IDENTIFICATION AND CHARACTERIZATION OF NICKEL TOLERANCE GENES IN *BETA MARITIMA*

A Thesis Submitted to
the Graduate School of Engineering and Science of
Izmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Molecular Biology and Genetics

by
Gönensin Ozan BOZDAĞ

December 2009
İZMİR

We approve the thesis of **Gönensin Ozan BOZDAĞ**

Assist. Prof. Dr. H. Çağlar KARAKAYA
Supervisor

Assoc. Prof. Dr. Talat YALÇIN
Committee Member

Assoc. Prof. Dr. Ahmet KOÇ
Committee Member

16 December 2010

Assoc. Prof. Dr. Sami DOĞANLAR
Head of the Department Molecular Biology and Genetics Department

Prof. Dr. Hasan BÖKE
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGMENTS

First of all, I would like to thank my advisor Assist. Prof. Dr. H. Çağlar KARAKAYA for giving me the chance to study in this motivating project and also for his support, guidance and helps in every part of this project.

I would like to thank my committee and laboratory member, Assoc. Prof. Dr. Ahmet KOÇ for his suggestion and offers.

I also would like to thank my committee member Assoc. Prof. Dr. Talat YALÇIN for his suggestions and contributions.

I would like to thank Prof. Dr. Dirk PRUFER at Universität Münster Germany.

I would like to thank Prof. Dr. Ahmet E. EROĞLU and Meral KARACA.

I also would like to thank all my lab. friends Önder KİMYON, Erhan BAL, A. Banu DEMİR, Alaattin KAYA, Elise HACIOĞLU, Beren ATAÇ and İrem ULUIŞIK.

I would like to thank my friend Yeliz COŞKUN

Finally, I would like to thank to my family for the supports that they showed in every stage of my life.

ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF NICKEL TOLERANCE GENES IN *BETA MARITIMA*

Nickel is an essential micronutrient for plant growth. However, an excess amount of nickel is toxic for plants and animals. The *Beta maritima* plant which is known crop as salt and manganese tolerant nature. In this study, we aimed to identify nickel detoxification metabolism genes of *Beta maritima* by screening plant's overexpressed cDNA library in yeast cells. After initial screening, three transformants were isolated that were able to grow in the presence of toxic nickel concentration. After the sequence analyses, three genes were named as *NIC3*, *NIC6* and *NIC8*. We tested the growth rates of the yeast cells those were overexpressing cDNAs in solid mediums and showed similar nickel detoxification patterns. Then intracellular nickel concentrations were measured to see whether mentioned cDNAs pump nickel out of the cell or not. We also analysed the gene expression levels of *NIC3* and *NIC6* genes after appllying nickel stress to the plants to see the relations between nickel treatment and transcription changes of these two genes. Our data suggest that *NIC3*, *NIC6* and *NIC8* genes, besides their other functions, confer yeast cells nickel tolerance and may play roles in heavy metal detoxification for plant cells.

ÖZET

BETA MARİTİMA BİTKİSİNDE NİKEL TOLERANS GENLERİNİN TANIMLANMASI VE KARAKTERİZASYONU

Nikel bitki büyümesi için dışarıdan temin edilmesi gereken bir mikrobesin maddesidir. Bununla birlikte, nikelin aşırı miktarları bitki ve hayvanlar için toksik etki yapmaktadır. İyi tanınan bir ekin olan *Beta maritima* bitkisi, doğal olarak sahip olduğu tuz ve manganaz direnci sebebi ile, nikel toksisite metabolizma genlerini çalışmak için ideal bir organizmadır.. Bu çalışmada, *Beta maritima* bitki cDNA kütüphanesini maya hücrelerinde tarayarak nikel detoksifikasyonu ile ilişkili genleri tanımlamayı amaçladık. İlk tarama sonrasında toksik nikel derişimlerinde büyümeyi başaran üç transformant izole edildi. Dizi analizlerinden sonra, üç gen *NIC3*, *NIC6* ve *NIC8* olarak adlandırıldı. cDNA'ları aşırı düzeylerde ifade eden maya hücrelerinin büyüme oranlarını katı besiyerlerinde test ettik ve genlerin tümü benzer bir eğilim gösterdiler. Ardından adı geçen cDNA'ların nikeli hücre dışına atıp atmadığını görmek için hücre içi nikel derişimleri ölçüldü. Ayrıca, nikel uygulanması ve genlerin transkripsiyon seviye değişimleri arasındaki ilişkiyi gözlemek için, bitkilere nikel stresi uyguladıktan sonra *NIC3* ve *NIC6* genlerinin ifadelenme seviyelerini inceledik. Tüm bunların sonunda, elde ettiğimiz veriler, *NIC3*, *NIC6* ve *NIC8* genlerinin, sahip oldukları diğer işlevler dışında, maya hücrelerine nikele karşı tolerans sağladığını ve bitkilerde ağır metallerin toksik etkilerinin giderilmesinde görevleri olabileceklerini göstermektedir.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. *Beta maritima* Plant

Sugar beet, *Beta maritima* is in the *Chenopodiaceae* family same as the *Beta vulgaris* (Plants for a Future 2008). Sugar beets prefer alkaline and saline habitats.

It is known as having salinity tolerance with the ability to survive under high salt containing habitats (Rao, et al. 2006). It has also been reported to have tolerance to manganese toxicity (Duke 1983). This naturally tolerant structure of sugar beet makes it a useful candidate for stress tolerance studies.

1.2. Nickel and Plants

At the end of the 1970's there were enough evidence for Nickel to be essential element for many species such as bacterias and animals (Eskew, et al. 1984). But for higher plants, this essentiality has been confirmed at the end of the 1980's, and from then nickel is said to be as an essential micronutrient for plant growth (Eskew, et al. 1984).

In the metabolism of living systems, the primary role of nickel is its characteristic to bind some specific enzymes as a co-factor, and turn them to functionally active state (Watt and Ludden 1999). Firstly identified nickel enzyme is urease and activity of this enzyme directly determines essentiality of nickel for higher plants (Dixon, et al. 1975). Urease converts urea to ammonia and carbamate by using water. But for performing this reaction, urease requires nickel at its active site.

When plants grown in soils containing urea as nitrogen source, nickel becomes essential for integrity of these plants. Because, without nickel supply, urease can not form its functionally active conformation, which leads to accumulation of urea as a highly toxic component (Eskew, et al. 1984). This well known function of nickel makes it an essential element for plants.

1.3. Nickel Toxicity

On the other hand, as all other heavy metals, nickel is toxic to plants when it presents in the excess amounts. Up to date, most researchs those dealing with the nickel toxicity and plants, focused on to nickel hyperaccumulators. These hyperaccumulators are capable of accumulating extremely high levels of nickel at their cell compartments. In nature, about 300 plant species are identified as nickel hyperaccumulators (Rengasamy and Doran 2002). And strategies to produce plants those are resistance to toxic levels of nickel mainly depending on to understand genetic and biochemical features of these hyperaccumulators.

High levels of metals inhibits the growth of the plants which therefore decrease the food production. What makes metal toxicity very harmful is these metals can not be metabolically transformed into non-toxic forms (Rengasamy and Doran 2002). And these toxic effects cause damages from cellular levels to tissue and organ levels.

Metals, commonly, bind to proteins, lead to structural and functional matters. The non-selectivity of these interactions affect whole metabolism of cell (Van Assche and Clijters 1990). Redox imbalance, metabolic imbalance, ionic disturbance are the result of the metal toxicity which includes metals such as Ni , Cd and As (Sharma and Dietz 2008).

Besides these direct damages of heavy metals, these elements also lead to a very harmful effect by via free radical formation that is called oxidative stress (Sharma and Karl-Josef 2008). Oxidative stress forming free radicals bind biological macromolecules such as lipids, DNA, mRNA and proteins. Thus ROS cause functional and structural damages, effects metabolic balance (Figure 1.1.). The main reactive oxygen species those formed in plant cells are superoxide, hydroxyl radicals and peroxides (Rengasamy and Doran 2002). Dietz et al. (1999) showed that nickel stress leads to oxidative stress via indirect ways. Therefore nickel is classified as a non-redox-active metal (Rengasamy and Doran 2002). But indirectly occuring reactive oxygen species also lead important oxidative damage inside the cells.

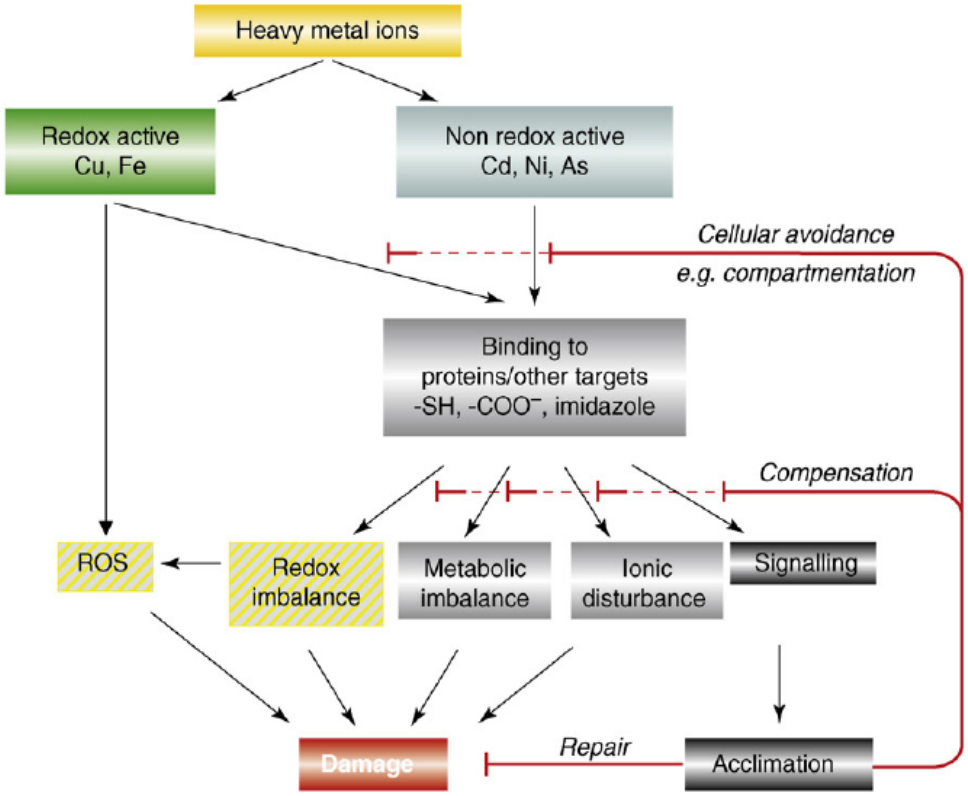


Figure 1.1. Heavy Metal induced damage pathways in plant cells (Source: Sharma and Dietz 2008).

