CLONING OF WHEAT TREHALOSE-6-PHOSPHATE SYNTHASE GENE AND MICROARRAY ANALYSIS OF WHEAT GENE EXPRESSION PROFILES UNDER ABIOTIC STRESS CONDITIONS

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submitted by **BERAY GENÇSOY ÜNSAL** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biochemistry Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Mesude İŞCAN _____
Head of Department, **Biochemistry**

Prof. Dr. Meral YÜCEL _____
Supervisor, **Biology Dept., METU**

Prof. Dr. Haluk HAMAMCI _____
Co-Supervisor, **Food Engineering Dept., METU**

Examining Committee Members

Prof. Dr. Emel ARINÇ _____
Biology Dept., METU

Prof. Dr. Meral YÜCEL _____
Biology Dept., METU

Prof. Dr. Hüseyin Avni ÖKTEM _____
Biology Dept., METU

Assoc. Prof. Dr Candan GÜRAKAN _____
Food Eng. Dept., METU

Assist. Prof. Dr. Füsun EYİDOĞAN _____
Education Dept., Başkent University

Date: 05.12.2008

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last Name: Beray Gençsoy ÜNSAL

Signature :

ABSTRACT

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ÜNSAL, Beray Gençsoy

Ph.D. Department of Biochemistry

Supervisor: Prof. Dr. Meral YÜCEL

Co-Supervisor: Prof. Dr. Haluk HAMAMCI

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The aim of this study was cloning of wheat (*Triticum aestivum* L. cv. Bayraktar) Trehalose-6-phosphate synthase gene and examining of gene expression pattern of wheat seedlings in response to salt and drought stress conditions using Wheat GeneChip (Affymetrix).

In this study, 10-days old wheat seedlings were subjected to the salt (350 mM NaCl) and drought stress (20% PEG) for 24 hours, then root and leaf tissues were used for wheat TPS gene cloning and microarray studies.

RACE (Rapid Amplification of cDNA Ends) was used to determine cDNA sequence of wheat *TPS* gene, *TaTPS*. The ORF of *TaTPS* encodes a putative protein of 859 amino acids with a predicted molecular weight (MW) of 96.7 kDa and an isoelectric point (pI) of 5.97. Based on tblastx, *TaTPS* showed great similarity with other plants *TPS* genes. In root tissue, expression of *TaTPS* increased under drought stress while

no change was observed under salt stress. In leaf tissue, both salt and drought treatments repressed the expression of *TaTPS*.

Microarray study was used to monitor transcript abundance in salt and drought stressed wheat. Data analyses were determined by using GCOS 1.4 and GeneSpring GX10. The genes encoding ferritin, Lipid transfer protein, LEA/Dehydrin, early nodulin, cold regulated protein and germin like proteins were upregulated at least 10-fold under salt and drought stress conditions. In addition, salt and drought stresses induced the expression of genes identified as *DREB*, *ERF*, *NAC*, *MYB*, and *HSF*, suggesting existence of various transcriptional regulatory pathways under salt and drought stresses.

Key words: trehalose, wheat, TPS, salt, drought, microarray

ÖZ

TREHALOZ-6-FOSFAT SENTAZ GENİNİN BUĞDAYDAN İZOLASYONU VE BUĞDAY GEN İFADE PROFİLLERİNİN ABİYOTİK STRES KOŞULLARINDA MİKROARRAY YÖNTEMİ İLE İNCELENMESİ

ÜNSAL, Beray Gençsoy
Doktora, Biyokimya Bölümü
Tez Yöneticisi: Prof. Dr. Meral YÜCEL
Ortak Tez Yöneticisi: Prof. Dr. Haluk HAMAMCI

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Bu çalışmanın amacı, buğday (*Triticum aestivum* L. cv. Bayraktar) Trehaloz-6-fosfat Sentaz (*TPS*) geninin klonlanması ve tuz ve kuraklık stresleri altında buğdayın genel gen ifade profillerinin mikroarray Wheat GeneChip (Affymetrix) kullanılarak incelenmesidir.

Bu çalışmada, tuz (350 mM NaCl) ve kuraklık (20% PEG) stresi 10 gün büyütülmüş buğday fidelerine 24 saat uygulanmıştır. Stres sonunda toplanan kök ve yaprak numuneleri kullanılarak *TPS* gen klonlaması ve mikroarray çalışmaları yapılmıştır.

Buğday TPS genine (*TaTPS*) ait cDNA dizisinin belirlenmesi için RACE (Rapid Amplification of cDNA Ends) yöntemi kullanılmıştır. Bu cDNA dizisinin açık okuma bölgesi, moleküler ağırlığı (MW) 96.7 kDa ve izoelektrik noktası (pI) 5.97 olarak tahmin edilen 859 amino asitlik bir proteini kodlamaktadır. Bu dizi tblastx sonuçlarına göre diğer bitki *TPS* genleri ile benzerlik göstermiştir. Buğday kök dokularında bu genin ifadesi kuraklık stresi altında artarken, tuz stresi altında

değişim göstermemiştir. Yaprak dokularında ise hem tuz hem de kuraklık uygulamaları bu genin ifadesini azaltmıştır.

Tuz ve kuraklık stresi uygulanmış buğday fidelerinde, transkript miktarlarındaki değişime mikroarray yöntemi kullanılarak bakılmıştır. Veri analizleri GCOS 1.4 ve GeneSpringGX 10 kullanılarak gerçekleştirilmiştir. Tuz ve kuraklık stresleri altında, ferritin, LTP, LEA/Dehidrin, Erken Nodulin, Soğuk Regüle Proteini ve Germin Benzeri proteinleri kodlayan genlerin ifadelerinde en az 10 kat artış belirlenmiştir. Bunlara ek olarak, tuz ve kuraklık stresleri *DREB*, *ERF*, *NAC*, *MYB* ve *HSF* olarak belirlenen genlerin ifadelerinde artışa neden olmuştur ki bu gözlem tuz ve kuraklık stresleri altında çeşitli transkripsiyon düzenleyici izyollarının varlığını göstermektedir.

Anahtar: Kelimeler: trehaloz, buğday, TPS, tuz, kuraklık, mikroarray

To my family

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AAO	ABA aldehyde oxidase
ABA	Abscisic Acid
ABC	ATP-binding cassette
ABFs	ABRE-binding factors
ABRE	ABA-responsive element
APGase	ADP-Glucose Pyrophosphoryase
APX	Ascorbate peroxidase
AREB proteins	ABRE binding proteins
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
CaM	Calmodulin
CDPK	Ca^{+2}-dependent protein kinase
CIPK	CBL-interacting protein kinase
CLB	Calcineurin B-like
COR	Cold regulated
DAG	Diacylglycerol
DEPC	Diethyl pyrocarbonate
DMA	Deoxymugineic acid
DMAS	Deoxymugineic acid synthase
DRE/CRT	Dehydration reponse element / C-repeat
DRE/CRT	Dehydration reponse element / C-repeat
DREB1/CBF	DRE Binding protein 1/ C-repeat Binding Factor
ENOD	Early Nodulin
ERD	Early responsive to dehydration
ERF	Ethylene-responsive-element-binding factor
ERF/AP2	Ethylene-responsive element-binding

	protein/APETALA2
GSH	Glutathione
GSP	Gene Specific Primer
H_2O_2	Hydrogen peroxide
HDZIP	Homeodomain-leucine zipper
HK	Histidine Kinase
HKT	High Affiity Potassium Transporter
HOG	High osmolarity glycerol
IP_3	Inositol 1,4,5-trisphosphate
JA	Jasmonic acid
KIN	Cold inducible
LEA	Late Embrogenesis-Abundant
LTP	Lipid transfer protein
MA	Mugineic acid
MAPK	Mitogen-activated Protein Kinase
MAPK	Mitogen-activated protein kinase
MG	Methylglyoxal
NA	Nicotianamine
NAAS	Nicotianamine aminotransferase
NAS	Nicotianamine synthase
NCED	9-*cis*-epoxycarotenoid dioxygenase
NIP	Nodulin-26 like intrinsic proteins
$O_2^{\cdot -}$	Superoxide radials
OH^-	Hydroxyl radical
ORF	Open reading frame
P5C	Δ^1-pyrroline-5-carboxylate
P5CDH	P5C dehydrogenase
P5CR	Δ^1-pyrroline-5-carboxylate reductase
P5CS	Δ^1-pyrroline-5-carboxylate synthase
PA	Phosphatidic Acid
PCA	Principle Component Analysis
PEG	Polyethylene glycol

PI	Phosphatidylinositol
PIP	Plasma membrane intrinsic proteins
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PI-PLC	Phosphatidylinositol specific phopholipase C
PLA1	Phospholipase A1
PLA2	Phospholipase A2
PLC	Phospholipase C
PLD	Phospholipase D
PPase	Phosphoprotein (serine/threonine) phosphatases
PR	Pathogenesis-related
ProDH	Proline dehydrogenase
PTPases	Protein tyrosine phosphatases
RAB	Responsive to ABA
RACE	Rapid Amplification of cDNA Ends
RACE	Rapid amplification cDNA ends
RCD1	Radical-induced cell death 1
RD	Responsive to dehydration
RLK	Receptor Like Kinase
RMA	Robust Multiarray Analysis
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SAM	S-adenosyl-L-methionine
SDR	A short-chain alcohol dehydrogenase/reductase
SLG	S-D-lactoylglutathione
SOD	Superoxide dismutase
SOS	Salt Overly Sensitive
T6P	Trehalose-6-Phosphate
TIP	Tonoplast intrinsic proteins
TPP	Trehalose-6-Phosphate phosphatase
TPS	Trehalose-6-Phosphate synthase
ZEP	Zeaxanthin epoxidase

CHAPTER 1

INTRODUCTION

1.1 Wheat

The most widely cultivated crop of the world is wheat. It occupies 17% of total cultivated area (in 2002, 210 million of hectares). Because of its caloric value and high protein content, wheat comprises most abundant component of human diet. Annual rate of 2% increase in wheat production is needed to meet human needs by 2050. This increase can be accomplished by protecting wheat from an estimated average annual loss of 25% caused by biotic (pets) and abiotic stresses (heat, frost, drought and salinity) (Gill *et al.*, 2004).

Bread wheat, *Triticum aestivum*, is a hexaploid (genomes AABBDD). It originated from hybridization of tetraploid wheat, *T. turgidum* (genomes AABB), with the diploid goatgrass *Aegilops tauschii* (genomes DD) (Akhunov *et al.* 2005 & references therein) (Figure 1.1).

1.2 Environmental Stresses

Plant growth and productivity are adversely affected by environmental stresses. Environmental stresses can be divided into two main groups that are abiotic and biotic stresses. Abiotic stresses are cold, heat, salinity, drought, excess water (flooding), radiation, chemicals, oxidative stress, wind and nutrient deprivation in

soil. Biotic stresses are pathogens, insects, herbivores and rodents (Mahajan and Tuteja, 2005).

Abiotic stresses cause reduction in the average yields of major crops by more than 50%. Among the abiotic stresses, drought and salinity are becoming widespread in many regions. By the year 2050, more than 50% of arable lands may become useless because of salinization resulted from drought and salinity (Wang *et al.*, 2003 and references therein).

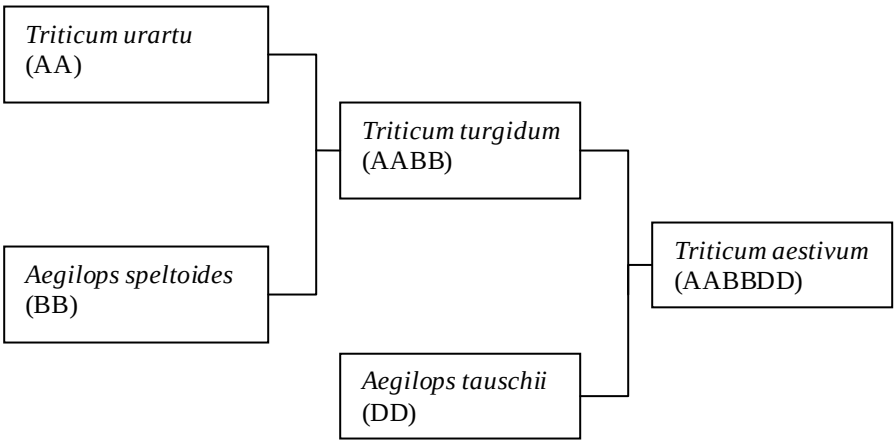


Figure 1.1 Evolution of Triticum aestivum

1.3 Salt and Drought Stress

Drought and salinity are abiotic stresses that adversely affect the growth and productivity of plants. Plants cope with these stresses by developing several strategies at molecular, cellular, physiological and biochemical levels. Both stresses lead to osmotic stress that activates similar cell signaling pathways and cellular response. Plant adaptation to them involve inhibition of shoot growth, transient increase in ABA levels, synthesis and accumulation of compatible solutes and

protective proteins, decreased photosynthesis and increased expression of stress-associated genes (Bartels and Sunkar, 2005).

Apart from osmotic stress, salt stress also causes ionic stress. The ions involved in ionic stress are Na^+ and Cl^-. Na^+ causes disruption of ionic equilibrium, reduction in photosynthesis, production of reactive oxygen species (ROS), membrane disorganization, reduction in growth and inhibition of the functioning of some enzymes (Hasegawa *et al.*, 2000; Mahajan and Tuteja, 2005).

Salt and drought stress signaling include three functional categories. These are (i) the restoration of cellular homeostasis under stress conditions by ionic and osmotic stress signaling, (ii) control and repair stress damages by detoxification signaling, and (iii) signaling to manage cell division and expansion to levels suitable for the particular stress conditions (Zhu, 2002) (Figure 1.2).

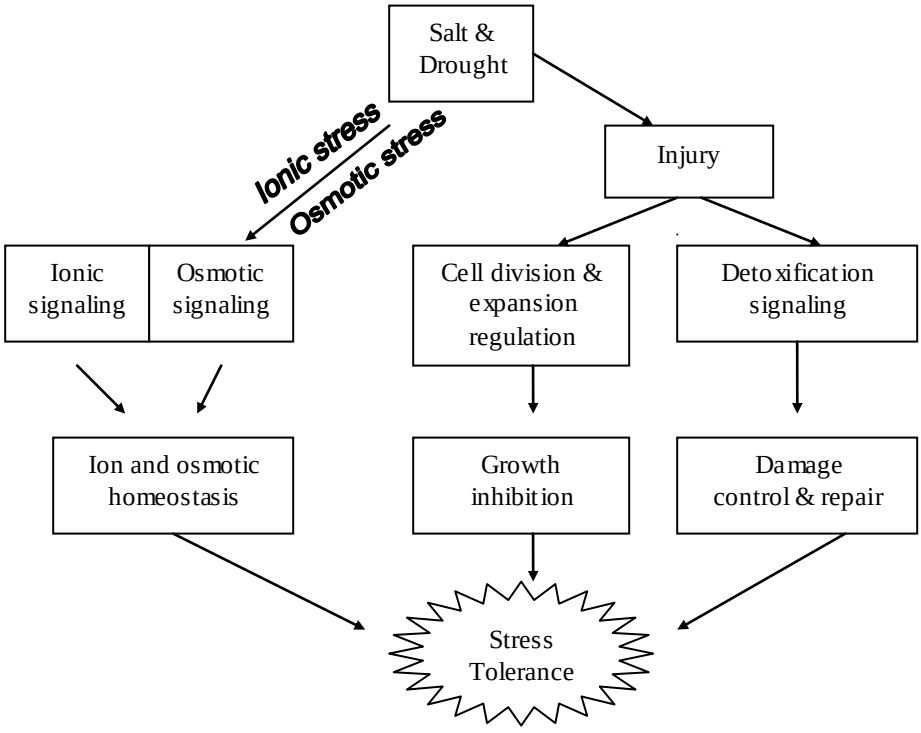


Figure 1.2 Salt and Drought Stress Signaling Pathways (Zhu, 2002)

1.4 Signal Transduction in Plants

Sensors, such as receptors, are molecules that perceive initial stress signaling. Sensors transmit the signal to downstream components, leading to generation of second messengers such as calcium, reactive oxygen species (ROS) and inositol phosphates. Salt, drought and cold stresses induce transient Ca^{+2} influx into the cell cytoplasm. Calcium sensors, calcium binding proteins, sense this perturbation in cytosolic Ca^{+2}. They interact with other proteins and initiate phosphorylation/dephosphorylation cascades. The downstream targets of these cascades are major stress responsive genes or transcription factors controlling these genes. These genes lead to stress tolerance and adaptation that provide survival under unfavorable conditions (Mahajan and Tuteja, 2005).

The various stress responsive genes are grouped as early- and late-response genes. After signal perception, early response genes are induced quickly and transiently. The induction of these genes does not require new protein synthesis since signaling components are already primed. Several transcription factors are in this group. In contrast, late response genes that are downstream of early response genes are induced after hours of signal perception. This group includes major stress response genes such as KIN (cold induced), COR (cold responsive), RD (responsive to dehydration) and LEA/dehydrin (Mahajan and Tuteja, 2005; Zhu, 2002) (Figure 1.3).

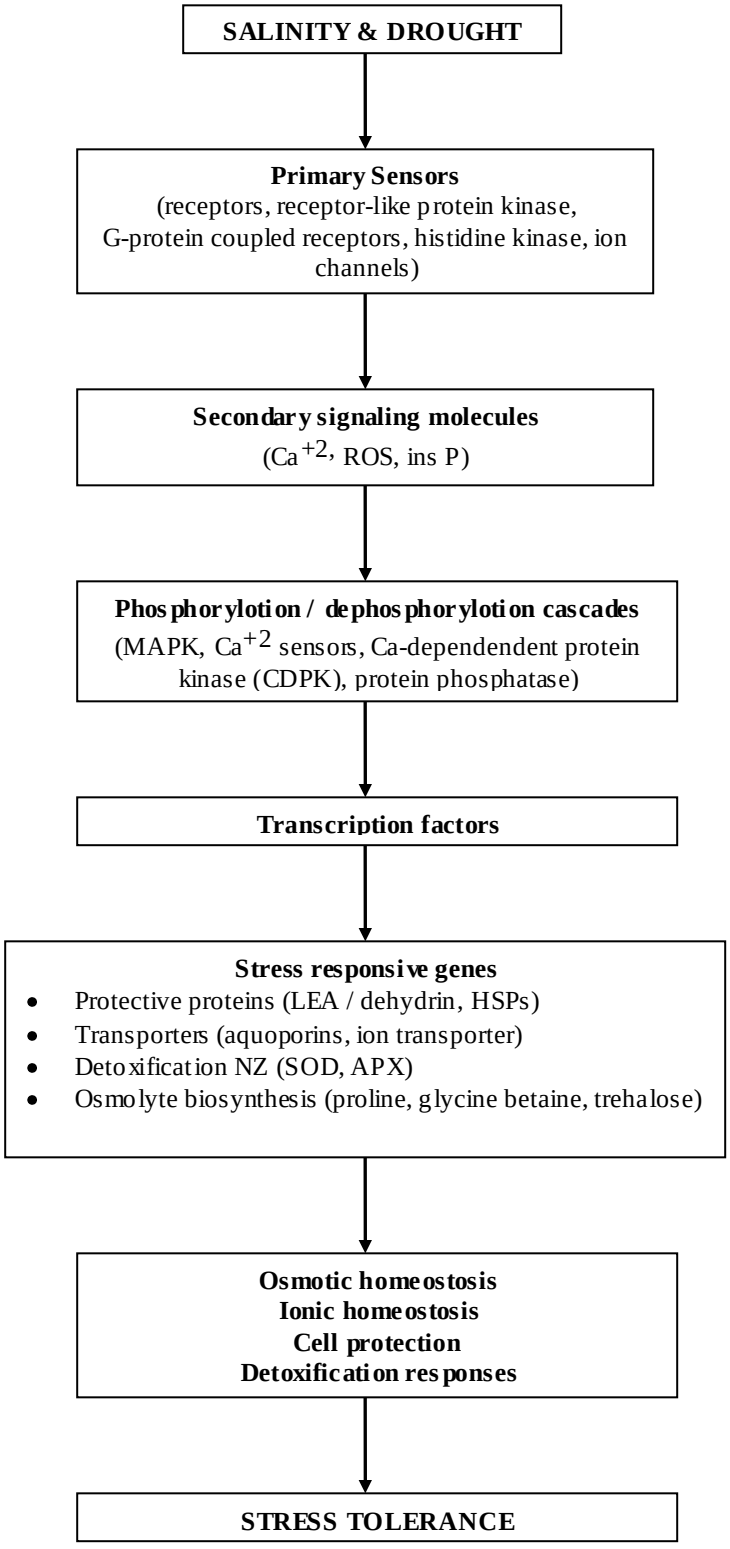


Figure 1.3 Signal transduction pathways for salt and drought stress signaling

1.5 SOS Pathway

In Arabidopsis, ion homeostasis is maintained by Salt Overly Sensitive (SOS) pathway under salinity. This pathway was discovered by identifying genes related to salt tolerance. Arabidopsis plants having mutations in SOS genes become sensitive to salinity.

Na^+ efflux from the cytosol and its compartmentalization into the vacuole are important for salt tolerance in plants. High salinity causes increased cytosolic Ca^{+2}, which initiates signal transduction (Knight *et al.*, 2000). Perturbation in cytosolic Ca^{+2} levels is sensed by SOS3, a myristoylated calcium binding protein. SOS3 activates SOS2, a serine/threonine protein kinase. Activated SOS2 phosphorylates and activates SOS1, a plasma membrane Na^+/H^+ antiporter. SOS1 transports Na^+ out of the cytosol. SOS2 also regulates the activity of tonoplast Na^+/H^+ antiporter (NHX). This antiporter functions in compartmentalization of excess Na^+ into the vacuole (Chinnusamy *et al.*, 2005) (Figure 1.4).

