CONSTRUCTION OF cDNA LIBRARY FROM *Hordeum marinum* TO IDENTIFY SALT TOLERANCE GENES

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ABSTRACT

CONSTRUCTION OF cDNA LIBRARY FROM *Hordeum marinum* TO IDENTIFY SALT TOLERANCE GENES

Salt is necessary for plants because of ion homeostasis; however, excess uptake of salt leads to damage in plant cells, which may even result in the death of the plant. *Hordeum marinum*, also known as sea barley, is a member of *Poaceae* family that resides in coastal areas, so it is thought that it may have a possible salt tolerance gene or genes. Therefore, this study aims to identify the genes involved in salt tolerance in *Hordeum marinum* by functional genomics method. After screening, seven transformant yeast colonies found and sequence analyses of these plasmids gave homology to hypothetical protein of *Bipolaris oryzae*. To confirm salt tolerance of this protein, salt sensitive yeast cell transfected by this candidate gene was checked in high salt concentration containing medium. Based on solid growth assay, these transgenic yeast cells could survive in 1M saline medium. Hence, it is hypothesized that *Hordeum marinum* and *Bipolaris oryzae* might have a symbiotic association. It is possible that in this association *Bipolaris oryzae* may play a role as endophytic fungus that might also confer salt tolerance in *Hordeum marinum*.

ÖZET

TUZ TOLERANS GENLERİNİN BELİRLENMESİ İÇİN *Hordeum marinum'* dan cDNA KÜTÜPHANESİ OLUŞTURULMASI

Tuz bitkilerde iyon dengesi için gerekli olup; aşırı alındığında bitkiye zarar veren hatta bitkinin ölümüne bile yol açabilecektir. Deniz arpası olarak bilinen *Hordeum marinum, Poaceae* ailesinin bir üyesi olup sahil bölgelerinde yaşabilmektedir. Taşıdığı bu özellikten, potansiyel olarak tuza tolerans genlerine sahip olabileceği düşünülmektedir. Bu tez çalışmasında, *Hordeum marinum*da tuz tolerasında rol oynayan genleri ortaya çıkarmayı, bunu da bitkinin cDNA kütüphanesini maya hücrelerinde tarayarak gerçekleştirmesi amaçlanmıştır. Tarama sonucunda, yedi maya kolonisi tespit edildi. Sekans sonuçlarına göre bitkiye tuz toleransını sağlayan gen, *Bipolaris oryzae* adlı fungus ile homoloji gösterdi. Sonraki aşamada, aday genle transfekte olmuş tuza hassas maya hücreleri yine yüksek tuzlu ortamda kontrol edildi. Kontrol sonucunda, bu hücreler 1Molarlık tuzlu ortamda yaşayabildi. Bu sonuçların ışığında, *Hordeum marinum* ile *Bipolaris oryzae*'nin simbiyotik bir birliktelik kurarak tuz toleransı sağladığı düşünülmektedir.

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CHAPTER 1

INTRODUCTION

1.1. *Hordeum marinum* Plant

Hordeum marinum, also known as sea barley, is in *Poaceae* family and a close relative to *Hordeum vulgare*. *H. marinum* is located on coastal lands of Anatolia and around the Mediteranean Sea and it survives at higher concentrations in saline soils up to 300 mM NaCl, in comparison to cultivatable *Hordeum vulgare* (Seckin, Turkan, Sekmen, & Ozfidan, 2010). This feauture of the plant makes it a suitable candidate for salt tolerance mechanism studies.

1.2. Salt and Plants

There is a wide range of stress sources for plants such as heat, drought, cold, and salt. One of the most substantial environmental effects that influence plant growth and development is soil salinity.

Deposition of salt in soil is caused by over irrigation, sea water, global warming and watering at wrong times, for example at noon. Salt accumulation in soil leads to water loss in plants. The solutes dissolved in water that may have crucial functions in metabolic processes can be lost, as well. The saline soils influence the plant negatively by reducing its water intake from the medium because of osmotic stress. In addition, the Na^+ and Cl^- ions obtained in large amounts by root hair leads to damage in intracellular metabolic processes, and diminishes photosynthetic activity, which eventually influences plant growth adversely (Flowers & Yeo, 1995) (Tester & Davenport, 2003) (Mäser, Eckelman, et al., 2002); (Munns & Tester, 2008); (Blumwald, 2000).

1.3. Salt Tolerant Plants Developed some Strategies to Cope with Salinity

Unlike animals, when plants are exposed to any kind of stress, they have to adapt to that environment. Otherwise, they might be in danger, and even die as they do not have any chance to escape like animals or leave the environment. As a result, some plants living in saline soils developed some strategies to cope with salinity.

1.3.1. HKT Gene (Na^+ Influx Transporter) Regulation in Plants

There are two types of HKT gene. One of them is HKT1 involved in selective Na^+ ions transport (Uozumi et al., 2000); (Mäser, Hosoo, et al., 2002).

The gene in *Arabidopsis thaliana* AtHKT1 serves to unload Na^+ ions from *Xylem vessels* into *Xylem parenchyma* cells. These gene products are located in *Xylem parencymacells*' plasma membrane. Their function is to draw Na^+ ions from *Xylem fluid* (sap) and store them inside the cells, which inhibits excess Na^+ ions concentration in *Xylem sap*, preserving the leaves from toxicity arising from salt stress (Sunarpi et al., 2005). The other one, called HKT2, is responsible for the transport of both Na^+ and K^+ ions (Rubio, Gassmann, & Schroeder, 1995)**.**

1.3.2. Na^+ Ions Are Compartmentalized (Sequestered) into Vacuole by NHX (Na^+-Hydrogen Exchanger)

Extreme Na^+ ions in cytoplasm normally disrupt the metabolic process in plant cells. The Na^+/H^+ antiports localized in tonoplast (vacuolar membrane) import Na^+ ions from cytoplasm into vacuole; and then they export H^+ ions from vacuole into cytosol. Salt tolerance can be achieved by overexpression of vacuolar Na^+/H^+ antiport in *Arabidopsis* (Apse, Aharon, Snedden, & Blumwald, 1999).

1.3.3 SOS1 (Na^+-H^+ antiporter) Transporter Located on Cell Membrane (SOS Pathway)

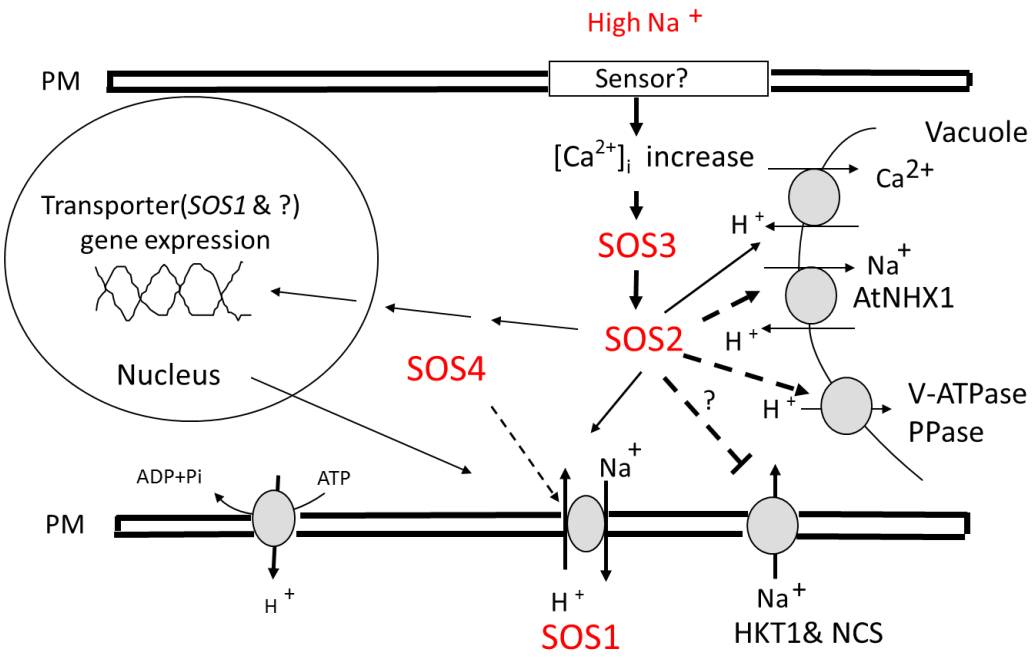

Figure 1.1. Sos Pathway.