1.4. Metal Detoxification

Cells have evolved many different metal detoxification pathways, but, these pathways are conserved in diverse species. Detoxification pathways are common for different heavy metals (Dameron and Harrison 1998).

For detoxification of heavy metals, all type of cells have three main mechanisms: First is the removal of toxic metals from inside of the cell through the extracellular region. Secondly, compartmentation of these toxic elements in some organelles, such as vacuoles, to keep them away from the active metabolism. And thirdly, by increasing the levels and activities of antioxidant enzymes, such as SOD (superoxide dismutase), CAT (catalase), cells, decrease the oxidative stress effects caused by those metals (Freeman, et al. 2005). These detoxification mechanisms can be classified in more topics as shown in Figure 1.2.

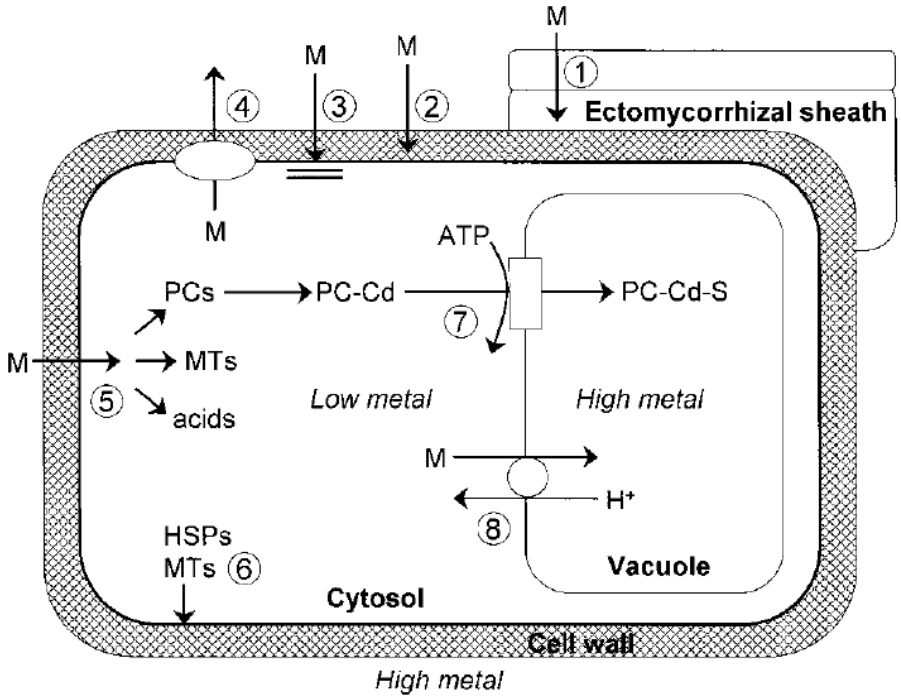


Figure 1.2. Heavy metal detoxification pathways in plant cells. Against oxidative stress effect of heavy metals, overexpression or increase in the activities of antioxidant enzymes also can be added. (Source: Hall 2001)

1.5. Transporter Dependent Detoxification

From unicellular to multicellular organisms, species have common and general metal detoxification patterns. Limited uptake is an effective way of metal detoxification. This occurs by limited expression of the metal specific transporter or inhibition of the uptake proteins (Dameron, et al. 1998).

Exporting and sequestring of the metal ions are very common and effective metal detoxification ways in diverse organisms. Sequestring or exporting of metals decrease the cytosolic concentrations of toxic metal, which therefore removes the metal ions to the metabolicaly less active organelles (Hall 2001).

Recent studies have identified some transporter genes from hyperaccumulators (Freemann, et al. 2005; Mizuno, et al. 2005). Transporters from the accumulator species have been tested for their nickel transport activities at unicellular model organisms, like *E. coli*, *Saccharomyces cerevisiae.* There are also some other nickel transporter genes identified at *Maize*, *Tobacco* and *Arabidopsis thaliana* (Forzani, et al. 2001; Arazi, et al. 1999; Wire´n, et al. 2006). One important point for these transporters is that they have also been tested for their abilities to transport other metal ions such as Zn, Cd, Mo, Fe,

Pb *etc.* and shown to be able to transport some of these metals (Mizuno, et al. 2005; Forzani, et al. 2001; Wire´n, et al. 2006).

Vacuole has a great importance at detoxification of elavated metal ions by compartmentation especially in hyperaccumulator plants. When it was compared the abilities of the vacuolar compartmentation in nickel hyperaccumulator and non-accumulator species, Kramer et al. (2000) have shown that Ni-tolerant hyperaccumulator *T. goesingense* is able to accumulate almost 18 % of its cellular nickel content inside the vacuoles, whereas non-accumulator *T. arverse* could only accumulate 8.3 % of its intracellular nickel content inside the vacuoles.

1.6. Secretory Pathway Dependent Detoxification

There are also other metal detoxification pathways differently than the ones mentioned above and called as secretory pathways. It is well studied that the secretory vesicles involve in protein folding, translocation, and with the endocytosis, taking inside the some required molecules from cells surrounding. But those secretory pathways are also involve in metal detoxification processes in cells. E.R., Golgi and small vesicles between these organelles leads to detoxification of the metals by removing heavy metals from cytosol and by exporting metals through the cell membrane (Wu, et al. 2002; Dürr, et al. 1998). For example ECA1 protein located on endoplasmic reticulum leads to the uptake of manganase and confers manganase tolerance for the cell (Wu, et al. 2002). Similar strategies for metal uptake also have been reported for Golgi and for other metals (Dürr, et al. 1998). But for yeast and also for plants, details of these pathways stil remains unknown.

1.7. Detoxification of Heavy Metal Induced Oxidative Stress

The other important harmful effect of heavy metal stress is, it also causes oxidative stress in plants. By its indirect effect, nickel dependent oxidative stress leads to oxidation of biological macromolecules. Those oxidation changes the structures of molecules which then causes to loss of activity (Rengasamy and Doran 2002). Researchs, those comparing nickel hyperaccumulators and non-accumulators showed that antioxidant enzyme activities, which protects cells against those type of metal

stress, were critically higher in hyperaccumulators. And after treatment with nickel , an increase in the activities of antioxidant enzymes such as SOD and CAT have been shown. Catalase activity were nearly 500 –fold higher in nickel hyperaccumulator (Rengasamy and Doran 2002). And this greater activity has very important role in nickel induced oxidative stress detoxification. Also, glutathione and SAT (serine acetyltransferase) levels have been shown to be important at increasing resistance to nickel related oxidative stress in *Thlaspi* nickel hyperaccumulator (Freeman, et al. 2004).

1.8. Role of S-Adenosyl-L-Methionine in Metal Induced Oxidative Stress

Glutathion (GSH) is a very important defensive molecule against free radicals. It is called as a "major" defensive tripeptide (Lieber 2002). It reacts with free radicals and converts them to non-toxic metabolic forms. So, the levels of GSH is very important at oxidative stress conditions. GSH contains cysteine amino acid which determines the rate of glutathione.

SAMe (S-adenosyl-L-methionine) has important role in GSH synthesis. It is a precursor molecule for homocystein, which then turns to cystein form. Thus the formation of GSH occurs (Lieber 2002).

Also the gene family of S-adenosylmethionine decarboxylase (SAMDC) is shown to confer tolerance to diverse abiotic stress conditions such as salt stress, acidic stress and cold stress. All these environmental stress conditions lead to oxidative stress (Wi, et al. 2006). With the increase at the decarboxylated SAM levels, transgenic tobacco plants showed increased resistance those environmental stess conditions. Wi et al. (2006) also measured the polyamine (PA) levels in stress tolerant tobacco cells and showed the increased levels of PAs. So, PAs also may play a role in stress tolerance in plants related to SAM levels.

SAMDC overexpressing plants have increased transcript levels for antioxidant enzymes such as ascorbate peroxidase (APx), mitochondrial manganese superoxide dismutase (Mn-SOD) and glutathion-S-transferase (GST) which also points out another important link between stress tolerance and SAM levels (Wi, et al. 2006).

CHAPTER 2

MATERIAL AND METHODS

2.1. Yeast Growth and Media

The yeast *Saccharomyces cerevisiae* strain BY4741 (*MATa*; *his3*; *leu2*; *met15*; *ura3*) was used in the experiments. YPD media (with 2 % glucose, 2 % peptone, 1 % yeast exract and 2 % agar) and YNB minimal media without uracil supplement were used for yeast growth.

2.2. Plant Growth and RNA Isolation

Beta maritima seeds firstly germinated in soil. And then were grown in half strength Hoagland Solution which consists of 3.5 mM $Ca(NO_3)_2.4H_2O$, 2.5 mM KNO_3, 1 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$, 22 µM H_3BO_3, 4.5 µM $MnCl_2.4H_2O$, 0.35 µM $ZnSO_4.7H_2O$, 0.2 µM $CuSO_4.5H_2O$, 0.07 µM $NaMoO_4$, 15 µM EDTA.2Na, 14 µM $FeSO_4.7H_2O$ and 0.5 mM KOH . Conditions of the growth chamber was as 12 hours dark and 12 hours light photoperiod with a 40 µmol m^{-2} s^{-1} light intensity. The temperature was 25 ^{0}C. Humidiity has been setted to 50,0 (as % rH). 75 µM $NiCl_2$ were applied for 7 days. Total RNA isolation was performed by Invitrogen RNA Isolation Kit according to manufacturer's instructions (Invitrogen, Germany). DNAse treatment by Fermentas DNase I Kit were performed (Fermentas, Germany).

2.3. Synthesis of Gateway® –Compatible cDNAs

Gateway –compatible cDNAs have been synthesized using the procedure that explained by Ni et al (2007). 4 µl total RNA and attB2 (5' ACCACTTTGTACAAGAAAGCTGGGT-3') primers (15 µM) have been mixed and following incubation as 72 ^{0}C for 2 miutes kept in ice for 2 minutes. 2 µl (5X) First st. Buffer, 1 µl DTT (20 mM), 1 µl dNTPmix (10 mM) and 1 µl Power Reverse

Transcriptase have been added to the initial mixture and incubated at 42 ^{0}C for 1 hour. After this incubation, 1 µl attB1 (5' ACAAGTTTGTACAAAAAAGCAGGCT-3') primer (15 µM) added lastly and the final mixture has been incubated at 42 ^{0}C for 1 hour.