Under salinity, Na^+ enters into the roots through cation transporter. Arabidopsis high affinity K^+ transporter (HKT) functions as a low affinity Na^+ transporter under salinity (Uozumi *et al.*, 2000). Mutation in *AtHKT1* suppresses the sos3 mutation (Rus *et al.*, 2001). According to these findings, Zhu (2002) suggested that SOS3-SOS2 complex may downregulate AtHXK1 activity under salinity, which prevents Na^+ entry and provide salt tolerance.

Katiyar *et al* (2006) showed that SOS1 interacts with RCD1 (Radical-induced cell death 1) under salt and oxidative stress. RCD1 is found in the nucleus under unstressed conditions whereas it is localized in both nucleus and cytoplasm under salt and oxidative stress. Wild type yeast and yeast mutant overexpressing Arabidopsis RCD1 showed enhanced tolerance in response to oxidative stress (Ahlfors *et al.*, 2004).

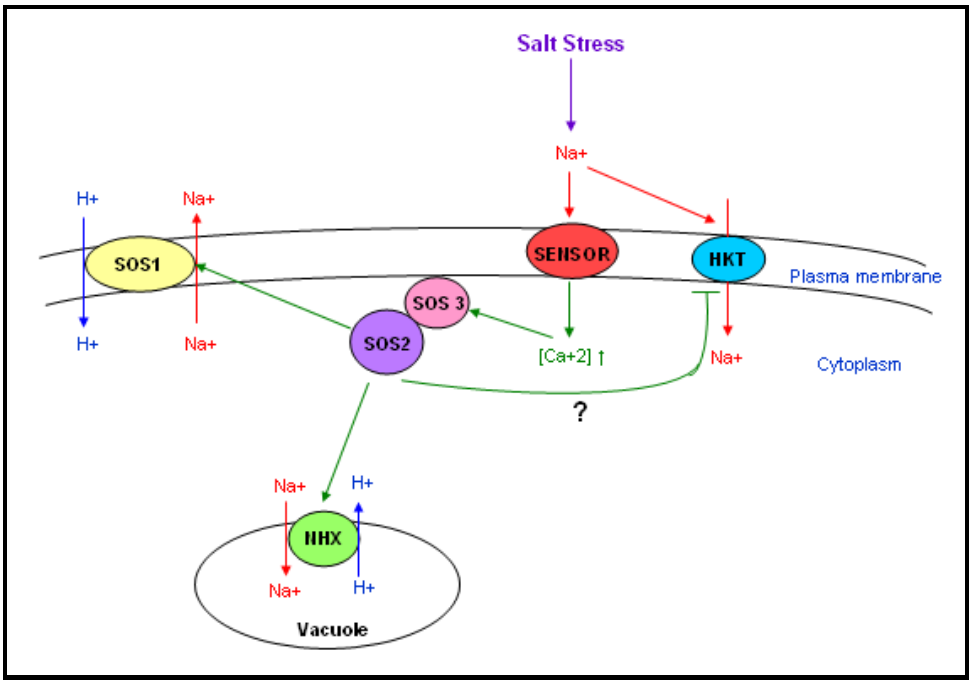


Figure 1.4 SOS Pathway in *Arabidopsis* under salinity.

1.6 Receptors in Plants

Environmental stimuli are considered to be first sensed by receptor molecules. In plants, receptors for salt and drought stress are not clearly identified. Receptor-like kinases (RLKs) may perceive osmotic stresses. These kinases have an extracellular part that may play role in ligand binding or protein-protein interaction, a transmembrane part and an intracellular kinase part (Xiong and Zhu, 2001).

The two component histidine kinases first identified in bacteria sense various environmental stimuli. When a signal is perceived by sensor of this kinase, the cytoplasmic histidine residue is autophosphorylated and subsequently the phosphate is transferred to an aspartate residue in the receiver. In yeast, SNL1, two component histidine kinase, was identified as an osmosensor. This kinase is responsible for the regulation of the high-osmolarity glycerol (HOG) MAPK cascade that leads to

glycerol (osmolyte) accumulation. Arabidopsis histidine kinase, AtHK1, displays structural homology with SNL1. *AtHK1* can complement yeast *sln1* mutant and therefore may have a role in osmotic stress in *Arabidopsis thaliana* (Xiong and Zhu, 2001).

1.7 Calcium Signaling In Abiotic Stress

In plants, salt, drought and cold stresses causes cytosolic Ca^{+} oscillations. This signal is sensed by specific proteins that induce wide ranges of downstream responses related to the protection of plant and the adaptation to the new environment (Knight *et. al.*, 2000).

In plants, many calcium sensors have been characterized. These are calmodulin (CaM), Ca^{+2}-dependent protein kinase (CDPK) and CBL (calcineurin B-like) protein. Calmodulins are calcium binding proteins. After activated by increased calcium concentrations, CaMs induce specific kinases. CBLs have 4helix-loop-helix calcium binding domains named as EF hands. CBLs interact with CBL-interacting protein kinase (CIPKs), leading to activation of downstream signaling components (Mahajan and Tuteja, 2005). The well-known example for CBL/CIPK pathway is SOS pathway. In Arabidopsis, SOS3 and SOS2 are also known as CBL4 and CIPK24, respectively (Mahajan and Tuteja, 2005). As mentioned in Section 1.5, SOS3/CBL4-SOS2/CIPK24 complex has an important role in the ion homeostasis under salt stress.

1.8 Abscisic Acid

1.8.1 Biosynthesis of Abscisic Acid

The first step in ABA biosynthesis pathway is the epoxidation of zeaxanthin and antheraxanthin to violaxanthin, which is catalyzed by a zeaxanthin epoxidase (ZEP). This step occurs in plastids. Oxidative cleavage of the major epoxycarotenoid 9-*cis*-

neoxanthin to xanthoxin is catalyzed by the 9-*cis*-epoxycarotenoid dioxygenase (NCED). The xanthoxin is then exported to the cytosol. A short-chain alcohol dehydrogenase/reductase (SDR), encoded by the *AtABA2* gene, catalyzes the conversion of xanthoxin to ABA aldehyde. ABA aldehyde oxidase (AAO) then catalyzes the last step in the biosynthesis pathway (Xiong and Zhu, 2003).

Drought and salt stresses induce the expression of genes encoding biosynthesis of ABA, which leads to accumulation of ABA. Expression studies with *ZEP*, *NCED*, *AAO3* showed that these genes are induced under salt and drought stress (Xiong and Zhu, 2003 and references therein).

1.8.2 Role of Abscisic Acid in Salt and Drought Stresses

ABA is involved in regulating plant water status through guard cells and induction of genes that encode protein and enzymes related to osmotic stress tolerance. ABA produced in dehydrated roots in drying soil is loaded into root xylem and transported to the shoot where ABA regulates stomatal opening and leaf growth. ABA mediates the expression of many stress inducible genes such as *MYB*, *EmBP-1* and *rd29B* (Zhang *et al.*, 2006). ABA-induced gene expression was discussed in detail in section 1.9.2.4.2.

1.9 Function of Drought and Salt Inducible Genes in Plants

Genes induced during abiotic stress have roles in stress tolerance and stress response. The products of these genes are classified into two groups. The first group comprises proteins that function in stress tolerance. Examples of these proteins are LEA proteins, detoxification enzymes, enzymes for osmolyte biosynthesis, water channel proteins and transporters. The second group comprises proteins that function in signal transduction and stress-responsive gene expression. Examples of second group proteins include transcription factors, enzymes involved in phospholipid metabolism and protein kinases (Shinozaki and Yamaguchi-Shinozaki, 2007).

1.9.1 Functional Proteins

1.9.1.1 Protective Proteins

1.9.1.1.1 Late Embryogenesis-Abundant Protein

Late Embryogenesis-Abundant (LEA) Proteins are found to accumulate in vegetative tissues in a wide range of plant species in response to abiotic stress (Xiong and Zhu, 2002). Expression of LEA genes are induced by salt, drought, cold and ABA (Bartels and Sunkar, 2005 and references therein). It has been suggested that LEA proteins function in stabilization of protein structure and membrane integrity and sequestration of ions from stress tissue (Wang *et al.*, 2003)

Dehydrins, group 2 LEA proteins, accumulate in plant tissues under osmotic stress (Rorat, 2006). The poplar dehydrin gene, *Peudhn1*, is induced by drought, salt and cold stress (Caruso *et al.*, 2002). Similarly, rice dehydrin gene, *OsDhn1*, is transcriptionally upregulated by salt, drought and ABA (Lee *et al.*, 2005).

Some of LEA/dehydrin type genes are named as RD (responsive to dehydration), ERD (early responsive to dehydration), KIN (cold inducible), COR (cold regulated), RAB (responsive to ABA) genes (Chinnusamy *et al.*, 2005; Zhu, 2002).

1.9.1.1.2 Heat Shock Proteins

Abiotic stresses causes dysfunction of proteins and enzymes. Under environmental stress conditions, it is important to sustain proteins in their functional conformations and prevent the aggregation of non-native proteins for cell survival. Heat shock proteins have an important function in the folding and assembly of proteins. Moreover, they stabilize proteins and membranes and can assist in protein refolding under stress conditions (Wang *et al.*, 2003)

1.9.1.1.3 Enzymes in Polyamine Biosynthesis

Polyamines, such as spermidine, spermine, and putrescine, are small organic cations. They are involved in a variety of plant processes such as development and senescence. In addition, they are thought to play role in response to abiotic stresses. Enzymes involved in the biosynthesis of polyamines are ornithine decarboxylase, arginine decarboxylase, S-adenosyl-L-methionine (SAM) decarboxylase and spermidine synthase (Bartels and Sunkar, 2005). Transgenic Arabidopsis plant overexpressing spermidine synthase showed enhanced tolerance to multiple environmental stresses. In addition, drought-inducible genes were induced in these transgenic plants. These results suggest that spermidine is one of the regulators of osmotic stress signaling (Kasukabe *et al.*, 2004).

1.9.1.2 Enzymes Involved in Osmolyte Biosynthesis

Plants accumulate compatible solutes or osmolytes in response to osmotic stress. These solutes do not interfere with normal metabolic reactions. They help to maintain osmotic balance under dehydration. Examples of osmolytes are sugars, polyols, proline and glycine betaine. Transgenic plants expressing osmolyte biosyntesis enzymes showed improved stress tolerance (Ramanjulu and Bartels, 2002).

1.9.1.2 Proline

Proline is thought to have diverse roles under osmotic stress conditions. Besides osmotic adjustment, it seems to function in stabilization of proteins, membranes and subcellular structures, and protecting cellular functions by scavenging reactive oxygen species (Kishor *et al.*, 2005). Proline is synthesized from glutamate under stress conditions. This reaction is catalyzed by Δ^1-pyrroline-5-carboxylate synthase (P5CS) and Δ^1-pyrroline-5-carboxylate reductase (P5CR). P5CS is the rate limiting step in the proline biosynthesis. In reverse reaction, proline dehydrogenase (ProDH)

and P5C dehydrogenase (P5CDH) metabolize the proline to glutamate (Wang *et al.*, 2003). Strategies involved in maintaining proline biosynthesis include overexpressing of P5CS and antisense suppression of proline degradation enzyme ProDH. Both strategies resulted in increase in proline concentration and enhanced abiotic stress tolerance (Hmida-Sayari *et al.*, 2005; Seki *et al.*, 2002).

1.9.1.2.2 Sugar

Oligosaccharides such as rafinose and galactinol are synthesized in response to drought. These compounds seem to function as osmoprotectants rather than providing osmotic adjustment. Mannitol, a sugar alcohol, functions to scavenge the ROS, hydroxyl radicals and it also stabilizes the macro molecular structure of enzymes. These osmolytes constitude hydrogen bonds with macromolecules under drought stress condition and prevent the formation of intramolecular hydrogen bonds. Trehalose is a non-reducing disaccharide of glucose and has been shown to stabilize membranes and macromolecules during drought stress (Mahajan and Tuteja, 2005 and references therein).

1.9.1.3 Detoxification Enzymes

Aerobic cellular reactions such as photosynthesis and respiration produce reactive oxygen species (ROS) including superoxide radials ($O_2^{\cdot -}$), hydroxyl radical (OH^-) and hydrogen peroxide (H_2O_2). Abiotic stresses cause increase in the level of ROS, which leads to oxidative damage to membrane lipids, proteins and nucleic acids. This phenomenon is called oxidative stress. Plants have an antioxidant system consisting of antioxidants (ascorbate, glutathione, α-tocopherol and carotenoids) and detoxifying enzymes such as superoxide dismutase (SOD), catalase, ascorbate peroxidase (APX) and glutathione reductase to eliminate ROS (Xiong and Zhu, 2002).

Abiotic stresses also increase the formation of methylglyoxal (MG). MG is known to be toxic at high concentration to plant cells. It reacts with RNA, DNA and proteins. Glyoxalase pathway, a two step enzyme catalyzed reaction, detoxifies MG by glutathione-based detoxification. The first step of the pathway catalyzed by glyoxalase1, lactoylglutathione lyase, leads to the formation of S-D-lactoylglutathione (SLG) from methylglyoxal (MG) and glutathione (GSH). In the second step, glyoxalase II, hydroxyacylglutathione hydrolase, hydrolyze SLG, leading to release of GSH and formation of D-Lactate (Yadav *et al.*, 2007).

Free iron causes damage in the cell. It is high reactivity with H_2O_2 and $O_2^{\cdot -}$, leading to Fenton reaction. $OH^{\cdot}$ radicals, product of Fenton reaction, react with DNA, lipids and proteins. Since free iron causes oxidative damage, it is sequestered by metal binding proteins and ferritins. Ferritins are multimeric protein complexes that consist of 24 subunits. They can store up 4000 iron atom, thereby sequestering excess iron (Hell and Stephan, 2003).

In plants, there are two strategies for iron uptake. Strategy 1 is used by all plants except Gramineae. Grasses take up iron by Strategy 2. Their root release mugineic acid family phytosiderophores (MAs), which bind Fe^{3+} in the rhizosphere. Fe^{+3}-MA complexes are taken into the root cell by specific transporters. Firstly, nicotianamine (NA) is synthesized by three molecules of methionine by nicotianamine synthase (NAS). Then, nicotianamine aminotransferase (NAAS) catalyses the deamination of NA, leading to 3''-keto intermediate. The reduction of this intermediate catalysed by deoxymugineic acid synthase (DMAS) leads to deoxymugineic acid (DMA), the first MA synthesized in this pathway. DMA is the substrate for the production of other MAs. Iron deficiency increases the synthesis and secretion of MAs (Haydon and Cobbett, 2007; Hell and Stephan, 2003).

1.9.1.4 Transporters

1.9.1.4.1 Aquaporins

Aquaporins are channel proteins found in the plasma and intracellular membranes of plant cells. They facilitate the diffusion of water, small neutral solutes (urea, boric acid, silicic acid) and gases (ammonia, carbon dioxide). They belong to major intrinsic proteins. Based on the sequence homology, plant aquaporins are divided into four groups. These are plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), nodulin-26 like intrinsic proteins (NIP) and small basic intrinsic proteins (SIP). PIP and TIP are localized in the plasma membrane and tonoplast, respectively. NIPs are found in different subcellular locations (Maurel *et al.*, 2008)

Aquaporins can regulate the water permeability of membranes. Therefore, under abiotic stress conditions, they may maintain water status of plants by minimizing water loss. There are several reports indicates that different aquaporin genes are up-regulated, downregulated or nonchanged under osmotic stress. Arabidopsis plasma membrane aquaporin gene, *rd28*, is induced under drought stress (Yamaguchi-Shinosaki *et al.*, 1992). In *C. plantagineum*, *CpPlPa2* gene is upregulated by drought and downregulated by salt stress (Smith-Espinoza *et al.*, 2003).

1.9.1.4.2 Lipid Transfer Proteins

Lipid transfer proteins are cationic proteins present in several plant species such as barley, wheat, and Arabidopsis. LTP can facilitate the transfer of phospholipids between membranes *in vitro*. Proposed biological roles for LTPs include cutin synthesis, β-oxidation, defense reaction against pathogens and adaptation of plants to environmental stresses (Kader, 1996; Sels *et al.*, 2008)

Epidermis, the outermost layer of cells covering all young plant organs, is site of direct contact with the environment. Because of this, epidermal cells secrete cutin that forms protective layer called cuticle. Under abiotic stresses, LTPs may have a protective role by participating in the cutin biosynthesis (Kader, 1996).

1.9.1.5 Cytochrome P450

Cytochrome P450 monooxygenase are heme-thiolate containing enzymes. In plants, they comprise the largest family of plant enzymes. Currently, 273 Arabidopsis, 457 rice P450 genes have been identified. Because of their abundance, function of the small portion of P450 is known. Plant P450s have roles in synthetic pathways of signaling molecules (ABA, gibberellin, brassinosteroids, jasmonic acid) (Bundock *et al.*, 2003; Schuler *et al.*, 2006) and biosynthesis of cutin and lignin (Werck-Reichhart and Feyeresien, 2000). They also participate in herbivore and pathogen resistance (Bundock *et al.,* 2003). In catabolic pathways, they are involved in breakdown of toxic compounds encountered in the environment. Narusaka *et al* 2004 showed that most *Arabidopsis* cytochrome P450 genes induced by biotic and abiotic stresses contains MYB and MYC recognition sites in their promoters.

1.9.2 Regulatory Proteins

1.9.2.1 Phospholipid signaling

The plasma membrane has an important function in the perception and transmission of environmental signals. Osmotic stress leads to changes in phospholipids composition in plants. The current hypothesis is that in osmotic stress signaling, phospholipids are to serve as precursors for the phospholipid-derived second messengers (DAG, IP_3, PA, etc). Phospholipid-based signaling pathways include phospholipase C (PLC), phospholipase D (PLD), phospholipase A1 and A2 (PLA1 and PLA2) (Bartels and Sunkar, 2005; Xiong and Zhu, 2001).

Phosphoinositide-specific phospholipase C (PI-PLC) cleaves phosphotidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 activates Ca^{+2} releases from internal stores. Several studies have demonstrated that IP_3 levels increase under osmotic stress in plants (Drobak and Watkins, 2000; Takahashi *et al.*, 2001). In Arabidopsis, inhibition of PI-PLC activity led to decrease in the level of IP_3, resulting in inhibition of hyperosmotic stress-responsive expression of some dehydration-inducible genes, such as rd29A (lti78/cor78) and rd17 (cor47) (Takahashi *et al.*, 2001).

There are several reports indicating that osmotic stress upregulates the expression level of plant PLC. The expression of Arabidopsis phospholipase C (*AtPLC1*) and potato PLC genes are induced by osmotic stress (Hirayama *et al.*, 1995; Kopka *et al.*, 1998).

1.9.2.2 MAP Kinase Pathways

MAP Kinase pathways are one of the main mechanisms for regulating cellular activities in response to environmental stimuli. MAPKKK phosphorylates serine/threonine residue of MAPKK. The MAPKK, upon activation, phosphorylates a MAPK on conserved threonine / tyrosine residues. The activated MAPK translocated into the nucleus where it phosphorylates transcription factors, which in turn activates stress genes (Xiong and Zhu, 2001).

In plants several MAP kinases are activated by hyperosmotic stress. SIMK from alfalfa and SIPK from tobacco, identified as MAP kinase, are activated by hyperosmotic stress (Mikolajczyk *et al.*, 2000; Munnik *et al.*, 1999). In Arabidopsis, AtMEKK1, a MAPKinase kinase kinase, and AtMPK3, a MAPKinase, is induced by cold, salt, drought and touch (Mizoguchi *et al.*, 1996).

1.9.2.3 Phosphatases

In a stress signaling cascade, the role of phosphatases is the dephosphorylation of phosphoproteins, acting opposition to kinases. There are two main groups of phosphatases: phosphoprotein (serine/threonine) phosphatases (or PPases) and phosphotyrosine (protein tyrosine phosphatases or PTPases). PPases are divided into four groups: PP1, PP2A, PP2B, and PP2C. The PTPases consist of three subgroups: receptor-like PTPases, intracellular PTPases, and dualspecific PTPases (Bartels and Sunkar, 2005).

1.9.2.4 Transcription Factors

In plants, several genes are induced in response to osmotic stress such as drought and salt. Several transcription factors are involved in drought and salinity signal transduction pathway. Transcription factors specifically bind *cis* acting elements in the promoter region of stress inducible genes and regulate their expression. In osmotic stress, stress responsive gene expression is regulated by ABA-dependent and ABA-independent pathways (Yamaguchi-Shinozaki and Shinozaki, 2006).

1.9.2.4.1 Transcription Factors Involved in ABA-Independent Pathway

DRE/CRT (dehydration reponse element / C-repeat) is an essential *cis*-element in the ABA-independent pathway. It is found in the promoter regions of many drought- and cold inducible genes. In Arabidopsis, transcription factors that bind to DRE/CRT were isolated and named as DREB1/CBF (DRE Binding protein 1/ CRT Factor), and DREB2. They belong to ERF/AP2 (Ethylene-responsive element-binding protein/APETALA2) family. DREB1/CBF and DREB2 are induced by cold and dehydration respectively. DREB1/CBF regulates the cold induced gene expression. By contrast, DREB2 regulates the drought-and salinity-induced gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006).

