CHARACTERIZATION OF POLLEN-E1 GENE MIGHT PLAY ROLE IN SALT TOLERANCE IN *Beta maritima*

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

MASTER OF SCIENCE

in Molecular Biology and Genetics

by

Özge UYSAL

July 2015

İZMİR

We approve the thesis of **Özge UYSAL**

Examining Committee Members:

_____
Assoc.Prof. Dr. H. Çağlar KARAKAYA
Department of Molecular Biology and Genetics,
Izmir Institute of Technology

_____
Prof. Dr. Ahmet KOÇ
Department of Molecular Biology and Genetics,
Izmir Institute of Technology

_____
Assoc.Prof. Dr.Melike BOR
Department of Biology, Ege University

22 July 2015

_____
Assoc.Prof. Dr. H. Çağlar KARAKAYA
Supervisor, Department of Molecular Biology and Genetics, Izmir Institute of Technology

_____
Prof. Dr. Ahmet KOÇ
Head of the Department of
Molecular Biology and Genetics

_____
Prof.Dr.Bilge KARAÇALI
Dean of the Graduate School
of Engineering and Sciences

ACKNOWLEDGEMENT

I would like to thank my advisor Assoc. Prof. Dr. H. Çağlar KARAKAYA for encouraging and motivating me in studies and also for his support and helps during my master.

I am very thankful to Yeast Lab members for their support. Also I am very grateful to İlkcan ERCAN and Ebru GÜRELME for their encouraging and friendship.

I would like to thank my committee members; Prof. Ahmet KOÇ, Assoc. Prof. Dr. Melike BOR, Assoc. Prof. Dr. A. Hediye SEKMEN ESEN and Assoc. Prof. Dr.Gülşah ŞANLI.

Finally , I also would like to especially thank you my family, Ahmet UYSAL, Mücella UYSAL and Simge UYSAL for their love and respect to my decisions throughout of my life.

ABSTRACT

CHARACTERIZATION OF POLLEN-E1 GENE MİGHT PLAY ROLE IN SALT TOLERANCE IN *Beta maritima*

Salinity stress has a negative impact on the growth of plants, which affects homeostasis and productivity. The uptake of non-essential salt ions change the osmotic balance of the cell and cause dehydration. Higher plants develop salt tolerance mechanisms to avoid dehydration.

In this project, we isolated and characterized salt tolerance genes in *Beta maritima* plant. For this purpose, functional genomics technique was used by over expressing cDNAs in yeast and colonies can grow toxic salt media isolated and characterized. We found several colonies and we focused on uncharacterized Pollen-E1 gene with an unknown function. Pollen-E1 cDNA confers salt tolerance to yeast cells. Intracellular sodium measurements of Pollen-E1 overexpressed in yeast cells showed decreased salt levels as compared to wild type suggesting that sodium was transported out of the cell. Pollen-E1 protein localized in endomembrane systems in the yeast cells. In mRNA expression analysis, Pollen-E1 mRNA levels induced immediately in leaves and later stages in root systems under salt stress. Our results showed that is the uncharacterized and unknown function Pollen-E1 gene might have some role of regulating salt tolerance in *Beta maritima*.

ÖZET

Beta maritima BİTKİSİNDE TUZ TOLERANSINDA ROL OYNADIĞI DÜŞÜNÜLEN POLEN-E1GENİNİN KARAKTERİZASYONU

Tuz stresi bitki büyümeseni etkileyen başlıca negative etkenlerdendir. Tuz stresi bitkide homeostasisi ve bitkinin gelişimini etkiler. Esansiyel olmayan iyonların hücre içine alımı ozmotik dengeyi değiştirir ve büyük oranda dehidrasyona sebep olur. Dehisrasyondan korunmak için gelişmiş bitkiler çeşitli tuz tolerans mekanizmaları geliştirmişlerdir.

Bu projede, *Beta maritima* bitkisinden tuz tolerans genlerinin karakterizasyonu ve izolasyonu gerçekleştirildi. Bu amaçla, bitki cDNAları mayada ekspres edildi ve koloniler toksik tuzlu ortamda büyütüldü. Ardından karakterizasyon ve izolasyon çalışmaları fonksiyonel genomik teknikler kullanılarak gerçekleştirildi. Elde edilen bir çok koloniden fonksiyonu bilinmeyen Pollen-E1 genine odaklanıldı. Pollen-E1 cDNA maya hücrelerine tuz toleransı sağlamıştır. Pollen-E1'i aşırı ifade eden maya hücreleri yabani tip mayayla karşılaştırıldıklarında, tuz düzeyinde düşüş gösterdi ve Pollen-E1'in sodyumun dışarı taşınmasında rol oynadığı düşünülmektedir. Pollen-E1 proteinleri maya hücrelerinde endomembran sistemlerinde lokalize olmuştur. mRNA transkript analizlerinde, Pollen-E1 mRNA seviyeleri yaprakta ve kökte tuz stresi altında indüklenmiştir. Sonuçlarımız, karakterizasyonu ve fonksiyonu bilinmeyen Pollen-E1 genininin *Beta maritima* bitkisinde tuz toleransında rol oynadığını göstermiştir.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Beta maritima

Beta maritima also known as wild type sea beet (LANGE, BRANDENBURG, & BOCK, 1999). *Beta maritima* grows in coastal areas so it can tolerate high concentrations of salt, additionally it can also grow in drought conditions (Srivastava, Shahi, Kumar, & Bhatnagar, 2000).

Plants are categorized according to their responses to salinity. While salt-sensitive plants are affected even at low salt concentrations, salt tolerant plants can absorb water from the saline soil. Salt-sensitive plants can be classified as sensitive and moderately sensitive to soil solinity(Blaylock, 1994; Chinnusamy, Zhu, & Zhu, 2006; Glenn, Brown, & Blumwald, 1999). Salt tolerant plants are known as halophytes(Parida & Das, 2005).

1.1.1. Soil Salinity

Salt is a natural component of all soils and ion content can be different according to the soil types. Sodium (Na^+), calcium (Ca^{2+}), magnesium (Mg2+), potassium (K^+) are general ions which found in all types of soils (Teakle & Tyerman, 2010). Salinity is the basic enviromental factor which effect the growth and productivity of a plant (Allakhverdiev, Sakamoto, Nishiyama, & Murata, 2000). High salinity effects plants negatively and most of the time salinity causes the death of the plant. However, some plants develop mechanisms for protection. The result of these mechanisms can change such as some plants exclude salt from cell, others tolarate the presence of salt in the cell. Energy and lipid metobolisms, protein synthesis and photosynthesis are major metabolisms which are affected from salt stress (Parida & Das, 2005).

According to research soil salinity is one of the agricultural problem and The United Nations Enviroment Program researchers show that nearly 20% of agricultural and 50% of crop lands are affected from salt stress (Rubio, Gassmann, & Schroeder, 1995). While the soil salinity influence ion toxicity, osmotic stress, nutrient limiting and oxidative stress directly, besides this plant productive, growth and symbiotic microbes are affected indirectly on plants from salinity (Chinnusamy et al., 2006).

First, osmotic adjustment has a crucial role in water stress responses in plants (Osakabe et al., 2013) water potential decreases called as water stress and results evoporation. Uptaken of water changes by soil salinity which soil contain soluble salt and this affect the water potential. Under this condition while water potantial decrease, osmotic potential decrease as a result of these decreasing, solute content increseade to resume turgor potential (Mudgal, Madaan, & Mudgal, 2010). Under salt stress transpiration rate, water retention and water use are imposed. Leaf water potential is affected negatively from salinity in halophyte. While the salt concentration increase both evoporation rate and leaf water potential rate decrease. (Chaudhuri & Choudhuri, 1997).

Second, salt stress induce increase of Reactive Oxygen Species(ROS) such as superoxide radical (•O2), hydrogen peroxide (H2O2), hydroxyl radical (OH•) and singlet oxygen (O2) (Dat et al., 2000)**.** Increasing in ROS content cause peroxidation of lipids, destroy skeleton srtructure and damage proteins and nucleic acids. Plants have complex antioxidant systems which contain ROS-scavenging enzymes, such as superoxide dismustase (SOD), catalase (CAT), ascorbate peroxidase, guaiacol eroxidase and glutahione reductase in order to be protected from these damages. Antioxidants systems eliminate reactive oxygen species effects. (Hu, Li, Zhang, Luo, & Fu, 2011; Mudgal et al., 2010) .

Last, in biochemical reactions non-essential ion Na+ and essential ion K+ replace and it cause metabolic imblance. Potasium play role in enzymatic reaction as a cofactor of enzyme, when Na+ take place of K+, this mechanism damaged and cause metobolic imblance (Chinnusamy et al., 2006).

1.2. Effect of Salinity on Plants

1.2.1. Effect on Planth Growth

Salinity limited the growth of plant negatively by changing the morphological, physiological and biochemical feature of a plant. Salt stress cause ticker, smaller and darker leaf formation. Additionally plants develop a strategy to protect younger leaves by carrying sodium from root to older leaves at first, if salt stress condition continue, younger leaves are affected from salt stress at last (Hu et al., 2011; Mudgal et al., 2010). Salt decrease the resistance of CO2 uptake and decrease leaf surface so decrease photosynthetic rate. CO2 diffusion limited the photosynthesis in leaf and it is crucial for resistance of stomatal and mesophyll (Longstreth & Nobel, 1979).

Root can not uptake sufficient water from saline soil. Accumulation of salt in the roots decrease available water and leaves loose water due to salinity stress. It is also called hyperosmotic stress (Gupta & Huang, 2014).