Double-stranded cDNA synthesis has been performed as 80 µl dH_2O, 10 µl (10X) Advantage 2 PCR buffer, 2 µl (10 mM) dNTPmix, 2 µl (15 µM) attB1 primer, 2 µl (15 µM) attB2 primer, 2 µl Adv. 2 Polymerase Mix and finally cDNA template from first step as 2 µl have been mixed. The PCR was performed on Aplied Biosystems Gen Amp® PCR System 9700 with the following cycling conditions: 1 min initial denaturation at 95 ^{0}C, 20 cycles of three temperatures as 15 seconds at 95 ^{0}C, 30 seconds at 64 ^{0}C, 6 minutes at 68 ^{0}C and finally one cycle 10 minutes at 68 ^{0}C.

The quality of synthesized cDNAs has been checked by performing agarose gel (1 %) electrophoresis for cDNA mixture.

2.4. Cloning of the cDNAs to Entry and Expression Clones with Gateway Technology®

Synthesized cDNAs have been loaded to agarose gel (1%) and electrophoresized at 100 V for 20 minutes. And >500 base pairs part of the gel excised and extracted by Fermentas DNA Extraction Kit (Fermentas, Germany).

2 µl (160 ng/µl) of extracted cDNA were introduced to 150 ng pDONR221 (Invitrogen, Germany) vector using 2 µl BP Clonase™ II enzyme with an overnight incubation at 25 ^{0}C. The reaction has been terminated by 2 µl protease K (Invitrogen, Germany) as 10 minutes at 37 ^{0}C and then 10 minutes at 70 ^{0}C.

The result of the cloning reaction transformed to the *E. coli* omnimax cells by heat shock method. Firstly the competent *E. coli* cells have been incubated in ice for 5 minutes. 2 µl of BP clonase reaction mixture added to omnimax competent cells and incubated at ice for 25 minutes. Then cells have been heat shocked at 42 ^{0}C for 30 seconds and 2 minutes at ice. 250 µl SOC medium added to cells and the mixture has been incubated for 1 hour at 37 ^{0}C 200 rpm. Then plated on LB + 50 µg/ml kanamycin containing plates. Grown colonies have been collected at 50 ml falcon tubes and the plasmids were isolated using PureLink™ Quick Plasmid Maxiprep Kit (Invitrogen, Germany).

LR Clonase reaction was performed with 2 µl isolated entry clones, 1.5 µl pAG426GPD (Invitrogen, Germany) expression vector, 3 µl TE buffer and 2 µl LR Clonase™ II enzyme mixture. Reaction performed overnight at 25 ^{0}C and 2 µl of the reaction mixture were transformed to the same cell line by the heat shock method which is described above. The transformation mixture has been plated on LB + ampicillin plates. Plasmids were isolated by PureLink™ Quick Plasmid Maxiprep Kit (Invitrogen, Germany).

2.5. Yeast Transformation of Cloned cDNA Library

Yeast transformation has been performed by LiAc method (Kaiser, et al. 1994). Yeast cells have been grown until mid-log phase at 5 ml YPD medium (with 2 % glucose, 2 % peptone, 1 % yeast exract) and washed by dH_2O. Pelleted cells were resuspenden by 0.1 M LiAc and transferred to 1.5 ml microcentrifuge tubes. Pelleted by 5 seconds top speed centrifuge and washed with 500 µl 0.1 M LiAc. After the removal of the LiAc following mixture has been added on cells: 240 µl PEG (50 % w/v), 36 µl (1 M) LiAc, 5 µl ssDNA, 10 µl DTT (0.3 mM), 20 µl plasmid DNA, 10 µl dH_2O, vortexed for 1 minute, incubated at 30 ^{0}C (160 rpm) for 30 minutes. Then taken to waterbath for 30 minutes at 42 ^{0}C. The cells have been plated on YNB-ura + 1600 µM $NiCl_2$ plates. Formed colonies numbered and were spreaded on YNB-ura plates to get more cells and then plasmid isolations have been. The isolated plasmids have been sent to the Biotechnology Center for the sequence analyses of the cloned *Beta maritima* genes.

2.6. Sequence Analyses of the Identified *Beta maritima* Genes

Blast searches were performed on BLAST service of NCBI and for the multiple protein sequence alignments ClustalW software were used. For phylogenetic tree of NIC6, software at http://iubio.bio.indiana.edu/soft/molbio/java/apps/trees/ have been used, by entering the newick format of the aligned sequences.

For NIC3 and NIC8 proteins, after blast search results, first four similar sequences have been chosen for multiple sequence alignment. After performing

alignment in ClustalW software, the tree c also have been taken from the result of the same software.

2.7. Solid Culture Experiments

Sequenced colonies have been tested for confirmation of their nickel resistance activities. Finally three of the colonies have been chosen for further analyses.

Wild type yeast cells transformed with void vector pAG426GPD or *NIC6*, *NIC3* and *NIC8* cDNAs containing pAG426GPD overexpression vectors. Overnight incubated yeast cells (shaking as 170 rpm, at 30 ^{0}C) were diluted to OD_{600}= 0,2; 0,02; 0,002; 0,0002 by distilled water and spotted as 5 µl on solid YNB (–ura; 2 % glucose) plates without $NiCl_2$ or with 1800 µM $NiCl_2$. Cells growth continued for 5 days at 30 ^{0}C.

2.8. Intracellular Nickel Measurements

Intracellular nickel concantrations were measured as described by T. Mizuno et al. (2005). Pre-cultured yeast cells containing empty-vector or vector overexpressing *NIC6*, *NIC3* and *NIC8* cDNAs were grown overnight in YNB –ura medium. Cells have been taken as 1/1000 ratio to new YNB –ura medium supplemented with 800 µM $NiCl_2$ and grown for 36 hours. Then cells were washed three times by 50 mM EDTA containing ultrapure water and dried overnight at 70 ^{0}C. 5 mg of yeast cells were extracted by 0.1 M HCl and filtrated with 0.20 µm Minisart® filters (Germany) . Ni measurements of the solutions were performed by atomic absorption spectrometer (AAS).

2.9. Real-Time PCR Analyses

75 µM $NiCl_2$ were applied to *Beta maritima* plats for gene expression analyses and in every 24 hours leaf and root samples have been collected. Same RNA isolation procedure applied for real-time analyses. cDNAs have been synthesized using Fermentas cDNA Synthesis kit (Fermentas, Germany). iQ5 real-time PCR cycler system were used for determining quantitave expression analyses (Bio-rad, Germany).

For the amplification 12.5 µl of Fermentas Maxima^{TM} SYBR Green qPCR Master Mix (2X) was used (Fermentas, Germany). For reverse and forward primers, 0.5 µl samples have been taken from 10 pmol primer stock solutions. 2 µl cDNA template has been used.

Real-time PCR primers for *NIC6* cDNA were: *NIC6* RT-F: 5'AGCCTATCGTCCCACCAAAGTTGT-3' and *NIC6* RT-R: 5' ACATGGGTGTTCCCTTTCAGACCA-3' and amplified 130 bp of cDNA.

Real-time PCR primers for Beta Actin cDNA were: BmActRTF 5'AGACCTTCAATGTGCCTGCT-3' and BmActRTR 5' TCAGTGAGATCACGACCAGC-3' and amplified 187 bp of *Beta maritima* beta actin cDNA.

Real-time PCR primers for *NIC3* cDNA were: Ni3RT-F 5'AGCAAGGTTGCTTGTGAGACTTGC-3' and Ni3RT-R 5'TGTCAGGACTCTGCTGCTCAATGT-3', amplified 196bp of the *NIC3* cDNA.

Real-time PCR primers for *NIC8* cDNA were: Ni8RT-F 5'AATAGGGCATCAGAACAGGCTCCA-3' Ni8RT-R 5'ACGAGTTCCAACGGTCAACTCAGA-3', amplified 144bp of the *NIC8* cDNA.

PCR amplification conditions were: Initial denaturation at 95 ^{0}C for 5 mins. as one cycle , and then 94 ^{0}C for 25 s, 59 ^{0}C for 25 s, 72 ^{0}C for 30 s as 40 cycles.

2.10. Differential pH Experiments for Ni6 cDNA Overexpressing Yeast Cells

Yeast cells containing void vector pAG426GPD or *NIC6* cDNA overexpressing pAG426GPD vector incubated overnight (shaking as 170 rpm, 30 ^{0}C) at liquid YNB-ura medium were diluted to OD_{600}= 0,2; 0,02; 0,002; 0,0002 by distilled water and spotted as 5 µl on solid YNB (–ura; 2 % glucose) plates without $NiCl_2$ or with 1800 µM $NiCl_2$ with the different pHs as 4.5, 6.5 and 7.5. For adjusting the different pH levels MOPS/TRIS and Acetate buffers. Cell growth continued for 5 days at 30 ^{0}C.

CHAPTER 3

RESULTS

3. 1. Identification and Cloning of the *NIC6*, *NIC3* and *NIC8* Genes

According to solid growth experiments, three *Beta maritima* cDNAs overexpressing yeast cells survived under toxic nickel containing mediums. After the isolation of plasmids from three colonies, plasmids sequenced using ABI 3130XL (Appendix A).

NIC3 open reading frame consists of 732 base pairs. And NIC3 is composed of 244 amino acids with a theoretical molecular weight of 26883,35 Da and isoelectric point as 4,87.

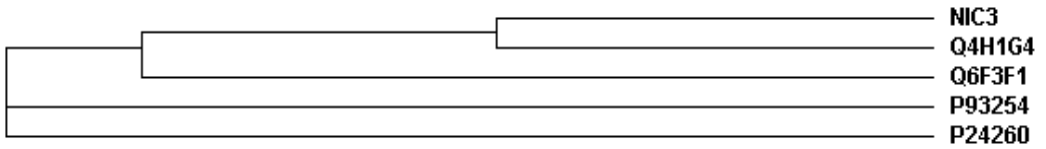
NIC6 open reading fraim is 582 base pairs length. NIC6 protein contains 194 amino acids. Theoretical molecular weight of NIC6 is 20891.91 Da and isoelectric point is 10,09.

And *NIC8* open reading frame consist of 603 base pairs. NIC8 protein is composed of 201 amino acids with a theoretical molecular weight of 22807.58 Da and isoelectric point of 5,89.

