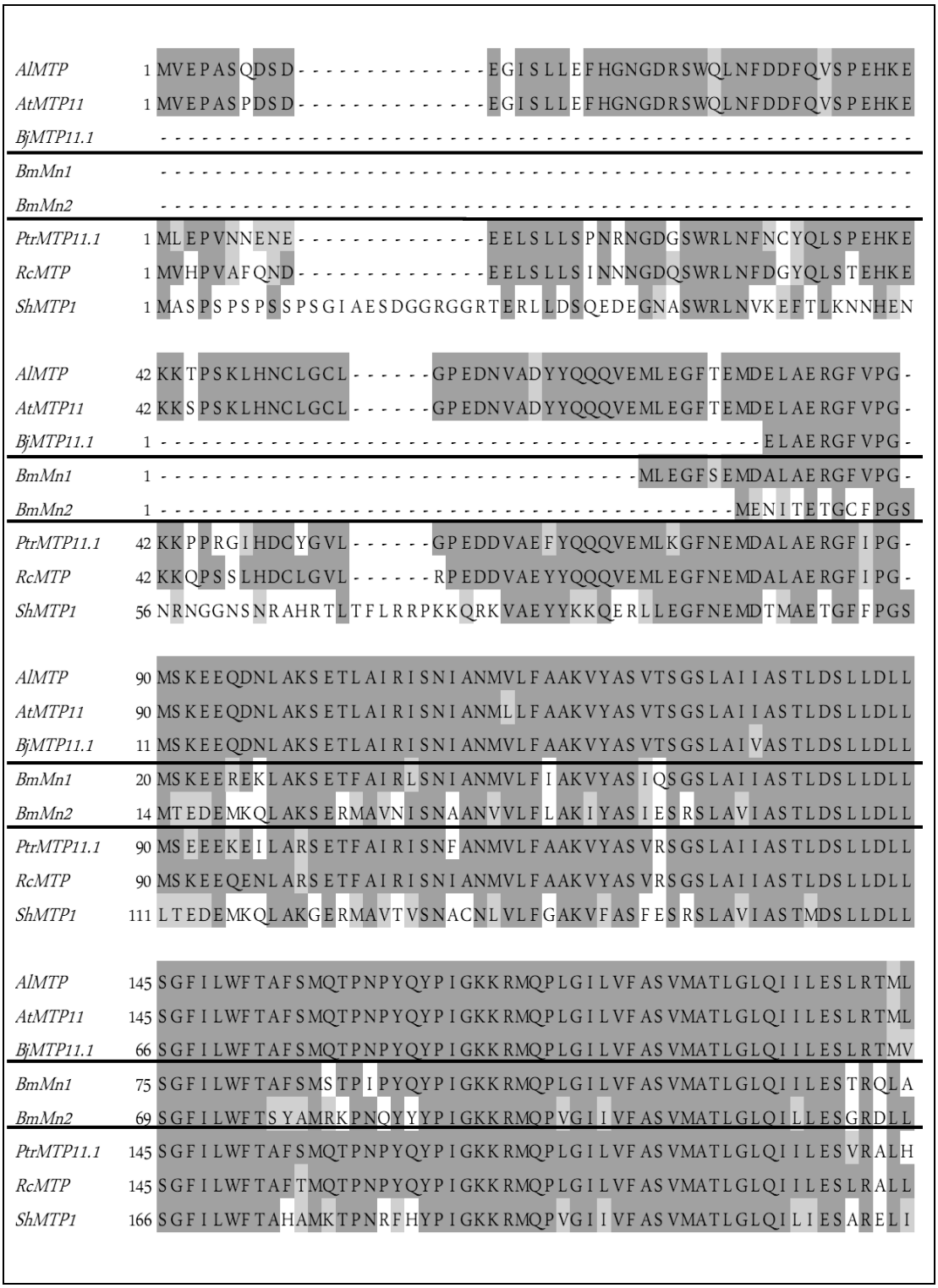

Figure 3.2. The result of multiple amino acid sequence alignments of the deduced BmMn1 and BmMn2**.** Same colors on columns indicate conserved amino acids between BmMn1, BmMn2 and other proteins. Same amino acids were highlighted according to Blosum 62 score with JALVIEW 2.6.1.

(cont. on next page)