Besides root and leaf, salt affect some important intracellular organels such as chloroplast, mitochondria and peroxisomes. These organelles play a role in producing of active oxygen species. They control the cellular levels of reactive groups and protect cell from damage. In addition, in salinity stress, ER and mitochondria swell then affect to golgi stacks to trigger produce larger vacuoles in plant cell (Mitsuya, Takeoka, & Miyake, 2000).

1.2.2. Effect on Plant Photosynthesis

Beside cell growth, the salt stress effects directly to photosynthesis. It can limit the difusion of CO2 to stoma and mesophyll, so photosynthetic metobolism can change (M. M. Chaves, J. Flexas, & C. Pinheiro, 2009)**.** In addition to photosynthesis, stress condition cause oxidative stress. Oxidative stress generally ocur in choloroplast because absorption sunlight cause ROS formation. So ROS damage to membrane, proteins and DNA(Stepien & Johnson, 2009)**.**

During salt regulation, specific constructions are produceed by cell such salt gland which secrete salt from leaves and they are needed for decrease ion concentration

level to maintain homeostasis. Some ions are accumulated in plants to arrange osmotic pressur (Parida & Das, 2005).

Salt in soil or saline soils affect the uptake of water in plants by reducing turgor pressure and prevent transpiration to conserve water by closing the stomas. Closing the stoma result reducing the photosynthesis because of blocking entry of CO2 (M. Chaves, J. Flexas, & C. Pinheiro, 2009), Hernandez et al. 1999 (Hernandez, Campillo, Jimenez, Alarcon, & Sevilla, 1999). At the same time soil salinity effect pigment biosynthesis. Chlorophylls, betaine and carotenoids are affected by salinity ,in addition total soluable proteins, free amino acids and sugars are affected differently under salt stress condition (Agastian, Kingsley, & Vivekanandan, 2000).

1.2.3. Effects on Plant Metabolism

Carbon and nitrate is essential elements for plant metabolism. Nitrate reductase(NR) is a crucial enzyme that is affected by enviroment factors and stress conditions which change the activity of nitrate reductase and change the plant growth and development. Cl- reduces nitrate (NO3-) uptaken , so NR activity is decrease under salt stress condition (Flores, Botella, Martinez, & Cerdá, 2000).

Nitrate and carbon metobolism are linked by NADP-specific isocitrate dhydrogenase (NAPD-ICDH, EC 1.1.1.42). NAPD-ICDH activity is change as a response of high concentration of salt stress (Popova, Ismailov, Popova, Dietz, & Golldack, 2002).

1.3. Salt tolerance Mechanisms in Plants

Halophytes can survive at high salt conditions and scientists propose that that they were evolved from glycophytes under salt stress condition (Winicov, 1998; Zhu, 2000).

Plants developed some mechanism to adapt salt stress. Adaptive mechanisms allow maintaince, activation and enhanced system of physiological function of plants. These plants can tolerate salt and continue to their life-cycle and even they can reproduce. The tolerancemechanisms differ from plant to plant and can be examined in

three catagories: 1- Plants can transport ions or uptake and accumulate them into specific locations; 2- morphological features and biomass distruption of plants can be controlled by traspiration and stoma closure; 3- Physiological and metobolical events can neutralize the affect of intracellular salt (Winicov, 1998).

Recent studies show that, biochemical and molecular mechanisms responsible for the tolerance to salt stress are based on expression of stress-related genes which can grouped in three catagories: 1- genes which play role in signaling cascade and control of transcription such as SOS kinases; 2- genes that has a function in protection such as heat-shock proteins; 3- ion transporters (Wang, Vinocur, & Altman, 2003).

The salt tolerance mechanisms divided into two groups. Low-complex mechanisms basically change the biochemical pathways , on the other hand high-complexicity mechanisms regulate major processes such as photosynthesis and resprition to protect plants from changes in chromosome and chromatine structures (Parida & Das, 2005).

1.3.1. Signaling Pathways of Salt Tolerance

Accumulation of Na+ into the cell inhibits activity of enzymes, cell division, distrurb osmotic imbalance and plant growth. Besides, high concentration of Na+ ions leads to the production of reactive oxygen photosynthesis. Sodium accumulation results in alteration of K+ levels in the cell which plays essential role in metabolism, cell growth, stress tolerance and it is directly related to the enzyme activities. In addition to Na+, K+ ions salinity affect of calcium (Ca+) ions which has a crucial role in cell growth and signaling pathways. Calcium ions are secondary messengers in signaling cascades (Knight, Trewavas, & Knight, 1997). Salt stress signals recognized by the cell membrane receptores than they reach to signaling (Fig.1.1). Ca+ levels are increase by two ways, first it can be uptaken from the extracellular sources by binding to EGTA and BAPTA (calcium-binding protein), second by the activation of PLC. Stress signals leading to increase in Ca+ levels in the cell and this increasement activate signaling cascade, so cell responce to stress (Knight et al., 1997).

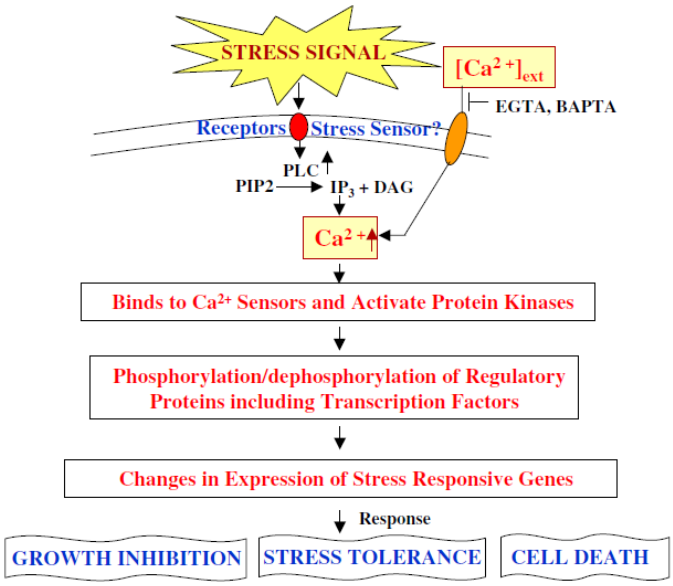


Figure 1.1. Genetic pathway for plant response to stress.
(Source:S.Mahajan et al./Archives of Biochemistry and Biophysics 471(2008) 146-158)

Salt overly sensitive (SOS) pathway mechanism is one of the signaling mechanism that is induced by salt, it contains SOS1, SOS2 and SOS3 which mediate cellular signaling under salt stress conditions and these mechanism regulate ion homeostasis (Ji et al., 2013). Mutation research in *sos1*, *sos2* and *sos3* genes shows that these 3 genes are hypersensitive to Na+ and Li+ (Zhu, 2000). SOS2 and SOS3 modulate SOS1 gene expression. SOS1 is plasma membrane Na+/H+ exchanger SOS3 which is a Ca-dependent protein and essential for SOS2 (protein kinase) (Knight et al., 1997; Qiu et al., 2004; Quintero, Ohta, Shi, Zhu, & Pardo, 2002). SOS1 is a plasma membrane Na+/H+ antiporter and the N-terminal region contain several transmembrane domains and a short hydrophilic cytoplasmic tail which interact with protein kinases,molecular chaperons and Ca-binding proteins. SOS1 is expresses only under NaCl stress ,neither absasic asid (ABA) nor cold stress induce SOS expression unlike other salt-stress responsive genes(Zhu, 2000). SOS2 is a Ser/Thr protein kinase with two functional domains. Kinase catalytic domain located in the N-terminal region and the C-terminal region has regulatory function by increasing with SOS3. The C-terminal is essential for the salt tolerance plants (Liu, Ishitani, Halfter, Kim, & Zhu, 2000; Quintero et al., 2002). After calcium signals recognition, SOS2 is activated by SOS3 and form a kinase complex. These complex phosphorylate SOS1 and efflux of excess Na+ ions. Additionally, SOS2-SOS3 complex limits Na+ entry to cytosol by inhibiting HKT1

activity (a low affinity Na+ transporter) and activates some vacuolar Na+/H+ exchanger to maintain ion homeostasis (Mahajan, Pandey, & Tuteja, 2008).

ABA is a phytohormone that one of the response mechanism to salt stress and drought in plants, it also regulates growth and development Biosynthesis of ABA is upregulated by salinity. This cause accumulation of reactive oxygen species (ROS). Calcium and ROS are seconder messengers of ABA-induced stotomal closure and gene expression. Additionally expression of *NHZ1(*Na+/K+ antiporters) is regulated by ABA in Arabidopsis. The SOS and ABA regulates *Arabidopsis* vacuolar Na+/K+ antiporter gene(*AtNHX1)* expression and activity of antiporter under salt stress (Fig 1.2) (Chinnusamy, Jagendorf, & Zhu, 2005).

Under salt stress condition , ABA biosynthesis is regulated by osmotic stress. Salt stress cause osmotic stress. Genes releated to ABA biosynthesis are induced by a calcium-dependent phosphorylation pathway. ABA is synthesized from *β*-carotene. ABA accumulation activate it's biosynthesis and catabolysis pathways through a calcium-siganling pathway (Tuteja, 2007).

Mutation in *Arabidopsis* abscisic acid insensitive1(ABA1) cause reducing seed dormancy, water loss and abnormal drought rhizogenesis (Leung et al., 1994).