Homology of the three nickel detoxifying cDNAs have been searched database using blast(x) program. Multiple sequence alignment result for *NIC3*, *NIC6* and NIC8 have been shown in Figure 3.1., 3.2. and 3.3, respectively.

```
NIC3          MAAPIDTFLFTSESVNEGHPDKMCDQISDAVLDACLAQDPESKVACETCTKTNLVMVFGE 60
Q4H1G4        MAAPIDTFLFTSESVNEGHPDKMCDQISDAVLDACLAQDPESKVACETCTKTNLVMVFGE 60
Q6F3F1        MAAAVDTFLFTSESVNEGHPDKLCDQISDAVLDACLAQDPESKVACETCTKTNLVMVFGE 60
P93254        ----MDTFLFTSESVNEGHPDKLCDQISDAVLDACLAQDAESKVACETCTKTNLVMVFGE 56
P24260        MAAAADTFLFTSESVNEGHPDKLCDQISDAVLDACLAQDAESKVACETCTKTNLVMVFGE 60
                   ****************:***************.******************

NIC3          ITTKGNVDYEKIVRQSCRDIGFVSADVGLDADNCKVLVYIEQQSPDIAQGVHGHLTKRPE 120
Q4H1G4        ITTKGNVDYEKIVRQTCRDIGFVSADVGLDADNCKVLVYIEQQSPDIAQGVHGHLTKRPE 120
Q6F3F1        ITTKANVDYEKIVRQTCRDIGFVSADVGLDADNCKVLVYIEQQSPDIAQGVHGHLTRRPE 120
P93254        ITTKANVDYEKIARDTCRNIGFVSDDVGLDADNCKVLVYIEQQSPDIAQGVHGHLTKRPE 116
P24260        ITTKANVDYEKIVADTCREIGFVSPDVGLDADNCKVLVYIEQQSPDIAQGVHGHLTKRPE 120
              ****.*******. ::**:***** ******************************:***

NIC3          EIGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCPWLRPDGKTQVTAEY 180
Q4H1G4        EIGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCPWLRPDGKTQVTVEY 180
Q6F3F1        EIGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCPWLRPDGKTQVTVEY 180
P93254        EIGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCPWLRPDGKTQVTVEY 176
P24260        DIGAGDQGHMFGYATDETPELMPLSHVLATKLGARLTEVRKNGTCAWLRPDGKTQVTVEY 180
              :*****************************************.***********.**

NIC3          YNDNGAMVPIRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDERPSFHLNPSG 240
Q4H1G4        YNDNGAMVPIRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDEKTIFHLNPSG 240
Q6F3F1        YNENGAMVPIRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDEKTIFHLNPSG 240
P93254        YNENGAMVPVRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDEKTIFHLNPSG 236
P24260        YNENGAMVPIRVHTVLISTQHDETVTNDEIAADLKEHVIKPVIPEKYLDENTIFHLNPSG 240
              **:******:****************************************.. *******

NIC3          RFVI------------------------------------------------------- 244
Q4H1G4        RFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIVAS 300
Q6F3F1        RFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIVAA 300
P93254        RFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSGKDPTKVDRSGAYIARQAAKSIVAA 296
P24260        RFVIGGPHGDAGLTGRKIIIDTYGGWGAHGGGAFSRKDPTKVDRSGAYIARQAAKSIVAS 300
              ****
```

a)

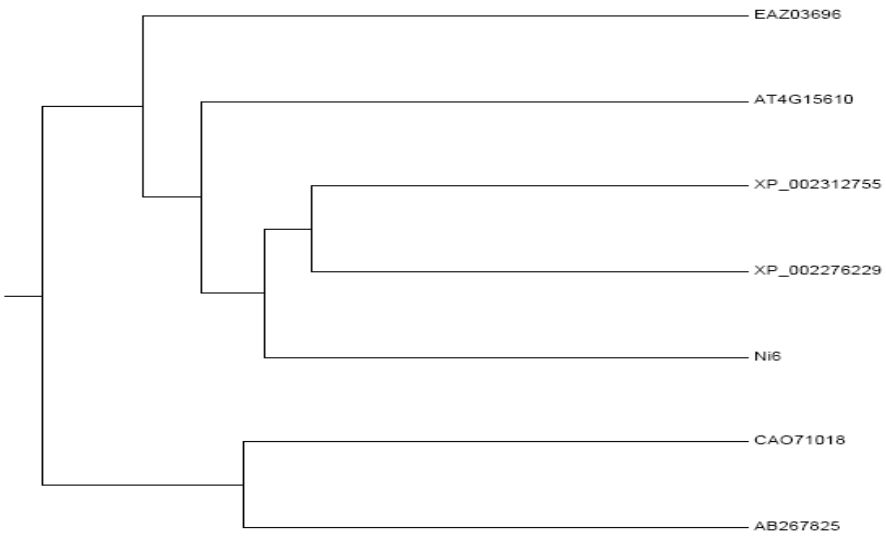


b)

Figure 3.1. Multiple amino acid sequence alignments of the deduced NIC3 protein sequence. A) Same colours on columns indicate conserved amino acids between *Beta vulgaris* (Q4H1G4), *Atriplex nummularia* (Q6F3F1), *Mesembryanthemum crystallinum*. (P93254), *Dianthus caryophyllus* (P24260) B) Phylogenetic tree of the proteins. All result have been performed using blastp program in blast.ncbi.nlm.nih.gov/Blast.cgi. And then alignments have been performed in ClustalW.

```
Ni6             MSSMETEKGAVP---TPQAPPVAPT----------DNKYRVVDVILRVLLLAASIASVVL 47
AB267825        ------------------------------------------------------------
XP_002276229    MSSVDTEKPAPPPLETEAPPPPPPPPPPPPPPPPPAGYSALDVVLRILLLGSAVASVVV 60
CAO71018        ------------------------------------------------------------
XP_002312755    MASTD--KPDRESIKSEEAPAAHPRR----------SNYSSVHVALRFLLFAASVTAVVV 48
AT4G15610       ---MGYETKSTLDTERSTAPGTGTTT----------KSCSMTQVVLRFVLFAATLTSIVV 47
EAZ03696        MATVDGTTAPSSGGKTATVASESGGGRYGGGPPP-PARCSGANLALRALLFAVSLSALVV 59

Ni6             MVTSKQTEII---VSPFG--SRPNAAKFQNSPAFIYLVAALSVAGLYSIITALVSLS-YM 101
AB267825        MVTSKETELISVKLDPFPPFMLPLTAKFTQSPAFIYFVAGLSVAGLYTIISTLASFYNLL 60
XP_002276229    MVTSVQTKLIA--VAGVP-VLVSNKAKFQNSPAFIYFVAALSVVGLYSIITTLASFI-FI 116
CAO71018        MVTSVQTKLIA--VAGVP-VLVSNKAKFQNSPAFIYFVAALSVVGLYSIITTLASFI-FI 56
XP_002312755    MVTAKQTKIVP--VPGFP-ISVPLEAKFSDSPAFIYFISALSVAGLYGILTTLAAIS-IV 104
AT4G15610       MVTSKQTKNIF--LPGTP--IRIPAAEFTNSPALIYFVVALSVACFYSIVSTFVTVS-AF 102
EAZ03696        LVTAKQTVMIPFVIRPPQFILAPVPAKYTHSPALIYLLAALCATCFYSLITAISSVR-LL 118
                :**: :*  :   :           *:: .***:**:: .*... :* ::::: :.   .

Ni6             RKPIVPPKLFWILLIHDVLLLGIVAAATGTAGGVGYIGLKGNTHVRWGKIRNVYDKFCRH 161
AB267825        IKPGFCPALVSHFIILDVVMLGIVGTATGAAGGVAYIGLKGNSHVGWTKVCNKYGKLCTH 120
XP_002276229    SKPSCSTKTILHLAIWDVLMLGLAASATGTAGGVAYVGLKGNSHVGWNKVCNTYDKFCRH 176
CAO71018        SKPSCSTKTILHLAIWDVLMLGLAASATGTAGGVAYVGLKGNSHVGWNKVCNTYDKFCRH 116
XP_002312755    LKPAYATRFLLHFALLDVLMLGIVASATGAAGGVAYVGLKGNSHVRWGKVCNVYDKFCQH 164
AT4G15610       KKHSCSAVLLLNLAIMDAVMVGIVASATGAGGGVAYLGLKGNKEVRWGKICHIYDKFCRH 162
EAZ03696        SSSACSAKTLFYLILLDVFYAAVMASATGTAGAVAWVGLKGNSHTRWNKICNVYGKFCRH 178
                 .    .  .  : : *..  .: .:***:.*.*.::*****... * *: : *.*:* *

Ni6             VGASIIVSLFAAAVLVLLVFVNANSLYRRIPKY 194
AB267825        LGASLAVSFFAFIVLLLLIILSIHSLSKKIPK- 152
XP_002276229    VGGSIAVALFASILLVLLVWLSLFTLYSRIRK- 208
CAO71018        VGGSIAVALFASILLVLLVWLSLFTLYSRIRK- 148
XP_002312755    VGSSIAVALFASVLLVLLTMLSVFSIYRKIPK- 196
AT4G15610       VGGAIAVSLFASVVLLLLSIISVLSLYKKIR-- 193
EAZ03696        IGSSTFLALIAAIVLVLLAFLNAYSLYRRSR-- 209
                :*.:  ::::*  :*:**  :.  ::  :
```

a)

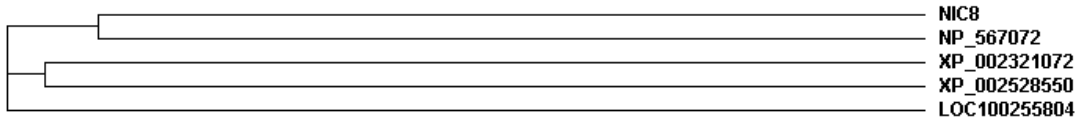


b)