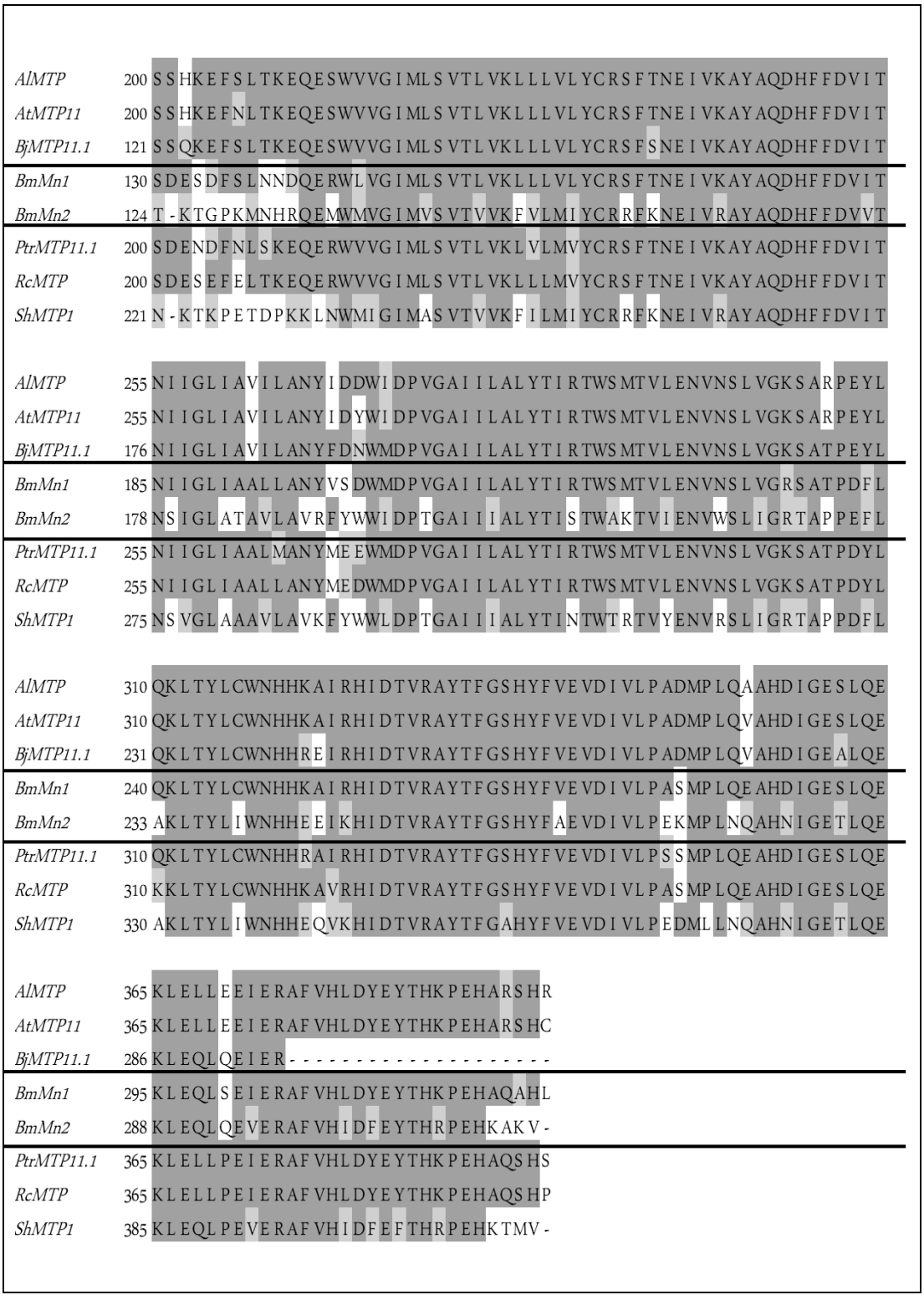

Figure 3.2. (cont.)

Table 3.1.The scores of multiple amino acid sequence alignment analysis.

Name (sequence A)	Length (aa)	Name (sequence B)	Length (aa)	Score %	Function *
BmMn1	324	RcMTP	394	89	Cation efflux protein/ zinc transporter
BmMn1	324	PtrMTP11.1	394	88	Mn-specific cation diffusion facilitator transporter
BmMn1	324	AlMTP	394	87	Cation efflux family protein
BmMn1	324	AtMTP11	394	86	Cation transmembrane transporter/ manganese ion transmembrane transporter
BmMn1	324	BjMTP11.1	295	86	Metal tolerance protein
BmMn1	324	BmMn2	316	67	
BmMn1	324	ShMTP1	415	65	Cation diffusion facilitator family/manganese tolerance
BmMn2	316	ShMTP1	415	80	Cation diffusion facilitator family/manganese tolerance
BmMn2	316	RcMTP	394	66	Cation efflux protein/ zinc transporter
BmMn2	316	PtrMTP11.1	394	66	Mn-specific cation diffusion facilitator transporter
BmMn2	316	AlMTP	394	66	Cation efflux family protein
BmMn2	316	AtMTP11	394	66	Cation transmembrane transporter/ manganese ion transmembrane transporter
BmMn2	316	BjMTP11.1	295	66	Metal tolerance protein

*Functions of the proteins were obtained by NCBI database

Sequences of isolated genes were identified (Appendix A). The functions of these genes were predicted after NCBI's blast search. It was observed that *BmMn1* and *BmMn2* were orthologous of cation or manganese transporter genes which were identified in previous studies.

The ORFs (open reading frame) of *BmMn1* and *BmMn2* were observed as 972 and 948 bp length. Nucleotide sequences of the genes were translated to protein sequences by using Expasy proteomic server (Appendix B). The protein sequences of

BmMn1 and BmMn2 were determined as 324 and 316 amino acid length. In addition, the structure of BmMn1 and BmMn2 were predicted to include five transmembrane helices by TMHMM 2.0 v.

The close relative proteins which were identified as Mn^{2+} transporters in previous studies of BmMn1 and BmMn2 were identified BLAST service of NCBI. The proteins were compared with CLUSTALW2 multiple align sequence online tool to identify conserved amino acids and highlighted with JALVIEW 2.6.1 multiple alignment editor (Figure 3.2). The similarity scores were shown in Table 3.1 Phylogenetic tree of this multiple alignment analysis were created in JALVIEW 2.6.1. and Fig Tree v1.3.1 were used for further changes (Figure 3.3)

The results of multiple alignment sequence analysis showed that BmMn1 and BmMn2 have highly conversed amino acids with the members of CDF (cation diffusion facilitators) family proteins.

According to the similarity scores of multiple alignment sequence analysis, BmMn1 revealed a remarkable homology with RcMTP (89%), PtrMTP11.1 (88%), AlMTP (87%), AtMTP11 (86%) and BjMTP11.1 (86%). BmMn2 also showed high homology with ShMTP1 (80%). On the other hand, only 67% identity was indicated between BmMn1 and BmMn2 suggesting 2 different transporter proteins identified.

Three different groups having different evolutionary relations were identified in the phylogenetic three. The members of first group were AlMTP, AtMTP11 and BjMTP11. It was not surprising that AlMTP and AtMTP11 show the closest relation between themselves because both of them are the members of Arabidopsis genus. The second group included BmMn1, RcMTP and PtrMTP11.1 which also showed a remarkable relation. On the hand, the third group was very distinct from the other two groups. The members of third group; BmMn2 and ShMTP1 also showed a close relation.