In rice homologous of genes coding DREB1/CBF and DREB2 were identified. Transgenic Arabidopsis overexpressing rice OsDREB1A showed increased tolerance to high salinity and drought (Dubouzet *et al.*, 2003).

Expression of *ERD1*, one of the early responsive to dehydration gene, is regulated by ABA-independent pathway in response to dehydration. This gene is induced by dehydration and natural senescence. Promoter analysis of this gene showed that two transcription factors, NAC and ZFHD, are necessary for the expression of *ERD1* (Simpson *et al.*, 2003)

ERF (ethylene-responsive-element-binding factor) transcription factors are only found in plants. They are a subfamily of ERF/AP2 transcription factors. They can bind two *cis* elements: GCC box and CRT/DRE motif. The GCC box is found promoter region of several *PR* (Pathogenesis-related) genes (Singh et al., 2002). As mentioned previously, CRT/DRE motif is located in the dehydration- and cold-inducible gene promoters. Transgenic tobacco over expressing *Tsi1* (tobacco ERF) showed improved salt tolerance and enhanced resistance to pathogen attack (Park *et al.*, 2001).

1.9.2.4.2 Transcription Factors Involved in ABA-Dependent Pathway

ABRE (ABA-responsive element) is found in the promoter region of many ABA inducible genes. It functions as a *cis* acting element in ABA-responsive gene expression. In Arabidopsis, bZIP transcription factors named as ABRE binding (AREB) proteins or ABRE-binding factors (ABFs) function as *trans*-acting activators that bind ABRE. Expression of AREB/ABF proteins are induced by ABA, dehydration and salinity stress (Yamaguchi-Shinozaki and Shinozaki, 2006). AREB/ABF activates the expression of genes whose promoters contain ABRE such as responsive to dehydration 29B (RD 29B).

In Arabidopsis, drought inducible gene *RD22* is upreulated by ABA. Two transcription factors, MYC transcription factor (AtMYC2) and MYB transcription factor (AtMYB2) bind to the *cis* elements, MYC-recognition site and MYB-recognition site in the promoter region of *RD22* and cooperatively activate the expression of *RD22*. Synthesis of MYC and MYB transcription factors requires endogenous accumulation of ABA, indicating that these transcription factors function in the late stage of stress response (Yamaguchi-Shinozaki and Shinozaki, 2006).

Arabidopsis *RD26* encoding NAC transcription factor is upregulated by drought, high salinity, ABA and jasmonic acid (JA) treatments (Fujita et al., 2004). In rice, NAC gene *SNAC1* (*STRESS-RESPONSIVE NAC1*) was isolated and characterized. Transgenic rice plant overexpressing *SNAC1* showed significantly increased drought and salt tolerance (Brodmann *et al.*, 2002). Another rice NAC gene, *OsNAC6*, is induced by cold, drought and high salinity. *OsNAC6* gene expression is also induced by biotic stress such as wounding and blast disease. Microarray analysis showed that many abiotic stress inducible genes were induced in rice overexpressing *OsNAC6* (Nakashima *et al.*, 2007).

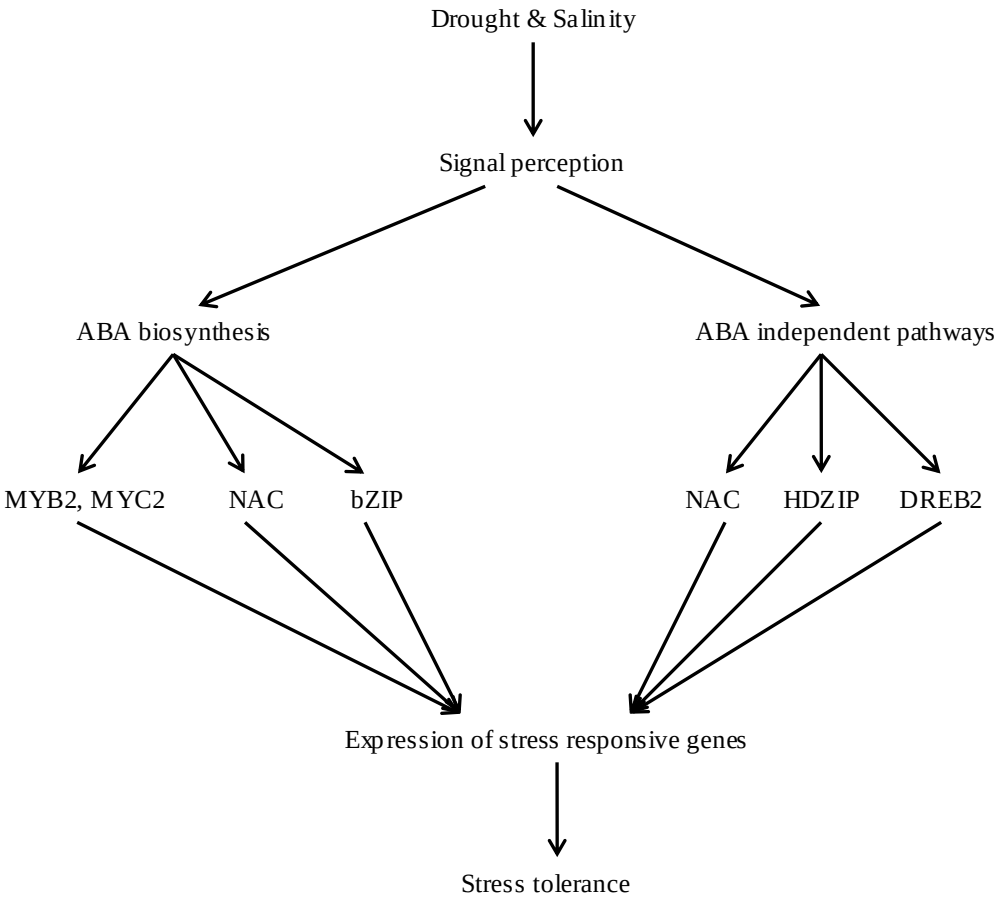


Figure 1.5 Transcription Factors involved in ABA dependent and ABA independent pathways

1.10 Trehalose

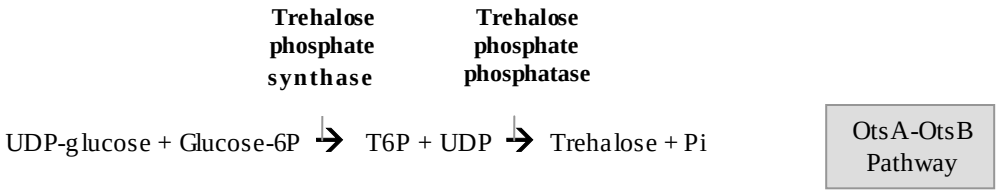
Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside) is a nonreducing disaccharide composed of two glucose unit linked by an α, α-1, 1 glucoside bond. It is found wide range of organisms such as bacteria, yeast, fungi, insects, invertebrates, and plants (Elbein *et al.*, 2003). It stabilizes membranes and proteins under stress conditions, especially drought stress (Wingler, 2002).

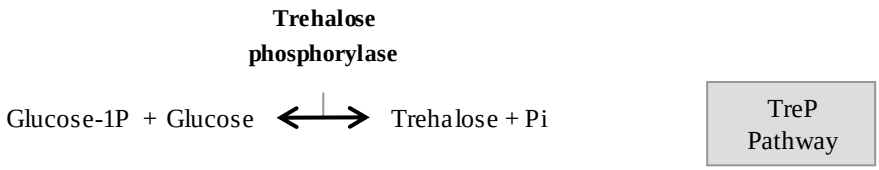
1.10.1 Biosynthetic Pathways of Trehalose

Up to now, five routes have been found for the biosynthesis of trehalose (Figure1.6). Among the five routes, OtsA-OtsB is the most widely distributed and is found in bacteria, fungi and plants. This pathway involves two enzymatic steps. The first step, catalyzed by Trehalose-6-phosphate synthase (TPS), is the transfer of glucose from the UDP-glucose to glucose-6-p to form trehalose-6-phosphate (T6P). Then trehalose-6-P phosphatase (TPP) dephosphorylate T6P to trehalose and inorganic P. In the second pathway, TreP pathway, trehalose phosphorylase catalyses the formation of trehalose from glucose 1-phosphate and glucose. It is found in some fungi. TheS pathway, the third pathway, is found in bacteria. In this pathway, isomerisation of α1-α4 bond of maltose to a α1-α1 bond of trehalose is catalysed by trehalose synthase. TreY-TreZ pathway found in bacteria and Archaea is the fourth pathway. It involves two enzymatic steps catalysed by trehalose synthase and maltooligosyl trehalose trehalohydrolase. The last pathway is TreT that is found in Archea . In this pathway, trehalose glycosyltransferring synthase catalyses the formation of trehalose from ADPglucose and glucose. (Avonce *et al.*, 2006; Paul *et al.*, 2008).

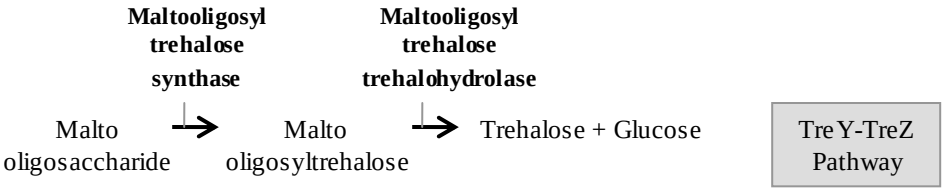
Trehalase, is an enzyme hydrolyzing trehalose into two glucose moieties. It is the only known route for the breakdown of trehalose in plants. Several prokaryotes and eukaryotes contain alternative enzymes such as Trehalose-6-P hydrolase (Phosphotrehalase) and trehalose phosphorylase (Argüelles, 2000).

a Eukaryotes and prokaryotes





b Prokaryotes



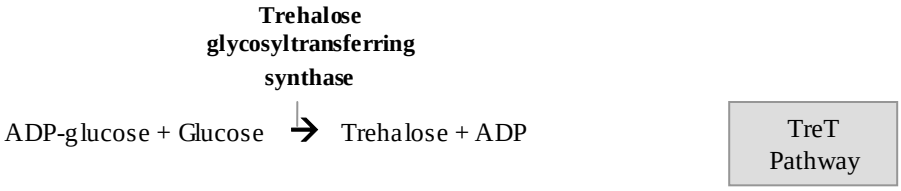


Figure 1.6 Biosynthetic Pathways of Trehalose

1.10.2 Occurance of Trehalose in Plants

Previously, it was believed that plants did not accumulate trehalose. Presence of trehalase was shown in many plants. The reason for the low level of trehalose in plants is probably due to trehalase activity. Arabidopsis plant grown on validamycin A, a potent inhibitor of trehalase, demonstrated increase in the level of trehalose, the result of which validates the minute amount of trehalose in plants. In early reports, trehalose was not detected in plants because sensitivity of assays was below the detection limit. Recently, trehalose has been determined in many plant species such as wheat, potato, tomato and Arabidopsis by using GC-MS analysis (El-Bashiti *et al.*, 2005; Roessner-Tunali *et al.*, 2003; Roessner *et al.*, 2000, Vogel *et al.*, 2001).

1.10.3 Trehalose Biosynthesis Genes in Plants

1.10.3.1 Trehalose-6-Phosphate Synthase

The genomes of Arabidopsis, rice and poplar contain 11, 11 and 12 TPS homologues, respectively (Leyman *et al.*, 2001; Lunn, 2007). Two plant TPS genes from *Arabidopsis* and *Selaginalla* was cloned and shown functional by complementation of *S. cerevisae* tps1 mutant (Blazquez *et al.*, 1998; Zentella *et al.*, 1999). Unlike *S. cereisiae* and *E. coli* TPS genes, *A. Thaliana* and *S. lepidophylla* TPS genes have specific N- and C-terminal extensions that seem to be specific to plant TPSs. N-terminal of plant TPS genes act as an autoinhibitory domain (Van Dijck *et al.*, 2002).

TPS genes from *Ginkgo Biloba* and *Gossypium hirsutum* L. were also cloned but functionality of these genes have not been shown yet (Kosmas *et al.*, 2006; Wu *et al.*, 2006).

1.10.3.2 Trehalose Phosphate Phosphatase

Analysis of *Arabidopsis thaliana* genome showed that there are 10 TPP genes (Eastmond *et al.*, 2003). AtTPPA and AtTPPB genes were identified from Arabidopsis by functional complementation of yeast *tpp* mutant (Vogel *et al.*, 1998). Moreover, plant TPP genes were cloned from rice, maize and tobacco (Pramanik and Imai, 2005; Satoh-Nagasawa *et al.*, 2006; Wang *et al.*, 2005).

1.10.3.3 Role of Trehalose in Abiotic Stress Tolerance

In recent years, there has been a growing interest in trehalose metabolism for the production of transgenic plants tolerant to abiotic stresses (Penna, 2003). Expression of *E.coli* or yeast *TPS1* in plants improved stress tolerance, especially tolerance to drought, but it leads to alteration in growth or morphology in plants (Cortina and Culianez-Macia, 2005; Holmström *et al.*, 1996; Romero *et al.*, 1997). These studies are given in Table 1.1.

It seems that phenotypic alterations in these transgenic plants are possibly due to T6P (Jang *et al.*, 2003; Miranda *et al.*, 2007; Paul *et al.*, 2008). Therefore, in order to avoid accumulation of free T6P, bifunctional *TPS-TPP* gene fusion was used in transgenic studies. Rice and Arabidopsis plants transformed with bifunctional *TPS-TPP* fusion displayed resistance to several abiotic stresses without any growth or morphological abnormalities (Garg *et al.*, 2002; Jang *et al.*, 2003; Miranda *et al.*, 2007).

There are also some biochemical studies that display the role of trehalose under abiotic stresses. The rice plants under salt and drought stresses accumulated trehalose. Exogenously applied trehalose improved the growth of rice under salt stress (Garcia *et al.*, 1997; Morsy *et al.*, 2007). In wheat, it has been also shown that under salt and drought stresses trehalose levels were increased both in shoots and root tissues (El-Bashiti *et al.*, 2005). These studies demonstrate the importance of trehalose pathway in plants during abiotic stresses.

Table 1.1 Transgenic plants expressing trehalose biosynthesis genes with improved abiotic stress tolerance

Transgenic Plants	Gene used	Effects	References
Tobacco	Yeast TPS	Drought tolerance, Developmental alteration	Holmström *et al.*, 1996
Tobacco	Yeast TPS1	Improve drought tolerance Morphological changes	Romero *et al.*, 1997
Tomato	Yeast TPS1	Improved tolerance to drought, salt, oxidative stress Developmental alteration	Cortina and Culianez-Marcia ,2005
Rice	E. coli TPSP fusion gene	Improve tolerance to drought, salt and cold, accumulation of trehalose, improve photosynthesis capacity, No growth inhibition	Garg *et al.*, 2002
Rice	E. coli TPSP fusion gene	Improve tolerance to drought, salt and cold No growth inhibition	Jang *et al.*, 2003
Arabidpsis	Bifunctional yeast TPS1-TPS2	Improve tolerance to drought, freezing, salt and heat No morphological or growth alteration	Miranda *et al.*, 2007

1.10.4 The Functions of Trehalose Metabolism in Plants

It was shown that Arabidopsis TPS1 is required for embryo maturation. The *tps1* mutant does not develop beyond the torpedo stage. The *tps1* mutants demonstrate decreased cell division, starch accumulation and altered cell wall structure compared to wild type. The author suggested that TPS1 may play a major role in coordinating cell wall biosynthesis and cell division during embryo development (Eastmond *et al*., 2002; Gomez *et al.*, 2006; Gomez *et al*., 2005).

Schluepmann *et al*. (2003) showed that T6P is required for embryo development in *Arabidopsis thaliana*. The embryo-lethal *tps1* mutants can be rescued by expressing *E. coli* TPS ortholog *OtsA*. Since *E.coli* TPS is quite different from the Arabidopsis counterpart at the amino acid level, T6P rather than TPS protein itself is responsible for the embryo-lethal phenotype.

In Arabidopsis, it has been shown that T6P levels control the carbohydrate utilization. Plants expressing *TPP* have low level of T6P content. They accumulate high level of sugar phosphates but have low ATP levels. The authors suggested that T6P controls the glycolysis in a manner similar to that in *S. cerevisiae* since yeast *tps1* mutant grown on glucose demonstrate the same metabolic profile (Shluepmann *et al.*, 2003). However, in contrast to yeast hexokinase, plant hexokinase is not inhibited by T6P (Eastmond *et al.*, 2002)

Recent studies have shown that T6P regulates the starch accumulation. Arabidopsis seedlings grown on 25 mM trehalose in the presence of Validamycin A, a potent inhibitor of trehalase, accumulate starch in their shoots. In these seedlings, expression of *ApL3* encoding large subunit of ADP-Glc pyrophosphorylase (AGPase, the key enzyme in the starch biosynthesis) was induced (Wingler *et al.*, 2000). Trehalose feeding resulted in accumulation of T6P (Schluepmann *et al*., 2004). T6P has a role in the posttranslational activation of ADGase through thioredoxin-mediated redox mechanism (Kolbe *et al*., 2005). Exogenously applied trehalose affect not only starch biosynthesis but also starch breakdown. It has been

demostrated that accumulation of starch in Arabidopsis grown on trehalose is also the result of inhibition of the expression of genes involved in starch breakdown such as *SEX1* and *BMY8/BAM3*, which are regulated by the transcription factor ABI4 (Ramon *et al.*, 2007).

Genetic modification of the trehalose pathway in tobacco demonstrates the role of T6P in photosynthesis. Increase in the T6P level leads to increase in the photosynthetic capacity per unit leaf area. Plants expressing *E.coli OtsA* , with high T6P level, has higher photosynthetic activity compared to that expressing *E.coli OtsB* and wild type coding TPP gene (Pellny *et al.*, 2004).

1.11 Microarray Technology

Microarray technology is a hybridization-based method. It uses thousands of highly arrayed probes on a solid surface to simultaneously examine targets, multiple RNA or DNA molecules, within individual samples. The samples to be tested are fluorescently labeled. When labeled samples are applied to the DNA microarray, they hybridize to their complementary DNA probes on the array. Fluorescent signal strength captured by the DNA probes is scanned and digitally quantified. In general, the signal strength represents (i) target abundance (transcript level, if the samples were RNA) or (ii) sequence similarity between probes and targets (Clarke and Zhu, 2006).

DNA microarrays are grouped into cDNA microarrays and oligonucleotide-based microarrays. In cDNA microarrays, DNA probes are PCR amplified DNA fragments. GeneChip produced by Affymetrix is an oligonucleotide-based array. It uses 25 bp long oligoncleotide probes, which are complementary to the 3' end of expressed sequences (Donson *et al.*, 2002).

1.11.1 Global Gene Expression Analysis of Drought- and Salt- Inducible Genes Using Microarrays

Microarray technology has allowed the examination of gene expression profile of plants subjected to abiotic stresses such as drought, salt, cold and ABA. It provides identification of novel stress responsive genes (Seki *et al*., 2003). Transcriptome analysis of abiotic stress response using microarray has been studied in many species such as Arabidopsis, rice, sorghum and barley (Buchanan *et al.*, 2005; Rabbani *et al*., 2003; Seki *et al*., 2002; Walia *et al*., 2006). Also, there are some reports related to wheat microarray (Table 1.2). Kawaura *et al* (2008), Kawaura *et al* (2006) identified wheat genes responsive to salt stress using oligonucleotide microarray. In addition, Mott and Wang (2007) monitored transcript profile of salt-stressed wheat using Wheat GeneChip (Affymetrix). Mohammadi *et al* (2007) and Mohammadi *et al* (2008) employed oligonucleotide microarray to identify drought-inducible genes in wheat.

Table 1.2 Transcriptome Analysis of drought- and salt- inducible wheat genes using microarray

Species	Type of stress	Tissue	Microarray Type	References
Wheat	Salt	Root Shoot	Oligo-DNA microarray	Kawaura *et al*., 2008
Wheat	Dehydration	Root	(Oligonucleotide-based Microarray)	Mohammadi *et al*., 2008
Wheat	Dehydration	Root	(Oligonucleotide-based Microarray)	Mohammadi *et al*., 2007
Wheat	Salt	Root Shoot	Affymetrix GeneChip	Mott and Wang, 2007
Wheat	Salt	Root Soot	Oligonucleotide microarray	Kawaura *et al*., 2006

1.13. Aim of the study

Salinity and drought adversely affect the growth and production of crop in worldwide. Plants cope with these stresses by developing several strategies at molecular, cellular, physiological and biochemical levels. At molecular level, plants regulate expression of stress-associated genes. Microarray is a powerful technique to analyze transcriptome profile of plants subjected to abiotic stresses.

Osmoprotectant role of trehalose has been shown in some plants. In recent years, there has been a growing interest in trehalose metabolism as a means of engineering stress tolerance in plants. Overexpression of TPS in some plants improved stress tolerance. Until now, there has been no known literature data about wheat TPS gene.

There are two aims of this thesis,

- Cloning and examining transcriptional regulation of wheat TPS under salt and drought stress conditions
- Analyzing transcriptome profile of wheat seedling treated with salt and drought stress using Wheat GeneChip (Affymetrix).

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemical Materials

The chemicals and kits with their suppliers were given in Appendix A.

2.1.2 Plant Material

In this study, bread wheat (*Triticum aestivum* L. cv. Bayraktar) was used. The wheat seeds were provided by Turkish Ministry of Agriculture and Rural Affairs, Central Research Institute for Field Crops, Ankara.