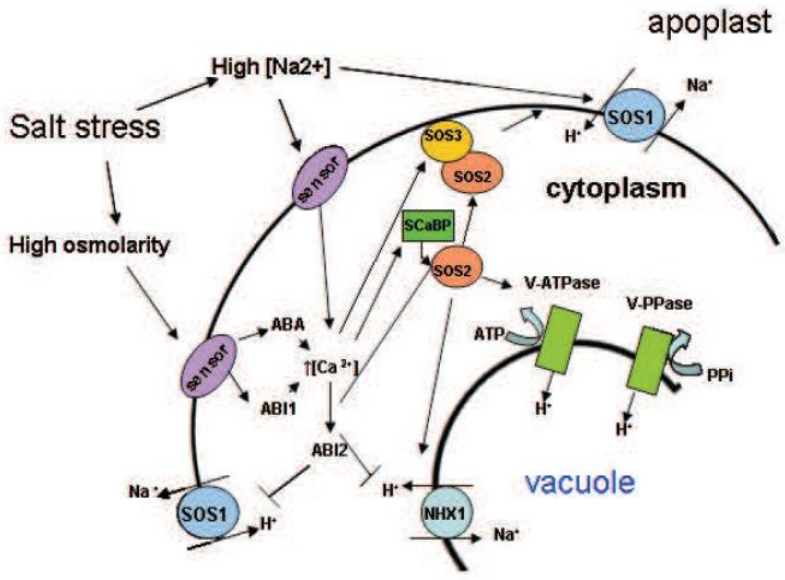


Figure 1.2. Signalling pathways in *Arabidopsis* under salt stress
(Source: Carillo, FuPontecorvo, Annunziata, & Woodrow, 2011)

1.3.2. Transport Mechanism

Increasing levels of Na+ and Cl- cause decrease in of K+ and Ca+ levels. This results in nutrient imbalance and ion toxicity and, plants develop various mechanism to deal with this toxicity (Mansour, Salama, & Al-Mutawa, 2003). Plasma membranes of root cells are the first to be affected injury (toxicity). Plasma membrane ATPases respond to the soil salinity. ATPases are integral transport proteins which transport ions across the plasma membrane and electrocehmical gradient is maintained. Salt stress induces P-ATPase (plasma membrane ATPases), V-ATPases (vacuolar ATPases), vacuolaror plasma membrane Na+/H+ antiporters, Cl-/2H+ symporters, K+/H+ antiporters, K+ channels and water channels (aquaporins) (Mansour et al., 2003; Mäser, Gierth, & Schroeder, 2002; Munns & Tester, 2008).

1.3.2.1. Ion Influx Mechanisms

Salinity affects K+/Na+ ratio and Na+ and Cl- ions concentration. Ionic balance maintanence is essential, especially K+, for plant cells. When the sodium and potassium hydrated, discrimination is very difficult because, sodium and potassium ionic radius are similar. This cause Na+ toxicity. K+ is necessary for enzyme activation, hovewer in this case K+ ions and Na+ ions compete to bind enzyme. Na+/K+ ration need to maintain in plant cells. There are four types of channels responsible for maintain Na+/K+ ratio. First one is K+ inward rectifying channels (KIRC), such as AKT1 which localize in plasma membrane and activate K+ uptaken, but doesn't play a role in Na+ uptataken. Second is K+ outward rectifying channels (KORCs) maintance the Na+ influx into plant cells and KORCs are highly sensitive to K+ than Na+. Third one is, voltage-independent cation channels (VICs) that have highly Na+/K+ sensitivity and they are not gated by voltage. Last one is non-selective outward-rectifying conductance (NORC) play role in diffusion of Na+ through the plasma membrane to down its electrochemical gradient (Blumwald, 2000; Blumwald, Aharon, & Apse, 2000).

KUP/HAK/KT transporters, HKT transporters, CNGC trasporters and LCT are cation transporters. They responsible uptaken of cations in the plant cell (Blumwald, 2000; Blumwald et al., 2000; Mäser et al., 2002).

HKT and KUP/HAK/KT are high affinity K transporter and transport to Na+ in several species like *Arabidopsis,* rice and wheat. HKT alleles are crucial under salt stress and knockout experiments show that HKT mutant cell are more sensitive to salt stress. In addition to that, HKT proteins are monovalent cation transporter, but recent studies indicate that some divalent cations are transported by HKT proteins. Some of HKT proteins are highly affinity to Na+, the others transport Na+ and K+ (Waters, Gilliham, & Hrmova, 2013).

KUP/HAK/KT mutant result in 'tiny-root-hair' phenotype and small leaves. When the KUP/HAK/KT gene HvHAK1 expressed in yeast, it show low affinity to Na+transport and high effinity to K+transport and another gene called AtHAK5 inhibited by Na+ (Mäser et al., 2002).

LCT is low affinity cation transporter and Na+ influx into plant cells which characterize in yeast and found that transport both Na+ and K+, as well as Ca+ and Cd+. Hovewer Ca+ inhibit the LCT1-mediated Na+ transport and neutralize Na+ toxicity (Mäser et al., 2002; Mäser et al., 2001).

CNGC transporter family firstly discovered in barley that gated by cGMP and CAMP and activition of cAMP and cGMP modulated by calcium and calmodulin. CNGC transporters exibite more comprehensive specifity to substrate such as K+, Na+ and Ca+ that have similar permeability. Taken up Na+ from root is inhibited by membrane-permeable cylic AMP and GMP analogs, so salt tolerance is increased in plant cells (Gobert, Park, Amtmann, Sanders, & Maathuis, 2006; Mäser et al., 2002; Mäser et al., 2001).

There are also anion transporter mechanisms such as Cl^- transport mechanism. Chloride is essential micronutrient that play role in enzyme activity, co-factor in photosynthesis and stabilize the membrane potential. Higher concentration of Cl^- result with toxicity in plants (Fig 1.3)(Teakle & Tyerman, 2010; White & Broadley, 2001).

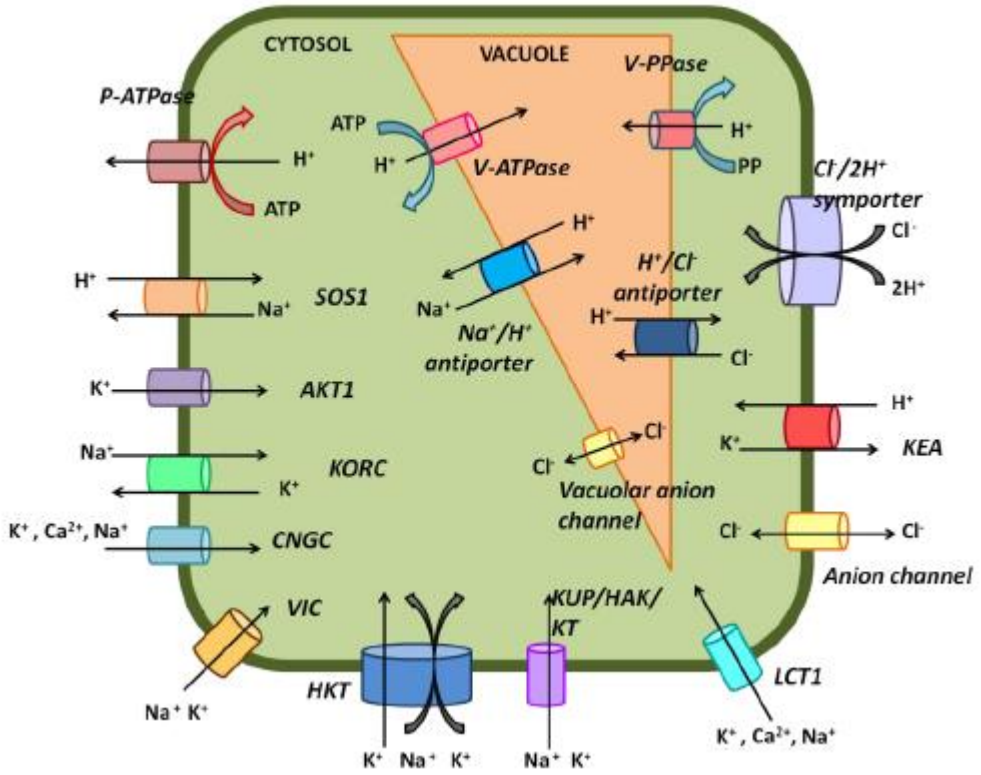


Figure 1.3. Ion transport systems on plasma and vacuolar membrane of plant cell (Source: The figure was modified from Maser, Gierth et al. 2002; Mansour, Salama et al. 2003; Munns and Tester 2008; Teakle and Tyerman 2010, ÇAKIROĞLU, 2012)

1.3.2.2. Ion Efflux Mechanisms

Plants have essential efflux mechanisms to remove potentially dangerous ions from the cytosol to maintain the electrochemical gradient of plant cell. The Na^+ export from the cytoplasm or vacuole are energized by H^+-coupled antiport(Blumwald, 2000; Brini & Masmoudi, 2012).

Plasma membrane ATPase, vacuolar pyrophosphatase and vacuolar ATPase mechanisms are used to extrusion of Na^+ which pump the proton across the plasma membrane. Under salt stress condition activation of H^+ pumps is increased and gene expression is induced. NHX1 gene play role in the Na^+/H^+ exchanger in yeast and response to sodium stress. NHX1 distruption researches indicate that NHX1 may have a role in vacuole biogenesis and vacuolar volume regulation. ENA1 is another gene play role in salt tolerance. Overexpression of vacuolar H^+-pryphosphatase(AVP1) in salt-sensitive *ena1* experiments results show that *ena1*mutant cells do not express plasma

membrane Na^+-ATPase mechanism and cytosolic Na^+ level rich the toxic level. Finally both plasma membrane Na^+-ATPase and plasma membrane Na^+/H^+ exchange essential for salt tolerance (Blumwald et al., 2000; Brini & Masmoudi, 2012; Mansour et al., 2003).