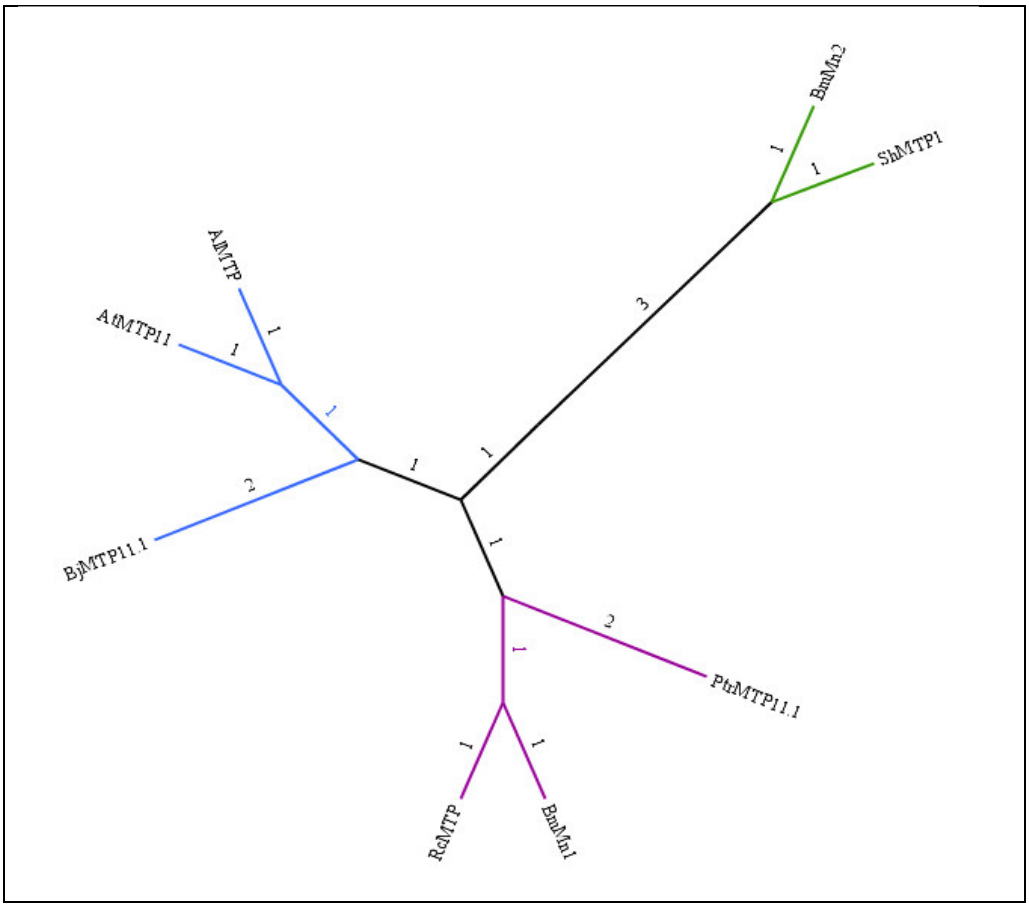

Figure 3.3. Phylogenetic tree of the BmMn1, BmMn2 and other proteins identified as Mn^{2+} transporters; RcMTP (XP_002533664.1), PtrMTP11.1 (XP_002315247.1), AlMTP (XP_002862450.1), AtMTP11 (NP_181477.1), BjMTP11.1 (ACO90095.1), ShMTP1 (AAO38709.1).

3.2. Metal Tolerance Assays

BmMn1 and *BmMn2* were selected to be used for further analysis by benefiting from the results of bioinformatics' analyses. Firstly, to understand the metal tolerance selectivity of *BmMn1* and *BmMn2*, metal tolerance assay were performed by using different yeast deletion mutants transformed with empty vector *pAG426GPD*, *BmMn1* or *BmMn2*. The yeast strains were diluted and spotted on solid YNB-ura medium plates including different metals (Figure 3.4).

It was observed that *BmMn1* and *BmMn2* tolerance Mn^{2+} but not other divalent cations such as Co^{2+}, Cd^{2+}, Zn^{2+}, B^{2+}. In addition this assay revealed that *BmMn1* provides insignificant Ni^{2+} tolerance. It was concluded that *BmMn1* and *BmMn2* are highly selective for Mn^{2+} tolerance and they have a role in manganese transport in cells.