Figure 3. 2. Multiple amino acid sequence alignments of the deduced NIC6 (indicated as Ni6) protein sequence. A) Same colours on columns indicate conserved amino acids between *Populus trichocarpa* (XP 002312755), *Vitis vinifera* (XP 002276229 and CAO71018), *Arabidopsis thaliana*. (AT4G15610), *Oryza sativa* (EAZ03696), *Ipomoea nil* (AB267825). B) Phylogenetic tree of the proteins.

```
XP_002321072      DPDSAEKDEGSLQVHRKLEASSNVLGPSLRNMMLRSATSIDRKLSLSHSEPNIATTFWRR 357
XP_002528550      DPDSAEKDE-SLQFHRKLEG--NVSGPALRNMMLRSATSIDRKLSLSHSEPNIATTFWRR 356
LOC100255804      DPDSTEKDE-GLQFQRRLEASSNVSGPSLRNVMLRSTPSIDRKLSLSHSEPNIATTFWRR 357
NP_567072         DHENAEKDE-NLSLHRKLDGSPNTSGPPSRNMLLRSASALERKLSFSQSESNMANEFWRQ 351
NIC8              --------------------------------MLMMLVHQD-QLVYQH------------ 15
                                                   :*      : :*  .:

XP_002321072      SRKKVIAEQRTASSSPEHPSFRGRGRSMLSGDRHSIRHYADDVAISSHRSEGASMS--EA 415
XP_002528550      SRKKVIAEQRTASSSPEHPSFRARGRSMLSGDRHSIRDYADDEAAP--RSVGASAS--ET 412
LOC100255804      SRRKVIAEQRTASSSPEHPSFRARGRSMLSGDRKSFRDYADDIAASSYRSDGASTSTSET 417
NP_567072         SRRKVIADQRTASSSPEHLSFRARTKSMLSGDKNLARDFTGDVATSSCKSVGGAKM--ET 409
NIC8              --QKLVDYGEEVSASPQRLVT-------------------------------------- 34
                    :*::   . .*:**::

XP_002321072      RRMRRRSISMTPEIGDDIVRAVRAMNETLKQNRLLMEQGDDRLFTNNLGDKDNGTDLQKN 475
XP_002528550      RRIRRRSISMTPEIGDDIVRAVRAMNESLKQNRLLRERDD----------KDNGTDFQRN 462
LOC100255804      RRIRRRSISITPEIGDDIVRAVRAMNETLKANRLMRDQGDDR-------AFSSNPDIQKN 470
NP_567072         KRIRRRSISITPEIGDDIVRAVRAMNEALKQNRLSKEQGDDDSSPNSPNDRTESSHLQKN 469
NIC8              -----------------ILRAVRAMNETLKQNRLLRERGEDRSLPTSPSSRNTGANADTN 77
                                  *:********:** ***  ::.:            ... : *

XP_002321072      VSNFSLDGRDEISGGRSALYTLERNRINSQKAISLPSSPHEYRSQTSERSGPSGFVADDQ 535
XP_002528550      VSNLDLDGHDDISGGRSALYTLQRDHINSQKAISLPSSPHQYRSHISDRRGPSGHAVNDE 522
LOC100255804      VSDFHLDGHGEISHGSSSMYTLPREQISSQKAISLPSSPHEFRSQTSGRSGTS-DIVNDE 529
NP_567072         VSGFHLDAHDQVSGGRS---TLSREPLDPQKAISLPSSPQNYRSQYE-QSGSS----HRN 521
NIC8              ASDFHLGSRDEISGARSALYALHGE-QNSHKAISLPSSPHQYRSLTPERNRASEQAPSDE 136
                  .*.: *..:.::* . *   :*  :  ..:*********:::**    :  .*      :

XP_002321072      LVSTWNKVLESPLFHNNPPLPFQEWHIDFSELTVGTRVGIGFFGEVFRGIWNGTEVAVKV 595
XP_002528550      LVSTWNKVLESPMFNNKPLLPFQEWNIDFTELTVGTRVGIGFFGEVFRGVWNGTDVAIKV 582
LOC100255804      MVSIWNRVLEKPMFHSKPLLPFQEWNIDFSELTVGTRVGIGFFGEVFRGIWNGTDVAIKV 589
NP_567072         ISHIWDKVLGSPMFQNKPLLPYEEWNIDFSELTVGTRVGIGFFGEVFRGIWNGTDVAIKV 581
NIC8              MVSTWEKILEARMYRDKSLLPYEEWNIDFSELTVGTRVGIGFFGEVFRGIWNGTDVAIKV 196
                  :   *:::*   ::..:. **:::**:***:********************:****:**:**
```

a)



b)

Figure 3.3. Multiple amino acid sequence alignments of the deduced NIC8 protein sequence. A) Same colours on columns indicate conserved amino acids between *Populus trichocarpa* (XP_002321072), *Ricinus communis* (XP_002528550), *Vitis vinifera* (LOC100255804), *Arabidopsis thaliana* (NP_567072) B) Phylogenetic tree of the proteins.

All results have been performed using blastp program in blast.ncbi.nlm.nih.gov/Blast.cgi. And then alignments have been performed in ClustalW.

The NIC3 protein gives 88% amino acid identities with the *Arabidopsis thaliana* S-Adenosylmethionine Synthetase 2 protein. NIC3 also shares a high sequence similarity (starting from 98 %) with other S-Asenosylmethionine Synthetase proteins (Figure 3.1.).

Blast result of the NIC6 protein gave similarities with integral membrane family proteins of *Arabidopsis thaliana* starting from 49% top identity with unknown function. The alignment results and phylogenetic relations between similar proteins of NIC6 have been shown in a phylogenetic three (Fig.3. 2.).

The NIC8 protein gives homology with protein kinase family proteins starting from 58% amino acid identity in *Arabidopsis thaliana* and in some other plant species (Figure 3.3.). There are also some identical sequences referred as hypothetical protein.

3.2. Solid Growth Tests

3 colonies which have been choosen from cDNA library screening on toxic nickel containing plates also tested again in solid mediums for confirmation of nickel tolerance. By spotting assays on solid YNB-ura medium, it was confirmed that three cDNAs, *NIC6*, *NIC3* and *NIC8* confer nickel tolerance to the yeast cells (as shown in Figure 3.3.), up to 1800 μM nickel levels. 1600 μM and 1800 μM nickel concentrations completely inhibits the growth of the empty vector containing yeast cells, while *NIC6*, *NIC3* and *NIC8* overexpressing cells survive under these highly toxic nickel levels.

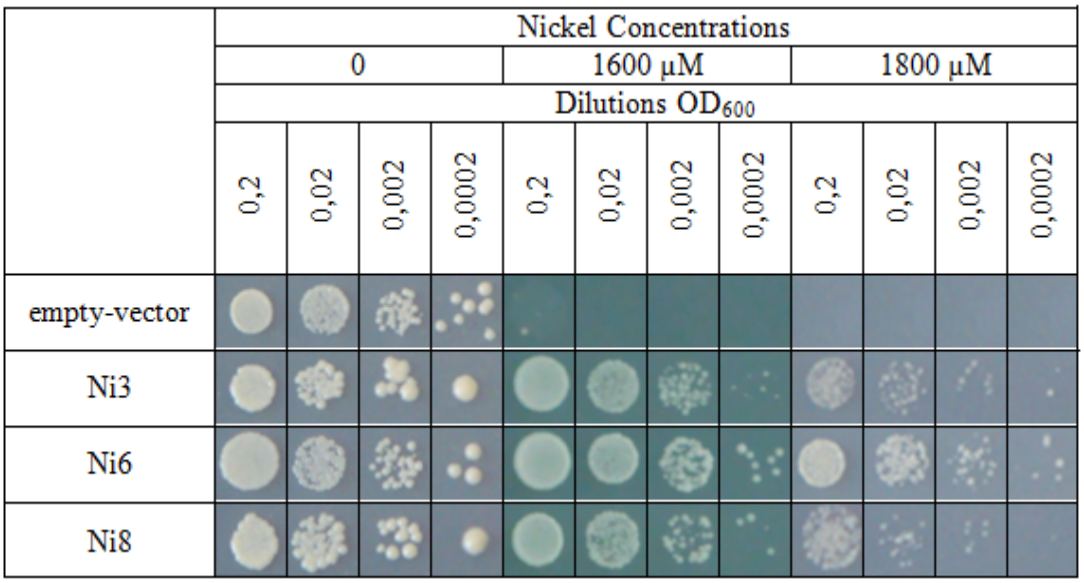


Figure 3.4. Solid growth tests. Four serial dilutions from O.D. $_{600}$= 0,2 to O.D. $_{600}$= 0,0002 were plated on minimal medium. Void vector containing and *NIC3*, *NIC6* and *NIC8* cDNAs overexpressing vector containing recombinant yeast cells were incubated 5 days at 30^0C.

Two of the most identical proteins of NIC6, At4g15610 and At3g06390 from *Arabidopsis thaliana* also have been tested to see whether these proteins can confer nickel resistance to the yeast cells or not. But both of these proteins did not confer nickel resistance to the yeast cells (*Data not shown*).

For NIC6 cDNA, it was also necessary to test it for other heavy metal stress conditions to see the affinity of this cDNA to nickel. We tested *NIC6* overexpressing cells for toxic cobalt and toxic cadmium levels. Solid growth tests showed that, *NIC6* also confers cobalt tolerance to yeast cells (Figure 3.4.). But *NIC6* do not have a detoxification effect for cadmium (*Data not shown)*.

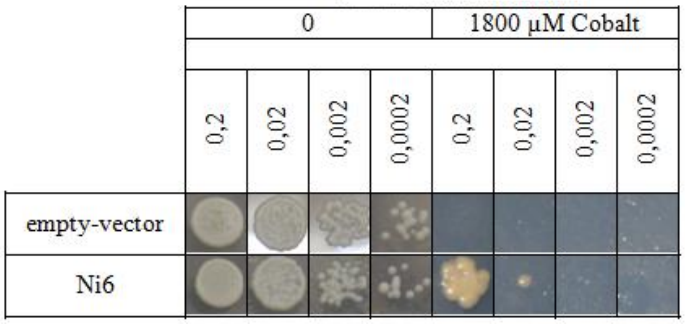


Figure 3.5. Solid growth test results for *NIC6* cDNA overexpressing yeast cells. The experiments were performed same as the nickel solid growth experiments. While 1800 µM Cobalt totally inhibits growth of empty vector containing cells, *NIC6* overexpressing cells could survive for first dilution of spotting.

3.3. pH Effects on the Growth of the *NIC6* cDNA Overexpressing Cells

NIC6 protein sequence also has been analysed for transmembrane domain prediction using TMHMM 2.0 software (at http://www.cbs.dtu.dk/services/TMHMM). NIC6 protein contains four transmembrane helices.

This result and the database search results lead us to test the NIC6 protein's nickel detoxification abilities at different pH values. The aim was to see whether NIC6 is a hydrogen dependent antiporter or not.

So effects of the different pH values on the growth of the yeast cells have been tested in the presence of the nickel stress (Fig 3.4.). At pH= 4.5 the growth of the cells were similar to the solid medium. At the pH= 6.5 a slight inhibition occured in the growth of the cells. But at the pH= 7.5 growth of the cells totally inhibited.

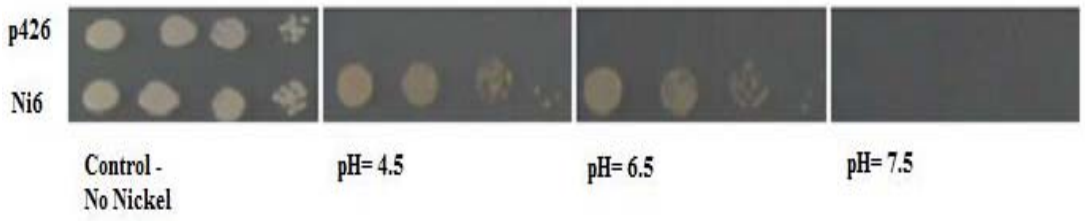


Figure 3. 6. pH Effects. Growth of the *NIC6* overexpressing and empty (pAG426GPD) containing cells at different pH values. Yeast cells grown in YNB –ura plates as four serial dilutions from 0.2 to 0.0002 (at O.D. 600). All mediums contain 1500 µM $NiCl_2$.