(Source: Zhu, 2000)

Salt overly sensitive 3 or SOS3 in short, is calcium binding protein (Ishitani et al., 2000) which first perceives salt-induced intracellular increased calcium ions. Afterwards, salt based-signal carrying SOS3 protein physically associates with SOS2 protein kinase, leading to activated SOS2 (Halfter, Ishitani, & Zhu, 2000). Plasma membrane-located SOS1, which is involved in efflux of sodium ions and influx of hydrogens, needs SOS2 and SOS3 (Qiu, Guo, Dietrich, Schumaker, & Zhu, 2002) and runs or fulfills its duty when phosphorylated by SOS3-SOS2 kinase complex. Furthermore, the function of activated SOS3 is to bring or direct the SOS3-SOS2 complex to plasma membrane in order to induce and activate SOS1. By this induction, Na^+ ions are pumped out of the cells, thereby conferring salt tolerance (Quintero, Ohta, Shi, Zhu, & Pardo, 2002).

1.3.4. Osmolytes

Osmolytes are solutes that protect cells from dehydration, especially when the cells are exposed to osmotic stress, by increasing intracellular dissolved substance concentrations, such as proline, glycine, betaine, and mannitol.

The sugar alcohole mannitol containing transgenic tobacco plants are more tolerant to salt stress than nontransgenic ones (Tarczynski, Jensen, & Bohnert, 1993).

*Delta-1-pyrroline-5-carboxylate synthetase enzy*me *(P5CS)* is responsible for proline synthesis. Mutated *P5CS1* gene in *Arabidopsis* leads to hypersensitivity for salt stress due to the lack of salt-caused abundancy of proline as well as overaccumulation of reactive oxygen species (Szekely et al., 2008).

1.3.5. Vacuole Pyrophosphatase V PPase, V ATPase, Located on the Vacuole Membrane, Function as Hydrogen Proton Pumping into Vacuole

ATP energy is derived from either glycolysis or oxidative phosphorylation. On the other hand, the sources of inorganic pyrophospahates are biosyhnthesis reactions, such as synthesis of RNA, DNA, proteins, fatty acyl-CoA etc. Enzymes named Vacuolar H^+ (proton) pumping PPase (pyrophosphatase) and Vacuolar ATPase (V-ATPase) coexist in vacuolar membrane called tonoplast (Masayoshi Maeshima, 2000).

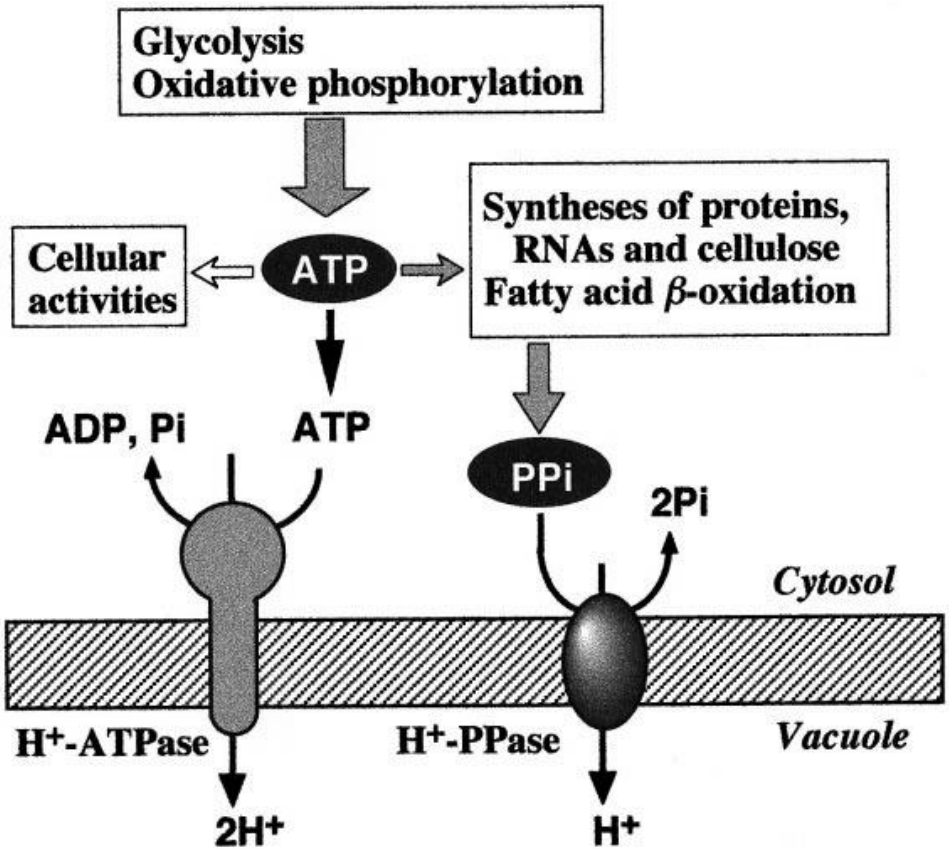

Figure 1.2. Derivation of ATP and PP_i, also utilization of both by Vacuolepyrophosphatase V PPase and V ATPase. (Source: Masayoshi Maeshima, 2000)

The first enzyme, V PPase, hydrolyzes cytosolic PPi (inorganic pyrophosphates), revealing energy in the course of hydrolysis which allows hydrogen ions to be pumped from cytoplasm into vacuole lumen (Silva & Gerós, 2009), (Belogurov et al., 2002), (M. Maeshima, 2001), (Masayoshi Maeshima, 2000),(Zancani, Skiera, & Sanders, 2007). That is to say, the released energy can be considered responsible for the movement of protons. In turn, the cytoplasm, in which the vital metabolic processes take place, gets rid of H^+ based acidity; however, these Hydrogen ions make the vacuole more acidic. Similarly the second enzyme Vacuolar ATPase (V-ATPase) utilizes the same mechanism, yet hydrolyses ATPs instead of pyrophosphates.

Furthermore, these proton pumps send hydrogen ions increasingly into vacuole; hereby creating hydrogen-based electrochemical gradient between cytoplasm and vacuole. This phenomenon can facilitate the act of vacuolar Na^+/ H^+ exchanger (Gaxiola, Fink, & Hirschi, 2002).

1.3.6. Halotropism

The growth direction of plants' root is modulated via *Auxin hormone* induced by salt in response to salinity. The plant roots refrain from salinity by changing its growth direction. In this event, the main aim is to reduce exposure of plant roots to salt, by which the plant roots refrain from salinity (Galvan-Ampudia et al., 2013).

1.3.7. ROS-Mediated Pathway

Reactive oxygen species and calcium ions can be considered as signal molecules for plants. For instance, the annexin protein located in the plant plasma membrane is responsible for calcium influx and regulated by ROS during the process of salinisation. Sodium ions enter the cell in excessive amounts during salinity, leading to a rise in cytosolic calcium ion concentration; subsequently, increased calcium ions activate NADPH oxidase creating ROS molecules at extracellular space such as hydrogen peroxide (H_2O_2) by transferring electrons from NADPH to oxygen molecules that are the precursors of hydrogen peroxide. The Reactive Oxygen Species affect annexin membrane proteins; hence, resulting in import of calcium ions from extracellular space to inside the cell. In turn, the resulting increased cytosolic calcium ions encourage S0S1 transcription that assists salt tolerance. In addition, the SOS1 mRNA stability is increased by salt-induced ROS (Chung, Zhu, Bressan, Hasegawa, & Shi, 2008; Laohavisit et al., 2013; Laohavisit et al., 2012) (Davies, 2014).