CHAPTER 2

MATERIAL METHODS

2.1. Yeast Growth and Media

The yeast strain,*Saccharomyces cerevisiae*, W303-1A (MATa; his3; leu2; met15; ura3) and its isogenic haploid transporter deletion mutant Ab11c(Ena1Δ, Nha1/4Δ, Nhx1Δ) obtain from Olga Zimmermannova (Academy of Sciences of the Czech Republic, Department of Bioenergetics Hlavni mesto Praha, Czech Republic). YPD media(with 2 % glucose, 2 % peptone, 1 %,yeast exract and 2 % agar) and SD media (2% glucose , 0.7 % YNB without aminoacid and 0.3 amino acid without uracil supplement) were used for yeast growth.

2.2. Plant Growth, RNA Isolation and Synthesis of Gateway® Compatible cDNAs

Beta maritima seeds sown in sterilized soil and after germination , they were watered regularly with 1/2nHoagland solution contained 3.5 mM Ca(NO3)2.4H2O, 2.5 mM KNO3, 1 mM KH2PO4, 1 mM MgSO4.7H2O, 22 µM H3BO3, 4.5 µM MnCl2.4H2O, 0.35 µM ZnSO4.7H2O, 0.2 µM CuSO4.5H2O, 0.07 µM NaMoO4, 15 µM EDTA.2Na, 14 µM FeSO4.7H2O and 0.5 mM KOH . Conditions of the growth chamber was as 12 hours dark and 12 hours light photoperiod with a 400 µmol m-2 s-1 light intensity. The temperature was 25 ^{O}C. Humidity has been set to 50,0 (as % rH).Salt tretment was started when plants were 1,3,5,7 hours . After these times plant harvested and 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).

We had *Beta maritima* CDNA library (Bozdag G. 2009 İYTE kütüphanesi Tez T000198 QH433.B79). This library used in pre-study.

2.3. Yeast Transformation of Cloned cDNA Library and Plasmid Isolation

Yeast transformation was performed by LiCl method (Burke, Dawson, and Stearns 1994). Yeast cells were grown overnight at 5ml liquid YPD medium. Sample was diluted , 1ml taken from sample and added 4ml new YPD mediaand incubated 3h at 30^{O}C incubator. Samples are santrifugated at 2500 rpm 5minutes. Pelleted cells were washed with dH2O three times . Pelleted cells were resuspended by 0.1M LiAc and transferred 1,5 ml microcentrifuge tubes. Centrifugated 5seconds at top speed and washed with 500 µl 0.1 M LiAc. Centrifugated at top seepd 5 minutes and removed LiAc . 240 µl PEG (50 % w/v), 36 µl(1 M) LiAc, 10 µl ssDNA, 5 µl DTT (0.3 mM), 20 µl plasmid DNA, 10 µl dH2O added to pellet and pipetting is done for 1 minute, incubated at 30 0C (160 rpm) for 30 minutes. Then taken to waterbath for 30 minutes at 42 0C. The cells have been plated on SD+ 800µM NaCl plates.Incubated at 35 0C etuve at 5-7 days an formed colonies are selected and spreaded on SD plates to get more plasmid. Colonies which growth in SD with NaCl platted SD liquet media and growth overnight and when OD rich 1 , they centrifugated (2500rpm ,24°C,7minute), washed with dH2O, 10 µl lyctase enzyme added to tube and incubated 25 minutes 37^{O}C 1800rpm. Then 10 µl %20 SDS added to microcentrifuge tubes and incubated at -20^{O}C 45 minutes and plasmid isolation were performed by Thermo GeneJET Plasmid MiniPrep Kiti.

After plasmid isolation , plasmids have been sent to the Biotechnology Center in IYTE to identify the sequence analyses of the cloned *Beta maritima* genes.

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

Sequenced genes from Beta maritima cDNA library were searched on BLAST service of NCBI and multiple protein sequence aligment analysis was performed by ClusterW2.

2.5. Salt Tolerance Assay

Identified gene was tested in different concentration and different salt stress: LiCl (40mM), KCl (1800mM) and NaCl (800mM). The wild type and mutant strain were transformed with empty vector *pAG426GPD*. Yeast cells incubated overnight in shaker at 30^{O}C ,170rpm and they were diluted to OD600= 0,2; 0,02; 0,002;0,0002 by distilled water. Dilutions were spotted as 5µl on solid SD plates with and without 800mM NaClied 70, 40mM LiCl and 1800mM KCl and growth for 5days at 30 ^{O}C.

2.6. Identification of NaCl, LiCl ve KCl concentration in Yeast Cells

After NaCl, LiCl and KCl treatment, salt concentration in Ab11c yeast cells and empty vector, were detected by reference to Mizuno et al.2005. Pre-cultured yeast cells were grown overnight in SD medium and the cells were diluted with the SD medium as the final concentration having 1/1000 diluted yeast cells and added NaCl(800mM),LiCl(40mM) and KCl(1800mM) and incubated for 48-60 hours at 30^{O}C. Then cells were washed with 10mM EDTA three times, dried overnight at 70^{O}C . Yeast cells (5mg) were extracted by 65% HNO3 and filtrated with 0.20 µm Minisart® filters (Gottingen, Germany). Extracteds were studied with Induced Coupled Plasma-Mass Spectrometry (ICP-MS) in the Environmental Development, Implementation and Research Center, IYTE to identified Na+, Li+ and K+.

2.7. Identification of Pollen-E1 protein localization (GFP)

Pollen-E1 gene is generated by PCR method with mutated stop codon. Stop codon was used to C-terminal fusion to the GFP gene and to obtain pAG415GFP-Pollen-E1 GFP, Gateway cloning system was used(Fig.2.1). The primers used were BmB1 forward (5’ GGGGACAAGTTTGTAAAAAAAGCAGGCTTCATGGTG AAATTGCAGGAATATTGT-3’) and reverse (5’-GGGGACCACTTTGTACAA GAAAGCTGGGTCCTA AACTTCATCTTCAGTGAGTTGA-3’). The PCR product inserted in pDONOR vector and generate entry vector then entry vector and crossing

with destination (pAG415GFP) vector to produce expression vector. Expression vector inserted in Ab11c strain growing in YNB-Leu media overnight and refreshed by YNB-Leu liquid and and growed 4hour. Then visualized by confocal microscopy.

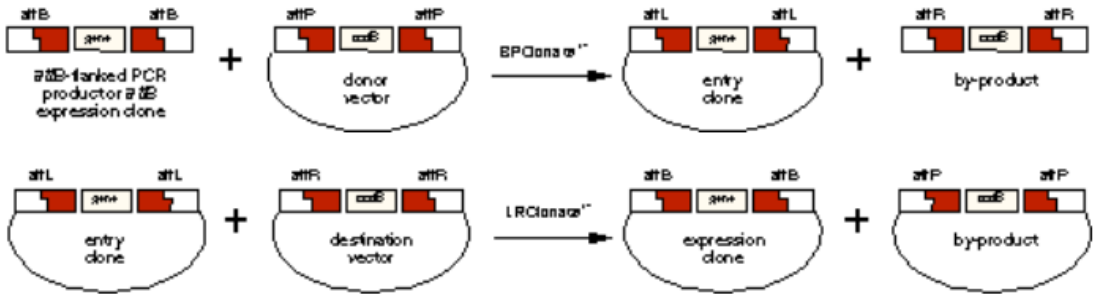


Figure 2.1. Gateway Cloning System

2.8. Real-Time PCR Analyses

Beta maritima plants were grown at 400mM NaCl concentration for gene expression analyses. Then leaf and roots were harvested at time 0 and after NaCl treatment at 1h, 3h, 5h and 7 hour. Before total RNA isolation , samples were stored at -80^{O}C. Then total RNA isolated from roots and leaves as described above. Genomic cDNA remnants were removed by DNAse treatment(Fermantase DNAse kit). cDNAs were synthesis by using Fermantes cDNA Synthesis Kit. Quantitive gene expression analyses were performed by IQ5 real-time PCR cycler system (Bio-rad, München, Germany). Ingradients added according to Table 2.1 12,5 µl master mix, 0,5 µl forward and reverse , 1µl cDNA template and 10,5 µl UP (ultra pure water) added and RT_PCR assay was performed according to Table 2.2 conditions.

Table2.1. Ingredients of RT-PCR

	µL
Fermantas MaximaTM SYBR Green qPCR Master Mix(2X)	12,5
BIRTF(15µL)	0,5
BIRTR(15µL)	0,5
cDNA	1
UP Water	10,5

Table2.2. RT-PCR conditions

STEP1	1 cycle	95^{O}C	03:00 min
STEP2	40 cycle	95^{O}C	00:20 min
		55^{O}C	00:30 min
		72^{O}C	00:30 min

Beta actin was used as positive control. BmAc2RTF 5'- used as forward prime 5'AGACCTTCAATGTGCCTGCT-3' and BmAct2RTR 5' TCAGTGAGATC ACGACCAGC-3 used for amplified 187 bp of *Beta maritima* beta actin cDNA.

The forward primer of Pollen-E1 gene was Pollen-E1RTF 5'-GTAGACCAGAGAAGAAGC CATAC-3' and reverse was Pollen-E1RTR 5'-GGCATTCCAACTTCACCTTTAC-3'. These primers were used to amplify 612 bp Pollen-E1 cDNA.

CHAPTER 3

RESULTS

3.1. Identification and Cloning of the *Pollen-E1* Gene

Beta maritima cDNA library transformed into Ab11c strain and several yeast strain colonies grown at 800mM NaCl concentration which was toxic for Ab11c cells within 4 days. Plasmid isolation was performed from these colonies and we focused on one cDNAs showed a homology in database.