3. 4. Intracellular Nickel Concentrations

Nickel contents of the recombinant cells also measured to understand whether these proteins, have a role as pumping the nickel out of the cell, or not.

Measurements of the intracellular nickel levels have been performed as described in T. Mizuno et al. (2005) using AAS. According to the results, there is no statistically significant differences between control cells with the void pAG426GPD vector and cloned cDNAs overexpressing yeast cells (Fig. 3.6.).

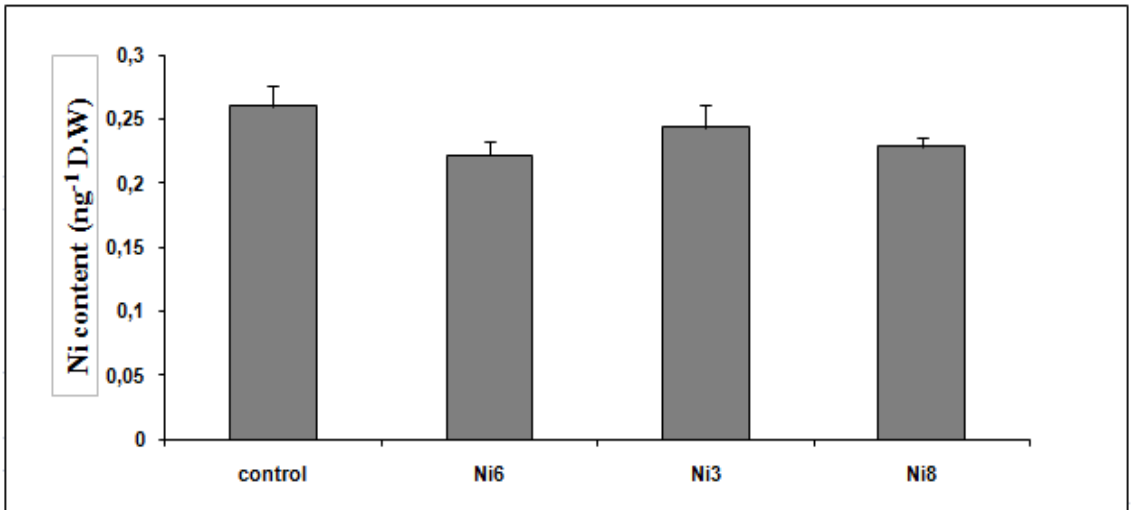


Figure 3. 7. Intracellular nickel concentrations of recombinant yeast cells. Control cells expressing empty pAG426GPD vector. NIC3, NIC6 and NIC8 indicated cells over expressing *NIC3*, *NIC6* and *NIC8* cDNAs. Cells were grown in 800µM $NiCl_2$ supplemented YNB-Ura medium for 36 hours at 30^0C. Values are the mean of three independent experiment and bars present the standart errors, N=5 and P <0,05.

According to the results that was shown in the Figure 3.6., *NIC6* containing cells accumulate less nickel comparing the mean values of all samples. Void vector containing cells accumulated 0,260 ng $^{-1}$ D.W nickel. *NIC3* overexpressing cells accumulated 0,244 ng $^{-1}$ D.W nickel. The nickel accumulation of *NIC8* overexpressing cells were 0,228 ng $^{-1}$ D.W. And *NIC6* cDNA overexpressing yeast cells accumulated 0,222 ng $^{-1}$ D.W nickel which was the least amount. But with the analyses of the standart deviations and the individual intracellular nickel levels for all the cells it becomes clear that there is no statistically differences between 4 types of cells, suggesting cells accumulate nickel in cytoplasm.

3. 5. Localization of the NIC6 Protein in Tobacco Cells

To determine the sub-cellular localization of NIC6 by expressing a Venus-NIC6 fusion protein in *Nicotiana benthamiana* epidermal cells, YFP experiments were performed. Therefore, the entire coding region of NIC6 was translationally fused to the carboxy-terminus of the fluorescent Venus protein and placed under the control of the double enhanced CaMV 35S promoter. The resulting construct was transformed into *N. benthamiana* leaves by agroinfiltration in the presence of the potent RNA gene silencing suppressor protein p19 from tomato bushy stunt virus and confocal laser scanning microscopy visualized fluorescence signals. Figure 3.7. shows three individual experiments. Fluorescence mediated by the Nib6-Venus fusion protein was specifically localized to the surface of multivesicular vesicles (Fig. 3.7. A-E). These structures are clearly localized within the cell cytoplasm as demonstrated by the co-expression of a CaMV35S-mRFP constructs, which led to an overall red fluorescence of the cytoplasm (Fig. 3.7. F-I). To distinguish the multivesicular vesicles from chloroplasts the auto-fluorescence of the chlorophyll is shown (blue fluorescence signal; Fig. 3.7. J).

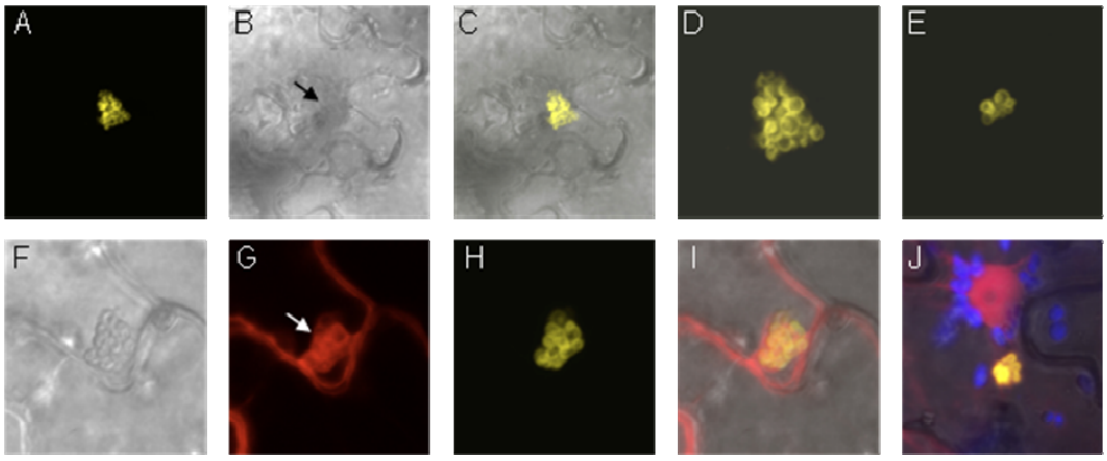


Figure 3.8. Sub-cellular localization of Venus-NIC6 fusion protein in epidermal cells of *Nicotiana benthamiana.* A) Fluorescence of vesicular structures observed after expression of pBatTL::*Venus-nic6.* B) Transmitted light image of (A), the vesicular structure is indicated by an arrow. C) Overlay of (A) and (B). D) Magnification of (A). E) Fluorescence of a small vesicular structures observed after expression of pBatTL::*Venus-nic6.* F-I) Localization of the vesicular structure inside the cytoplasm of a epidermal cell. F) Transmitted light image of the vesicular structure. G) Fluorescence observed after expression of pBatTL::*mRFP,* localization of the vesicular structure shown in (F) is indicated by an arrow. H) Venus-Fluorescence of vesicular structures. I) Overlay of (F), (G) and (H). J) Overlay of fluorescence

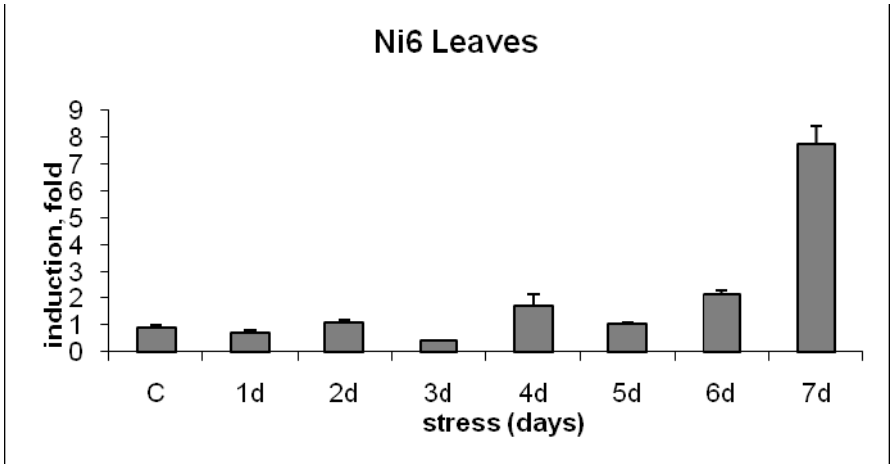
patterns resulted from Venus-tagged NIC6, cytoplasmic mRFP and transmitted light image. The auto fluorescence of chloroplasts is shown in blue.

3. 6. Expression Analysis for the *NIC3* and *NIC6* Transcripts

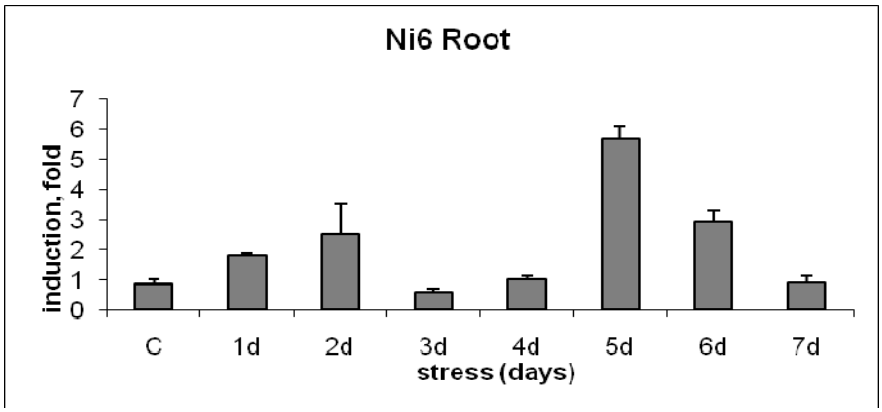
To see the expressions of the *NIC6* and *NIC3* are connected to direct nickel induced regulation or not, we performed quantitative PCR analyses. Applied nickel stress as 75 μM to the *Beta maritima.* Isolated total RNAs of leaf and root tissues from 1 day to 7 days nickel supplement. Also collected tissue samples from no nickel supplemented plant for control measurements.