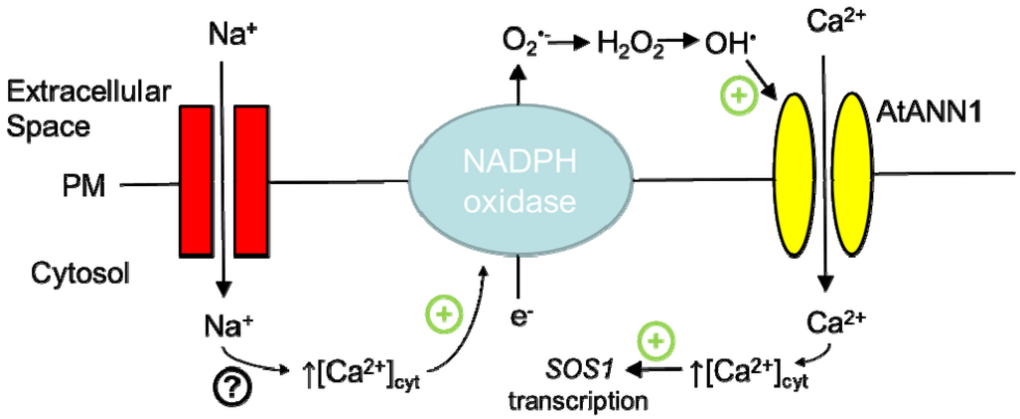

Figure1.3. Calcium transport protein *At annexin1* is regulated by extracellular ROS. (Source: Davies, 2014)

Salt responsive ERF1 (SERF1) is a transcription factor in *Oryza sativa* activated by reactive oxygen species and its transcription contents rapidly increase after exposure to salt. During salinity, this transcription factor participates in each expression of Mitogen–activated protein kinases including MAPK5, MAP3K6, in addition to dehydration–responsive element (DREB2A), Zinc finger Protein (ZFP179), and also its own tanscription by binding their promoter region (R. Schmidt et al., 2013).

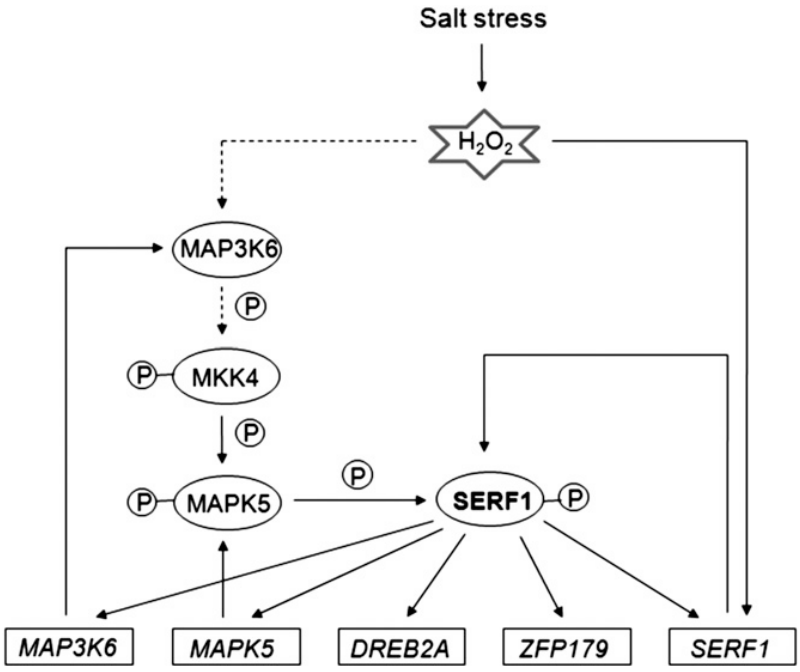

Figure 1.4. Function of SERF1 under salinity.

(Source: R. Schmidt et al., 2013)

There is a positive feedback between MAPK5 and SERF1 since the activity of SERF1 is modulated through MAPK5, which phosphorylates at the position of 105 serine residue of SERF1. Unphosphorylated SERF1 becomes less active, leading to naturally less transcription of Mitogen-activated protein kinases including MAPK5. Furthermore, downregulated SERF1 is more vulnerable to salt stress than overexpressed SERF1 (R. Schmidt et al., 2013), which means the more expression of SERF1 there is the more salt tolerance there will be.

Overexpression of Zinc finger protein 179 results in aggradation of salt-caused proline and soluble sugar at higher level in comparison to the wild type plant when treated with salt (Sun et al., 2010).

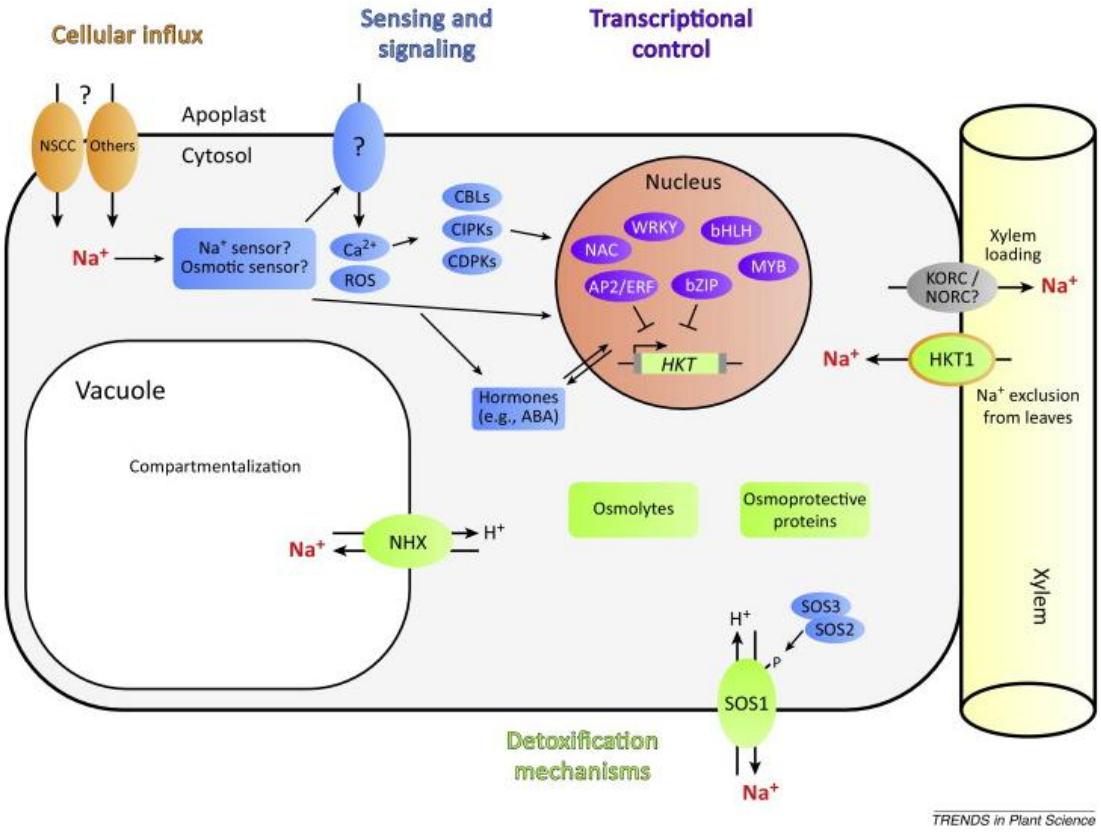

Figure 1.5. Summary of general mechanism involved in salinity.

(Source: Deinlein et al., 2014)

1.3.8. Symbiotic Associations such as Those between *Endophytic Fungus* and Plant Can Provide Salt Tolerance to Some Extent

Arbuscular mycorrhiza infested seedlings of trifoliate orange indicated relatively enhanced growth activity, substantially increased leaf and root soluble sugar contents; improved root structure compared to non-infested seedlings under 0, 100, and 200 mM NaCI salt concentrations (YongChao & QiangSheng, 2013).