2.2 Methods

2.2.1 Growth of Plants

The seeds were surface sterilized with sodium hypochloride (20% v/v) for 20 minutes, then rinsed with distilled water. Seeds were grown in a growth chamber at 25^{o}C with 16 hours light and 8 hours dark photo cycle. They were watered with ½ strenght Hoagland's Solution (Appendix B).

2.2.2 Application of Drought and Salt Stress

The seedlings grown for 10 days - the two-leaf-stage- were used for different treatments. For drought stress, the seedlings were treated with ½ Hoagland Solution containing 20% (w/v) PEG 6000 for 24 hours. In the salt treatment, the seedlings were watered with ½ Hoagland Solution containing 250mM NaCl. Both treated and nontreated plants are kept in the growth chamber at the same growth condition. Samples of root and leaf tissues were collected, immediately frozen in liquid nitrogen and stored at -80^{o}C before RNA extraction.

2.2.2 Cloning of Wheat Trehalose-6-Phosphate Synthase

2.2.2.1 RNA Isolation

2.2.2.1.2 Precautions

All equipments; reagents, glass and non-glass equipments should be free of any RNase contamination. Therefore, all equipments and solutions used for isolation of RNA were treated with dH_2O containing 0.1 % (v/v) DEPC (diethyl pyrocarbonate) and then autoclaved at 121^{o}C for 20 min.

2.2.2.1.3 Total RNA Isolation

Plant leaf and root tissue samples in amounts of 50-100 mg were powdered in a mortar in the presence of liquid nitrogen. Then powdered tissue samples were transferred to precooled 1.5 mL eppendorf tubes and treated with 1 mL Trizol Reagent (Appendix C). To permit the complete dissociation of nucleoprotein complexes, the tubes were vortexed for 15 min at room temperature. Samples were then centrifuged at 21,000 g (MPW 65) for 5 min at room temperature. Following centrifugation, 900 µL of the supernatants were transferred to a new tube. Then 200 µL of chloroform was added. The mixtures were shaked vigorously for 15 sec and

incubated at room temperature for 3 min. After incubation, samples were centrifuged for 15 min at 4°C at 21,000 g. Following centrifugation, 400 µL of upper phases were transferred to new tubes, mixed with 200 µL chloroform. Then the mixtures were shaked vigorously for 15 sec and incubated at room temperature for 3 min. Samples were centrifuged for 5 min at room temperature at 21,000 g. After centrifugation, 320 µL from the upper phases were transferred into a new 1.5 mL eppendorf tube, mixed with 1 volume isopropanol and then incubated for 10 min at room temperature. Samples were centrifuged for 10 min at room temperature at 21,000 g. The supernatant were removed and the RNA pellet was washed once with 1 mL 75% ethanol. Samples were mixed and centrifuged for 5 min at room temperature at 21,000 g. After centrifugation, supernatant were removed. The RNA pellet was dried briefly at room temperature for 10 min. Finally, the RNA pellet was dissolved in 40 µL of DEPC-treated H_2O.

2.2.2.1.4 Determination of RNA Quality and Quantity

The RNA concentration was determined by measuring absorbance at 260 nm on a double beam spectrophotometer (Carry 100 UV-Vis) in 10 mM Tris/HCl pH 8.0 (one absorbance unit = 40µg/mL RNA). The A260/A280 ratio around 2.0 was considered as high quality total RNA sample.

Concentration determination of RNA samples was carried out according to the equation given bellow

Conc. of RNA (µg/ mL) = A260 x dilution factor x 40 µg/mL

The integrity of the RNA samples were checked by agarose gel electrophoresis using Rnase-free 1% agarose gels in TAE or TBE buffer. The gel was run for 1 h at 80 V.

2.2.2.1.5 DNase Treatment of Total RNAs

Total RNA was treated with DNase in order to remove DNA contamination. For DNase treatment, 10 µL of reaction mixture contained 1µg RNA, 1 U/µL of DNase, 1 µL 10X Reaction Buffer with $MgCl_2$, 1 U/µL Ribonuclease Inhibitor. The mixture was incubated at 37°C for 40 min. DNase was stopped by adding phenol-chloroform-isoamylalcohol (25:24:1). This mixture was incubated 10 min in ice. Then it was centrifuged at 21,000 g at 4°C for 20 min. Following centrifugation, the upper phase containing total RNA was transferred into a new eppendorf tube. The solution was mixed with 1/10 volumes 3 M NaOAc (pH 5.2) and 3 volumes of absolute ethanol. The mixture was incubated at -80°C for 20 min or –at 20°C overnight. After incubation, samples were centrifuged at 21,000 g at 4°C for 30 min. The supernatant was removed and the RNA pellet was washed once with 1 mL 75% ethanol. The sample was centrifuged at 21,000 g at 4°C for 25 min. After centrifugation, supernatant was removed. The RNA pellet was dried briefly at room temperature for 10 min. Then, the RNA was dissolved in 40 µL of DEPC-treated H_2O.

2.2.2.1.6 Synthesis of First Strand cDNA

After DNase treatment, first strand cDNA synthesis was carried out by using RevertAid First Strand cDNA synthesis Kit. An aliquot of 5 µg of total RNA was mixed with oligo (dT)18 primer (0,5 µg/µl). The reaction mixture was incubated at 70°C for 5 min. Then it was mixed with 4 µL 5X reaction buffer, 1 µL RiboLock Ribonuclease Inhibitor (20 U/µL), and 2 µL dNTP mix (10 mM). The mixture was incubated at 37°C for 5 min. Following incubation, 1 µL of M-MuLV Reverse Transcriptase (200 U/µL) was added. The mixture was incubated at 42°C for 60 min. The reaction was stop by heating at 70°C for 10 min.

2.2.2.1.7 Internal Conservative Fragment Cloning

Gene specific primers (Table 2.1) were designed based on conserved amino acid domains of TPS sequences using the following accession numbers: *Arabidopsis thaliana*, Y08568; *Selaginella lepidophylla*, U96736; *E.coli*; X69160; *Saccharomyces cerevisiae* X68214 (Appendix D). COnsensus-DEgenerate Hybrid Oligonucleotide Primers (CODEHOP) software was used for this purpose (http://bioinformatics.weizmann.ac.il/blocks/codehop.html) (Rose *et al.*, 2003). The fragment was obtained with two steps Reverse Transcription-Polymerase Chain Reaction (RT-PCR). After cDNA synthesis, the following PCR program was carried out: 94^{o}C for 3 min followed by 35 cycles of amplification (94^{o}C for 30s, 58^{o}C for 30s, 72^{o}C for 30s). The amplified PCR product was electrophoretically cheked and extracted from % 0.75 (w/v) agarose gel using GeneMark Gel Elution Kit Extraction Kit. The extracted product was sent to RefGen Gen Araştırmaları ve Biyoteknoloji Ltd. Şti. for sequencing. Sequence was analysed in tBLASTx in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul *et al.*, 1997).

Table 2.1 Degenerate Primers (k: G or T; n: A or G or T or .C; r: A or G; y:C or T).

TPS	Left primer	5'-GCGACCTGGTGGGCttycanacnka -3
	Right primer	3'-tyaarygnccncGGCGGGTCAGCGA -5'

2.2.2.1.8 Rapid Amplification of cDNA Ends (RACE)

For 5 RACE and 3 RACE reactions, primers were designed based on the sequence of the internal conservative fragment. The 5 RACE-Ready cDNA and 3 RACE-Ready cDNA were synthesized by using the SMART RACE cDNA Amplification Kit. For

each cDNA preparation, 1 µg of total RNA was reverse transcribed according to manufacturer's directions.

5-RACE ready cDNA and 3-RACE ready cDNA were prepared in separate microcentrifuge tubes. The contents of tubes were given below:

5-RACE ready cDNA	**3-RACE ready cDNA**
1 µg of total RNA	1 µg of total RNA
1 µL 5-CDS primer A	1 µL 3-CDS primer A
1 µL SMART II A oligo	

Both mixtures were completed to a final volume of 5 µL by adding sterile H_2O. The contents of mixtures were mixed and spinned briefly. Then they were incubated at 70°C for 2 min. The tubes were cooled on ice for 2 min. The contents of the tubes were collected at the bottom by centrifugation. Then 2 µL 5X First-Strand Buffer, 1 µL DTT, 1 µL dNTP, 1 µL BD PowerScript Reverse Transcriptase were added to each tubes. The contents of the tubes were mixed by gently pipetting, and then collected at the bottom by centrifugation. The tubes were incubated at 42°C for 1.5 h. The first strand reaction product was diluted with 100 µL Tricine-EDTA buffer. The tubes were heated at 72°C for 7 min. Samples were stored at -20°C.

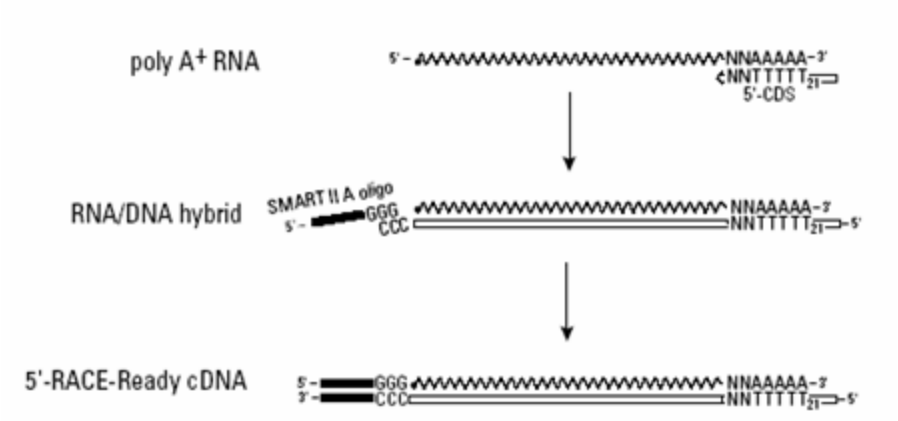


Figure 2.1 5'-RACE cDNA synthesis (taken from SMART RACE cDNA Amplification Kit User Manual)

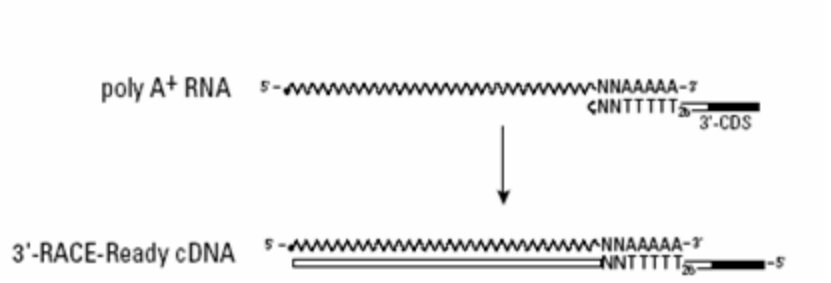


Figure 2.2 3'-RACE cDNA synthesis (taken from SMART RACE cDNA Amplification Kit User Manual)

For 5'-RACE PCR and 3'-RACE PCR, gene specific primers (GSP) were designed from internal fragment of wheat TPS gene using Primer 3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The primers used in these PCR's were given in Table 2.2.

Table 2.2 RACE Primers

5-RACE	**5'-AATCAGCGCAGAGCACGGAGCGAAG-3'** **5'-GATGTCATGCTCCTTGAGGCACCTGG-3'**
3-RACE	5'-CCCAGGTGCCTCAAGGAGCATGACATC-3'

The PCR mixture prepared according to manufacturer's directions was given below.

5-RACE ready cDNA / 3-RACE ready cDNA	**2.5 µL**
UPM (10X)	5 µL
GSP (10 µM)	1 µL
10X Advantage 2 PCR Buffer	5 µL
dNTP (10 mM)	1 µL
50X Advantage 2 Polymerase Mix	1 µL
PCR-Grade H2O	34.5 µL
Final Volume	50 µL

5'-RACE PCR and 3'-RACE PCR was performed as the following procedure: 94^{o}C for 3 min followed by 5 cycle 94^{o}C for 30 sec, 72^{o}C for 3 min; 5 cycle 94^{o}C for 30 sec, 70^{o}C for 30 sec, 72^{o}C for 3 min; 35 cycle 94^{o}C for 30 sec, 68^{o}C for 30 sec 72^{o}C for 3 min.

The amplified products was purified by GeneMark Gel Elution Kit and cloned into pGEMT-Easy vector (Promega) for sequencing. Vector containing amplified product

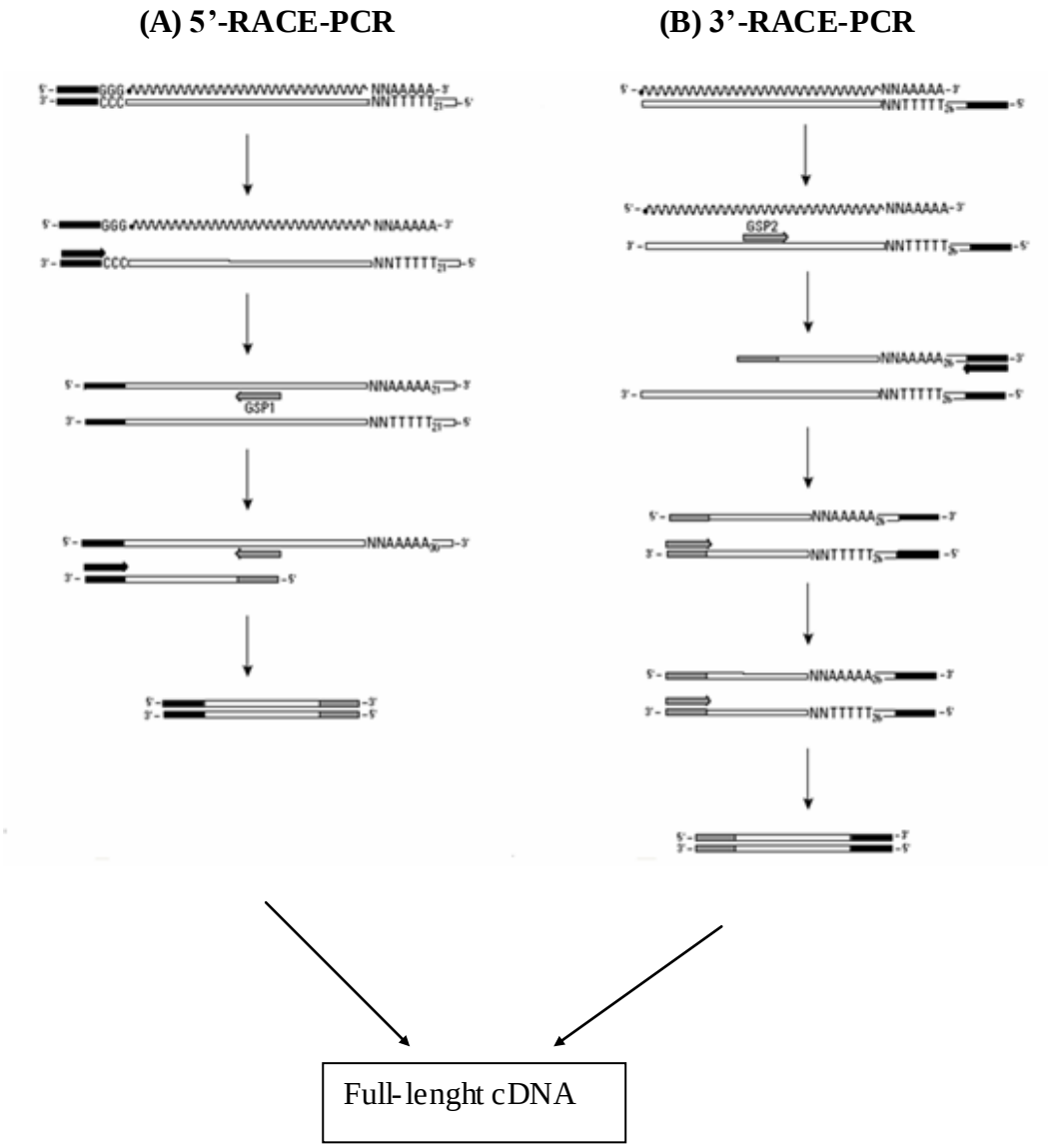


Figure 2.3 Mechanism of (A) 5'-RACE-PCR and (B) 3'-RACE-PCR (Taken from SMART RACE cDNA Amplification Kit User Manual)

2.2.3 Microarray Analysis

Complementary RNA (cRNA) synthesis, biotin labeling, hybridization and scanning were performed according to the protocols described in the Affymetrix GeneChip Expression Analysis Technical Manual.

2.2.3.1 First Strand cDNA Synthesis

One cycle cDNA synthesis kit was used for synthesis of first strand cDNA. This procedure was performed in three steps and thermal cycler (Applied Biosystem GeneAmp PCR 9700) was used for all incubation reactions. In the first step, 15 μg total RNA was mixed with 2 μL of the diluted poly-A RNA control, 2 μL of 50 μM T7-Oligo(dT) primer and RNase-free water to complete the total volume to 11 μL. The tube was gently flicked a few times to mix and then centrifuged briefly. The reaction mix was incubated at 70°C for 10 min. Following the incubation, the sample was cooled at 4°C for at least 2 min. and centrifuged briefly. In the second step of the synthesis reaction, the first strand master mix was prepared in a separate tube. This master mix was composed of 4 μL of first strand reaction mix (5X), 2 μL of DTT (0.1M), and 1 μL of dNTP (10mM). 7 μL of master mix was transfered to each RNA/oligo (dT) primer mix and incubated at 42°C for 2 minutes. In the last step, 1 μL of SuperScript II enzyme was added to the reaction and incubated at 42°C for 1 h. Finally the sample was cooled to 4°C.

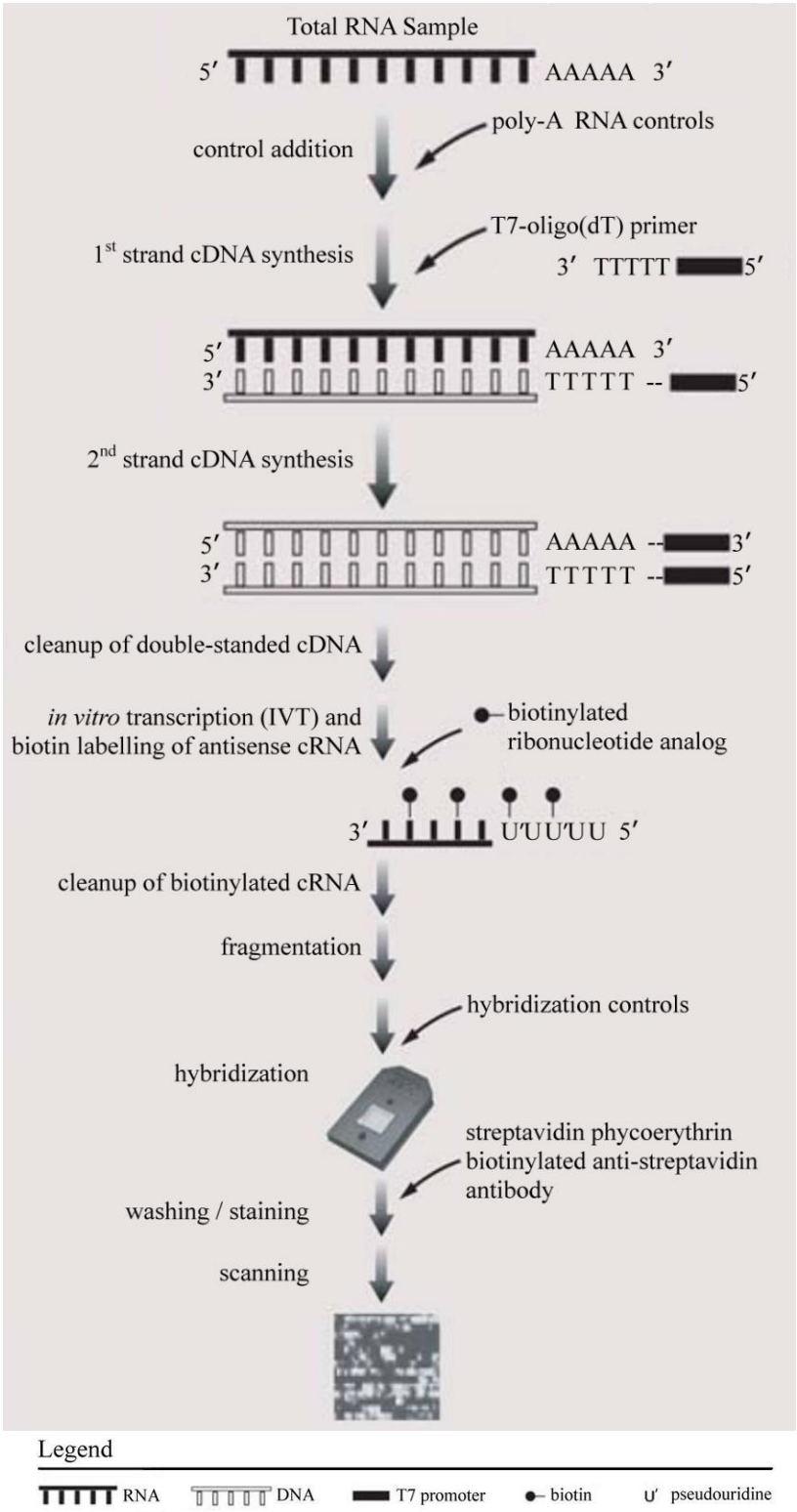


Figure 2.3 The methods used for gene expression profiling. (Adapted from Affymetrix GeneChip Expression Analysis Technical Manual)

2.2.3.2 Second Strand cDNA synthsesis

Second strand master mix wasprepared for all of the samples. It consists of 91 µL RNase free H_2O, 30 µL 5X 2nd Strand Reaction Mix, 3 µL dNTP (10 mM), 1 µL *E. coli* DNA ligase, 4 µL *E. coli* DNA Polymerase I and 1 µL RNase H. 130 µL of Second-Strand Master Mix was addd to each first-strand synthesis sample from*: First-Strand cDNA Synthesis* for a total volume of 150 µL. Samples were mixed gently and then centrifuged briefly (~5 seconds) to collect the reaction at the bottom of the tube. Then they were incubated for 2 hours at 16°C. End of the 2 hour, 2 µL of T4 DNA Polymerase was added to each sample and each sample was incubated for 5 minutes at 16°C. After incubation with T4 DNA Polymerase 10 µL of EDTA (0.5M) was added. Double strand cDNA was cleane up using GeneChip Sample Cleanup Module.