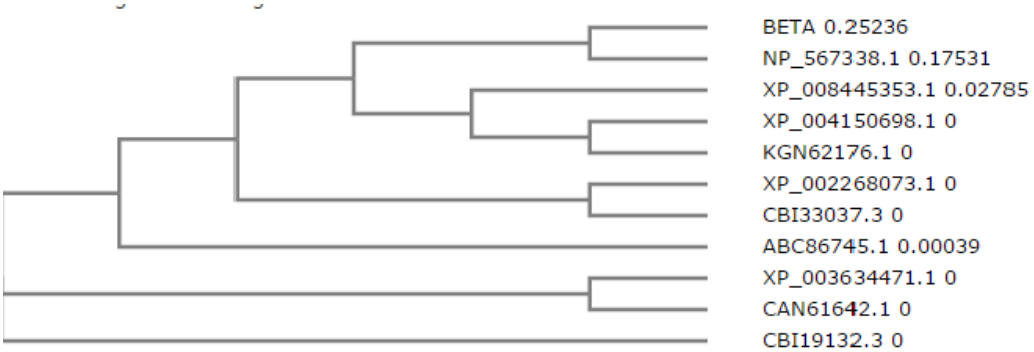
Pollen-E1 open reading frame consist of 662bp and composed of 61 amino acids. Teorical molecular weight is 17666.13674 Da and isoelectric point is 6,45. Blast (X) database program was used to search homology of *Pollen-E1* and multiple sequence aligment result for *Pollen-E1* (Fig.3.1).

```
BETA              MAKIAGILFLLCVLPVLAMAGRPEKKPYCVRGKVYCDTCRAGFETPASTYLEGVKVKLEC
NP_567338.1       MSK-AVLLVALCFLPALAIAARPNKNPFVVRGRVYCDTCLAGFETPASTYISGAVVRLEC
XP_008445353.1    MAR-LVILFALIMLPALAVASRPVRTPFVVRGKVFCDTCLAGFETSATTYIPGAKVRIEC
XP_004150698.1    MAR-VIILFALIMLPALALASRPVRTPFVVRGKVFCDTCLAGFETSATTYIPGAKVRIEC
KGN62176.1        MAR-VIILFALIMLPALALASRPVRTPFVVRGKVFCDTCLAGFETSATTYIPGAKVRIEC
XP_002268073.1    MAK-LLMSIALCLLLVYVSEARPMRKPFVLHGRVYCDTCRAGFETSATTYIAGARVRIEC
CBI33037.3        MAK-LLMSIALCLLLVYVSEARPMRKPFVLHGRVYCDTCRAGFETSATTYIAGARVRIEC
ABC86745.1        MGR-LMLLVALCVLPALVSAGRPVSQPFVLQGRVYCDTCRAGFETSATTYIAGAKVRVEC
XP_003634471.1    MGR-LMLLVALCVLPALVSAGRPVSQPFVLQGRVYCDTCRAGFETSATTYIAGAKVRVEC
CAN61642.1        MGR-LMLLVALCVLPALVSAGRPVSQPFVLQGRVYCDTCRAGFETSATTYIAGAKVRVEC
CBI19132.3        MGR-LMLLVALCVLPALVSAGRPVSQPFVLQGRVYCDTCRAGFETSATTYIAGAKVRVEC
                  *.:   : . * .* . .  .**   *: ::*:*:**** ***** *:**: *. *::**

BETA              RHRQTQEVLYSAVATTDRTGSYKIFVENDQKENICDTMLLSSPHRRCKLADPGRDRSRVV
NP_567338.1       KDRRTMELTYSHEARTDSTGSYKILVNEDHDEQFCDAMLVRSSQLRCSNVSPGHDRARVT
XP_008445353.1    KDRNSMEVRYTHEATTDSTGSYTLLVSEDHGDELCDAVLVSSPQEKCSSVAEGRDRARVI
XP_004150698.1    KDRNSMELQYTHEATTDSTGSYTLLVNEDHGDELCDAVLVSSPQEKCSSVSEGRDRARVI
KGN62176.1        KDRNSMELQYTHEATTDSTGSYTLLVNEDHGDELCDAVLVSSPQEKCSSVSEGRDRARVI
XP_002268073.1    KDRNSLQLVYSVEGVTDSTGTYKFSIADDHGDQMCDAVLVKSPQPDCAKVDAGRDRSRLS
CBI33037.3        KDRNSLQLVYSVEGVTDSTGTYKFSIADDHGDQMCDAVLVKSPQPDCAKVDAGRDRSRLS
ABC86745.1        KDRNSMQLLYSIEGITDSTGTYKIMVTEDHEDQLCDAVLVSSPQSDCASVDPGRDRAAVI
XP_003634471.1    KDRNSMQLLYSIEGITDSTGTYKIMVTEDREDQLCDAVLVSSPQSDCASVDPGRDRAAVI
CAN61642.1        KDRNSMQLLYSIEGITDSTGTYKIMVTEDREDQLCDAVLVSSPQSDCASVDPGRDRAAVI
CBI19132.3        KDRNSMQLLYSIEGITDSTGTYKIMVTEDREDQLCDAVLVSSPQSDCASVDPGRDRAAVI
                  :.*.: :: *:  . ** **:*.: : :*: :::**::*: * :  *  .  *:**: :

BETA              LTSNNGVVSNDRYANNMGFTVEEPMSYCAQLMQQYQLTEDEV
NP_567338.1       LTRFNGIASDDRFANNMGFLRDAAMPGCADIMKLYQETED--
XP_008445353.1    LTRYNGIASNDRYVNAMGFAIDEPMSGCNQVMSQYQDIED--
XP_004150698.1    LTRYNGIASNERYVNAMGFAMDEPMSGCNQVMSQYQDIED--
KGN62176.1        LTRYNGIASNERYVNAMGFAMDEPMSGCNQVMSQYQDIED--
XP_002268073.1    LTRSNGLVSDTRFANAMGFMKDEPASGCTQLLKQYQESDD--
CBI33037.3        LTRSNGLVSDTRFANAMGFMKDEPASGCTQLLKQYQESDD--
ABC86745.1        LTRYNGIVSDNRYANSMGFLKDHPMSECTQLLQQYQEFED--
XP_003634471.1    LTRYNGIVSDNRYANSMGFLKDHPMSECTQLLQQYQEFED--
CAN61642.1        LTRYNGIVSDNRYANSMGFLKDHPMSECTQLLQQYQEFED--
CBI19132.3        LTRYNGIVSDNRYANSMGFLKDHPMSECTQLLQQYQEFED--
                  **  **:.*: *:.* ***  :     * :::. **  :*
```

a)



b)

Figure 3.1.Multiple amino acid sequence aligment of *Pollen-E1* protein sequence. a) some colurs indicate conserved amino acids between *Beta vulgaris subp. Vulgaris*(XP_010676978.1) *Vitis pseudoreticulata* (ABC86745.1), *Vitis vinifera* (XP_003634471.1), *Vitis vinifera* (CBI19132.3), *Vitis vinifera* (XP_002268073.1), *Vitis vinifera*(CBI33037.3), *Cucumis melo* (XP_008445353.1), *Cucumis sativus* (XP_004150698.1), *Cucumis sativus*(KGN62176.1), *Arabidopsis thaliana* (NP_567338.1) b) phylogenetic tree the proteins.

Blast program was used to indicate results. Then Cluster omega has been performed for aligment.

The Pollen-E1 protein gives 57% amino acid identities with *Arabidopsis thaliana* Sah7 (NP_567338.1) protein which called Sinapis Arabidopsis Homolog 7.

3.2. Solid Growth Tests

One colony is choosen from screening of cDNA library on plates which contain 800mM NaCl. First of all, NaCl tolerance spotting assay was performed on solid YNB-Ura medium to confirm, POLLEN-E1 provide NaCl tolerance at 800mM NaCl level to mutant Ab11c yeast cells (as shown in Fig. 3.2).

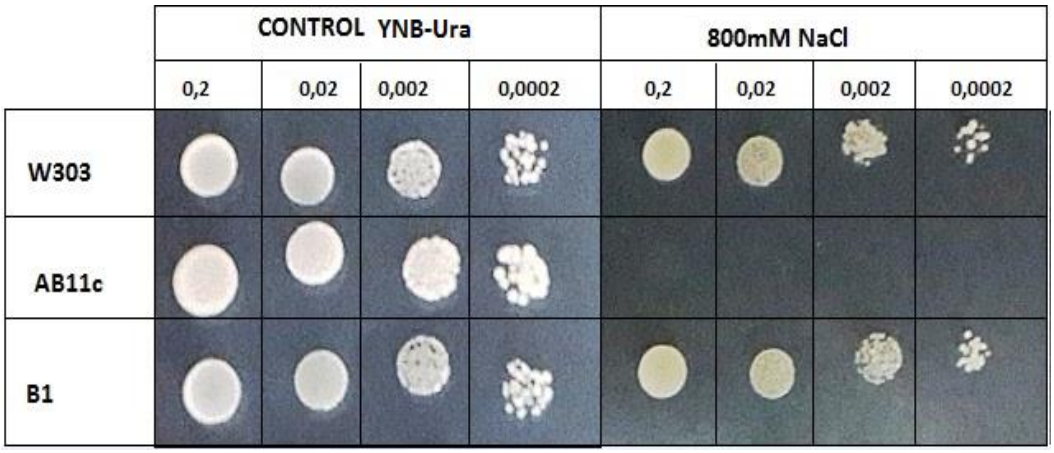


Figure 3.2. Solid growth test. Four serial dilution from O.D. 600= 0,2 to O.D. $_{600}$ = 0,0002 were plated on minimal medium. W303 is wild type strain which contain pAG426GPD plasmid. A Pollen-E1 1c is mutant strain that contain pAG426GPD plasmid and POLLEN-E1 that mutant cells contain *POLLEN-E1* cDNA overexpressing vector .