3.6.1. Results of the *NIC6* Expressions

For *NIC6*, mRNA amount in leaves for first 5 days, does not change significantly, comparing with the control result (Figure. 3. 8. A.). But for 6th day 2,4 - fold induction occured compared to the untreated control. The highest induction was at day 7 as 8,7 –fold.



a)



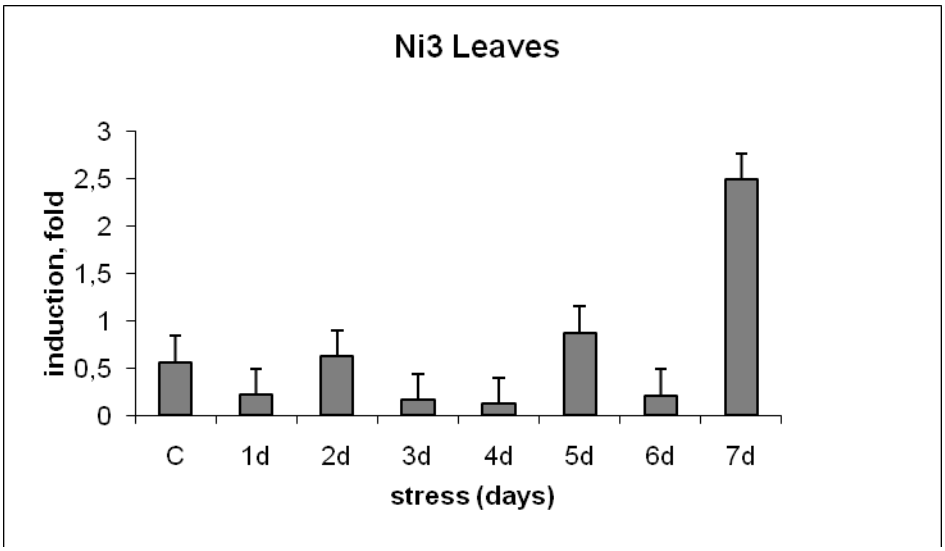
b)

Figure 3. 9. Expression levels of *NIC6*. (A) shows the expression of the *NIC6* (indicated in figures as Ni6) cDNA from control (without Ni) to 7 days nickel treatment in the leave, and (B) shows the expression of the *NIC6* cDNA in the root. For (a) and (b), 75 µM Nickel stress were applied. Values are the mean of four expriments and bars present standart errors, N=5 and P <0,05.

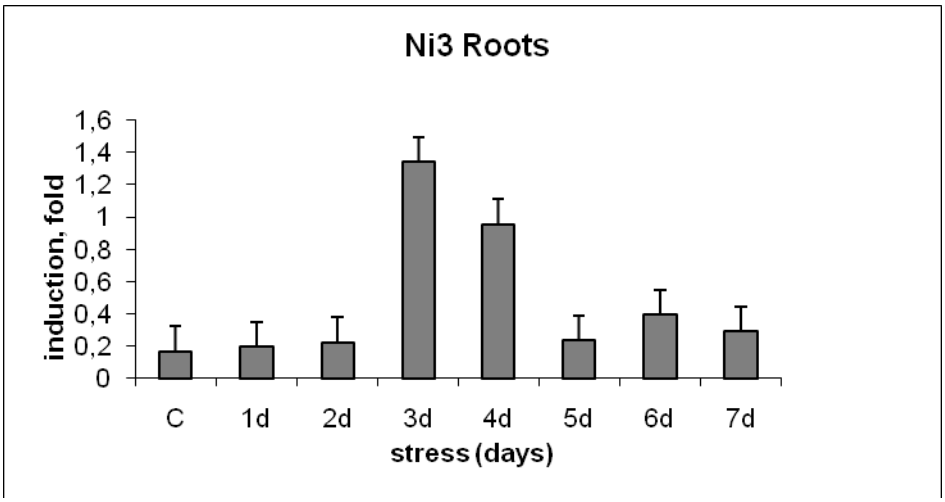
There has been more dynamic regulation with the nickel supplement in the roots. For 1th and 2th days, compared to the control result there is an overexpression of the *NIC6* as 2,1 –fold and 2,9 –fold, respectively. Surprisingly 3th and 4th days a decrease in the expression of the gene occured, below 2 –fold for each. But at the 5th and 6th days, there is a significant overexpression for the *NIC6* gene. (Fig. 3. 8. B.). 5th day, induction was 6,5 -fold compared to the untreated control. 6th day the induction level was 3,3 –fold.

3.6.2. Results of the *NIC3* Expressions

For *NIC3*, in leaves, there were a dynamic change between the days. But the most induction occured at the day 7 as 2,4 –fold compared to the control sample. There were also an induction for 2th and 5th days (Figure 3.8.).



a)



b)

Figure 3. 10. Expression levels of *NIC3* (indicated as Ni3 in figures). For the root levels of *NIC3* mRNA the results were more regular than the leaves' results. There were similar narrow induction for most of the days except 3th and 4th days, but all were not statistically significant again. At the 3th day induction was 1,3 –fold and at the 4th day the induction was 0,9 –fold compared to the control samples.

CHAPTER 4

DISCUSSION

In this study, we screened cDNA library of *Beta maritima* to identify plant genes that confer nickel tolerance to yeast *Saccharomyces cerevisiae* cells. Survival capacities for all three different cDNA expressing cells were similar, while void vector containing cells' growth totally inhibited at 1600 μM $NiCl_2$ concentrations. Looking at the solid growth test results, it can be concluded that *NIC3*, *NIC6* and *NIC8* genes confer yeast cells nickel tolerance.

With the sequencing analyses, it was searched for homolog genes those functions are known and related to nickel detoxification function. For *NIC3*, blastp results showed that this protein has a high (88 % amino acid identity) homology to *Arabidopsis thaliana* S-Adenosylmethionine Synthetase protein sequence which was not mentioned for a nickel specific function in the literature. But, nickel stress indirectly induces oxidative stress in plant cells (Sharma and Karl-Josef 2008).

The related product, S-adenosyl methionine (SAM) has been shown to play significant roles in antioxidant defense systems against cellular ROSs (Reactive Oxygen Species). Firstly the enzyme converts SAM to cysteine which therefore forms glutathione (GSH). GSH is a very important antioxidant tripeptide, reacts with cellular ROSs and turns them to non-toxic metabolic forms (Lieber 2002).

At another study, SAMDC enzyme has been shown to have important roles at abiotic stress tolerance in tobacco plants (Wi, et al. 2006) by increasing the polyamine (PA) levels. The roles of polyamines in antioxidant defence system is not clearly understood. But these compounds have been shown to decrease the levels of ROSs inside the cell. For example, it has been reported that spermine (one of the polyamines) has a role in free radical H_2O_2 scavenging role (Ha, et al. 1998). So, it is clear that SAM homolog *NIC3* cDNA and its products confer our yeast cells tolerance against nickel induced oxidative stress.

Looking at the protein alignment results of NIC3, blastp results gave us that similar sequences of S-Adenosyl methionine synthetase protein has a role as metal binding. Cobalt, magnesium, potassium and ATP binding roles also have been given in

the blastp result by cofactor similarity (Tabuchi, et al. 2006). So, we can also speculate that NIC3 protein might have a nickel binding function. This feature of the protein may provide additional metal detoxification function besides other antioxidant related functions.

From the blastp result of NIC6, we found homolog genes in *Arabidopsis thaliana* with unknown function. Only homolog gene which's function has been studied was *InPSR26* gene from Japanese morning glory (*Ipomoea nil*) showed sequence identity as 51 % at amino acid level (Shibuya, et al. 2009). The function of this gene has been shown as having regulatory role in programmed cell death.

Since NIC6 has a plasma membrane localization we want to see wheter NIC6 protein pumps nickel out of the cell, so decreases the intracellular nickel levels, or not. According to the results, *NIC6* cDNA overexpressing yeast cells accumulated similar intracellular nickel levels with the void vector containing yeast cells. Suggesting NIC6 does not localizes in the outer membrane of cells and pumps nickel out of the cells.

So, to find out more detailed localization of NIC6 protein, cDNA was cloned to the upstream regions of GFP and YFP fusion proteins and then transformed to the tobacco epidermal cells. From the results, it can be concluded that NIC6 protein localizes multivescular protein bodies, which could be derived from E.R., tonoplast, peroxisomes or liposomes. But more work is needed to identify exact source of these membrane structures that NIC6 localizes on.

But, although there are limited literature information, it is well known that coated vesicles, endomembrane systems/structures have been shown to transport some metal ions such as Ca^{2+} and Mn^{2+} (Wu, et al. 2002) and confer Mn^{2+} tolerance. With the transport of metals through this endomembrane structures, firstly cells remove these toxic elements from cytoplasm where active metabolism takes place. And in some cases, with the transfer of these endomembrane systems through the plasma membrane, contents of this structures are being removed even from the cell itself. It might be possible that *NIC6* gene's function seems to be parallel with these kind of detoxification function.

We also tested *NIC6* cDNA for other heavy metal stress conditions in yeast cells to find out the affinity of this cDNA to other heavy metal. *NIC6* cDNA overexpressing cells could not survive under toxic cadmium level but a slight tolerance to the toxic cobalt levels have been shown in the solid growth experiments. For the 0,2 dilution spotting, *NIC6* cDNA overexpressing cells could survive on the plates. But, it must be

notted that, this cobalt tolerance effect of *NIC6* is not as much as effective for its nickel tolerance effect. So, we can conclude that *NIC6* cDNA's metal detoxification role is not specific only to nickel. But it's affinity to nickel is significantly higher than it's affinity to other metals such as cobalt.

To see the gene expression levels of isolated cDNAs we performed Real-Time PCR experiments with the nickel stress application to *Beta maritima* plants. According to *NIC3* expression results, there were not a significant overexpression for *NIC3* transcript occured. With nickel treatments from 1st day to 7th days, we observed a dynamic change, not a regular overexpression. But looking at the –fold induction values, it can be said that there is not a direct change occurs after nickel treatment for *NIC3* mRNAs. So, *NIC3* gene is not directly regulated with nickel induced signal through cell nucleus suggesting posttranslational regulation.

Similar results have been seen for *NIC6* gene expression. But, this time, the change in the expression with the stress application were more as induction -fold values compared to the *NIC3* induction –fold results. And the changes were more dynamic. Also, looking at the induction –fold values, the expression changes were affected with nickel stress which can point out that *NIC6* gene has a more related role in nickel toxicity.

NIC8 gave homology to protein kinase family proteins in diverse species that leads us to conclude that NIC8 may play role in nickel detoxification signal transduction pathway which needs to be investigated in the future.

CHAPTER 5

CONCLUSION

In this study, we construct plant cDNA library to find out nickel detoxifying genes using heterelogous expression system in yeast. *Beta maritima* plant cDNAs , reported salt and manganese tolerant plant, have been used The yeast *Saccharomyces cerevisiae* has been used as a model organism to screen the library for its fast resulting life cycle.