2.2.3.3 Synthesis of Biotin-Labeled cRNA for the One-Cycle Target Labeling Assays

GeneChip IVT Labeling Kit is used for this step.Nuclease-free water, buffers and pipette tips were used in all steps. According to the instructions in the kit manual, 12 µL of cDNA was used. This amount was decided according to the amount of total RNA as starting material. 4 µL of IVT Labeling Buffer (10X), 12 µL of IVT Labeling NTP mix, 4 µL of IVT Labeling Enzyme mix was transfered on cDNA and the total volume was completed to 40 µL by adding RNase-free water. Since spermidine in the 10X IVT Labeling Buffer can lead to precipitation of the template cDNA, this step was not performed on ice. This mixture was incubated at 37°C for 16 hours. After incubation, cRNA was cleanup using GeneChip Sample Cleanup Module. Quantification of labeled cRNA was performed spectrophotometrically at 260 and 280 nm. Twenty µg of cRNA product was fragmented by metal-induced hydrolysis at 94°C for 35 minutes. The efficiency of the fragmentation procedure was checked by analyzing the size of the fragments on an agarose gel.

2.2.3.4 Eukaryotic Target Hybridization

Each fragmented cRNA sample was used to prepare 200 µL of hybridization cocktail containing 100 mM MES, 1 M NaCl, 20 mM ethylenediamine tetraacetic acid, 0.01% Tween-20, 0.1 mg mL^{-1} herring sperm DNA (Promega), 0.5 mg mL^{-1} bovine serum albumin (Invitrogen), 0.1% DMSO, hybridization controls and 10 µg of fragmented sample. Samples were then hybridized for 16 h to Wheat GeneChip (Affymetrix) which contains 61,127 probe sets. in Hybridization Oven 640 (Affymetrix) at 45°C and 60 rpm.

2.2.3.5 Washing, Staining and Scanning

After hybridization, arrays were washed in Fluidics Station 450 (Affymetrix) and stained with streptavidin-phycoerythrin (Invitrogen) and biotinylated anti-streptavidin antibody (Sigma), according to the appropriate standard protocol for each array type. Arrays were then scanned with GeneChip Scanner 3000 (Affymetrix). Hybridization, scanning and preliminary analyses with GeneChip Operating Software 1.4 were performed at METU Central Laboratory.

2.2.3.6 Microarray Data Processing and Analysis

Data from all hybridizations were further analyzed using GeneSpringGX 10.0 (Agilent) and the probe annotations for the Wheat GeneChip were updated prior to analysis. Expression values, computed from .CEL files, were processed first by Robust Multiarray Analysis (RMA) which is a model of normalization over multiple arrays. RMA uses only perfect match (PM) probes and includes probe-specific background correction, normalization across all arrays, and median polishing (Irizarry et al., 2003). Filtering on expression levels and fold changes (≥ 2) were performed for determination of differentially expressed genes. Statistical analyses were done using one-way ANOVA at $P < 0.05$ – with asymptotic P-value computation – followed by Tukey HSD post hoc test. Fold change of at least 2 was

considered as an indication of differential expression, where P-value of at most 0.05 was considered as an indication of significant alteration in expression. The significantly different probe sets were annotated using HarvEST:Wheat (version 1.54) (http://harvest.ucr.edu).

CHAPTER 3

RESULTS

3.1 Cloning of Wheat Trehalose-6-Phosphate Synthase

Rapid amplification of cDNA ends (RACE) is a polymerase chain reaction (PCR)-based technique which is used to clone full-length cDNA 5′- and 3′-ends after a partial cDNA sequence has been determined by other methods (Schaefer, 1995).

In this study, wheat TPS was cloned by using RACE. As sequence of wheat *TPS* was not available in GeneDatabase, internal conservative fragment was cloned by using degenerate primers. Degenerate primers were designed from conserved amino acid regions of *Arabidopsis thaliana*, *Selaginella lepidophylla*, *Saccharomyces cerevisia*, and *E.coli*. The gel photograph of internal conservative fragment amplified by degenerate primers was given in Figure 3.1

The sequence of internal conservative fragment was given in Appendix E. The tblastx analysis of this fragment revealed that this product shared high identity with known plant *TPS* genes such as *Arabidopsis thaliana* (86%) and *Selaginella lepidophylla* (84%). The results were given in Appendix F.

Based on this fragment, 3'- and 5'-RACE primers were designed. At the end of 3'RACE and 5'RACE reactions, 2928- bp cDNA fragment was amplified. This fragment has a poly (A) tail in its 3'end.

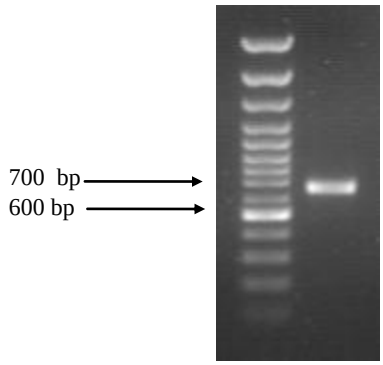


Figure 3.1 PCR product amplified by degenerate primers. Line1: Marker (Fermentas SM371), Line2: PCR product

For determination of open reading frame (ORF), ORF Finder was used (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The cDNA contains open reading frame of 2580 bp long ORF which encodes a putative protein of 859 amino acids with a predicted molecular weight (MW) of 96.7 kDa and isoelectric point (pI) of 5.97 (http://www.expasy.ch/tools/pi_tool.html) (Bjellqvist *et al.*, 1993). This cDNA sequence was deposited in GeneBank with accession number FJ167677.1 (Appendix G). ORF of wheat *TPS*, *TaTPS*, was amplified using RT-PCR. The fotograph is given in Figure 3.2.

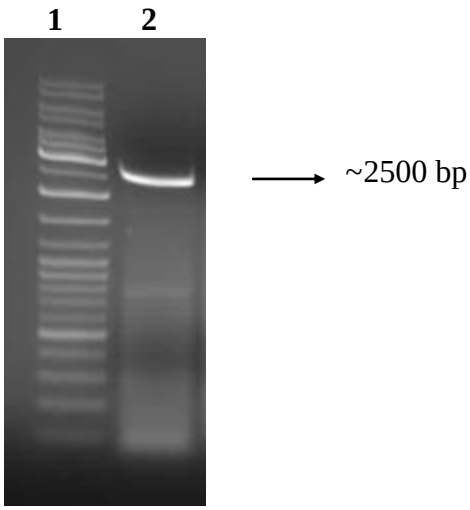


Figure 3.2 ORF of wheat TPS. Line 1 marker; Line 2 Amplified PCR Product

The amino acid sequence of wheat TPS was analyzed by Pfam 23.0 in order to find conserved domain (http://pfam.sanger.ac.uk/search) (Finn *et al.*, 2008). This analysis revealed the presence of two conserved regions: TPS domain (3-470) and TPP domain (504-754). *Arabidopsis thaliana* and *Selaginella lepidophylla TPS* genes also contain these two conserved regions.

Table 3.1 Amino acid Sequence of putative wheat TPS

MKQRLLVVANRLPVSANRRGEDQWSLEISAGGLVSALLGVKDVDAKWIGWAGVNVPDEVGQQALTNAL
AEKRCIPVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRNFESQFDAYKRANQMFADVVYQ
HYQEGDVIWCHDYHLMFLPRCLKEHDINMKVGWFLHTPFPSSEIYRTLPSRSELLRSVLCADLVGFHT
YDYARHFVSACTRILGLEGTPEGVEDQGKLTRVAAFPIGIDSDRFKRALDIDAAKRHVNELKQRFAGR
KVMLGVDRLDMIKGIPQKILAFEKFLEENPEWIDKVVLLQIAVPTRTDVPEYQKLTSQVHEIVGRING
RFGTLSAVPIHHLDRSLDFHALCALYAVTDVALVTSLRDGMNLVSYEYVACQGSKKGVLILSEFAGAA
QSLGAGAILVNPWNITEVADSIKHALTMTSDEREKRHRHNYAHVTTHTAQDWAETFVCELNDTVAEAL
MRTRQVPPDLPSRTAIQQYLQSKNRLLILGFNSTLTEPVESSGRRGGDQVKEMELKLHPDLKGPLRAL
CEDESTTVIVLSGSDRSVLDENFGEFNLWLAAEHGMFLRPTDGEWMTTMPEHLNMDWVDSAKHVFEYF
TERTPRSHFEHRETSFVWNYKYADVEFGRLQARDMLQHLWTGPISNAAVDVVQGSRSVEVRSVGVTKG
AAIDRILGEIVHSKSMVTPIDYVLCIGHFLGKDEDIYVFFDPEYPSEPKVKPDGASVSVDRRQNGRPS
NGRSNSRNSQARTQKPQVAPPPPERSSSSSDHSTANNNSHHDWREGSSVLDLNGDNYFSCAVGRKRSN
ARYLLNSSEDVVSFLKEMAESTTPRAGGLPPGAAADYMFLDRQ

The amino acid sequence of TaTPS was analysed by BLASTP (Altschul *et al*., 1997). Based on BLASTP, it shares identities 77% with *Arabidopsis thaliana* TPS (NP_177979), 78% with *Solanum lycopersicum* TPS (ABO61742), 88% with *Zea mays* TPS (NP_001123593), 69% with *Selaginella lepidophylla* TPS (AAD00829), 87% with *Saccharum* hybrid cultivar TPS (ACE79166), 80% with *Musa balbisiana* TPS (ABF70084) and 51% with *Saccharomyces cerevisiae* TPS (ABL11219). Multiple sequence alignment was carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Table 3.2 Multiple sequence alignment

```
Arabidopsis     --------------MPGNKYNCSSSHIPLSRTERLLRDRELREKRKSNRARN-PNDVAGS 45
Solanum         --------------MPGNKYTG-NQAVASTRLERLLRERELRKSSKVSHFPNESTDNNRG 45
Musa            MRPASGQPPDEQRGMQGSKYGG---SRNPTRVERLLRDRELRKINRAFQQDEAGGNGGRE 57
Selaginella     ----MPQPYPSSSSTSNAKEAGGGAAAAGGGGGGAFSLPPSLASSRVERLVRERQLRNQR 56
Triticum        ------------------------------------------------------------
Zea             --------------------------MGEGAGDRVLSR---LHSVRERIGDSLSAHPNEL 31


Arabidopsis     SENSENDLRLE--GDSSRQYVEQYLEGAAAAMAHDDACERQEVRPYNRQRLLVVANRLPV 103
Solanum         NELSDHDFRQGEADNGGVSYVEQYLEG--AALAYNEGWERPDGKP-TRQRLLVVANRLPV 102
Musa            AESSEH-LPEDCGREVGGEPDEEIVDGSLAPVITTGQGSRRSGGRPMKQRLLVVANRLPV 116
Selaginella     QEDEPEDEQQALEAEEAAVAATEVPDAVAAATPSLSD-EPSKISSGRGQRLLVVANRLPL 115
Triticum        ----------------------------------------------MKQRLLVVANRLPV 14
Zea             VAVFTRLKNLGKGMLQPHQIIAEYNNAIPEAEREKLKDGAFEDVLRAAQEAIVIPPWVAL 91
                                                              *. :*:.  :.:

Arabidopsis     SAVRRGEDSWSLEISAGGLVSALLGVKEFEARWIGWAGVNVPDEVGQKALSKALAEKRCI 163
Solanum         SAVRRGEESWSLEISGGGLVSALLGVKEFEARWIGWAGVNVPDEAGQRALTKALAEKRCI 162
Musa            SAVRRGEDSWSLEISAGGLVSALLGVKDVDAKWIGWAGVSVPDEVGQRALTKALAEKRCI 176
Selaginella     SATRKGETEWNLEMSAGGLVSALLGVKQFEVTWIGWPGVYVQDEKGEKSLRGALEEKGFV 175
Triticum        SANRRGEDQWSLEISAGGLVSALLGVKDVDAKWIGWAGVNVPDEVGQQALTNALAEKRCI 74
Zea             -AIRPRPGVWEYVRVN----VSELAVEELRVPEYLQFKEQLVEEGPNNNFVLELDFEPFN 146
                 * *     *.          : *.*::. .         : :*  :. :   *  :

Arabidopsis     PVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRSFQSQFAAYKKANQMFADVV 223
Solanum         PVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRSFQSQFAAYKKANQMFADVV 222
Musa            PVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRSFQSQFDAYKRANQMFADVV 236
Selaginella     PVLLDEATVDQYYNGYCNNVLWPLFHYIGLRQEDRLAATRSLLSQFNAYKRANRLFAEAV 235
Triticum        PVFLDEEIVHQYYNGYCNNILWPLFHYLGLPQEDRLATTRNFESQFDAYKRANQMFADVV 134
Zea             ASFPRPSLSKSIGN--------------GVQFLNRHLSSKLFHDKESMYPLLNFLRAHN- 191
                . :      ..  *              *:   :*  ::: : .:   *   * : *.

Arabidopsis     NEHYEEGDVVWCHDYHLMFLPKCLKEYNSKMKVGWFLHTPFPSSEIHRTLPSRSELLRSV 283
Solanum         NEHYEEGDVVWCHDYHLMFLPKCLKDYNSQMKVGWFLHTPFPSSEIHRTLPSRSELLRAV 282
Musa            NQHYQEGDVVWCHDYHLMFLPKCLKEYNSKMKVGWFLHTPFPSSEIHRTLPSRSELLRSV 296
Selaginella     FNFYQEGDVVWCHDYHLMFLPSYLKEKDSQMKVGWFLHTPFPSSEIYRTLPLRAELLQGV 295
Triticum        YQHYQEGDVIWCHDYHLMFLPRCLKEHDINMKVGWFLHTPFPSSEIYRTLPSRSELLRSV 194
Zea             ----------WVQDYHLMFLPKCLKDHDINMKVGWFLHTPFPSSEIYRTLPSRLELLRSV 241
                          * :********  **: : :*****************:**** * ***:.*

Arabidopsis     LAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGRLTRVAAFPIGIDSDRFIRALE 343
Solanum         LAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGRLTRVAAFPIGIDSERFIRALE 342
Musa            LAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGRLTRVAAFPIGIDSDRFKRALE 356
Selaginella     LAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGKNTRVAAFPVGIDSERFIEAVE 355
Triticum        LCADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGKLTRVAAFPIGIDSDRFKRALD 254
Zea             LCADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGRLTRVAAFPIGIDSDRFKRALE 301
                *.************************************: *******:****:** .*::

Arabidopsis     VPEVIQHMKELKERFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENANWRDKVVLLQIA 403
Solanum         VTQVQEHIKELKERFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPYWRDKVVLLQIA 402
Musa            LPAVKEHINELTHRFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPSWRDKVVLLQIA 416
Selaginella     TDAVKKHMQELSQRFAGRKVMLGVDRLDMIKGIPQKLLAFEKFLEENSEWRDKVVLVQIA 415
Triticum        IDAAKRHVNELKQRFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPEWIDKVVLLQIA 314
Zea             LPAVKRHVSELTERFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPDWNNKVVLLQIA 361
                   . .*:.**..**********************:**********. * :****:***

Arabidopsis     VPTRTDVPEYQKLTSQVHEIVGRINGRFGTLTAVPIHH--------LDRSLDFHALCALY 455
Solanum         VPTRTDVPEYQKLTSQVHEIVGRINGRFGTLTAVPIHH--------LDRSLDFHALCALY 454
Musa            VPTRTDVPEYQKLTSQVHEIVGRINGRFGTLTAVPIHHLVCLTTILMDRSLDFHALCALY 476
Selaginella     VPTRTDVLEYQKLTSQVHEIVGRINGRFGSLTAVPIHH--------LDRSMKFPELCALY 467
Triticum        VPTRTDVPEYQKLTSQVHEIVGRINGRFGTLSAVPIHH--------LDRSLDFHALCALY 366
Zea             VPTRTDVPEYQKLTSQVHEIVGRINGRFGTLTAVPIHH--------LDRSLDFHALCALY 413
                ******* ********************:*:******        :***:.*  *****

Arabidopsis     AVT--------DVALVTSLRDGMNLVSYEFVACQEAKKGVLILSEFAGAAQSLGAGAILV 507
Solanum         AVT--------DVALVTSLRDGMNLVSYEFVACQELKKGVLILSEFAGAAQSLGAGAILV 506
Musa            AVTVYIFHFFSDVALVTSLRDGMNLVSYEFVACQGSKKGVLILSEVHLGKQLEKYLTIFI 536
Selaginella     AIT--------DVLLVTSLRDGMNLVSYEFVACQKDKKGALILSEFAGAAQSLGAGSILI 519
Triticum        AVT--------DVALVTSLRDGMNLVSYEYVACQGSKKGVLILSEFAGAAQSLGAGAILV 418
Zea             AVT--------DVALVTSLRXGMNLVSYEYVACQGSKKGVLILSEFAGAAQSLGAGAILV 465
                *:*        ** ****** ********:****  ***.*****.  . *     :*::
```