CHAPTER 2

MATERIAL AND METHODS

2.1. Yeast Growth and Media

Saccharomyces cerevisiae strain W303 (haploid wild type) and Ab11c (*ena1-4Δ::HIS3 nha1Δ::LEU2 nhx1Δ::TRP1*) yeast mutant cells were utilized in this study.

For the growth and selective amplification of yeast cells, yeast extract peptone dextrose that is known as YPD medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) and Yeast Nitrogen Base that is abbreviated as YNB minimal media with necessary amino acids devoid of uracil were exploited.

2.2. Plant Growth and RNA Isolation

Hordeum marinum seeds were sowed in the soil. Subsequent to the germination of *Hordeum marinum* seedlings, they were transplanted into pots with semi-strong hoagland solution in growth chamber which is 12 hours dark and 12 hours light at the intensity of 40 μmol $m^{-2}.s^{-1}$, a tempurature of 25 ^{o}C and 50% moistness to perform sufficient growth. Then these satisfactorily grown seedlings were exposed to 200 mM NaCl solution for 7 days. After exposure, samples extracted from *Hordeum marinum* leaves and roots were instantly treated with liquid nitrogen to be frozen and kept in -80 ^{o}C. The Invitrogen RNA Isolation Kit was used to perform total RNA isolation with respect to manufacturer's instructions (Invitrogen, Germany).

2.3. Synthesis of Gateway® –Compatible cDNA Library

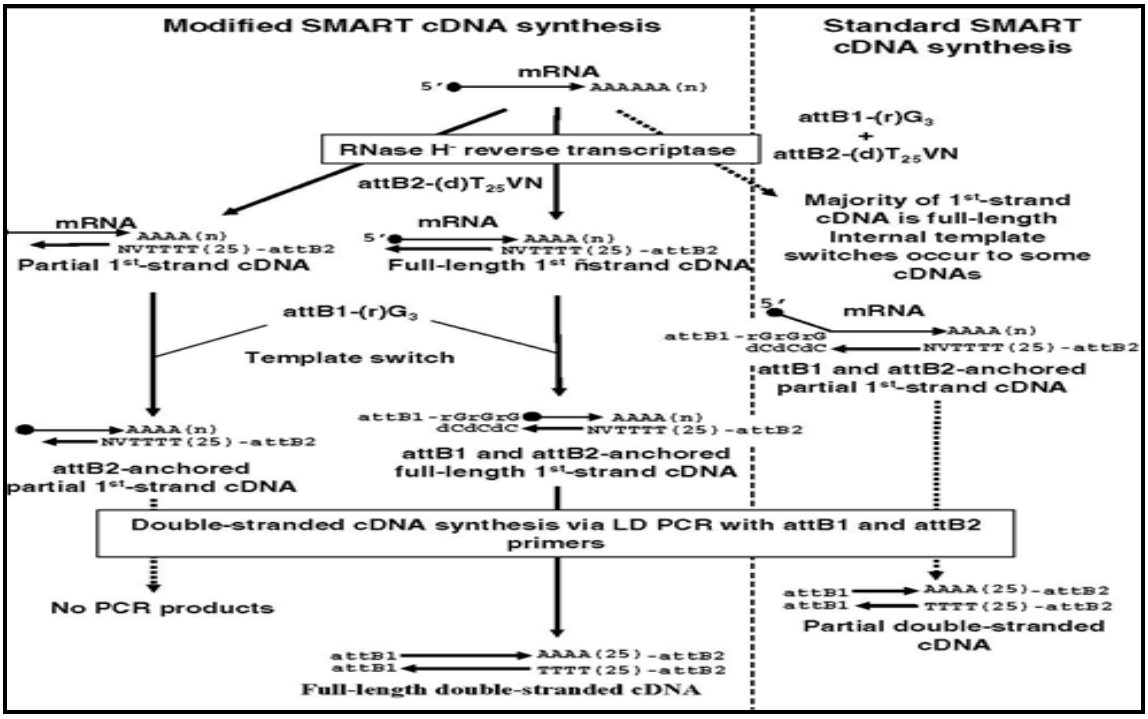

Figure 2.1. SMART cDNA synthesis.
(Source: Ni, Lei, Chen, Oliver, & Xiang, 2007)

By following the procedure mentioned by (Ni et al., 2007) , Gateway–compatible cDNAs have been produced. To achieve synthesis of single stranded cDNA, the steps below were followed:

First of all, 4 µl total RNA and (15 µM) attB2 with oligo(d)T25 primer (5′ACCACTTTGTACAAGAAAGCTGGG T25TVN-3′) were mixed, after which they were firstly incubated at 72 °C for 2 minutes before they were chilled in ice for 2 minutes. Two µl (5X) First Strand Buffer, 1 µl DTT (20 mM), 1µl dNTP mix (10 mM) and 1 µl Power Reverse Transcriptase were incorporated into the initial blend and incubated at 42 °C for 1 hour. Subsequent to incubation, (15 µM) 1 µl oligo attB1-(r) G3 primer (5′ACAAGTTTGTACAAAAAAGCAGGC TrGrGrG-3′) was ultimately incorporated into the mixture, then the resulting mixture was incubated at 42 °C for 1 hour.

Production of double-stranded cDNAs was executed via PCR amplification on applied Biosystems Gen Amp® PCR System 9700. The ingredients required for PCR

amplification were 80 µl dH2O, 10 µl (10X) advantage 2 PCR buffer, 2 µl (10 mM) dNTP mix, 2 µl (15 µM) attB1 primer, 2 µl (15 µM) attB2 primer, 2 µl Adv. 2 Polymerase mix and the 2 µl cDNA template were derived from the first step. Applied Biosystems Gen Amp® PCR System 9700 was adjusted with respect to the circumstances below to carry out PCR reaction, initial denaturation for 1 minute at 95 °C, 20 cycles of three temperatures as 15 seconds at 95 °C, 30 seconds at 64 °C, 6 minutes at 68 °C and finally, one cycle 10 minutes at 68 °C. To determine the qualification of newly synthesized cDNAs, cDNA library were run on the (1%) agarose gel by electrophoresis.

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

Polyethylene glycol 8000 with 30 mM $MgCl_2$ Solution were utilized to eliminate primers, primer dimers and cDNA fragments smaller than 300 base pair in size from PCR products. 2 µl Purified cDNAs (160 ng/µl) were inserted into 150 ng pDONR221 entry vector (Invitrogen, Germany) by incorporation of 2 µl BP Clonase™ II enzyme, and subsequently an overnight incubation at 25 ^{0}C. These reactions were arrested by supplement of 2 µl protease K (Invitrogen, Germany) into the mixture, which was then incubated initially for 10 minutes at 37 ^{0}C and then for another 10 minutes at 70 ^{0}C.

E.coli omnimax competent cells were transfected by products of BP reactions via the heat shock method by fulfilling the following steps. Initially, *OmniMAX™ 2T1 Phage-Resistant (T1R)* cells of *Escherichia coli* strain (Fermentas, St. Leon-Rot, Germany) were cooled in ice for 5 minutes. Next, 2 µl of BP clonase reaction mixture were supplemented onto the competent cells within eppendorph tube, which was kept in ice for 25 minutes. Then, competent cells were exposed to heat shock at 42 °C for 30 seconds to facilitate the entrance of BP products from pores into the cells and after that heat shock, these competent cells were incubated on ice for 2 minutes, and after that, 250 µl SOC medium was supplemented onto mixture of the transfected cells and lastly these transformed cells were placed into a shaker for proliferation for 1 hour at 37 °C 200 rpm. Then, the mixture formed by transfected cells was plated on petri dishes including LB with 50 µg/ml kanamycin.