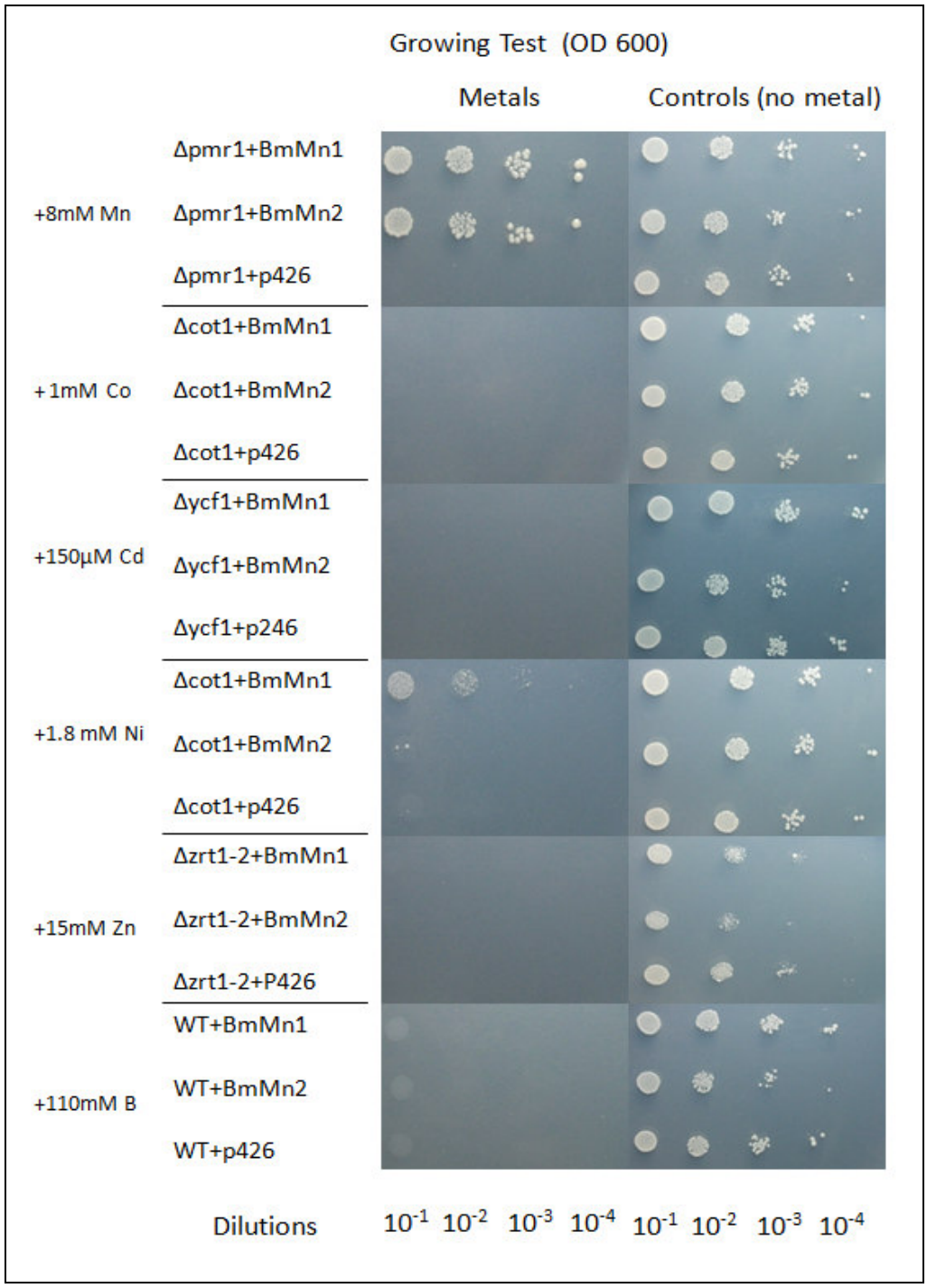

Figure 3.4. Solid growth tests for transformed yeast strains. Four serial dilutions from O.D. $_{600}$= 0,2 to O.D. $_{600}$= 0,0002 were plated on minimal medium. Transformed yeast strains with *BmMn1*, *BmMn2* or *p426 (pAG426GPD)* were incubated 5 days at 30 °C.

3.3. Identification of Manganese Concentration in Yeast Cells

The manganese concentrations in Δ*pmr1* yeast strains transformed with *BmMn1*, *BmMn2* or empty vector *pAG426GPD* were detected by using ICP-MS after that the cells were exposed to 1 mM Mn^{2+} toxic stress (Figure 3.5).

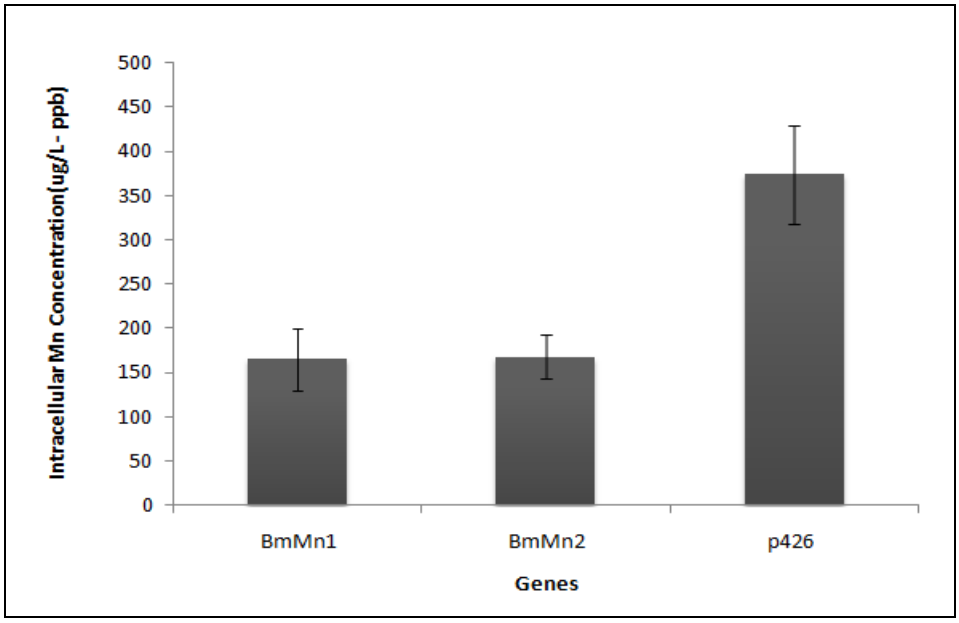

Figure 3.5. The results of manganese concentrations in transformed Δ*pmr1* strains**.** pAG426GPD (p426) represents control group. The cells were grown in 1 mM Mn^{2+} supplemented YNB-ura medium for 48-60 hours at 30 °C. Values are the mean of four independent experiments and the bars represent standard error of mean with 95% confidence.

Measurement of intracellular Mn concentration showed that Mn^{2+} concentrations in transformed Δ*pmr1* strains with *BmMn1* or *BmMn2* were significantly lower than transformed Δ*pmr1* strain with *pAG426GPD* (374 ppb). It was observed that Mn^{2+} accumulations in Δ*pmr1* strain with BmMn1 (165 ppb) and Δ*pmr1* strain with BmMn2 (168ppb) are quite similar. These result indicated that both BmMn1 and BmMn2 have a role in transport of excess Mn^{2+} out of the cells.

3.4. Identification of BmMn1 and BmMn2 Localization

Localization in yeast of BmMn1 and BmMn2 with a C-terminal GFP fusion revealed a punctuated pattern (Figure 3.6) resembled that of Golgi localized proteins, such as Pmr1p (Mandal, Rulli, and Rao 2003) and Gyp1p (Du and Novick 2001).