Table 3.2 (Continued)

```
Arabidopsis      NPWNITEVAAS-IGQALNMTAEEREKRHRHNFHHVKTHTAQEWAETFVSELNDTVIEAQL 566
Solanum          NPWNITEVAAS-IGQALNMSAEEREKRHRHNFLHVTTHTAQEWAETFVSELNDTVIEAQQ 565
Musa             G--AILQMGGDGKSYALNMSPDEREKRHRHNYAHVTTHTAQDWAETFVSELNDTVVEAQL 594
Selaginella      NPWNIIESSNA-IADALNMPEEEREERHRHNFMHITTHSAQVWAETFISELNDSILEAEL 578
Triticum         NPWNITEVADS-IKHALTMTSDEREKRHRHNYAHVTTHTAQDWAETFVCELNDTVAEALM 477
Zea              NPWNITEVADS-IRHALTMPSDEREKRHRHNYAHVTTHTAQDWAETFVFELNDTVAEALL 524
                 .   * : .      **.*. :***:*****: *:.**:** *****: ****:: **

Arabidopsis      RISKVPPELPQHDAIQRYSKSNNRLLILGFNATLTEPVDNQGRRG-DQIKEMDLNLHPEL 625
Solanum          RIRKVPPRLNISDAIERYSFSNNRLLILGFNSTLTESVDTPGRRGGDQIKEMELKLHPEL 625
Musa             RTRQVPPLLPTNIAIERYLQSMNRLLILGFNATLTESVESSGRRGGDQIKEMELKLHPEL 654
Selaginella      RTLHIPPQLPLDKAVAKYSESKNRLVILGFNSTLTAQVEAPRGRAPDQIREMKIRLHPSI 638
Triticum         RTRQVPPDLPSRTAIQQYLQSKNRLLILGFNSTLTEPVESSGRRGGDQVKEMELKLHPDL 537
Zea              RTRQVPPGLPSQMAIQQYLRSKNRLLILGFNSTLTEPVESSGRRGGDQIKEMELKLHPDL 584
                 *  ::** *    *: :*  * ***:*****:***  *:    *. **::**.:.***.:

Arabidopsis      KGPLKALCSDPSTTIVVLSGSSRSVLDKNFGEYDMWLAAENGMFLRLTNGEWMTTMPEHL 685
Solanum          KESLLAICNDPKTTVVVLSGSDRNVLDDNFSEYNMWLAAENGMFLRSTNGVWMTTMPEHL 685
Musa             KVPLTTLCNDAQTTVVVLSGSDRSVLDDNFGEYNMWLAAENGMFLRHTGGDWMTTMPEHL 714
Selaginella      KDILNVLCSDPKTTIVILSGSERVALDEVFGEFDLWLAAENGMFLRHTQGEWMTTMPEHL 698
Triticum         KGPLRALCEDESTTVIVLSGSDRSVLDENFGEFNLWLAAEHGMFLRPTDGEWMTTMPEHL 597
Zea              KGPLRALCEDERTTVIVLSGSDRSVLDENFGEFKMWLAAEHGMFLRPTYGEWMTTMPEHL 644
                 *  * .:*.*  **:::****.* .**. *.*:.:*****:***** * * *********

Arabidopsis      NMEWVDSVKHVFKYFTERTPRSHFETRDTSLIWNYKYADIEFGRLQARDLLQHLWTGPIS 745
Solanum          NMDWVDSVKHVFEYFTERTPRSHFEQRETSLVWNYKYADVEFGRLQARDMLQHLWTGPIS 745
Musa             NMDWVDSVKHVFEYFTERTPRSHFEHRETSLVWNYKYADVEFGRLQARDMLQHLWTGPIS 774
Selaginella      NMDWLESVQLVFDYFCERTPRSFVETRETSLVWNYKYADVEFGRVQARDMLQHLWTGPIS 758
Triticum         NMDWVDSAKHVFEYFTERTPRSHFEHRETSFVWNYKYADVEFGRLQARDMLQHLWTGPIS 657
Zea              NMDCVDSVKHVFEYFTERTPRSHFEHRETSFVWNYKYADVEFGRLQARDMLQHLWTGPIS 704
                 **: ::*.: **.** ******..* *:**::*******:****:****:**********

Arabidopsis      NASVDVVQGSRSVEVRAVGVTKGAAIDRILGEIVHSKSMTTPIDYVLCIGHFLGKDEDVY 805
Solanum          NASVDVVQGLRSVEVRAVGVTKGAAIDRILGEIVHSKAIATPIDYVLCIGHFLGKDEDVY 805
Musa             NTAVDVVQGSRSVEVRSVGVTKGAAIDRILGEIVHSKSMVTPIDYVLCVGHFLGKDEDIY 834
Selaginella      NAAVDVVQGGKSVEVRPVGVSKGSAIDRILGEIVHSKHMTIPIDYVLCIGHFLSKDEDIY 818
Triticum         NAAVDVVQGSRSVEVRSVGVTKGAAIDRILGEIVHSKSMVTPIDYVLCIGHFLGKDEDIY 717
Zea              NAAVDVVQGSRSVEVRSVGVTKGAAIDRILGEIVHSENMITPIDYVLCIGHFLGKDEDIY 764
                 *::****** :*****.***:**:************: :  *******:****.****:*

Arabidopsis      TFFEPELPSDMPAIARSRPSSDSGAKSSSGDRRPPSKSTHNNNKSGSKSSSSSNSNNNNK 865
Solanum          TFFEPELPSDCIGMPRSKVS---DAPKVPGERRSVPKLPS------SRTSSKSSQNRNRP 856
Musa             TFFEPELPAEPASSTRMKIS---ETSKASPDKRSTGRSSN------IRNNSRMSHVRPQR 885
Selaginella      TFFEPELPLLDRDSSTSNGG------KPLGGKLPIDRKSS-------KSSSRMKPPVSSP 865
Triticum         VFFDPEYPSEPKVKPD--------GASVSVDRRQNGRPSN------GRSNSRNSQARTQK 763
Zea              VFFDPEYPSESKVKPEGG--------SASLDRRPNGRPPSN-----GRSNSRNPQSRTQK 811
                 .**:** *      .           .    :    : .        :..*

Arabidopsis      SSQRSLQSERKSGSNHSLGNSRRPSPEKISWN----VLDLKGENYFSCAVG-RTRTNARY 920
Solanum          VSN----SDKKTS------NGRRPSPENVSWN----VLDLKKENYFSCAVG-RTRTNARY 901
Musa             APVG---SERRMPANHNILTGWRSPQETMSWREGSSVLDLKGDNYFSCAVG-RKRSNARY 941
Selaginella      KSPG-----RGSE-------QQQQAEEASRWE-GSSVLDLQGENYFSCAVGTMKRSLARY 912
Triticum         PQVAPPPPERSSSSSD---HSTANNNSHHDWREGSSVLDLNGDNYFSCAVG-RKRSNARY 819
Zea              AQQAA--SERSSS------SSHSSTSSNHDWREGSSVLDLKGENYFSCAVG-RKRSNARY 862
                         :               .   *.    ****: :********  .*: ***

Arabidopsis      LLGSPDDVVCFLEKLADTTSSP-------------------------------------- 942
Solanum          LLSTPDDVVAFLRELAEAPISNGTS---------------------------------- 926
Musa             LLNTSDDVVVFLREMAEAACQGALAALF------------------------------- 969
Selaginella      CLTSSEEVVTFLTSLTSTVAAAAGAGAGARATGSGAAGAGAGAGAGGDHEAPGSPIRKSD 972
Triticum         LLNSSEDVVSFLKEMAESTTPRAGGLPPGAAADYMFLDRQ------------------- 859
Zea              LLSSSEEVVSFLKELATATAGFQATCADYMHVLG-------------------------- 896
                  * :.::** ** .:: :

Arabidopsis      ----------------------
Solanum          ----------------------
Musa             ----------------------
Selaginella      SFKTSGWHSPTPRSPKLAPAVQ 994
Triticum         ----------------------
Zea              ----------------------
```

3.2 Expression Profile of Wheat TPS under Salt and Drought Stresses

In plants, trehalose biosynthesis genes were reported to be induced under abiotic stresses. Expression of cotton *TPS* in root and leaves was increased under drought stress (Kosmas *et al.,* 2006). Ginkgo biloba *TPS*, *GbTPS*, was found to be upregulated by cold, drought, salt and mannitol (Wu *et al.,* 2006).

In this study, microarray results were examined whether TPS related probe sets were regulated differentially. Affymetrix GeneChip contains 13 probe sets related to TPS, 8 probe sets related to TPP and 12 probe sets related to trehalose-6-P synthase/phosphatase (Appendix H).

Based on blastn analysis carried out using HarvEST Wheat 1.54, *TaTPS* (FJ167677.1) shares identities 98% with one unigene (Ta.6534.1) This unigene was represented by two probe sets (Ta.6534.1.S1_at and Ta.6534.1.S1_s_at) in GeneChip. Fold change of wheat *TPS* in root and leaf tissues was found using GeneSpring GX10 (Table 3.3).

Table 3.3 Expression profile of wheat TPS in stress-treated leaf and root tissues

Tissue	Probe Set ID	Fold change ([Drought] vs [Control])	Regulation ([Drought] vs [Control])	Fold change ([Salt] vs [Control])	Regulation ([Salt] vs [Control])	p value
ROOT	Ta.6534.1.S1_at	1.46	up	1.07	No change	0.084
ROOT	Ta.6534.1.S1_s_at	1.68	up	1.09	No change	0.054
LEAF	Ta.6534.1.S1_at	1.61	down	2.02	down	0.35
LEAF	Ta.6534.1.S1_s_at	1.49	down	2.36	down	0.36

In root tissue, expression of this gene increased in abundance under drought stress while no change was observed under salt stress. In leaf tissue, both salt and drought treatments repressed the expression of this gene.

3.3 Microarray Analysis of Wheat Seedlings Exposed to Drought and Salt Stress

In the present work, 10 day-old wheat seedlings were treated with 350 mM NaCl and 20% PEG for 24 hours. Affymetrix GeneChip Wheat Arrays were used to identify differentially expressed genes in root and leaf tissues under salt and drought stress conditions. In order to diminish biological variability and to increase significance of the results statistically, three replicates per condition were used. Data obtained from arrays were analyzed by using GeneChip Operating Software 1.4 and GeneSpring GX 10.

3.3.1 Data Analysis

In the present study, root and leaf data files were analyzed separately. Significantly expressed probe sets with p-values lower than 0.05 were determined by One way ANOVA. Among significantly expressed probe sets, fold change of at least two was considered as differentially expressed probe sets. Significantly different probe set lists ($p<0.05$; $FC\geq2$) were determined for both root and leaf data files. Under salt and drought stresses, numbers of probe sets found significantly different compared with control were given Table 3.4.

Table 3.4 Numbers of probe sets determined by One-way ANOVA and filtering according to Fold Change. Numbers in bold are significantly different probe sets

		One-way ANOVA ($p \leq 0.05$)	Fold Change ($\geq$2-fold)
ROOT	Salt vs Control	4240	**1574**
	Drought vs Control	2499	**246**
LEAF	Salt vs Control	1188	**185**
	Drought vs Control	700	**44**

Significantly different probe sets lists were used to test accuracy of biological replicates. Accuracy of biological replicates was determined by Principle Component Analysis (PCA). PCA allows viewing of variation among groups of replicates. For this analysis, entity list was taken from One way ANOVA. Leaf and root PCA analysis results were given Figure 3.3 and Figure 3.4, respectively. PCA results for both tissues showed that replicates within a group clustered together and separately from arrays in other groups.

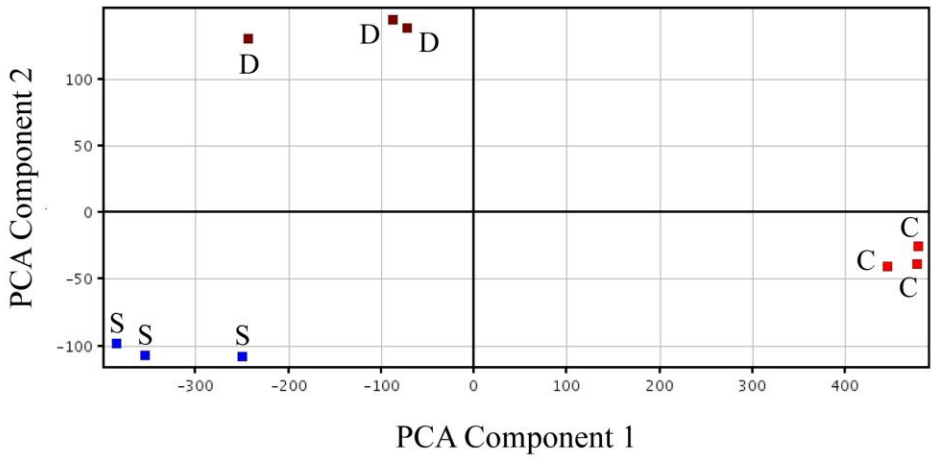


Figure 3.3 Principle Component Analysis (PCA) for leaf samples. PCA on conditions with 1748 entities (entities from one-way ANOVA) (C: Control, S: Salt, D: Drought)

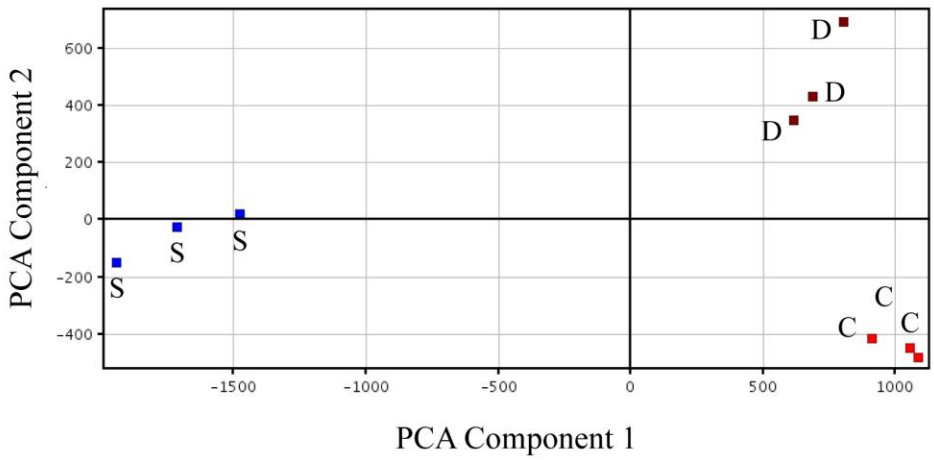


Figure 3.4 Principle Component Analysis (PCA) for root samples. PCA on conditions with 6141 entities (entities from one-way ANOVA) (C: Control, S: Salt, D: Drought)

3.3.2 Differentially Expressed Genes in Drought- and Salt-Stressed Leaf Tissues

The transcripts of genes represented with 123 and 22 probe sets increased in abundance at least 2 fold compared with control after salt and drought treatments, respectively. In contrast, genes represented with 62 and 22 probe sets were repressed in response to salt and drought stress conditions, respectively (Figure 3.5). Based on Venn diagram analysis (Figure 3.6), some up- and downreulated genes were found common for both treatments. These genes may be related to osmotic stress.

Table 3.5 Number of significantly different probe sets that changed more than 2-fold response to salt and drought treatment in the leaf

Fold Change	SALT	DROUGHT
Up-regulated		
2-4	80	17
4-10	29	3
10>	14	2
Total	123	22
Down-regulated		
2-4	50	16
4-10	11	5
10>	1	1
Total	62	22

Highly induced genes (FC>10) in response to salt stress were annotated as Lipid transfer protein, Dehydrin, Ferritin and early nodulin. Genes identified as early nodulin and dehydrin were also highly upregulated under drought stress. Expression of one gene represented by Ta.16731.1.S1_x_at was repressed more than 10- fold under both treatments. No homologs were found for this gene. It may be a wheat specific gene.

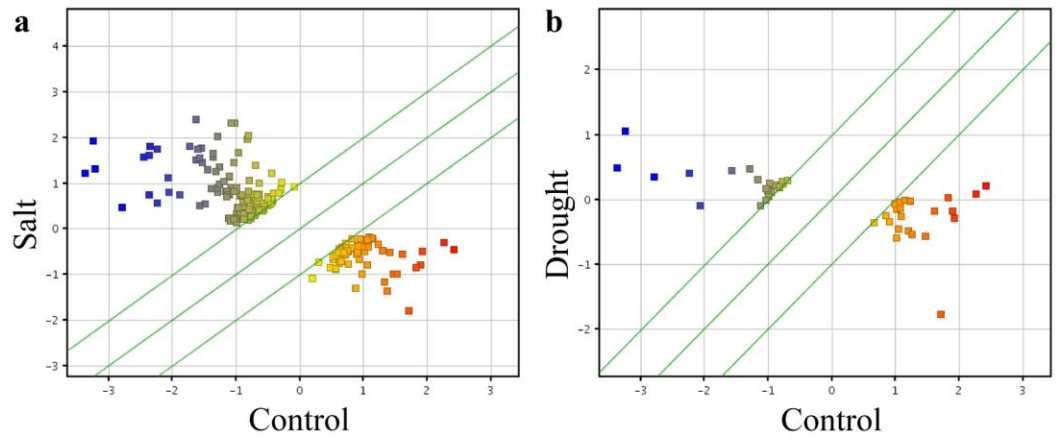


Figure 3.5 Scatter Plots for significantly different ($p<0.05$, $FC\geq2$) probe sets in drought and salt-stressed leaf tissues. (a) Scatter plot for salt stress, (b) Scatter plot for drought stress

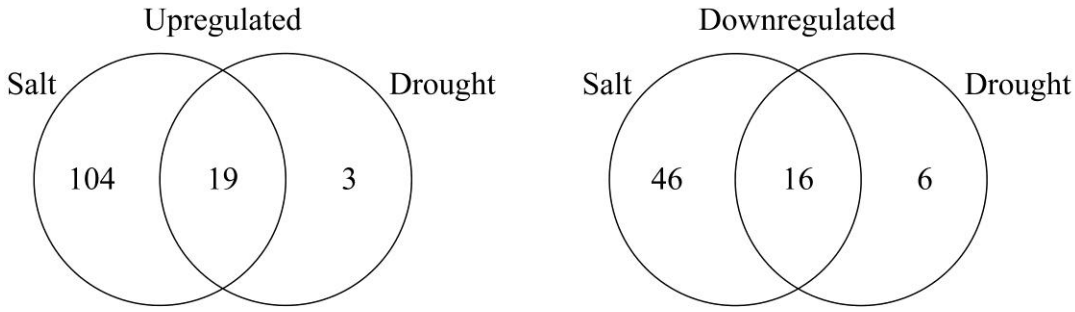


Figure 3.6 Venn Diagram for significantly different probe sets which showed up-and down-regulation in response to drought and salt stresses in leaf tissue

Significantly different genes were annotated with HarvEST WheatChip version 1.54. Annotated genes were functionally categorized in groups such as metal binding, transporters, protective proteins, proteins involved in signaling, stress related proteins, detoxification, cytochrome P450s, hormone biosynthesis, transcription factors, defense related proteins, and early nodulins. Selected annotation lists for up- and down-regulated genes were given in Table 3.6 and Table 3.7, respectively.

In leaf tissue, genes represented with 126 probe sets were differentially upregulated in response to 350 mM NaCl and PEG 20 % treatment. Upregulated genes include (1) metal binding proteins-metallothionein (2) Transporters- ABC transporter, Lipid transfer proteins, (3) Protective proteins- dehydrin, chaperonin, heat shock protein (4) transcription factors- homeodomain-leucine zipper transcription factor, DRE binding factor, heat shock transcription factor (5) protein involved in signaling-phosphatidyl inositol 3- and 4- kinase (6) stress related proteins- universal stress protein, dehydration induced protein RD22 (7) detoxification- peroxidase, ferritin, and glutathione transferase (8) Hormone biosynthesis- 9-cis-epoxycarotenoid dioxygenase 2.

In leaf tissue, genes represented with 68 probe sets were differentially downregulated in response to 350 mM NaCl and PEG 20 % treatment. Downregulated genes include (1) Stress related proteins-universal stress protein, cold regulated protein, cold acclimation proteins (2) pathogenesis related protein- Pathogenesis-related protein (3) Transporter- Iron-phytosiderophore transporter, glucose transporter (4) Transcription Factor- WRKY transcription factor (5) Signaling- EF hand family (6) cytochrome P450s

Table 3.6 Selected list of upregulated genes in drought- and salt-stressed leaf tissue

Probe ID	E-value	Matching Organism	Functional Annotation	Fold change salt	Fold change drought
transcription factors					
TaAffx.52188.1.S1_at	3.00E-11	*Oryza sativa*	Leucine zipper protein	4.77	
Ta.9223.1.A1_at	2.00E-11	*Oryza sativa*	dehydration responsive element binding protein	2.08	
TaAffx.108538.1.S1_at	3.00E-40	*Oryza sativa*	homeodomain-leucine zipper transcription factor TaHDZipI-1	4	
TaAffx.34778.1.S1_at	5.00E-76	*Oryza sativa*	Putative heat shock transcription factor 8	3.98	2.8
stress related proteins					
Ta.21021.2.S1_at	9.00E-12	*Oryza sativa*	protein universal stress protein, putative, expressed	3.16	
Ta.5390.2.S1_at	3.00E-28	*Oryza sativa*	protein stress-related protein, putative, expressed	2.63	
Ta.28209.2.S1_x_at	2.00E-15	*Oryza sativa*	protein dehydration-induced protein RD22-like protein 2	2.09	
tranporters					
Ta.27343.1.S1_at	0	*Oryza sativa*	Transmembrane amino acid transporter protein	4.31	
Ta.28012.1.S1_a_at	9.00E-49	*Oryza sativa*	protein potassium transporter 8, putative, expressed	2.02	
Ta.26177.2.S1_a_at	1.00E-111	*Oryza sativa*	Amino acid transporter-like	8.09	
Ta.24899.1.A1_at	2.00E-32	*Arabidopsis thaliana*	ABC transporter	3.16	
TaAffx.39438.1.A1_at	2.00E-46	*Oryza sativa*	Putative amino acid transporter Oryza sativa (japonica	2.17	
Lipid transport protein					
Ta.1360.1.S1_at	9.00E-27	*Oryza sativa*	Putative lipid transfer protein	**18.09**	-
TaAffx.97181.1.S1_s_at	2.00E-26	*Oryza sativa*	Nonspecific lipid-transfer protein 2, putative	2.43	2.11
Ta.13070.1.S1_at	1.00E-48	*Triticum aestivum*	Non-specific lipid transfer protein 1 precursor	2.72	2.21
Hormone					
TaAffx.76007.1.S1_at	9.00E-65	*Hordeum vulgare*	9-cis-epoxycarotenoid dioxygenase 2	7.07	6.32
Protective					
TaAffx.128555.1.S1_at	9.00E-86	*Hordeum vulgare*	Dehydrin DHN3	**36.18**	**19.91**

Table 3.6 (Continued)

detoxification					
Ta.29814.1.S1_at	6.00E-60	*Triticum monococcum*	Peroxidase 8	4.75	-
Ta.962.1.A1_at	2.00E-94	*Triticum monococcum*	Peroxidase 8	7.95	-
Ta.28354.1.S1_at	1.00E-137	*Triticum aestivum*	Glutathione transferase	2.05	-
Ta.681.2.S1_a_at	1.00E-130	*Triticum aestivum*	Ferritin	**16.44**	-
signaling					
Ta.2968.1.S1_at	0	*Oryza sativa*	Phosphatidylinositol 3-and 4-kinase family-like	2.68	-
Ta.8677.1.S1_at	2.00E-96	*Zea mays*	Putative ras-like small GTP binding ptotein	2.37	-
Early nodulin					
Ta.3573.3.S1_a_at	9.00E-49	*Oryza sativa*	protein early nodulin 75 protein	9.67	8.89
Ta.3341.1.S1_at	2.00E-57	*Oryza sativa*	protein early nodulin 75 protein	**24.25**	**14.61**

Table 3.7 Selected list of downregulated genes in drought- and salt-stressed leaf tissue

Probe ID	E-value	Matching Organism	Functional Annotation	Fold change salt	Fold change drought
Stress related genes					
Ta.21135.1.S1_at	1.00E-56	*Hordeum vulgare*	Universal stress protein	2.35	-
Ta.18487.1.S1_x_at	2.00E-47	*Hordeum vulgare*	Cold-regulated protein 2	2.67	-
Ta.28917.1.S1_x_at	4.00E-57	*Triticum aestivum*	Cold acclimation protein WCOR518	5.78	4.47
TaAffx.3623.1.A1_at	1.00E-29	*Triticum aestivum*	Jasmonate-induced protein	2.59	2.07
Pathogenesis related					
Ta.24501.1.S1_at	5.00E-65	*Hordeum vulgare*	Pathogenesis-related protein 1A/1B precursor	2.49	-
Transporter					
Ta.25540.1.S1_x_at	2.00E-63	*Oryza sativa*	Putative COPT5	2.02	-
Ta.27951.1.A1_x_at	5.00E-83	*Oryza sativa*	solute carrier family 2, facilitated glucose transporter member	2.33	-
TaAffx.113315.1.S1_at	1.00E-45	*Hordeum vulgare*	Iron-phytosiderophore transporter	2.64	2.33
Transcription factor					
Ta.8614.1.S1_at	1.00E-42	*Oryza sativa*	WRKY transcription factor 45	5.42	4.02
Signalling					
Ta.5490.3.S1_at	1.00E-40	*Oryza sativa*	EF hand family	2.04	-
Cytochrome P450					
Ta.3108.1.S1_at	5.00E-37	*Oryza sativa*	cytochrome P450	4.46	-
Ta.11588.1.S1_at	4.00E-82	*Oryza sativa*	cytochrome P450 family protein	2.34	-

3.3.3 Differentially Expressed Genes in Drought- and Salt-Stressed Root Tissues

In comparison with leaf tissues, there were more up-and down-regulated genes in the root tissues exposed to 350 mM NaCl and 20% PEG. In root, genes represented with 879 and 157 probe sets were upregulated in response to salt and drought stresses, respectively. In contrast, 695 and 89 probe sets were repressed in response to salt and drought stress conditions, respectively (Figure 3.7). Venn diagram analysis (Figure 3.8) showed that some up- and down-regulted genes overlapped in both treatments. Overlapping genes may be related to osmotic and oxidative stress.