Grown colonies were picked up and located in 50 ml falcon tubes and isolation of plasmids was executed by the assistance of *PureLink™ Quick Plasmid Maxiprep Kit* (Invitrogen, Karlsruhe, Germany). Entry clones originating from the BP reaction and (150ng) *pAG426GPD* destination vectors (Invitrogen, Karlsruhe, Germany) reacted with each other in the presence of 3 µl TE buffer and 2 µl LR Clonase™ II enzyme to generate expression clones. This reaction was executed overnight at 25 °C. The same cell line (competent cells) was transfected by 2 µl from product of LR clonase reaction via the heat shock method mentioned above.

The mixture formed by transfected cells was plated on petri dishes including LB with 100 µg/ml ampicillin. Isolation of plasmids was executed by the assistance of PureLink™ Quick Plasmid Maxiprep Kit (Invitrogen, Karlsruhe, Germany).

2.5. Yeast Transformation of Cloned cDNA Library

Yeast transformation was carried out by Lithium Acetate (LiAc) method (Burke, Dawson, and Stearns 1994). Yeast cells were grown until mid-log phase at 5 ml YPD medium, then amplified yeast cells were rinsed by autoclaved water. This mixture was centrifuged and resulting precipitated cells were treated with 1ml (0.1 M) LiAc. The treated cells were transported into new 1.5 ml micro centrifuge tubes. Lithium acetate was removed by pipette via centrifugation and the cells were retreated with 0.5 ml LiAc, and removal of LiAc by pipette via centrifugation took place again. The ingredients below were respectively supplemented onto the precipitated yeast cells: 240 µl PEG (50 % w/v), 36 µl (1 M) LiAc, 5 µl ssDNA, 10 µl DTT (0.3 mM), 20 µl plasmid DNAs, 10 µl dH_2O. The resulting mixture was vortexed in turn for 1 minute and placed into shaker for incubation at 30 °C (160 rpm) for 30 minutes. For the transfer of cDNA containing plasmids into yeast cells, yeast cells were exposed to heat shock in water bath for 30 minutes at 42 ^{o}C. Then transformed cells were plated on YNB-ura medium including 1000 mM NaCl. Plasmids of colonies that were able to survive in saline medium were isolated by utilizing PureLinkTM Quick Plasmid Miniprep Kit (Invitrogen, Karlsruhe, Germany). The isolated plasmids were sent to Biomer to reveal the sequence of cloned *Hordeum marinum* genes.

2.6. Sequence Analyses of the Salt Tolerance Gene from *Hordeum marinum* cDNA Library

The detected gene sequence of *Hordeum marinum* cDNA library was investigated by using BLAST service of NCBI. ClustalW software was utilized to compare the multiple protein sequence alignments.

2.7. Solid Culture Experiments

The Hm7 gene considered to confer salt tolerance was checked for the verification of its salt resistance performance. W303 Wild Type, Ab11c (salt sensitive yeast cells) transfected with pAG426GPD empty vector for control, as well as Ab11c transfected by Hm7 cDNA containing pAG426GPD expression vectors, in short all yeast cells were incubated overnight in the shaker (170 rpm, at 30 ^{o}C). Then yeast cell concentrations were measured at 600 nm and OD600= 0.2, 0.02, 0.002, 0.0002 yeast concentrations were created by dilution. Next, cells were spotted as 5 µl on solid YNB – ura plates without NaCl or with 1000 mM NaCI. To examine their growth, these yeast cells were incubated at 30 ^{o}C for 5 days. Also, this assay were repeated to observe Hm7 gene performance in other salts such as KCl and LiCl.

CHAPTER 3

RESULTS

3.1. Construction of cDNA Library

Hordeum marinum can survive in saline soils in accordance with its natural location. To reveal salt tolerance genes, cDNA library was constructed via SMART cDNA synthesis method.

The advantage of Reverse Transcriptase derived from Moloney Murine Leukemia used İn SMART cDNA synthesis is that it adds several (dCTP)s at 3'end of newly synthesized full length cDNA when the enzyme arrives 5'end of mRNA template (W. M. Schmidt & Mueller, 1999) leading to the existence of a deoxycytidine stretch at 3' end of cDNA, by which a potential matching site is created.

After addition of template switching oligonucleotide sequence including oligo ribo (G) to the reaction, these oligonucleotides with several ribo (G) can easily match with oligo deoxycytidines stretch at 3' end of cDNA.

Then, template switching oligonucleotides (TS oligo sequence) attached to 5' end of mRNA is exploited as template by the reverse transcriptase, which results in synthesis of complementary nucleotides, leading to extension 3'end of cDNA compatible with switched 5'end of mRNA, thereby allowing cDNA amplification simply by facilitating primer annealing at PCR applications (Matz et al., 1999) ,(Chenchik *et al.*, 1998)

To construct cDNA library, total RNAs were isolated from *Hordeum marinum* leaves and roots and these total RNAs were run on the gel as shown in Figure 3.1.

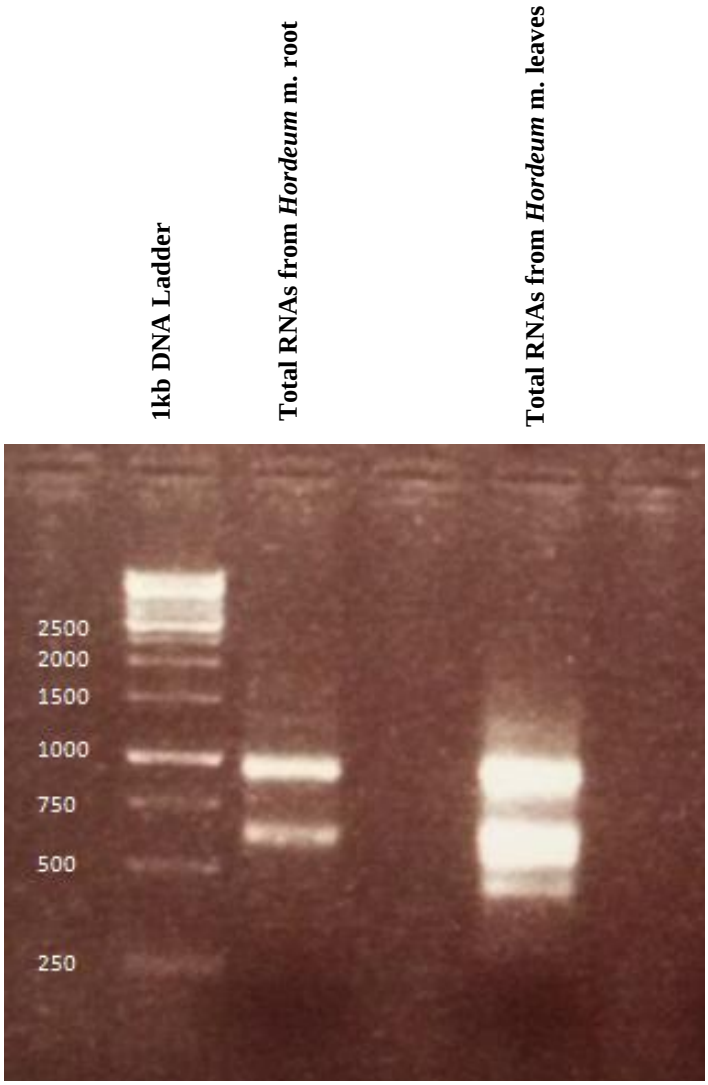

Figure 3.1. Total RNAs from *Hordeum marinum*.

mRNAs from *Hordeum marinum* were converted into cDNAs and amplified by SMART PCR method. In order to test whether the mRNAs were properly converted into cDNAs, the cDNAs were run on the gel. Subsequent to gel electrophoresis, it was observed smear cDNAs, indicating that the mRNAs were precisely converted into cDNAs.