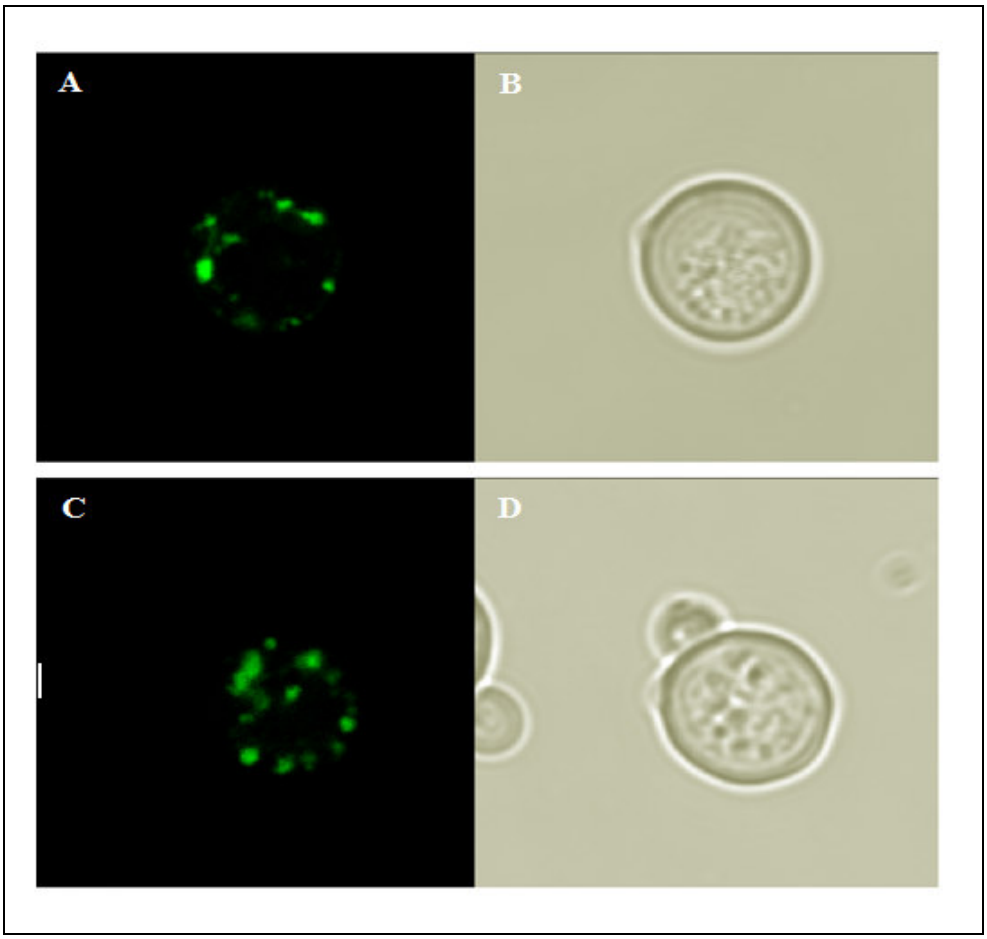

Figure 3.6. BmMn1 and BmMn2 localization in yeast cells. BmMn1:GFP and BmMn2:GFP fusion proteins localize in a punctuated pattern resembling the Golgi apparatus. Confocal Laser Scanning Microscopy of InvSc1 yeast cells expressing BmMn1:GFP(A) and BmMn2:GFP(C) were performed after 5 h of induction. Bright-field images (B and D) are shown for the cells shown in (A) and (C), respectively.

3.5. Gene Expression Analysis

Beta vulgaris subsp. maritima plants were exposed to 2 mM Mn^{+2} for 12 hours and every 3 hours, leaves and roots samples were harvested. Total RNAs were isolated from the samples and before cDNA synthesis the quality of the total RNAs were controlled by performing agarose gel (1%) electrophoresis (Figure 3.7). cDNAs were synthesized from the total RNAs and they were used for gene expression analysis performing with qRT-PCR.

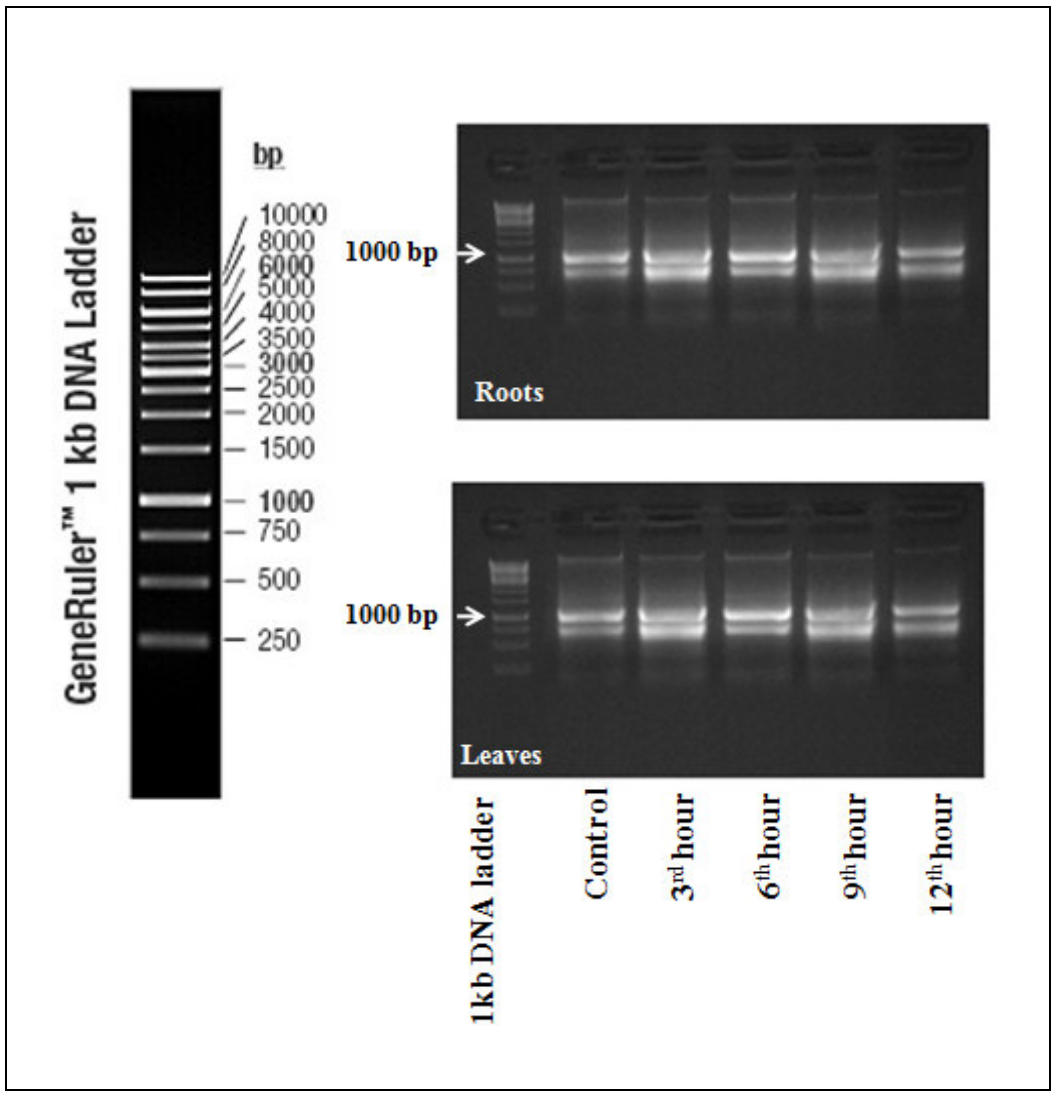

Figure 3.7. The quality control of total RNAs. They were obtained from leaves and roots samples which were harvested in every 3 hours under 2 mM Mn^{2+} exposing.

3.5.1. *BmMn1* Expression

The mRNAs abundance of *BmMn1* in roots showed no significant regulation under Mn^{2+} toxicity (Figure 3.8). Although increment of the mRNAs was observed in 6th hour as 0,5 fold and in 9th hour as 0,67 fold (the highest fold induction), these results did not show any remarkable mRNA induction under Mn^{2+} toxicity when they are compared to control group.