Table 3.8 Number of significantly different probe sets that changed more than 2-fold response to salt and drought treatment in the roots

Fold Change	SALT	DROUGHT
Up-regulated		
2-4	608	98
4-10	220	54
10>	51	5
Total	879	157
Down-regulated		
2-4	544	80
4-10	128	9
10>	23	-
Total	695	89

Under salt stress, highly induced genes (FC>10) were annotated as LEA, dehydrin, RAB (Response to ABA), wheatwin, cytochrome P450, early nodulin, lipid transfer protein, aquaporin NIP, WRAB1 leucine zipper protein, cold regulated protein and germin like proteins.

The genes represented by 4 probe sets (Ta.5216.1.S1_x_at, Ta.24547.1.S1_at, Ta.24547.1.S1_x_at, Ta.24542.1.S1_at) were common both salt and drought treatments. They were annotated as lipid transfer protein, early nodulin and leucine zipper protein. They may be involved in osmotic stress and/or oxidative stress.

Salt stress repressed the expression of genes represented by 23 probe sets more than 10 fold. The genes represented by these probe sets were identified as HKT1, nicotianamino synthase, nicotianamine transferase, calmodulin binding protein, aquaporin TIP. Some transcripts were not annotated. They may be wheat specific genes.

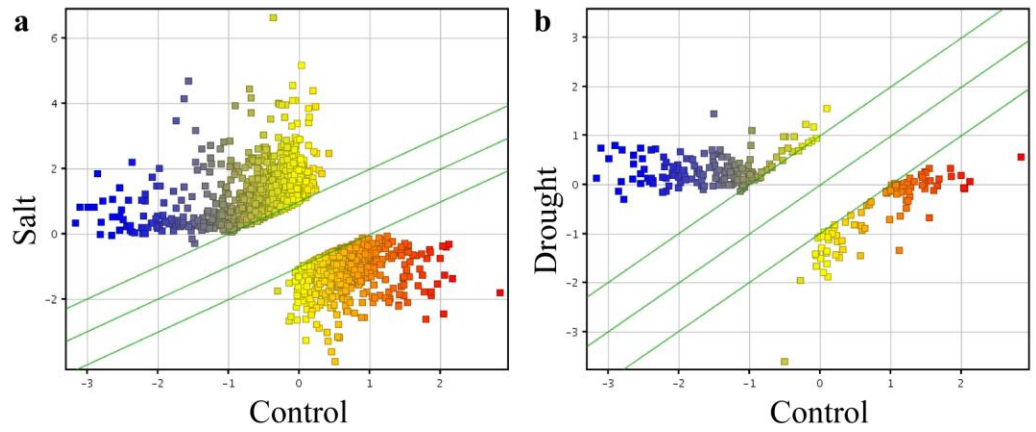


Figure 3.7 Scatter Plots for significantly different probe sets ($p<0.05$, $FC\geq2$) in drought and salt-stressed root tissues. (a) Scatter plot for salt stress, (b) Scatter plot for drought stress

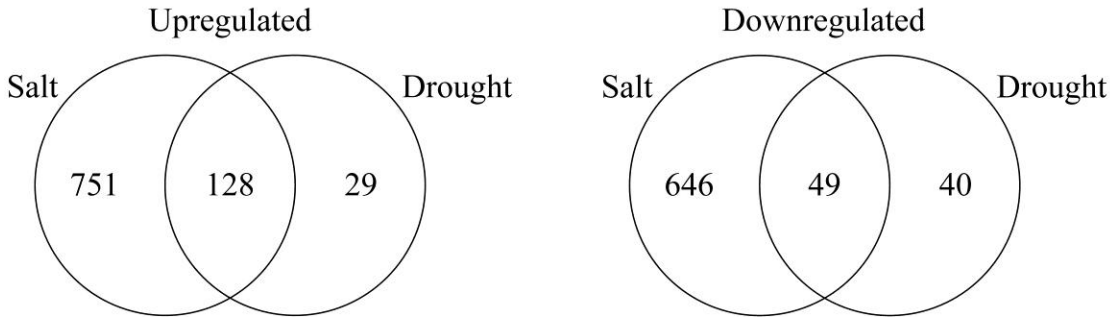


Figure 3.8 Venn Diagram for significantly different probe sets which showed up-and down- regulation in response to drought and salt in root tissue

In root tissue, genes represented by 908 probe sets were differentially upregulated in response to 350 mM NaCl and 20% PEG treatment. Upregulated genes include (1) metal binding proteins- ferritin, metallothionein (2) Transporters- ABC transporter, Lipid transfer proteins, proline transporters (3) Protective proteins- dehydrin, LEA, heat shock protein, spermidine synthase 1 (4) transcription factors- NAC, MYB, ERF, DRE binding factor, heat shock transcription factor (5) protein involved in signaling- calcium binding protein, histidine kinase, RCD1, (6) stress related proteins- universal stress protein, cold regulated protein, WCOR715, WCOR615 (7) detoxification- peroxidase, glutathione transferase, lactoylglutathione lyase family protein / glyoxalase I (8) Hormone biosynthesis-1-aminocyclopropane-1-carboxylate oxidase, 12-oxo-phytodienoic acid reductase (9) cytochrome P450s (10) cell wall related-Putative cinnamate 4-hydroxylase, Phenylalanine ammonia-lyase, Caffeic acid O-methyltransferase (11) defense related- pathogenesis related proteins, germin like proteins, chitinase, xylanase inhibitor, wheatwin.

In root tissue, genes represented by 535 probe sets were differentially downregulated in response to 350 mM NaCl and %20 PEG treatment. Downregulated genes include (1) Iron metabolism- nicotianamine synthase, nicotianamine aminotransferase, deoxymugineic acid synthase1 (2) Transcription factors-MYB, NAC (3) Transporters-HKT1, iron transporters, aquaporin TIP (4) Signalling- calmodulin binding protein, CBLK (5) Cytochrome P450 (6) Amino acid metabolism- Proline dehydrogenase family protein

Table 3.9 Selected list of upregulated genes in drought- and salt-stressed root tissue

Probe ID	E-value	Matching Organism	Functional Annotation	Fold change salt	Fold change drought
Early nodulin					
Ta.24547.1.S1_at	1.00E-47	*Oryza sativa*	protein early nodulin 93	**13.41**	**13.15**
Ta.24155.2.S1_s_at	5.00E-44	*Oryza sativa*	protein early nodulin 93	**13.97**	5.64
Protection					
Dehydrin/LEA					
Ta.2704.1.S1_x_at	3.00E-75	*Hordeum vulgare*	Dehydrin 9	**12.12**	3.37
Ta.25026.1.S1_at	1.00E-70	*Triticum turgidum*	Dehydrin	**14.25**	4.85
Ta.14247.1.S1_at	4.00E-61	*Hordeum vulgare*	Dehydrin DHN2	**37.76**	-
Ta.13255.1.S1_at	1.00E-73	*Triticum aestivum*	Dehydrin WZY1-1	**78.08**	-
Ta.124.1.S1_x_at	2.00E-54	*Triticum aestivum*	LEA D-11 dehydrin -	6.44	2.84
Ta.727.1.S1_x_at	1.00E-103	*Triticum aestivum*	LEA3	7.58	-
Ta.28605.1.S1_at	5.00E-87	*Triticum aestivum*	LEA2	**16.22**	4.12
Ta.23797.1.S1_x_at	5.00E-82	*Triticum aestivum*	LEA, group 3	**26.63**	6.63
Osmoprotectant					
TaAffx.58849.1.S1_at	7.00E-91	*Arabidopsis thaliana*	trehalose-phosphatase	3.28	2.71
Ta.7091.1.S1_at	0	*Oryza sativa*	protein delta 1-pyrroline-5-carboxylate synthetase	4.08	-
Polyamines					
Ta.7196.1.S1_at	1.00E-154	*Oryza sativa*	spermidine synthase 1	2.84	-
Ta.13941.1.S1_at	1.00E-124	*Oryza sativa*	ornithine decarboxylase	2.01	-

Table 3.9 (Continued)

Cell Wall					
Lignin synthesis					
TaAffx.51547.1.S1_at	1,00E-76	*Oryza sativa*	Putative cinnamate 4-hydroxylase	3,46	-
Ta.28046.1.A1_at	3,00E-62	*Triticum aestivum*	Phenylalanine ammonia-lyase	2,81	-
Ta.27338.1.S1_at	8,00E-92	*Lolium perenne*	Caffeic acid O-methyltransferase	2,03	-
Stress related protein					
Ta.21107.1.S1_at	2,00E-73	*Hordeum vulgare*	Universal stress protein	5,27	8,45
Ta.24665.1.S1_x_at	3,00E-63	*Hordeum vulgare*	Universal stress protein	4,15	4,8
Ta.9939.1.S1_at	8,00E-68	*Oryza sativa*	Universal stress protein family protein	5,82	4,73
Ta.759.1.S1_at	1,00E-118	*Triticum aestivum*	Cold acclimation protein WCOR413	3,39	2,36
Ta.2541.1.S1_s_at	4,00E-77	*Triticum aestivum*	Cold acclimation protein WCOR615	-	2,79
Ta.18239.2.S1_a_at	2,00E-44	*Triticum aestivum*	WCOR719	6,79	-
Ta.123.1.S1_x_at	3,00E-44	*Triticum aestivum*	Cold acclimation protein WCOR80	2,83	-
TaAffx.131747.1.S1_x_at	6,00E-41	*Triticum aestivum*	Cold acclimation protein WCOR80	2,25	-
Ta.13183.1.S1_x_at	1,00E-90	*Triticum aestivum*	Cold regulated protein	**29,77**	-
Transcription Factor					
Ta.27501.1.S1_at	1,00E-119	*Oryza sativa*	MYB94	2,09	-
TaAffx.34778.1.S1_at	5,00E-76	*Oryza sativa*	heat shock transcription factor 8	4,94	-
TaAffx.123327.1.S1_x_at	1,00E-34	*Oryza sativa*	ethylene-responsive factor-like protein	3,62	3,36
Ta.22822.1.S1_x_at	2,00E-39	*Oryza sativa*	ethylene responsive element binding protein 2	4,55	3,48
Ta.22338.2.S1_a_at	3,00E-33	*Oryza sativa*	ethylene responsive element binding protein 2	5,98	3,91
TaAffx.128871.1.S1_at	7,00E-13	*Oryza sativa*	DREB 2	2,31	-
TaAffx.77891.1.S1_at	2,00E-41	*Oryza sativa*	DREB2	2,48	-
Ta.10319.1.A1_s_at	8,00E-41	*Oryza sativa*	myb-like DNA-binding domain containing protein	2,43	-
Ta.9497.1.S1_at	1,00E-115	*Oryza sativa*	NAC domain-containing protein 68	5,25	-

Table 3.9 (continued)

Oxidative stress					
Ta.303.1.S1_x_at	2,00E-17	*Triticum aestivum*	Glutathione S-transferase 2	3,53	-
Ta.3118.1.S1_at	6,00E-82	*Zea mays*	Glutathione S-transferase GST 31	2,93	-
TaAffx.25966.1.S1_x_at	2,00E-16	*Hordeum vulgare*	Catalase isozyme 2	2,23	-
Ta.20582.2.S1_x_at	4,00E-51	*Araidopsis thaliana*	lactoylglutathione lyase family protein / glyoxalase I family protein	3,55	-
Ta.681.1.S1_at	1,00E-130	*Triticum aestivum*	Ferritin	2,54	-
Defense					
Ta.21342.1.S1_x_at	1,00E-175	*Triticum aestivum*	Endochitinase precursor	6,89	-
Ta.224.1.S1_at	1,00E-163	*Triticum aestivum*	Chitinase IV	5,05	-
Ta.26340.1.A1_at	3,00E-44	*Hordeum vulgare*	Chitinase	4,13	-
Ta.278.1.S1_at	2,00E-84	*Hordeum vulgare*	Pathogenesis-related protein PRB1-2 precursor	7,95	-
Ta.9226.1.S1_at	3,00E-82	*Triticum aestivum*	Wheatwin-2 precursor	**11,89**	-
TaAffx.104648.1.S1_at	2,00E-06	*Hordeum vulgare*	Pathogenesis-related protein precursor	**20,14**	-
Ta.22619.1.S1_at	4,00E-26	*Hordeum vulgare*	Pathogenesis-related protein 10	9,09	-
Ta.87.1.S1_x_at	1,00E-106	*Triticum aestivum*	Oxalate oxidase GF-2.8 precursor	7,73	
TaAffx.15880.1.S1_at	3,00E-82	*Oryza sativa*	Germin-like protein 7	5,01	
TaAffx.53596.1.S1_x_at	1,00E-31	*Hordeum vulgare*	Oxalate oxidase-like protein or germin-like protein	**10,85**	
Cytochrome P450					
TaAffx.55601.1.S1_at	2,00E-15	*Oryza sativa*	Cytochrome P450	**18,03**	-
Ta.8262.1.S1_at	2,00E-64	*Oryza sativa*	cytochrome P450	**13,53**	-
Ta.29826.1.S1_at	1,00E-101	*Oryza sativa*	cytochrome P450	8,44	-
TaAffx.53868.1.S1_at	6,00E-37	*Oryza sativa*	cytochrome P450	4,78	-

Table 3.9 (Continued)

hormone biosynthesis					
Ta.9107.1.S1_x_at	1,00E-124	*Phyllostachys pubescens*	1-aminocyclopropane-1-carboxylate oxidase	2,9	-
TaAffx.100446.1.S1_at	4,00E-61	*Saccharum officinarum*	1-aminocyclopropane-1-carboxylate oxidase	3,6	-
TaAffx.59867.1.S1_at	1,00E-138	*Oryza sativa*	1-aminocyclopropane-1-carboxylate oxidase -	4,54	-
Ta.30735.1.S1_at	1,00E-175	*Zea mays*	12-oxo-phytodienoic acid reductase	3,36	-
Transporters					
Lipid transfer protein					
Ta.5216.1.S1_x_at	3,00E-38	*Oryza sativa*	Lipid transfer protein-like protein	**14,12**	**11,63**
Ta.1360.1.S1_x_at	9,00E-27	*Oryza sativa*	lipid transfer protein	**15,06**	2,05
Proline transporters					
Ta.10058.1.A1_at	4,00E-90	*Hordeum vulgare*	Proline transporter	3,89	-
Ta.30603.1.S1_s_at	1,00E-124	*Hordeum vulgare*	Proline transporter	2,98	-
ABC transporter					
Ta.26152.1.A1_s_at	1,00E-42	*Oryza sativa*	multidrug resistance-associated protein 14	4,08	-
Ta.21281.1.S1_at	3,00E-70	*Oryza sativa*	PDR-like ABC transporter	4,02	-
Signaling					
Ta.4760.1.S1_at	0	*Oryza sativa*	PM type calcium-transporting ATPase 9,	2,26	-
Ta.23268.2.S1_a_at	1,00E-107	*Oryza sativa*	RCD1 (RADICAL-INDUCED CELL DEATH1)	2,51	2,66
Ta.23268.3.S1_a_at	1,00E-107	*Oryza sativa*	RCD1	2,35	2,58
Ta.10092.1.S1_x_at	1,00E-48	*Oryza sativa*	EF hand family protein	3,46	2,75
Ta.24121.1.S1_x_at	2,00E-54	*Oryza sativa*	EF hand family protein	6,65	3,06
Ta.12348.1.A1_at	8,00E-53	*Oryza sativa*	protein phosphatase 2C ABI2	3,78	2,59
TaAffx.97084.1.S1_at	0	*Oryza sativa*	protein histidine kinase 2	2,12	-

Table 3.10 Selected list of <u>downregulated</u> genes in drought- and salt-stressed root tissue

Probe ID	E-value	Matching Organism	Functional Annotation	Fold change salt	Fold change drought
Transporters					
TaAffx.4071.1.A1_at	2.00E-53	*Oryza sativa*	Putative SLC11A3 iron transporter	5.24	3.15
TaAffx.55509.1.S1_at	1.00E-53	*Oryza sativa*	Iron transport protein 2	3.52	2.43
TaAffx.64742.1.S1_at	1.00E-67	*Oryza sativa*	Putative SLC11A3 iron transporter	6.54	3.46
Ta.113.1.S1_at	0	*Triticum aestivum*	HKT1	**10.25**	-
Ta.23848.3.S1_at	6.00E-75	*Hordeum vulgare*	HAK2	3.14	-
TaAffx.111463.1.S1_at	3.00E-63	*Phragmites australis*	High-affinity potassium transporter	2.12	-
Oxidative Stress					
Ta.21094.1.S1_at	0	*Hordeum vulgare*	Nicotianamine aminotransferase	**11.61**	-
Ta.25114.1.S1_at	0	*Hordeum vulgare*	Nicotianamine aminotransferase	2.08	-
Ta.4977.2.A1_x_at	6.00E-27	*Hordeum vulgare*	Nicotianamine aminotransferase	**12.32**	-
Ta.5145.1.S1_x_at	1.00E-136	*Hordeum vulgare*	Nicotianamine synthase 8	**12.08**	-
Ta.5335.3.S1_a_at	1.00E-140	*Triticum aestivum*	Deoxymugineic acid synthase2	4.93	2.81
signaling					
TaAffx.31813.1.S1_x_at	3.00E-22	*Oryza sativa*	calmodulin binding protein	**20.97**	-
Ta.18735.1.S1_at	4.00E-12	*Oryza sativa*	CBL-interacting serine/threonine-protein kinase 15	4.31	-
Ta.4936.1.S1_at	0	*Oryza sativa*	CBL-interacting serine/threonine-protein kinase 15	2.56	-
Ta.8465.1.S1_at	1.00E-149	*Oryza sativa*	CBL-interacting serine/threonine-protein kinase 15	4.58	-
Amino acid					
Ta.3696.3.S1_a_at	0	*Oryza sativa*	Proline dehydrogenase family protein	9.64	-

Table 3.10 (Continued)

Transcription factors					
Ta.28092.1.S1_s_at	4.00E-70	*Oryza sativa*	MYB2	2.04	-
Ta.3031.1.A1_at	5.00E-43	*Oryza sativa*	MYB2	2.08	-
Ta.11631.1.A1_at	4.00E-12	*Oryza sativa*	NAC domain-containing protein 21/22	2.41	-
Cytochrome P450					
TaAffx.119714.1.A1_at	8.00E-61	*Triticum aestivum*	Cytochrome P450	2.61	-
TaAffx.84302.1.S1_at	3.00E-38	*Triticum aestivum*	Cytochrome P450	5.32	-
Ta.29962.1.A1_at	1.00E-47	*Oryza sativa*	Cytochrome P450	2.30	-

CHAPTER 4

DISCUSSION

Abiotic stresses adversely affect crop production worldwide. Developing abiotic stress tolerant plants is important since more than 50% of arable lands may become useless because of salinization resulted from drought and salinity by the year 2050 (Wang *et al.*, 2003 and references therein). In recent years, there has been a growing interest in trehalose metabolism for the production of transgenic plants tolerant to abiotic stresses (Penna, 2003). Overexpression of *TPS* in some plants improved stress tolerance. Until now, there has been no known literature data about wheat TPS gene. In the present study, wheat *TPS*, *TaTPS* was cloned and characterized under salt and drought stress conditions.

Microarray technology has allowed the examination of gene expression profile of plants subjected to abiotic stresses such as drought, salt and ABA. Identification of stress related genes by microarray analysis provides the improvement of the stress tolerance of agronomically important plants by gene manipulation. Microarray studies of wheat are important since wheat genome has not been completely sequenced yet. In the present study, Wheat GeneChip (Affymetrix) was used to investigate gene expression profile of 10-days old wheat seedlings exposed to 350 mM NaCl and 20% PEG for 24 hours. Data from this study provided the information related to stress tolerance mechanisms of wheat under salt and drought stress conditions.

4.1 Cloning of Wheat Trehalose-6-Phosphate Synthase

In this study, partial cDNA sequence encoding putative trehalose-6-phosphate synthase was cloned from wheat by using RACE method. Initially, internal conservative fragment was cloned by using degenerate primers. Then, RACE primers were designed from this conserved sequence.