After the initial screening, three *Beta maritima* cDNAs, named as *NIC3*, *NIC6* and *NIC8* have been shown to confer nickel tolerance to the yeast cells. With the further experiments, nickel detoxifying functions of these genes have been discussed.

REFERENCES

Arazi, T., S. Ramanjulu, K. Boaz and F. Hillel. 1999. A tobacco plasma membrane calmodulin-binding transporter confers Ni^{2+} tolerance and Pb^{2+} hypersensitivity in transgenic plants. *The Plant Journal*. 20: 171-182.

Dameron, T. C. and D. H. Mark. 2000. Mechanisms for protection against copper toxicity. *American Journal of Clinical Nutrition.* 67: 1091-1097.

Dietz, K-J., B. Mau and U. Krämer. 1999. Free radicals and reactive oxygen species as mediators of heavy metal toxicity in plants. *Prasad MNV, Hagemeyer J, eds. Heavy metal stress in plants: from molecules to ecosystems.* Berlin: Springer-Verlag, 73–97.

Dixon N. E., G. Carlo, J. James, L. B. Robert and Z. Burt. 1975. Jack Bean urease (EC 3.5.1.5). A metalloenzyme. A simple biological role for nickel? *J. Am. Chem. Soc.* 97: 4131-4133.

Duke, James A. 1983. *Handbook of Energy Crops*. Electronic Book. http://www.hort.purdue.edu/newcrop/duke_energy/dukeindex.html (accessed November 10, 2009).

Dürr, G., S. Jochen, P. Richard, K. K. Saskia, C. Patrice, W. H. Dieter and R. Hans. 1998. The medial-Golgi Ion Pump Pmr1 Supplies the Yeast Secretory Pathway with Ca^{2+} and Mn^{2+} Required for Glycosylation, Sorting, and Endoplasmic Reticulum-Associated Protein Degradation. *Mol. Biol. Cell* 9: 1149-1162.

Eskew D. L., M. W. Ross and A. N. Wendell. 1984. Nickel in higher plants. Further evidence for an essential role. *Plant Physiology*. 76: 691-693.

Forzani C., L. Clarisse, L. Stephane, B. Jean-François and L. Michel. 2001. Nickel resistance and chromatin condensation in *Saccharomyces cerevisiae* expressing a maize high mobility group I/Y protein. *The Journal of Biological Chemistry*. 276: 16731-16738.

Freeman J. L., W. P. Michael, N. Ken and E. S. David. 2005. Nickel and cobalt resistance engineered in *Escherichia coli* by overexpression of serine acetyltransferase from the nickel hyperaccumulator plant *Thlaspi goesingense*. *Applied and Environmental Microbiology*. 71: 8627-8633.

Ha, H.C., S. S. Nilantha, K. Periannan, L. Z. Jay, M. W. Patrick and A. C. Robert 1998. The natural polyamine spermine functions directly as a free radical scavenger. *Proc. Natl. Acad. Sci. USA*. Vol. 95: 11140–11145.

Hall, J.L. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany*. Vol. 53-336: 1-11.

Kaiser, C., S. Michaelis and A. Mitchell. 1994. *Lithium Acetate Yeast Transformation, in: Methods in Yeast Genetics*. NewYork: Cold Spring Harbor Laboratory Press. 133–134.

Koch, W.D.J. 2008. Plants for a future. Retrieved from http://www.pfaf.org/database/plants.php?Beta+vulgaris+cicla. (accessed November 7, 2009).

Lieber, C.S. 2002. S-Adenosyl-L-methionine: its role in the treatment of liver disorders. *Am J. Clin. Nutr*. 76:1183-1187.

Mizuno T., U. Koji, H. Kenji, N. Shiro, M. Naoharu and O. Hitoshi. 2005. Cloning of three ZIP/Nramp transporter genes from a Ni hyperaccumulator plant *Thlaspi japonicum* and their Ni^{2+}-transport abilities. *Plant Physiology and Biochemistry*. 43: 793-801.

Ni, W-S, L. Zhi-Yong, C. Xi, J. O. David and X. Cheng-Bin. 2007. Construction of a Plant ransformation-ready Expression cDNA Library for *Thellungiella halophila* Using Recombination Cloning. *Journal of Integrative Plant Biology*. 49 (9): 1313–1319.

Rao, K. V. Madhava, A. S. Ragha Vendra, K. Janardhan Reddy. 2006. *Physiology and Molecular Biology of Stress Tolerance in Plants*. Springer Press.

Rengasamy B. and P. M. Doran. 2002. Ni-induced oxidative stress in roots of the Ni hyperaccumulator, *Alyssum bertolonii. New Phytologist*. 156: 205-215.

Sharma, S.S and Karl-Josef D. 2008. The relationship between metal toxicity and cellular redox imbalance. *Trends in Plant Science*. 14(1): 43-50.

Shibuya, K., Y. Tetsuya, S. Tomoko, S. Keiichi and I. Kazuo. 2009. InPSR26, a putative membrane protein, regulates programmed cell death during petal senescence in japanese morning glory. *Plant Physiology*. 149: 816-824.

Tabuchi, T., O. Tomoyuki, T. Yuhei, A. Tetsushi, N. Takashi and Y. Takeshi. 2006. Transcriptional response of glycinebetaine-related genes to salt stress and light in leaf beet. *Plant Biotechnol*. 23: 317-320.

Van Assche, F. and H. Clijsters. 1990. Effects of metals on enzyme activity in plants. *Plant, Cell and Environment.* 13: 195–206.

Watt R. K and P. W. Ludden. 1999. Nickel-binding proteins. *Cell. Mol. Life Sci*. 56: 604-625.

Wi, S.J., T. K. Woo and K.Y. Young. 2006. Overexpression of carnation S-adenosylmethionine decarboxylase gene generates a broad-spectrum tolerance to abiotic stresses in transgenic tobacco plants. *Plant Cell Rep*. 25: 1111–1121.

Wu, Z., L. Feng, H. Bimei, C. Y. Jeff, R. S. Michael, F. H. Jeffrey and S. Heven 2002. An Endoplasmic Reticulum-Bound Ca^{2+}/Mn^{2+} Pump, ECA1, Supports Plant Growth and Confers Tolerance to Mn^{2+} Stress. *Plant Physiol.* 130: 128-137.

APPENDIX A

SEQUENCE RESULTS

1. *NIC3* (732 bp):

ATGGCCGCCCCAATTGACACCTTCCTTTTCACCTCCGAATCTGTGAAC
GAGGGACACCCCGACAAGATGTGCGATCAGATCTCTGATGCAGTGCT
TGATGCTTGCCTTGCGCAAGACCCTGAGAGCAAGGTTGCTTGTGAGA
CTTGCACAAAGACCAACTTGGTCATGGTCTTTGGTGAGATCACCACC
AAGGGGAACGTAGACTATGAGAAGATTGTCCGACAGTCTTGCCGCGA
CATCGGATTTGTGTCCGCTGACGTGGGTCTTGATGCTGACAACTGCA
AGGTCTTGGTTTACATTGAGCAGCAGAGTCCTGACATTGCCCAGGGT
GTCCACGGTCATCTTACCAAGCGTCCTGAGGAGATCGGTGCTGGTGA
CCAGGGTCACATGTTTGGCTATGCCACTGATGAGACCCCAGAATTGA
TGCCCCTCAGCCATGTCCTCGCCACCAAGCTTGGTGCCCGCCTCACTG
AGGTTCGCAAGAACGGTACCTGCCCATGGTTGAGGCCTGATGGCAAG
ACCCAAGTCACTGCTGAGTACTACAATGACAACGGTGCCATGGTTCC
CATCCGTGTTCACACCGTCCTCATCTCAACCCAGCACGATGAAACTGT
TACCAACGACGAGATTGCAGCTGACTTGAAGGAGCACGTGATCAAGC
CTGTGATCCCTGAGAAGTACCTTGATGAGAGACCATCTTTCCACCTTA
ACCCATCTGGGCGTTTCGTCA

2. *NIC6* (582 bp):

ATGTCATCGATGGAGACAGAAAAGGGAGCAGTGCCAACACCTCAAG
CTCCTCCGGTAGCACCAACGGATAACAAATATCGCGTCGTTGATGTG
ATTTTGAGGGTCTTGTTGTTGGCTGCATCTATAGCCTCAGTTGTACTT
ATGGTTACCAGCAAACAGACAGAGATTATTGTTTCACCTTTTGGTTCC
CGTCCCAATGCTGCAAAATTTCAGAATTCTCCCGCGTTTATATACTTA
GTGGCAGCACTATCTGTTGCCGGTCTGTACAGCATCATCACAGCACTT
GTGTCTTTGTCTTACATGAGGAAGCCTATCGTCCCACCAAAGTTGTTC

TGGATTTTGCTCATCCATGACGTGCTTCTATTGGGAATCGTAGCAGCA
GCAACGGGAACAGCTGGGGGGGTTGGATACATTGGTCTGAAAGGGA
ACACCCATGTACGTTGGGGTAAAATTCGCAATTATATGATAAGTTTT
GCCGACACGTCGGAGCCTCAATTATTGTTTCATTGTTTGCTGCTGCGG
TGCTCGTGCTTCTCGTCTTTGTCAACGCCAACTCTCTTTACCGTCGCAT
CCCAAAATACTAG

3. *NIC8* (603 bp):

ATGCTGATGATGCTGGTTCATCAAGATCAGTTGGTGTACCAACATCA
GAAGCTCGTAGATTACGGCGAAGAAGTATCAGCATCACCCCAGAGAT
TGGTGACGATATTGAGGGCTGTACGAGCAATGAATGAGACATTGAAG
CAGAATCGTCTTTTGAGAGAGCGGGGTGAGGATAGGTCTCTTCCCAC
TTCGCCCAGCAGTAGAAACACTGGTGCCAATGCTGATACAAATGCTT
CTGATTTCCATCTTGGGAGTCGAGATGAAATTTCTGGGGCCAGATCT
GCATTATATGCTCTGCATGGTGAACAAAATTCTCATAAGGCTATATC
ATTGCCTTCATCTCCTCATCAATACAGGAGTCTAACTCCAGAGAGGA
ATAGGGCATCAGAACAGGCTCCAAGTGACGAAATGGTATCAACATG
GGAGAAAATTTTGGAAGCACGCATGTATCGTGACAAATCCTTACTTC
CTTATGAAGAATGGAATATTGATTTCTCTGAGTTGACCGTTGGAACTC
GTGTTGGAATTGGTTTCTTTGGGGAGGTTTTCCGAGGAATTTGGAATG
GAACTGATGTTGCCATCAAAGTGTTTCTGGAGCAGATT