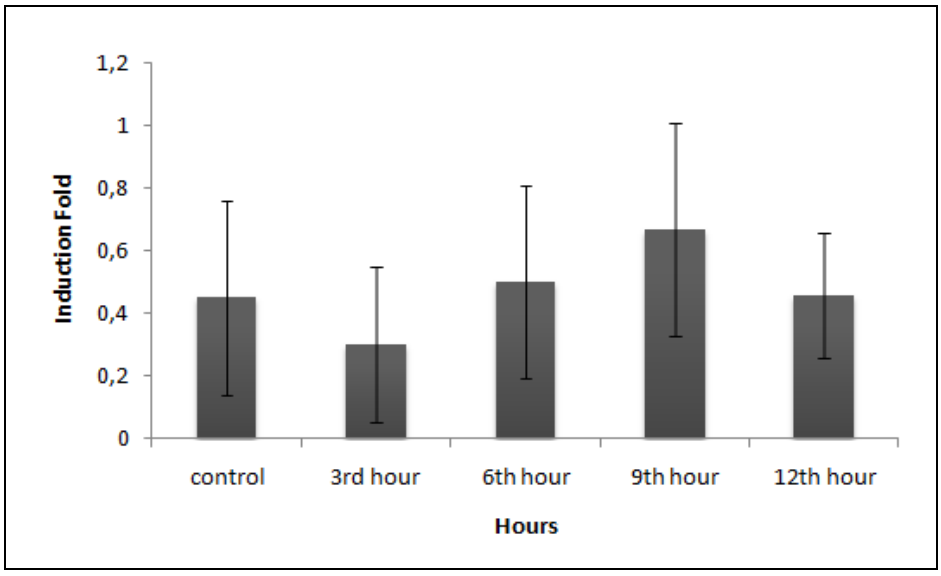

Figure 3.8. Expression levels of *BmMn1* in the roots. The cDNAs from the roots exposed to Mn^{2+} and the cDNAs from the roots of the control group(not exposed to Mn^{2+}) obtained in every three hours. 2 mM Mn^{2+} stress were applied. Values are the mean of three independent experiments and the bars represent standard error of mean with 95% confidence.

3.5.2. *BmMn2* Expression

The expression level of *BmMn2* in the roots decreased until at the end of 6^{th} hour but then, it increased at 9^{th} and 12^{th} hours (Figure 3.10). The remarkable gene expression was observed at 9^{th} with 5 folds under Mn^{2+}toxicity. From this result it may concluded that *BmMn2* was induced to express only after 9^{th} hour of treatment.

The mRNA concentration for *BmMn2* in the leaves was different compared to the result of *BmMn2* root expression (Figure 3.11). The first induction of the gene was observed at 3^{rd} hours remarkably (with 10,52 induction fold). However, this induction reduced dramatically from the 3^{rd} hour.

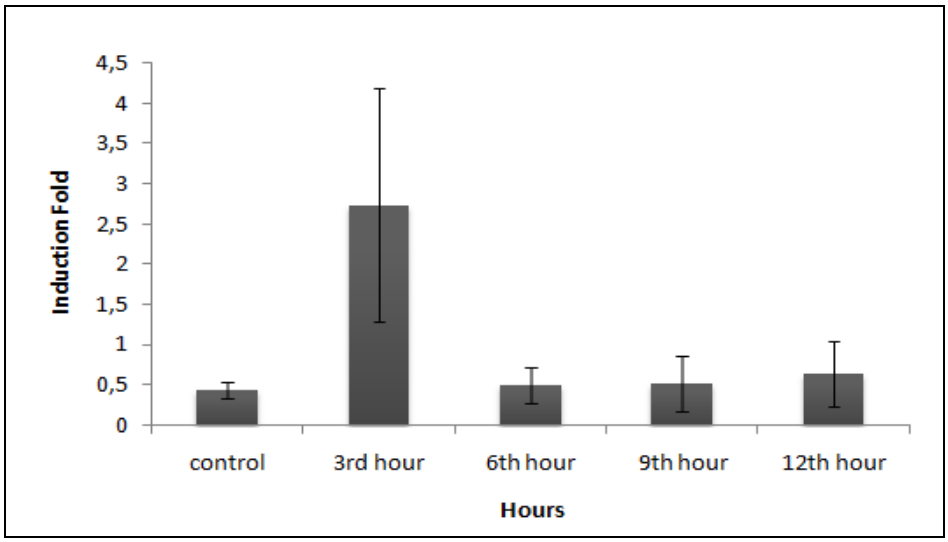

Figure 3.9. Expression levels of *BmMn1* in the leaves. The cDNAs from the leaves exposed to Mn^{2+} and the cDNAs from the leaves of the control group (not exposed to Mn^{2+}) obtained in every three hours. 2 mM Mn^{2+} stress were applied. Values are the mean of three independent experiments and the bars represent standard error of mean with 95% confidence.

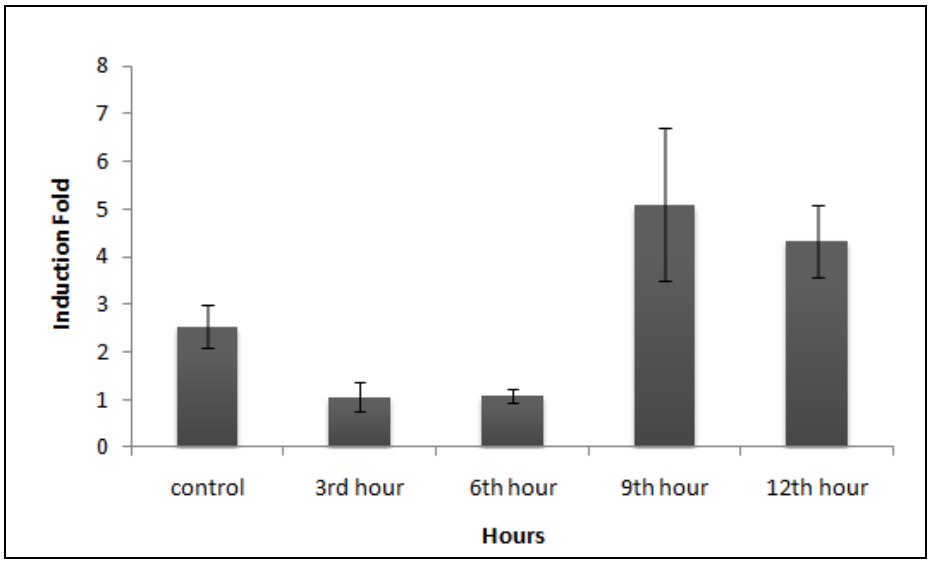

Figure 3.10. Expression levels of *BmMn2* in the roots. The cDNAs from the roots exposed to Mn^{2+} and the cDNAs from the roots of the control group(not exposed to Mn^{2+}) obtained in every three hours. 2 mM Mn^{2+} stress were applied. Values are the mean of three independent experiments and the bars represent standard error of mean with 95% confidence.