To understand different salt tolerance selectificity of *Pollen-E1* and *Sah7* which homolog to *Pollen-E1* in *Arabidopsis*, salt tolerance assay was performed by using KCl, LiCl and NaCl in different concentration that respectively 1M, 40 mM and 800mM (Fig3.3). In 1 M KCl, 40 mM LiCl and 800 mM NaCl media while *SAH7* and *Pollen-E1* show the same growth, Ab11c (pAG426GPD) didn't grow under same condition.

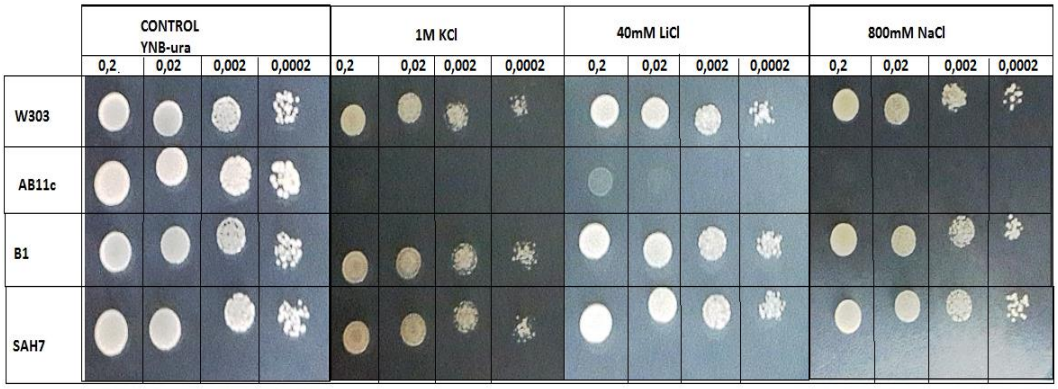


Figure 3.3. Solid growth test under different type and concentration of salt containing medium. After four serial dilution (O.D. $_{600}$ = 0,2 to 0,0002) cella were plated on minimal medium. Transformed yeast cells with *POLLEN-E1* and *Sah7* and pAG426GPD were incubated at 30^{O}C,5 days

3.3. Gene Expression Analysis

Beta maritima plants growth under 400mM NaCl condition. Leaves and roots are harvested before exposed salt tolerance, and after NaCl induction at 1st hour, 3rd hour, 5th hour and 7th hours. The total RNAs were isolated from the harvested samples and quality of total RNAs were controlled by performing agarose gel (1%) electrophoresis (Fig. 3.4). From the total RNAs , cDNAs were synthesized and they used to performed gene expression analysis by Real Time PCR.

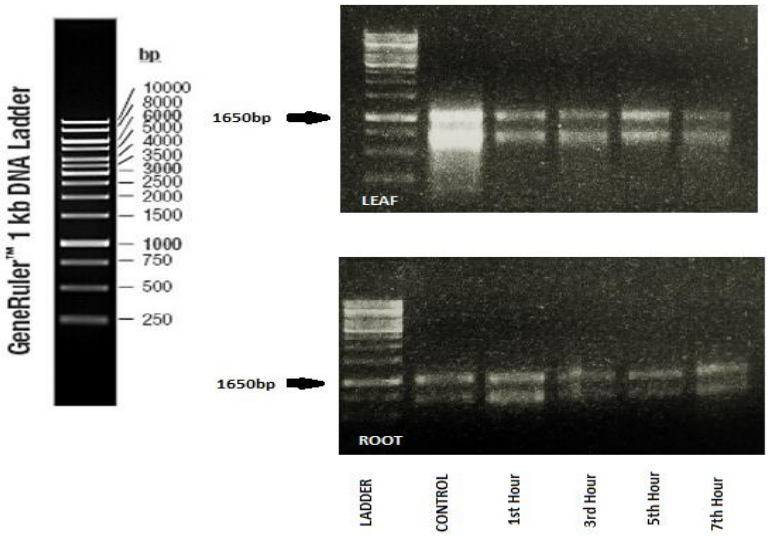
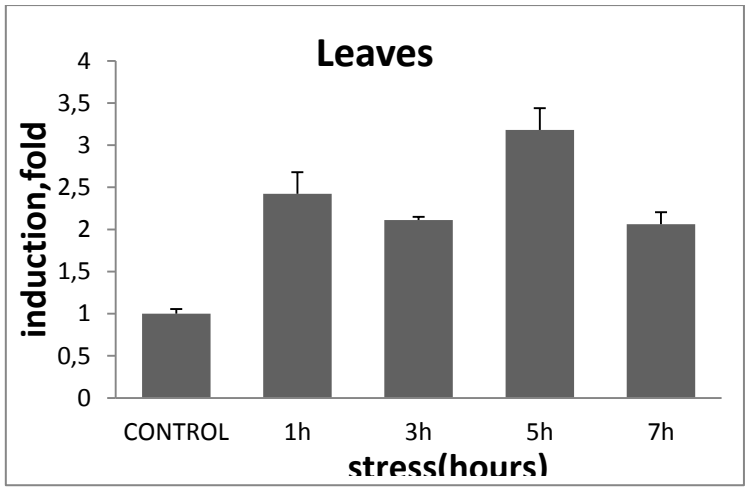


Figure 3.4. Agorose gel wiev to quality control of total RNAs. Leaves and roots sample that harvested at 1h, 3h, 5h and 7h under 400 mM NaCl stress.

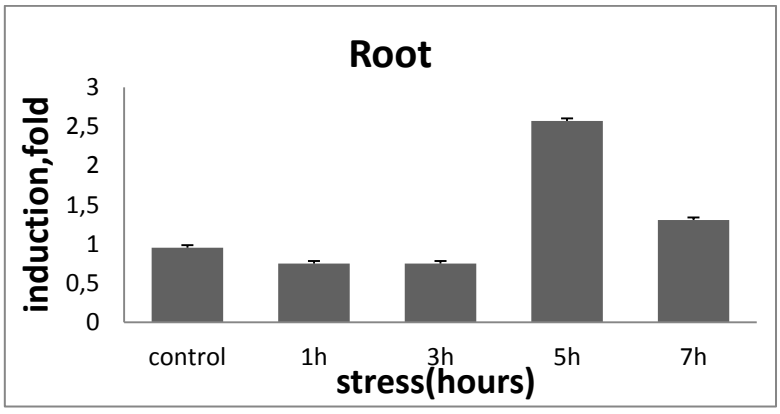
3.4. Pollen-E1 Gene Expression Results

Pollen-E1 mRNA amount in leaves, harvested at 1st h, 3rd h, 5th h, 7th h salt treatment, shows significant change (at least 2 fold induction) comparing with control group (Figure 3.5.a) suggesting Pollen-E1 gene might have some regulatory role under salt stress in leaves of *B. maritima*.

Pollen-E1 mRNA amount in root did not show statistically differences when we compare with control at 1h and 3h salt treatment, however gene expression was dramitically increase (nearly 2,5 fold) at 5h salt treatment (Figure 3.5.b) suggesting Pollen-E1 mRNA have some role in root after 5h salt treatment.



a)



b)

Figure 3.5. Expression level of POLLEN-E1 . (a) shows expression of POLLEN-E1 cDNA from leaves exposed with NaCl.(b) shows expression of POLLEN-E1 cDNA from roots exposed with NaCl. Plants exposed to 400mM NaCl and values are the mean of three repeats.

3.5. Intracellular Sodium Concentratitons

Sodium concentration of the yeast cells, which were overexpressing, Pollen-E1 was measured to understand whether Pollen-E1 protein have a role as pumping the sodium out of the cell.

Intracellular Na^+ levels were measured by Induced Coupled Plasma-Mass Spectrometry (ICP-MS). According to the results there was a significant differencess between control cells (W303 strain contain pAG426GPD) and mutant cells which contain Pollen-E1 genes (Fig.3.6)

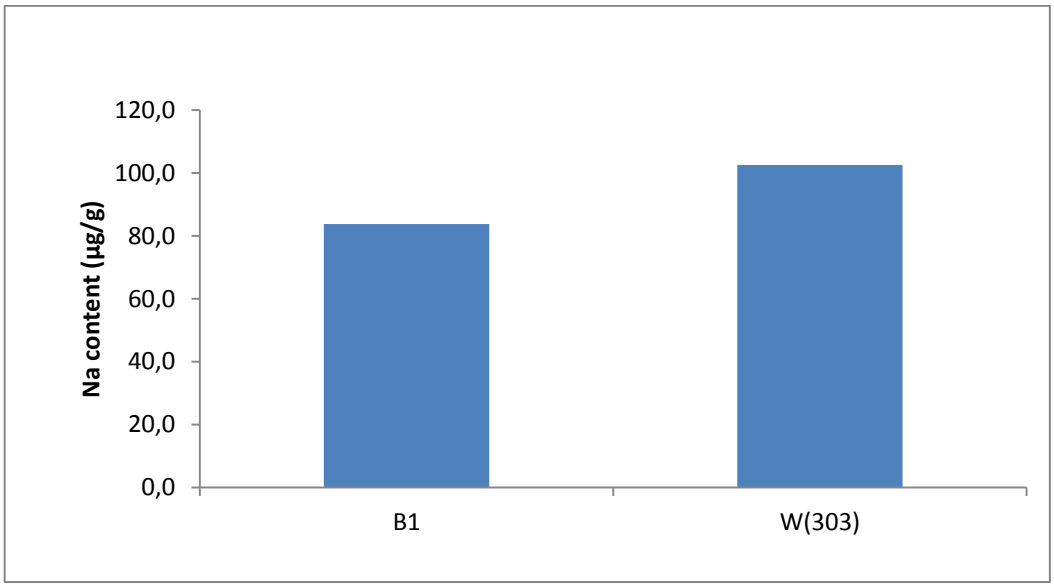


Figure 3.6. Intracellular sodium concentration of transformed APollen-E1 1c strains. W303 represent control group. The cells were grown in 400 mM NaCl containing YNB-ura media for 36 hours at 30^{O}C.