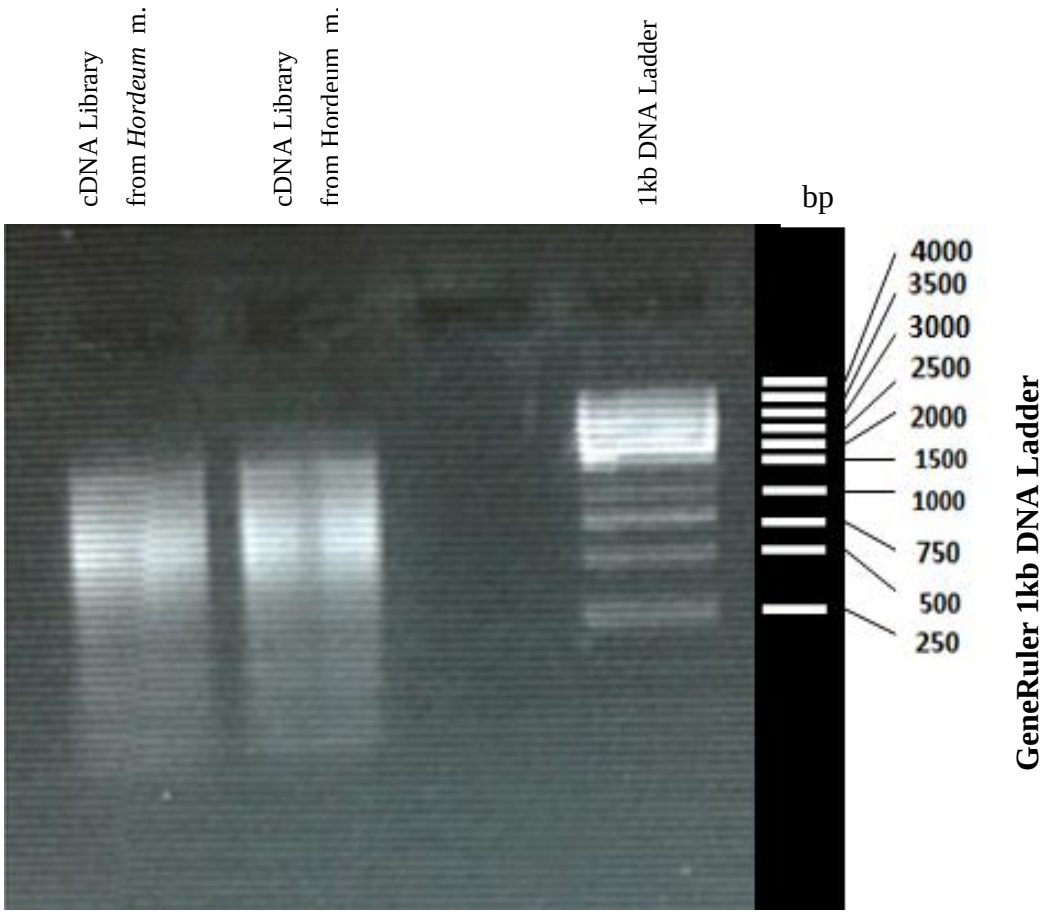

Figure 3.2. cDNAs from *Hordeum marinum* were viewed as smear on agarose gel.

To eliminate primers, primer dimers, and DNA fragments smaller than 300 base pairs in size from PCR products, 30% Polyethylene glycol 8000 with 30 mM $MgCl_2$ solution was utilized. PCR products exposed to PEG were run on the gel to assess whether unwanted DNA fragments <300 bp in size were removed. These unnecessary fragments were eliminated.

The purified cDNAs flanked by attB1 and attB2 were exposed to BP and LR reactions, respectively. After LR reaction, Ab11c salt sensitive yeast cells were transfected by cDNAs- from *Hordeum marinum*– containing pAG426GPD expression vectors.

Ab11c (*ena1-4Δ::HIS3 nha1Δ::LEU2 nhx1Δ::TRP1*) is a mutant yeast cell whose cation transporter genes were knocked out. Ena1 gene encoding P-ATPase serves as sodium ion efflux protein (Haro, Garciadeblas, & Rodriguez-Navarro, 1991), NHA gene- Na^+/H^+ antiporter- is responsible for the exclusion of sodium and potassium

ions across the plasma membrane (Banuelos, Sychrova, Bleykasten-Grosshans, Souciet, & Potier, 1998), NHX1 protein– sodium hydrogen exchanger–located in vacuole membrane is necessary for the uptake of Na^+ ions from cytoplasm into the vacuolar space, leading to the trapping of Na^+ ions into the vacuole (Nass, Cunningham, & Rao, 1997)]. Mutant Ab11c cells becomes hypersensitive and vulnerable to salt stress, which makes this cell a suitable tool for genetic studies related to identification of candidate salt tolerant genes introduced into this host cell via transformation under saline medium because survival of these cells under high salt concentrations is based on a foreign gene or genes.

These transformed Ab11c cells were plated to solid YNB-Ura medium rich in salt of 1000 mM NaCI, and then these petri dishes were incubated at 30 ^oC for 5 days. Seven yeast colonies were observed and they were able to grow in saline medium. To expose which cDNAs of *H. marinum* conferred salt tolerance, plasmid isolations of seven yeast colonies were executed and sequenced.

3.2. Identification of Hm7 gene

Sequence results of these genes showed 58% similarity to hypothetical protein COCMIDRAFT_101862 of *Bipolaris oryzae* ATCC 44560. After performing blast search, the first four protein sequences which relatively highly resemble Hm7 protein sequence have been selected for comparison and to indicate matching amino acids. The comparison of Hm7 gene and related proteins is shown in Figure 3.3. The scores of matching amino acid sequence alignments are shown in Table 3.3. Phylogenetic tree of the proteins is shown in Figure 3.4.

CTTTTGGGGCCAAGAAAGGTCCAACTATCCTCATCGGCCTTCTTGCTGCTCGAGCCC
TCAGCAATCCTGTTGATGGCGGCCGTGTTGCTTTGAGAGGCGAATGCGGCGAACTC
GGGGTC**ATG**GATTGGGACCTGAACGCTCTCCCAGAGGACACCGATGTCTCGGAGC
TTCGTCGTTGCAAGAAGCACCCATCCGAACTCGGCGTCACTAGCCCTCTGTTGAATC
CCGACGCATCACCAGAGGATCTCGATAGCTCCTACGGGAAGAGCGAAGAACTTGG
AACCTCGCCCAAGGAGGAACGGGGCCTAGACAAGAGATATTCTTGTCCTGACGGCT
CCGGTCGTGGCCGTGGTCAGTTTGAGTATGATTATGGTTGTGATAAGGGCTGGTGCT
GGAGGAACTGTGATGTGAATGTCGGGTCACCAGGCGGGGGAGAGGGCTTAAGGAA
GCCGTGGTGTTGGCTGGCATACGAAGGTGGACACGGAGGCTGGACGCCCTGCGGA
CGCTGGCAGGATTGCGAGTGGTCTTACAATAACAAAAACGCAAAGTGTGGAAAGG
GTAACTGCAAGGCTTGCGGCTGTGGCTGT**TAA**GGCAAATCTGAGCGCCTTCGTCG
TTTTGCTTGGAGATTGGTGAATCAAGGTTGCTTCTTTCCAGTATCATCATGATTGTG
TTTGTCTTGCAGTATTCACATTCGTCTAGTCTGATTTTACGCAACTATAACTTTGTTG
CCCAAGTTTG**AAAAAAAAAAAAAAAAAAAAAAAAA**CCCCAGCTT
TTCTTTTAAAAAGTGGTGGATGGGCTGCAGGAATTCAATATCAGGCTTATTCGATAC
CGCCACCTTCGAATCCAGGTAATTTAGT

Table 3.1. Hm7 gene sequence. Start, stop codon and poly A tail are in bold.