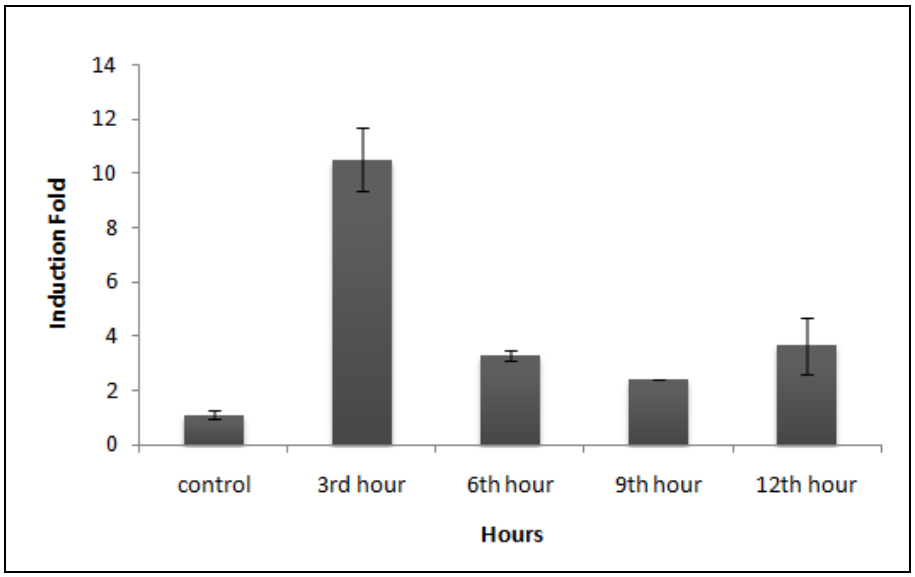

Figure 3.11. Expression levels of *BmMn2* in the leaves. The cDNAs from the leaves exposed to Mn^{2+} and the cDNAs from the leaves of the control group (not exposed to Mn^{2+}) obtained in every three hours. 2 mM Mn^{2+} stress were applied. Values are the mean of three independent experiments and the bars represent standard error of mean with 95% confidence.

CHAPTER 4

DISCUSSION

Plants do not have a power of locomotion so that they have to adapt challenging environments therefore the chance of living under such environments is higher than other organisms. However, some plants show greater adaptation to the environment when they have adequate genotypes (Lidon 2002) like *Beta vulgaris subsp. maritima.* It has many stress resistance genes providing tolerance to different stressful conditions such as salinity (Doney 1993).

Manganese is very abundant in earth's crust (Aubert and Pinta 1977). It is an essential nutrient for plants. However, some conditions such as increasing acidity in soils stimulate manganese absorption from roots (Maas, Moore, and Mason 1968) leading accumulation of excess level of Mn^{2+} which causes a toxicity.

Metal tolerance proteins are insurances of plants which protect them in case of exposing excess of metals. Transporter proteins provide metal homeostasis in plant cells. Many transporter proteins giving manganese tolerance to plants have been identified. These proteins are localized only one part of plants unlike, some of them is synthesized in different parts of plants such as roots or leaves.

Manganese is uptake into cells by AtIRT1 (Korshunova et al. 1999), OsYSL2 (Koike et al. 2004; Ishimaru et al. 2010) or AtNRAMP1 (Cailliatte et al. 2010). ShMTP1 (Delhaize et al. 2003) and AtCAX2 (Hirschi et al. 2000) transport manganese to vacuole. Other vacuole transporters AtNRAMP3 and AtNRAMP4 (Thomine et al. 2003; Lanquar et al. 2010; Oomen et al. 2009) provides efflux of Mn^{2+} out of vacuole to be used and AtCCX3 vacuolar membrane protein (Morris et al. 2008) keeps manganese homeostasis in cell.

Some proteins have a role in the secretory pathway. AtECA1 (Wu et al. 2002) is localized in ER and provides sequestration of manganese to reduce its concentration into cell. AtECA3 (Mills et al. 2008) and AtMTP11 (Delhaize et al. 2007; Peiter et al. 2007) is different to AtECA1 and they assure manganese homeostasis in Golgi.

In this study, we aimed to identify Mn^{2+} transporter genes which are able to provide a tolerance of excess manganese in *Beta vulgaris subsp. maritima.*

One of the accepted ways of identification of transporters in plants is screening of cDNA library in yeast deletion mutants (Korshunova et al. 1999). This opportunity has been used for identification of many transporter genes due to an easy way to implement.

PMR1 a member of P-type ATPase family identified as manganese and calcium ion transporter provides manganese and calcium transport into Golgi in yeast (Lapinskas et al. 1995; Mandal, Woolf, and Rao 2000). In this study, Δ*pmr1* yeast strain was transformed with the cloned cDNA library from *Beta vulgaris subsp. maritima* and we screened the cDNA library with 2, 7 mM Mn^{2+} to identify the genes which are able to tolerate excess of manganese. Two genes, *BmMn1* and *BmMn2* being able to tolerate toxic amount of Mn^{2+} (8 mM) were identified. In addition, metal tolerance assay with other metals pointed that these two genes provide significant Mn^{2+} tolerance in contrast to Co^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+} and B^{2+}.

Similarity analysis between BmMn1, BmMn2 and other manganese transporter proteins showed that PtrMTP11.1 (88% identity) and AtMTP11 (86% identity) are significantly similar to BmMn1. In addition, ShMTP1 (80%identity) revealed high similarity with BmMn2. The phylogenetic tree pointed that AtMTP11, PtrMTP11.1 and BmMn1 come from the same ancestor like BmMn2 and ShMTP1 therefore they are probably orthologous.