According to the intracellular Na^+ concentrations in transformed Ab11c strains with Pollen-E1 genes were remarkable decrease than wild type W303 strains. While the Pollen-E1 overexpression cells were accumulate 83,8 µg/g Na^+ , W303 cells were acculmulate 103 µg/g Na^+ . These results show that Pollen-E1 have a role in transport of excess Na^+ out of the cell.

3.6. Subcellular Localization Pollen-E1

The subsellular localization assay was performed according to Pollen-E1 genes fusion with pAG415GFP plasmid. The inserted cells were grown in YNB-Leu medium and visualize by confogal microscopy (Fig.3.7). POLLEN-E1 are localize in a punctuated pattern resembling in the endomembrane system.

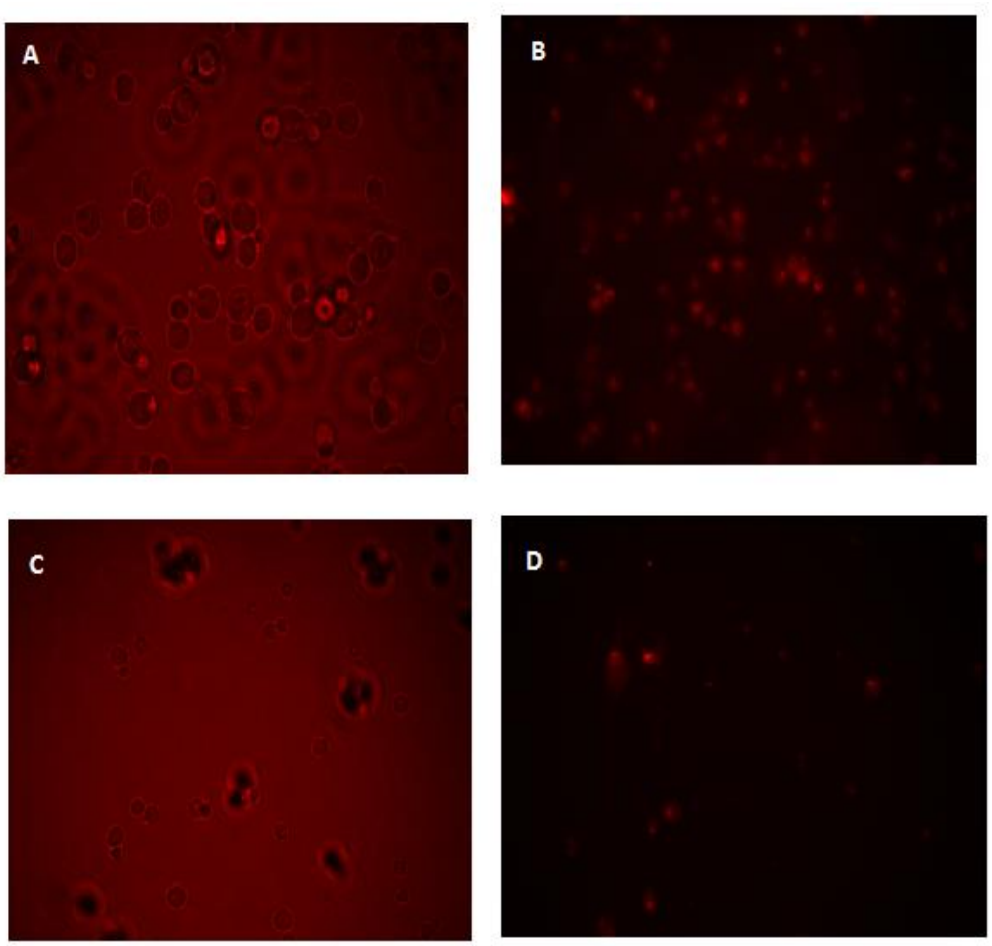


Figure 3.7. POLLEN-E1 localization in yeast cells. POLLEN-E1 : *POLLEN-E1* gene that inserted in pAG415GFP plasmid and proteins are localize in a punctuated pattern resembling in the endomembrane. A) POLLEN-E1 fusion protein , C) non-inserted pAG415GFP protein location B and C) bright-field images are shown for the cells shown in (A) and (C), respectively. Results indicate that POLLEN-E1 proteins localize in endomembrane in the yeast cells.

CHAPTER 4

DISCUSSION

We screen cDNA library of *Beta maritima* to identify salt tolerance genes in mutant Ab11c strain of *Saccharomyces cerevisiae* cells. Looking at the solid growth test results, *Pollen-E1* gene was showed promising salt tolerance to yeast cells. According to the sequence and homology analyses, *Sah7* gene in *Arabidopsis thaliana* is close homolog of the *Pollen-E1* gene in *Beta maritima. Sah7* is called Sinapis Arabidopsis Homolog 7 and firstly isolated in polen tube with unknown function and overexpress under some stress condition such as salt and antioxidant stress (Zhou et al., 2013).

According to solid growth test , *Pollen-E1* and *Sah7* growth show same result. Both grow under toxic level of KCl, LiCl and NaCl contained medium (Fig.3.3).

In subcellular localization assay, *Pollen-E1* protein localized in endomembrane system of the cell in yeast. Similarly endomembrane localized N-glycosylation proteins regulate salt tolerance, cellulose biosynthesis and protein quality control in plants (Kang et al., 2008). Research indicate that maturation of N-glycans necessary for salt stress adaptation in *Arabidopsis.* In Golgi apparatus N-glycan maturation confersalt sensitivity (Kang et al., 2008; von Schaewen, Frank, & Koiwa, 2008).

Intracellular Na^+ concentration measurement, *Pollen-E1* may play role in transport of excess Na^+ out of the cell compare with wild type yeast strain. While wild type yeast strains accumulate Na^+, *Pollen-E1* yeast strains transport sodium out of the cell and decrease toxic Na^+ concentration.

CHAPTER 5

CONCLUSION

In this study we screened *Beta maritima* cDNA library by using *Saccharomyces cerevisiae* yeast strain as a model organism to find out sodium detoxifying genes.

After screening assays, we found out a gene named as *Pollen-E1* confers salt tolerance to yeast cells. Intracellular sodium measurements of *Pollen-E1* overexpressed in yeast cells showed incline in salt levels compared to wild type suggesting sodium was transporting out of the cell. Pollen-E1 protein localized in endomembrane systems in yeast cells. In mRNA expression analysis, POLLEN-E1 mRNA levels induced immediately in the leaves and late stages in root systems under salt stress. Our results show that POLLEN-E1, is an uncharacterized gene with an unknown function might have a role in regulating salt tolerance in *Beta maritima*.

REFERENCES

Agastian, P., Kingsley, S., & Vivekanandan, M. (2000). Effect of salinity on photosynthesis and biochemical characteristics in mulberry genotypes. *Photosynthetica, 38*(2), 287-290.

Allakhverdiev, S. I., Sakamoto, A., Nishiyama, Y., & Murata, N. (2000). Inactivation of photosystems I and II in response to osmotic stress in Synechococcus. Contribution of water channels. *Plant Physiology, 122*(4), 1201-1208.

Blaylock, A. D. (1994). *Soil salinity, salt tolerance, and growth potential of horticultural and landscape plants*: University of Wyoming, Cooperative Extension Service, Department of Plant, Soil, and Insect Sciences, College of Agriculture.

Blumwald, E. (2000). Sodium transport and salt tolerance in plants. *Current opinion in cell biology, 12*(4), 431-434.

Blumwald, E., Aharon, G. S., & Apse, M. P. (2000). Sodium transport in plant cells. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1465*(1), 140-151.

Brini, F., & Masmoudi, K. (2012). Ion transporters and abiotic stress tolerance in plants. *International Scholarly Research Notices, 2012*.

Carillo, P., Fuggi, A., Pontecorvo, G., Annunziata, M. G., & Woodrow, P. (2011). *Salinity stress and salt tolerance*: INTECH Open Access Publisher.

Chaudhuri, K., & Choudhuri, M. (1997). Effects of short-term NaCl stress on water relations and gas exchange of two jute species. *Biologia plantarum, 40*(3), 373-380.

Chaves, M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot, 103*(4), 551-560.

Chaves, M. M., Flexas, J., & Pinheiro, C. (2009). Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot, 103*(4), 551-560. doi: 10.1093/aob/mcn125

Chinnusamy, V., Jagendorf, A., & Zhu, J.-K. (2005). Understanding and improving salt tolerance in plants. *Crop Science, 45*(2), 437-448.

Chinnusamy, V., Zhu, J., & Zhu, J.-K. (2006). Salt stress signaling and mechanisms of plant salt tolerance *Genetic engineering* (pp. 141-177): Springer.