Hm7 gene open reading frame constitutes of 468 nucleotides and it corresponds to 156 amino acids. Its molecular weight is 17196 and its isoelectric point is 5.11

A A R A L S N P V D G G R V A L R G E C G E L G V **Met** D W D L N A L P E D T
D V S E L R R C K K H P S E L G V T S P L L N P D A S P E D L D S S Y G K S E E
L G T S P K E E R G L D K R Y S C P D G S G R G R G Q F E Y D Y G C D K G W
C W R N C D V N V G S P G G G E G L R K P W C W L A Y E G G H G G W T P C
G R W Q D C E W S Y N N K N A K C G K G N C K A C G C G C **Stop**G K S E R L
R R F A W R L V N Q G C F F P V S S **Stop** L C L S C S I H I R L V **Stop** F Y A T I
T L L P K F E K K K K K K K K

Table 3.2. Hm7 amino acids sequence is shown with red.

Figure 3.3. Comparison of multiple amino acid sequence alignments of Hm7 and hypothetical proteins including Cocmidraft 101862 (*Bipolaris oryzae*), Cocsadraft 38604 (*Bipolaris sorokiniana*), Cochedraft 1019009 (*Bipolaris maydis*), and Coccadraft 95603 (*Bipolaris zeicola*).

Table 3.3. The scores of amino acid sequence alignment similarity.

SeqA	Name	Length	SeqB	Name	Length	Score
1	Hm7protein	156	2	Cocmidraft101862	183	63.46
1	Hm7protein	156	3	Cocsadraft38604	183	62.82
1	Hm7protein	156	4	Cochedraft1019009	183	59.62
1	Hm7protein	156	5	Coccadraft95603	183	61.54
2	Cocmidraft101862	183	3	Cocsadraft38604	183	97.27
2	Cocmidraft101862	183	4	Cochedraft1019009	183	94.54
2	Cocmidraft101862	183	5	Coccadraft95603	183	95.63
3	Cocsadraft38604	183	4	Cochedraft1019009	183	95.63
3	Cocsadraft38604	183	5	Coccadraft95603	183	95.08
4	Cochedraft1019009	183	5	Coccadraft95603	183	92.9

The scores of multiple amino acid sequence alignments similarity among Hm7 and hypothetical proteins: Cocmidraft 101862 (*Bipolaris oryzae*), Cocsadraft 38604 (*Bipolaris sorokiniana*), Cochedraft 1019009 (*Bipolaris maydis*), and Coccadraft 95603 (*Bipolaris zeicola*).

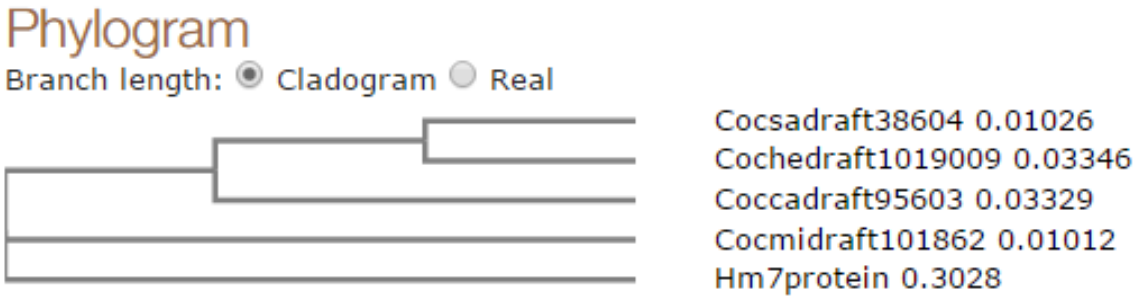

Figure 3.4. Phylogenetic tree of the proteins.

3.3. Solid Growth Assay

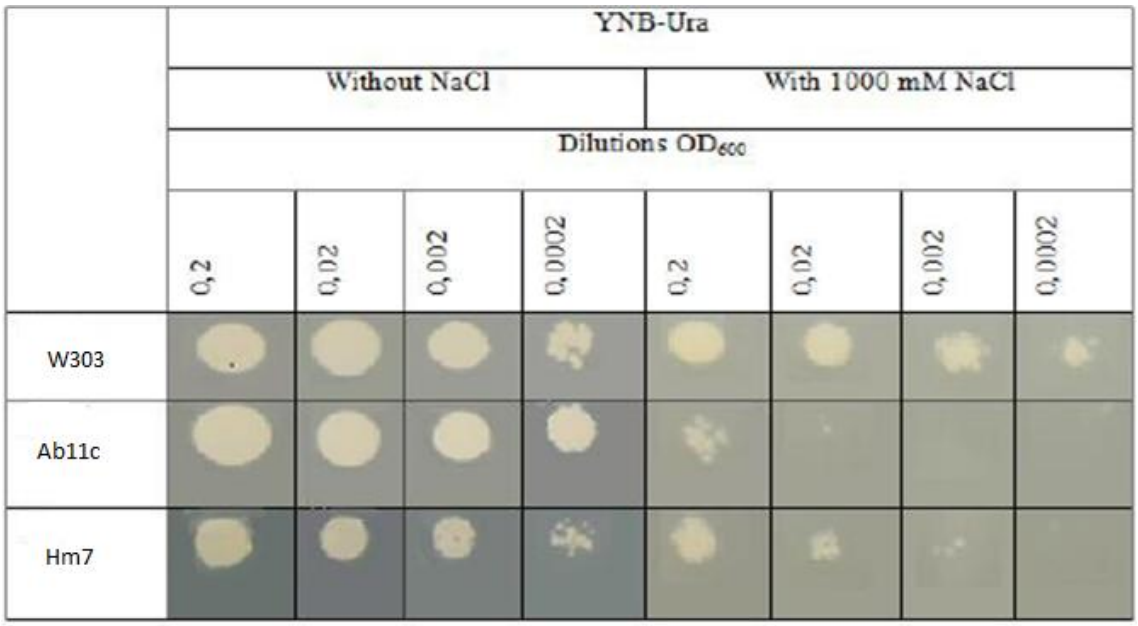

Figure 3.5. Solid growth assay for wild type (W303) cells, pAG426GPD empty vector containing Ab11c cells, Hm7 - within pAG426GPD expression vector-containing Ab11c cells.

After sequence results, to verify whether this Hm7 gene was actually related to salt tolerance or not, spotting method was utilized by plating 5µl transformed and wild type yeast cells on YNB minus Ura with 1000 mM NaCI medium and YNB-Ura that was exploited as control. Hm 7 containing yeast cells provided salt tolerance at least to a certain extent in comparison to Ab11c yeast cells; however, this salt tolerance was not as much as wild type yeast cells did.

It was also examined whether Hm7 protein is only associated with Na^+ ions tolerance or any other ions. Hm7 gene containing yeast cells were grown at both KCl and LiCl contained media as shown in Figure 3.6. and Figure 3.7.

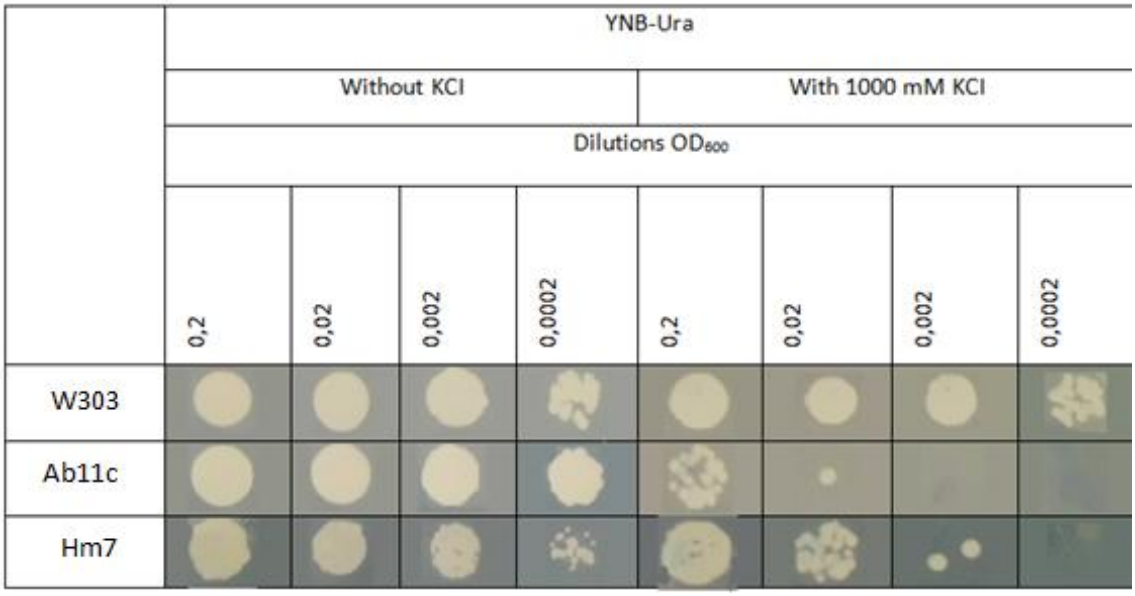

Figure 3.6. Solid Growth Test for KCl wild type (W303) cells, pAG426GPD empty vector containing Ab11c cells, Hm7- within pAG426GPD expression vector- containing Ab11c cells.