The localization of proteins were predicted as they localize in Golgi like AtMTP11 (Peiter et al. 2007) or in plasma membrane as integral membrane proteins because the manganese concentrations in transformed Δ*pmr1* yeast strains revealed that *BmMn1* and *BmMn2* decrease excess manganese into cell. Therefore GFP localization in yeast cells for BmMn1 and BmMn2 were observed and the results indicated that BmMn1 and BmMn2 are located in Golgi.

In addition, the gene expressions for *BmMn1* and *BmMn2* were identified by qRT-PCR after application of 2 mM Mn^{2+} to *Beta vulgaris subsp. maritima*. The root expression level of *BmMn1* showed no significant regulation. The highest mRNA expression level was observed at 9^{th} hour. BmMn1 transcripts in leaves were found to be 8 times more abundant in samples harvested at 3^{rd} hour than control group. Moreover, *BmMn1* expression in leaves was observed to be 6 times higher than in roots. The *BmMn2* transcripts in root samples were found 2 times abundant than control group at 9^{th} hour. Similar to the expression results for BmMn1 in leaves, the highest *BmMn2* was observed at 3^{rd} hour as 9 times higher than control group.

The leaves expression levels of *BmMn2* was observed similar to the leaves expression of *BmMn1*. The highest gene expression was revealed at 3rd hour. *BmMn1* expression in leaves was observed to be 2 times higher than in roots. It was observed that the expression levels of *BmMn2* in both roots and leaves were significantly higher than the expression levels of *BmMn1* under 2 mM Mn^{2+} stress.

The gene expression levels in previous studies were compared to our results. The expression levels for *AtNRAMP1* were observed 10 times higher in roots than leaves. Moreover, as a manganese importer in plasma membrane, the expression level of *AtNRAMP1* was significantly increased under Mn^{2+} deficiency in roots (Cailliatte et al. 2010). Therefore, it was concluded that *AtNRAMP1* are significantly synthesized in roots and provides Mn^{2+} absorption into cell under Mn^{2+} deficiency from roots. Moreover, it is known that *AtMTP11* is expressed in both leaves and roots and has a role in transport of excess Mn^{2+} out of the cell via vesicular mediated exocytosis. However, according to (Delhaize et al. 2007), after application of Mn^{2+} between 4,5 µM and 2 mM concentration, no significant changes for the gene expression were observed in whole parts of seedlings. In addition, according to (Peiter et al. 2007), the *AtMTP11* expression in roots and shoots of wild type *Arabidopsis* transformed with *AtMTP11* were lower when it was compared to control group (0 mM Mn^{2+}) under 2mM Mn^{2+} application and the gene expression in shoot is higher than roots. Furthermore, (Peiter et al. 2007) also performed another gene expression showing the gene regulation with different hours under 1mM Mn^{2+} application and it was seen that the shoot expression was decreased for between 0-48 hours. On the other hand, the roots expression showed a dynamic regulation. The maximum gene induction was observed after 6 hour Mn^{2+} stress application.

In this study, *BmMn1* and *BmMn2* providing manganese tolerance in *sea beet* were identified after scanning the cDNA library in yeast. The protein localization in yeast showed that BmMn1 and BmMn2 located in Golgi. In addition, it was identified that these two genes are highly selective for manganese transport by metal tolerance assay. Moreover, manganese concentration in transformed yeast cells with the genes and empty vector as a control group revealed that *BmMn1* and *BmMn2* have a role in manganese efflux out of cells. Although these experiments provided the identification of *BmMn1* and *BmMn2* in yeast, the similar experiments should be performed in plant cells. In addition, it must be noted that, protein analyses is needed to perform for identifying exact role of these proteins in manganese transport pathway in plants.

CHAPTER 5

CONCLUSION

In this study, two genes; *BmMn1* and *BmMn2* were identified in *Beta vulgaris subsp. maritima.* Similarity analysis and phylogenetic tree of these two proteins showed that they were orthologous of AtMTP11, PtrMTP11.1 and ShMTP1. Intracellular manganese measurements of these two genes revealed that they efflux Mn^{2+} out of the cell. BmMn1 and BmMn2 are localized in Golgi as coated-vesicle proteins in yeast. The gene expression results by qRT-PCR showed that these two proteins are localized in both roots and leaves. It is concluded that *BmMn1* and *BmMn2* isolated from *Beta vulgaris subsp. maritima* have a role in manganese detoxification.

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APPENDIX A

NUCLEOTIDE SEQUENCES

BmMn1 nucleotide sequence

*Start and stop codons are underlined.

BmMn2 nucleotide sequence

*Start and stop codons are underlined.

APPENDIX B

PROTEIN SEQUENCES

BmMn1 protein sequence

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  1 M L E G F S E M D A L A E R G F V P G M S K E E R E K L A K S E T F A I R L S N I
 42 A N M V L F I A K V Y A S I Q S G S L A I I A S T L D S L L D L L S G F I L W F T
 83 A F S M S T P I P Y Q Y P I G K K R M Q P L G I L V F A S V M A T L G L Q I I L E
124 S T R Q L A S D E S D F S L N N D Q E R W L V G I M L S V T L V K L L L V L Y C R
165 S F T N E I V K A Y A Q D H F F D V I T N I I G L I A A L L A N Y V S D W M D P V
206 G A I I L A L Y T I R T W S M T V L E N V N S L V G R S A T P D F L Q K L T Y L C
247 W N H H K A I R H I D T V R A Y T F G S H Y F V E V D I V L P A S M P L Q E A H D
288 I G E S L Q E K L E Q L S E I E R A F V H L D Y E Y T H K P E H A Q A H L
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BmMn2 protein sequence

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  1 M E N I T E T G C F P G S M T E D E M K Q L A K S E R M A V N I S N A A N V V L F
 42 L A K I Y A S I E S R S L A V I A S T L D S L L D L L S G F I L W F T S Y A M R K
 83 P N Q Y Y Y P I G K K R M Q P V G I I V F A S V M A T L G L Q I L L E S G R D L L
124 T K T G P K M N H R Q E M W M V G I M V S V T V V K F V L M I Y C R R F K N E I V
165 R A Y A Q D H F F D V V T N S I G L A T A V L A V R F Y W W I D P T G A I I I A L
206 Y T I S T W A K T V I E N V W S L I G R T A P P E F L A K L T Y L I W N H H E E I
247 K H I D T V R A Y T F G S H Y F A E V D I V L P E K M P L N Q A H N I G E T L Q E
288 K L E Q L Q E V E R A F V H I D F E Y T H R P E H K A K V
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