ÇAKIROĞLU, Ç. (2012). *IDENTIFICATION OF SALT STRESS RESPONSIVE PROTEINS IN WILD SUGAR BEET (BETA MARITIMA) USING 2D-PAGE WITH MALDI-TOF/TOF SYSTEM*. Izmir Institute of Technology.

Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., & Van Breusegem, F. (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS, 57*(5), 779-795.

Flores, P., Botella, M., Martinez, V., & Cerdá, A. (2000). Ionic and osmotic effects on nitrate reductase activity in tomato seedlings. *Journal of plant physiology, 156*(4), 552-557.

Glenn, E. P., Brown, J. J., & Blumwald, E. (1999). Salt tolerance and crop potential of halophytes. *Critical reviews in plant sciences, 18*(2), 227-255.

Gobert, A., Park, G., Amtmann, A., Sanders, D., & Maathuis, F. J. (2006). Arabidopsis thaliana cyclic nucleotide gated channel 3 forms a non-selective ion transporter involved in germination and cation transport. *Journal of Experimental Botany, 57*(4), 791-800.

Gupta, B., & Huang, B. (2014). Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. *International Journal of Genomics, 2014*, 1-18. doi: 10.1155/2014/701596

Hernandez, J., Campillo, A., Jimenez, A., Alarcon, J., & Sevilla, F. (1999). Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. *New Phytologist, 141*(2), 241-251.

Hu, T., Li, H.-y., Zhang, X.-z., Luo, H.-j., & Fu, J.-m. (2011). Toxic effect of NaCl on ion metabolism, antioxidative enzymes and gene expression of perennial ryegrass. *Ecotoxicol Environ Saf, 74*(7), 2050-2056.

Ji, H., Pardo, J. M., Batelli, G., Van Oosten, M. J., Bressan, R. A., & Li, X. (2013). The Salt Overly Sensitive (SOS) pathway: established and emerging roles. *Mol Plant, 6*(2), 275-286. doi: 10.1093/mp/sst017

Kang, J. S., Frank, J., Kang, C. H., Kajiura, H., Vikram, M., Ueda, A., . . . Fujiyama, K. (2008). Salt tolerance of Arabidopsis thaliana requires maturation of N-glycosylated proteins in the Golgi apparatus. *Proceedings of the National Academy of Sciences, 105*(15), 5933-5938.

Kim, S. H., Kim, S. H., Palaniyandi, S. A., Yang, S. H., & Suh, J. W. (2015). Expression of potato S-adenosyl-L-methionine synthase (SbSAMS) gene altered developmental characteristics and stress responses in transgenic Arabidopsis plants. *Plant Physiol Biochem, 87*, 84-91. doi: 10.1016/j.plaphy.2014.12.020

Knight, H., Trewavas, A. J., & Knight, M. R. (1997). Calcium signalling in Arabidopsis thaliana responding to drought and salinity. *The Plant Journal, 12*(5), 1067-1078.

LANGE, W., BRANDENBURG, W. A., & BOCK, T. S. D. (1999). Taxonomy and cultonomy of beet (Beta vulgaris L.). *Botanical journal of the Linnean Society, 130*(1), 81-96.

Leung, J., Bouvier-Durand, M., Morris, P.-C., Guerrier, D., Chefdor, F., & Giraudat, J. (1994). Arabidopsis ABA response gene ABI1: features of a calcium-modulated protein phosphatase. *Science, 264*(5164), 1448-1452.

Lieber, C. S. (2002). S-adenosyl-L-methionine: its role in the treatment of liver disorders. *The American journal of clinical nutrition, 76*(5), 1183S-1187S.

Lim, C. C., Liu, J. Z., & Pua, E. C. (2002). Characterization of S-adenosylmethionine synthetase genes and its expression is associated with ethylene synthesis in mustard (Brassica juncea). *Physiologia Plantarum, 116*(4), 522-530.

Liu, J., Ishitani, M., Halfter, U., Kim, C. S., & Zhu, J. K. (2000). The Arabidopsis thaliana SOS2 gene encodes a protein kinase that is required for salt tolerance. *Proc Natl Acad Sci U S A, 97*(7), 3730-3734. doi: 10.1073/pnas.060034197

Longstreth, D. J., & Nobel, P. S. (1979). Salinity effects on leaf anatomy consequences for photosynthesis. *Plant Physiology, 63*(4), 700-703.

Mahajan, S., Pandey, G. K., & Tuteja, N. (2008). Calcium-and salt-stress signaling in plants: shedding light on SOS pathway. *Archives of biochemistry and biophysics, 471*(2), 146-158.

Mansour, M., Salama, K., & Al-Mutawa, M. (2003). Transport proteins and salt tolerance in plants. *Plant Science, 164*(6), 891-900.

Mäser, P., Gierth, M., & Schroeder, J. I. (2002). Molecular mechanisms of potassium and sodium uptake in plants. *Plant and Soil, 247*(1), 43-54.

Mäser, P., Thomine, S., Schroeder, J. I., Ward, J. M., Hirschi, K., Sze, H., . . . Sanders, D. (2001). Phylogenetic relationships within cation transporter families of Arabidopsis. *Plant Physiology, 126*(4), 1646-1667.

Mitsuya, S., Takeoka, Y., & Miyake, H. (2000). Effects of sodium chloride on foliar ultrastructure of sweet potato (Ipomoea batatas Lam.) plantlets grown under light and dark conditions in vitro. *Journal of plant physiology, 157*(6), 661-667.

Mudgal, V., Madaan, N., & Mudgal, A. (2010). Biochemical mechanisms of salt tolerance in plants: a review. *International Journal of Botany, 6*(2), 136-143.

Munns, R., & Tester, M. (2008). Mechanisms of salinity tolerance. *Annu Rev Plant Biol, 59*, 651-681. doi: 10.1146/annurev.arplant.59.032607.092911

Osakabe, Y., Arinaga, N., Umezawa, T., Katsura, S., Nagamachi, K., Tanaka, H., . . . Yamaguchi-Shinozaki, K. (2013). Osmotic stress responses and plant growth controlled by potassium transporters in Arabidopsis. *Plant Cell, 25*(2), 609-624. doi: 10.1105/tpc.112.105700

Parida, A. K., & Das, A. B. (2005). Salt tolerance and salinity effects on plants: a review. *Ecotoxicol Environ Saf, 60*(3), 324-349. doi: 10.1016/j.ecoenv.2004.06.010

Popova, O. V., Ismailov, S. F., Popova, T. N., Dietz, K. J., & Golldack, D. (2002). Salt-induced expression of NADP-dependent isocitrate dehydrogenase and ferredoxin-dependent glutamate synthase in Mesembryanthemum crystallinum. *Planta, 215*(6), 906-913. doi: 10.1007/s00425-002-0827-0

Qiu, Q. S., Guo, Y., Quintero, F. J., Pardo, J. M., Schumaker, K. S., & Zhu, J. K. (2004). Regulation of vacuolar Na+/H+ exchange in Arabidopsis thaliana by the salt-overly-sensitive (SOS) pathway. *J Biol Chem, 279*(1), 207-215. doi: 10.1074/jbc.M307982200

Quintero, F. J., Ohta, M., Shi, H., Zhu, J. K., & Pardo, J. M. (2002). Reconstitution in yeast of the Arabidopsis SOS signaling pathway for Na+ homeostasis. *Proc Natl Acad Sci U S A, 99*(13), 9061-9066. doi: 10.1073/pnas.132092099

Rubio, F., Gassmann, W., & Schroeder, J. I. (1995). Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. *Science, 270*(5242), 1660-1663.

Srivastava, H., Shahi, H., Kumar, R., & Bhatnagar, S. (2000). Genetic diversity in Beta vulgaris ssp. maritima under subtropical climate of north India. *Journal of sugar beet research, 37*(3), 79-87.

Stepien, P., & Johnson, G. N. (2009). Contrasting responses of photosynthesis to salt stress in the glycophyte Arabidopsis and the halophyte Thellungiella: role of the plastid terminal oxidase as an alternative electron sink. *Plant Physiology, 149*(2), 1154-1165.

Teakle, N. L., & Tyerman, S. D. (2010). Mechanisms of Cl(-) transport contributing to salt tolerance. *Plant Cell Environ, 33*(4), 566-589. doi: 10.1111/j.1365-3040.2009.02060.x

Tuteja, N. (2007). Mechanisms of High Salinity Tolerance in Plants. *428*, 419-438. doi: 10.1016/s0076-6879(07)28024-3

Von Schaewen, A., Frank, J., & Koiwa, H. (2008). Role of complex N-glycans in plant stress tolerance. *Plant signaling & behavior, 3*(10), 871-873.

Wang, W., Vinocur, B., & Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta, 218*(1), 1-14. doi: 10.1007/s00425-003-1105-5

Waters, S., Gilliham, M., & Hrmova, M. (2013). Plant high-affinity potassium (HKT) transporters involved in salinity tolerance: structural insights to probe differences in ion selectivity. *International journal of molecular sciences, 14*(4), 7660-7680.

White, P. J., & Broadley, M. R. (2001). Chloride in soils and its uptake and movement within the plant: a review. *Ann Bot, 88*(6), 967-988.

Winicov, I. (1998). New molecular approaches to improving salt tolerance in crop plants. *Ann Bot, 82*(6), 703-710.

Zhou, C.-P., Qi, Y.-P., You, X., Yang, L.-T., Guo, P., Ye, X., . . . Chen, L.-S. (2013). Leaf cDNA-AFLP analysis of two citrus species differing in manganese tolerance in response to long-term manganese-toxicity. *BMC genomics, 14*(1), 621.

Zhu, J.-K. (2000). Genetic analysis of plant salt tolerance using Arabidopsis. *Plant Physiology, 124*(3), 941-948.