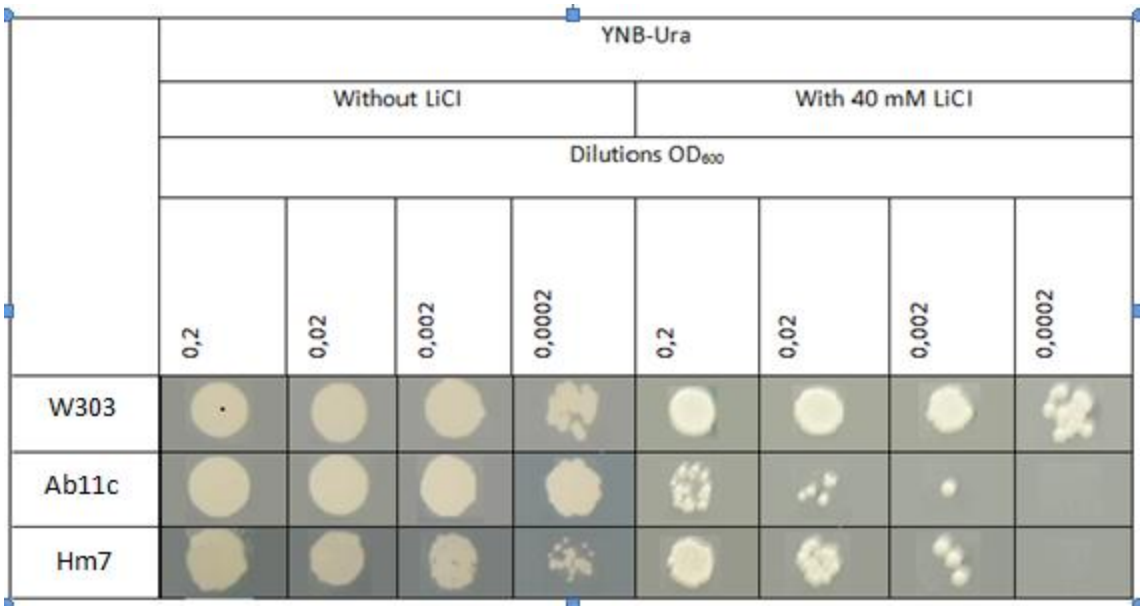

Figure 3.7. Solid Growth Test for LiCl wild type (W303) cells, pAG426GPD empty vector containing Ab11c cells, Hm7- within pAG426GPD expression vector- containing Ab11c cells.

According to solid growth assay, it was deduced that Hm7 gene may also confer salt tolerance for both K^+ and Li^+ ions besides Na^+ ions. However, the Hm7 gene was found to be more selective for Na^+ and K^+ transport than Li^+ ions. As well as, in every stage of these three stress conditions, it is observed that the number of Hm 7 gene containing yeast cells is higher than salt sensitive yeast cells.

CHAPTER 4

DISCUSSION

The major purpose of this study was to identify salt tolerance genes from *Hordeum marinum* but sequence results showed to hypothetical protein of *Bipolaris oryzae*.

Bipolaris oryzae is a fungus located in *Dematiaceae* family leading to brown spot disease, thereby resulting in loss of yield in rice production (Shabana, Abdel-Fattah, Ismail, & Rashad, 2008).

Similarly, *Fusarium culmorum* is a fungus that negatively affects cereal crops by causing head blight on wheat, in turn giving rise to a reduction in grain yields and enwrapment of grains with mycotoxins produced by *F.culmorum* (Scherm et al., 2013).

On the other hand, *Leymus mollis* known as dune grass can survive on coastal areas with the support of *F.culmorum*, indicating that *F.culmorum* is nonpathogenic in that association. To learn whether *F.culmorum* is required for salt tolerance of dune grass, an assay was designed. In this assay, a distinct number of dune grass was inoculated with this fungi, but other certain dune grass was not inoculated. These specimens, both symbiotic and non symbiotic plants, were subjected to 500mM NaCI for 14 days in laboratory conditions. In spite of salt stress, symbiotic dune grass with *FcRed1* did not desiccate, whereas nonsymbiotic ones wilted and died. The salt tolerance providing effect of *F. culmorum* is not only limited to dune grass if rice plants representative of *Monocotyledones* and tomato plants, which are members of *Dicotyledones* are infested with *FcRed1* fungus. They also indicate tolerance against salt stress. However, of the noninfested ones, neither rice nor tomato plants could survive under salt stress (Rodriguez et al., 2008).

In addition, barley plants (*Hordeum vulgare*) inoculated with *P.indica* , is a type of Mycorrhiza, had higher grain yield than noninoculated ones on account of growth promoting effect of this fungus. Also, in contrast to noninfested barley, *P. indica* infested barley can survive at moderate 100 mM NaCl concentration but not at higher concentrations such as 300 NaCl. Similarly, shoot weight of infested barley was heavier

than noninfested under salt treatment of 100 mM NaCl concentration (Waller et al., 2005).

The main question to be responded is by which mechanism this endophytic fungus overcomes salt stress and how it works.

Priformospora indica can synthesize auxin (indole-3-acetic acid: IAA) and that fungal auxin may be involved in plant root branching, thereby profusely branched roots lead to increased and better soil utilization (Sirrenberg et al., 2007). *Priformospora indica* endophytic fungus leads to salt tolerance by elavating the level of antioxidants in barley root. Also, this endophytic fungus alleviates the level of lipid peroxidation and fatty acid desaturation based on salt stress in barley leaves (Baltruschat et al., 2008).

In this study, it is hypothesized that hypothetical protein of *Bipolaris oryzae* that can be thought to be endophytic fungus that is also considered to probably have a habitat (saline medium) - adaptive feature can confer salt tolerance to its host (*Hordeum marinum*) just as *Fusarium culmorum* provides a habitat-adaptive feature to dune grass. In addition, this protein (Hm7) may be responsible for increasing the amount of antioxidant molecules. To illustrate, ascorbate or glutathione molecules that serve as scavengers of reactive oxygen species that is the natural result and indicator of salt stress by donating its hydrogen atoms to unstable radicals to inactivate them. Otherwise, these reactive oxygen molecules possessing an unpaired electron during salt stress could react with nucleic acids and damage them or abstract the hydrogen atoms from unsaturated fatty acids within the plasma membrane, thereby leading to the formation of lipid peroxidation, which, in turn, may destroy membrane integrity and even cause the death of the plant cells (Baltruschat et al., 2008); (Rodriguez et al., 2008)

CHAPTER 5

CONCLUSION

In this study, it is determined that Hm7 gene probably belongs to *Bipolaris oryzae*. It is also hypothesized that *Hordeum marinum* and *Bipolaris oryzae* might have a symbiotic association based on mutually beneficial relations. In this association, *Bipolaris oryzae* may possibly play a key role as endophytic fungus that also might be partly conferring salt tolerance. In addition, it can be concluded that Hm7 gene may confer salt tolerance associated with K^+ to a major extent and to a minor extent with Li^+ besides Na^+ ions.

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