The cDNA of *TaTPS* (GeneBank Accession No: FJ167677.1) is 2928 bp long, including poly (A) tail and has a ORF of 2580 bp long. The TaTPS protein was found 859 amino acids with a predicted molecular weight (MW) of 96.7 kDa and isoelectric point (pI) of 5.97. Based on BLASTP analysis, the amino acid sequence of wheat TPS shows great similarity with the other plant TPS from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Zea mays*, *Selaginella lepidophylla*, *Saccharum* hybrid cultivar, and *Musa balbisiana*. Like *Arabidopsis thaliana* and *Selaginella lepidophylla*, putative *TaTPS* determined in this study contains "family GT-20" TPS domain at N-terminal and TPP domain at C-terminal. These results suggest that this cDNA may encode functional wheat TPS protein.

4.2 Expression of Wheat TPS under Salt and Drought Stress Conditions

Expression profile of wheat *TPS*, *TaTPS*, under salt and drought stress was examined by using microarray data.

Trehalose is known to stabilize proteins and membranes under dehydration stress (Wingler, 2002). The first step catalyzed by TPS is the transfer glucose from the UDP-glucose to glucose-6-phosphate to form T6P. Then T6P phosphatase dephosphorylate T6P to trehalose and inorganic P. El-Bashiti *et al* (2005) showed the accumulation of trehalose under salt and drought stress in root tissue. In this study, expression of TPS gene increased in abundance in drought stressed root tissue while no change was observed in salt stressed roots. Induction of *TPS* under salt stress may be earlier or later than 24 hours. In addition, expression of TPP was induced both salt and drought stress conditions, suggesting trehalose accumulation in roots. Upon

exposure to salt and drought stress, wheat root may accumulate trehalose as an osmoprotectant in order to adapt osmotic stress.

Overexpressing of yeast *TPS* in transgenic plant showed growth retardation (Holmström *et al.*, 1996). It seems that phenotypic alterations in these transgenic plants are possibly due to T6P. It has been shown that T6P play critical role in plant growth and development (Paul *et al.*, 2008). Levels of T6P are likely to be tightly regulated by the activities of TPS and TPP (Eastmond *et al.*, 2003). In leaf tissue, both salt and drought treatments repressed the expression of *TPS*. Under salt and drought stresses, regulation of T6P may be critical for leaf growth and development.

4.3 Differentially Expressed Genes in Root Tissue under Salt and Drought Stress

From the data analysis, transcripts of genes represented by 1820 probe sets were found to differ ($p \leq 0.05$) at least 2 fold between stressed and control roots of wheat. These genes were functionally catagorized. In the present study, overlapping up- and down- regulated genes were found in response to salt and drought stresses, indicating the existance of cross talk between salt and drought stresses.

4.3.1 Osmoprotectant Biosynthesis

4.3.1.1 Proline

Proline known as a compatible solutes is believed to play role in osmotic adjustment, hydroxyl radical scavenging and protection of plasma membrane integrity (Bartels and Sunkar 2005 & references therein). Its accumulation increases in response to environmental stresses. There are two biosynthetic pathways for proline: the ornithine dependent pathway and the glutamate-dependent pathway. In the present study, salt stress induced the expression of genes related to proline biosynthesis. The genes were annotated as delta 1-pyrroline-5-carboxylate synthetase (P5CS)

(Ta.7091.1.S1_at) and ornithine aminotransferase (Ta.12337.1.S1_at, Ta.21613.1.S1_a). Ornithine aminotransferase and delta 1-pyrroline-5-carboxylate synthetase (P5CS) are enzymes in the ornithine dependent and glutamate-dependent pathway, respectively. Both salt and drought stresses downregulated the expression of proline dehydrogenase (Ta.3696.3.S1_a_at), the enzyme in the proline catabolism. It has been shown that salt stress induces the expression of proline transporter in barley roots (Ueda *et al.*, 2001). In the present study, 5 genes annotated as proline transporter were differentially expressed in response to salt stress. Among these genes, 4 of them were upregulated and 1 gene was downregulated. Taken together, these results suggest that under salt stress, wheat roots may accumulate proline. Proline might be an osmoprotectant or/and an osmoticum in wheat root.

4.3.2 Protective Proteins

4.3.2.1 Enzymes Involved in Polyamine Biosynthesis

Transgenic Arabidopsis plants overexpressing spermidine synthase displayed enhanced tolerance to multiple environmental stresses. Also, several drought stress related genes were upregulated in these transgenic plants (Kasukabe *et al.*, 2004). In the present study, genes related to polyamine synthesis were identified as differentially expressed under salt stress. These genes are spermidine synthase (Ta.7196.1.S1_at), and ornithine decarboxylase (Ta.13941.1.S1_at). Wheat may use spermidine as a regulator in the salt stress.

4.3.2.2 LEA/Dehydrins

LEA proteins are osmotic stress responsive proteins. They are involved in protecting/stabilizing macromolecules. They are induced by salt, drought, cold and ABA. Among the stress inducible genes identified, the ones related to LEA/dehydrins were highly upregulated under salt stress. Dehydrin WZY1-1

represented by Ta.13255.1.S1_at increased 78.08 fold in salt stressed roots. LEA group 3 represented by Ta.23797.1.S1_x_at was upregulated 26.63 fold in salt stressed roots compared with controls. In wheat, LEA/dehydrin genes may have a protective function under salt stress.

4.3.3 Transcription Factors

DRE-binding factors 2 (DREB2) specifically binds to DRE/CRT sequence found in the promoter region of many stress inducible genes. In Arabidopsis, expression of DREB2 genes is induced by dehydration and high salinity. Salt stress induced the expression of genes represented by 2 probe sets (TaAffx.128871.1.S1_at, TaAffx.77891.1.S1_at) and annotated as DREB2. DREB2 induces the expression of some stress-inducible genes such as LEA through ABA-independent pathway. DREB2 may be responsible for the induction of some LEA genes identified in this study.

NAC, a plant specific transcription factor, was shown to be involved in abiotic stress response and tolerance. Arabidopsis *RD26* (NAC gene) and rice *OsNAC6* were shown to be induced by abiotic stresses such as salt and drought (Nakashima *et al.*, 2007). In the present study, salt stress induced the expression of NAC domain containing protein represented by Ta.9497.1.S1_at. However, another gene (Ta.11631.1.A1_at) annotated as NAC domain containing protein 21/22 was downregulated 2.41-fold. There are variations in the timing of induction of genes. Expression of Ta.11631.1.A1_at may be an early response gene whose expression is rapid and transient in response to abiotic stresses.

Expression of *ERF* is induced by abiotic stresses and pathogens. Transgenic plants overexpressing tomato *ERF* gene, *TERF1*, or tobacco *Tsi* were shown to enhance abiotic stress tolerance. In addition, overexpressing *ERF* in transgenic plants induced the expression of *PR* (Huang *et al.*, 2004; Park *et al.*, 2001). Salt and drought stress upregulated *ERF* genes represented by Ta.22338.2.S1_a_at; Ta.22822.1.S1_x_at; TaAffx.123327.1.S1_x_at. These genes may induce the expression of downstream

genes related to stress tolerance. In the present study, *PR* genes were also induced. Since ERF binds to GCC box found in the promoter region of *PR*s, some of genes identified as PR in this study may be downstream target of ERF.

HSF (Heat shock transcription factor) controls the activation of heat shock genes. HSPs have role as a molecular chaperons. HSPs stabilize proteins and membranes and can assist in protein refolding under stress conditions and prevent the aggregation of nonnative proteins (Wang *et al.*, 2003). Plants overexpressing Arabidopsis HSF, *HsfA2,* demonstrated increased tolerance to combined environmental stresses (Nishizawa *et al.*, 2006). In the present study, salt stress induced HSF genes represented by Ta.11671.1.S1_at and TaAffx.34778.1.S1_at. Increase in the expression of HSFs leads to increase in the expression of HSPs, which in turn help protein refolding under stress conditions.

4.3.4 Transporters

4.3.4.1 High Affinity Potassium Transporters

High Affinity K^+ transporters (HKT) were identified in many plant species such as barley, Arabidopsis, wheat and reed plants (Santa-Marcía *et al.*, 1997; Uozumi *et al.*, 2000; Schachtman and Schroeder, 1994; Takahashi *et al.*, 2007). Besides transport of K^+, they also mediate the transport of Na^+. They behave as low affinity Na+ transporters. Transgenic wheat plants expressing antisense *HKT1* displayed enhanced growth under salinity when compared with the control plants. Moreover there was a significant decrease in Na^+ uptake in these transgenic lines (Laurie *et al.*, 2002). It seems that decrease in the expression of *HKT1* is associated with salt tolerance in plants.

Under salt stress, genes represented by Ta.113.1.S1_at, Ta.23848.2.A1_a_at, Ta.23848.3.S1_at, TaAffx.111463.1.S1_at were identified as *HKT*. Expression of gene represented by Ta.113.1.S1_at decreased 10.25 fold under salt stress. Since

HKTs are low affinity Na^+ transporters, the decrease in the abundance of these transcripts under salinity may be indicative of improvement of plant salt tolerance.

4.3.4.2 Lipid Transfer Proteins

LTPs were shown to be induced in response to drought and salt stress (Mott and Wang, 2007; Seki *et al*., 2002). Moreover, expression of wheat LTP, *TaLTP1*, was shown to be upregulated under drought stress (Janga *et al*., 2004). In the present study, 12 genes were annotated as LTPs. Expression of gene represented by Ta.5216.1.S1_x_at increased 14.12 and 11.63 fold under salt and drought stress respectively. The role of LTPs is presently unclear. It is thought to be involved in formation of cuticle, a protective layer around the epidermis. Cuticle prevents water loss from the epidermis. Cameron *et al* (2004) showed that in tree tobacco, expression of LTP increased concomitant with increased cuticular wax deposition. In wheat, LTP may be involved in cuticle formation, thus preventing water loss during abiotic stresses.

4.3.4.3 ATP- binding Cassette Transporters

ATP-binding cassette (ABC) transporters transport molecules through the membrane with concomitant hydrolysis of ATP. In plants, ABC transporters are involved in wide range of processes such as auxin transport, lipid transport, stomata regulation and cadmium and aluminum tolerance (Schulza and Kolukisaoglu, 2006). Proteomic analysis of wheat roots under salt stress demonstrated that one ABC transporter was upregulated in response to salt stress (Wang *et al*., 2008). Lee *et al* (2004) suggested that *AtMRP5*, Arabidopsis ABC transporter gene, is involved in K^+ homeostasis and also takes part in the NaCl stress response. In the present study, 11 genes identified as ABC transport family protein were induced in the ranges of 2.06- to 4.08- fold in response to salinity. The expression of multidrug resistance-associated protein (MRP) gene represented by Ta.26152.1.A1_s_at increased 4.08 fold under salt stress. Wheat ABC transporters may be involved in K^+ homeostasis under salt stress.

4.3.5 Cytochrome P450

Plant P450s have roles in synthetic pathways of signaling molecules (ABA, gibberellin, brassinosteroids, and jasmonic acid) and defense compounds (Bundock *et al.*, 2003; Schuler *et al.*, 2006). They are also involved in cutin and lignin biosynthesis (Werck-Reichhart and Feyereisen, 2000). Narusaka *et al* (2004) showed that most *Arabidopsis* cytochrome P450 genes is induced by biotic and abiotic stresses. In the present study, 29 genes were identified differentially expressed under salt stress. Of 29 genes, 14 of them were upregulated. Transcripts of genes represented by TaAffx.55601.1.S1_at and Ta.8262.1.S1_at increased in abundance by 18.03- and 13.53-fold, respectively. Among the downregulated P450 genes, transcripts represented by Ta.21108.1.S1_at and Ta.18643.1.S1_x_at decreased in abundance by 8.81- and 6.98- fold, respectively. Increase in the expression of P450 transcripts may be related to the hormone synthesis since genes related to JA and GA biosynthesis were also upregulated in response to salinity. In addition, some transcripts may be involved in cutin formation or pathogen resistance. P450 transcripts decreased in response to salinity may have functions in unstressed (normal) conditions.

4.3.6 Early Nodulin

Early nodulin (ENOD) genes are induced during early stages of nodule development in legumes. However, they are also expressed in non-symbiotic organs of legumes. Genomes of dicots and monocots contain also homologues of ENOD genes. Some legume ENOD gene products are proline-rich cell wall proteins, putative metal binding proteins and membrane sulfate transporters (Reddy *et al.*, 1999 & references therein). Increased in ENOD genes was reported in wheat root exposed to salinity (Mott and Wang, 2007). In the present study, total 26 ENOD genes were upregulated in response to salt and drought stress conditions. Genes represented by TaAffx.52191.1.S1_x_at, Ta.24547.1.S1_at and Ta.24547.1.S1_x_at were highly induced under both treatments. These genes may be related to osmotic stress. In wheat, ENOD genes may be involved in stress tolerance.

4.3.7 Stress-Responsive Proteins

In the present study, genes identified as universal stress protein, cold shock factor, WCOR719, WCOR615, WCOR413, WCOR80, cold regulated protein and cold acclimation induced proteins were induced in response to salt and drought stress conditions. In previous studies, upregulation of these genes in wheat roots were also reported in response to drought and salt stress conditions (Mohammadi *et al.*, 2007; Mott and Wang, 2007). In wheat roots, these genes may have roles in adaptation to abiotic stress conditions.

4.3.8 Cell-Wall Related Proteins/Enzymes

4.3.8.1 Lignin Biosynthesis

Lignin is integrated into secondary cell walls of the plants. It reduces the water loss in vascular plants (Gavnholta and Larsen, 2001). Jbira *et al* (2001) showed lignification in root cells of wheat species in response to 100 mM NaCl. Authors suggested that lignification protects the cell from salt invasion. In the present study, genes related to lignin biosynthesis were induced in response to salt stress in root tissue. These are cinnamate 4-hydroxylase, phenylalanine amino-lyase, caffeic acid O-methyltransferase. Transcripts of caffeic acid O-methyltransferase and cinnamate 4-hydroxylase were also reported to increase in abundance in wheat root exposed to salt stress (Mott and Wang 2007 & their Supplementary Table 1). These results suggest that lignin formation may be a stress adaptation for wheat roots exposed to salt stress. Lignification may reduce flow of Na^+ ions with water *via* apoplastic route to roots (Salekdeha *et al.*, 2002).

4.3.9 Signaling

4.3.9.1 Ca^{+2} Signaling

Abiotic stress conditions leads to increase in cytosolic Ca^{+2} levels. Ca^{+2} triggers many signal transduction pathways such as regulation of enzyme activity and gene expression leading to diverse cellular response (Bartels and Sunkar, 2005). Ca^{+2} signaling is sensed by protein phosphatase 2C (PP2C) and EF hand family proteins such as calmodulin, Ca^{+2}-dependent protein kinase (CDPK) and calcineurin B-like (CBL) proteins. Transgenic rice plants overexpressing *OsCDPK7* induced the expression of a stress responsive gene *rab16A* in response to drought and salt stress. In addition, these transgenic plants showed enhanced tolerance to high salt and drought stress conditions (Saijo *et al.*, 2000). In the present study, transcripts of 5 EF hand family protein and 2 calmodulin binding and 1 PP2C increased in abundance under salt and drought stress conditions. They may enhance osmotic stress tolerance by inducing phosphorylation/dephosphorylation cascade resulting in regulation of gene expression.

Ca^{+2}-ATPase plays a role in maintaining Ca^{+2} level in plant cells. It has been reported that NaCl induced the expression of endoplasmic reticulum (ER) and plasma membrane Ca^{+2}-ATPase in Arabidopsis and soybean, respectively (Chung *et al.*, 2000; Geisler *et al.*, 2000). Transcript of plasma membrane type Ca^{+2}-ATPase represented by Ta.4760.1.S1_at was upregulated 2.26 fold in response to salt stress. Chung *et al* (2000) suggested that upregulation of Ca^{+2} pumps in response to salt stress enhance the efflux of Ca^{+2}, thereby decreasing the duration of a given stimulus.

Salt stress repressed the expression of CBL- interacting protein kinase (CBLK) and calmodulin genes. These genes may be related to ionic stress caused by salt stress and their expression may be required for ion homeostasis. At 24 h, ionic homeostasis may be established. Therefore, there may be no more need for expression of these genes.

In Arabidopsis, SOS1 (Na^+/H^+ antiporter) functions in efflux of Na^+ from the cell. Katiyar *et al* (2006) showed that under salt and oxidative stress SOS1 interacts with RCD1 known to be involved in oxidative stress tolerance. In the present study, one gene identified as RCD1 were induced in response to salt and drought. Increase in the transcript level of RCD1 in response to abiotic stress may be related to stress tolerance in wheat.

4.3.9.2 Kinases

Protein phosphorylation is one of the mechanisms of signal transduction in response to environmental stresses and pathogens in plants. In the present study, transcripts of several types of kinases and receptor kinases were found to be increase in abundance in respose to salt stress in root tissue.

4.3.9.2.1 Ankyrin Protein Kinase

Ankyrin protein kinases have N-terminal region carrying ankyrin repeat that recognize several ligands such as protein receptors and signaling molecules. Alfalfa *APK* was induced in response to osmotic stress (Chinchilla *et al*., 2003). Transcript of ankyrin protein kinase gene represented by Ta.3322.3.S1_a_at was induced 2.36-fold. Under salt stress, ankyrin protein kinase may be involved in salt stress signaling and may be required for salt tolerance in wheat.

4.3.9.2.2 Histidine Kinase

Arabidopsis histidine kinase gene, *AHK1*, is a receptor like kinase. Tran *et al* (2007) showed that overexpression of *AHK1* enhanced drought tolerance in Arabidopsis. Based on microrray analysis of *ahk1* mutant, authors suggested that AHK1 functions upstream of important TFs such as ANAC, DREB2A and their downstream genes. In this study, transcript of histidine kinase increased 2.12 fold in response to salt stress. Since genes annotated as NAC and DREB2 were also determined, wheat histidine

kinase may be upstream of NAC and DREB2. Moreover, it may regulate the expression of many stress-inducible genes.

4.3.10 Defense Related Genes

In this study, 40 defense related genes were identified as differentially induced genes under salt stress. These are 28 Pathogenesis-related genes, 4 xylanase inhibitors and 8 germin like genes. These results are consistent with previous wheat microarray studies performed after drought and salt stress treatments (Mohammadi *et al.*, 2007; Mott and Wang, 2007). Chitinase is a pathogenesis related protein. Recently, it has been shown that Arabidopsis plants overexpressing chitinase displayed enhanced tolerance to salt and increased resistance to bacterial disease (Hong and Hwang, 2006). Taken together, these results suggest that there is a cross talk between abiotic and biotic stresses.

4.3.11 Hormone Biosynthesis

Ethylene (E) and Jasmonic acid (JA) cooperatively regulate stress responses. They play role in the upregulation of the expression of defense-related genes such as plant defensin and basic chitinase. These hormones induce synergistically the transcriptional expression of ERF (Shinshi, 2008). In the present study, genes related to biosynthesis of E and JA were identified as differentially regulated under salt stress. Expression of gene encoding 12-oxo-phytodienoic acid reductase, the enzyme in the biosynthesis of JA, was upregulated 3.36-fold. For E biosynthesis, gene encoding 1-aminocyclopropane-1-carboxylate oxidase was identified. As mentioned in section 4.3.3, genes identified as ERF were also upregulated. These results suggest that under salt stress, biosynthesis of E and JA is induced, leading to transcriptional activation of ERF whose downstream target is PR proteins. In addition, these results also show the existence of crosstalk between abiotic and biotic stresses.

4.3.12 Detoxification Enzymes

Abiotic stresses increase the level of ROS and MG. Plants have antioxidant defense system and glyoxalase pathway to detoxify ROS and MG, respectively. In this study, salt stress induced the expression of genes encoding ROS scavenging enzymes. These are catalase, glutathione-S-transferase, glutathione peroxidase, and peroxidases. Moreover, lactoylglutathione lyase gene, the first enzyme in the glyoxalase pathway, was upregulated under the salt stress. Unlike salt stress, drought stress did not induce genes related to these pathways. It seems that salt stress cause severe oxidative stress compared with drought stress.

In this study, NAS, NAAS and DMAS genes related to iron transport into root were downregulated under salt stress. Also, transcripts of iron transporters represented by TaAffx.64742.1.S1_at and TaAffx.55509.1.S1_at were repressed. On the contrary, ferritin (Ta.681.1.S.1_at) was upregulated. Since iron causes Fenton reaction under oxidative stress, iron transport may be inhibited. Ferritin, a chelator of iron, may prevent Fenton reaction, thereby having a protective role under oxidative stress generated by salt stress. Expression of ferritin was also shown to be induced in other microarray studies under abiotic stresses (Rabbani *et al.*, 2003; Seki *et al.*, 2002). Drought stress repressed the expression of DMAS and iron transporters. However, ferritin was not induced under drought stress.

4.4 Differentially Expressed Genes in Leaf Tissue under Salt and Drought Stress

From the data analysis, genes represented with 229 probe sets were found differentially expressed under salt and drought stress conditions. Like root tissue, genes encoding ferritin, ENOD, LEA/dehydrin, and LTP were upregulated.

4.4.1 Protective Proteins

4.4.1.1 LEA/Dehydrin

In previous studies, LEA/Dehydrin related transcripts were reported to increase in abundance under abiotic stresses. In leaf tissue, expression of dehydrin gene represented by TaAffx.128555.1.S1_at was upregulated 36.18- and 19.91-fold in response to salt and drought stress conditions, respectively. As its expression was induced highly in both stresses, this transcript may play role in osmotic stress.

4.4.1.2 Heat Shock Protein/Chaperonin

Abiotic stresses cause deleterious structural and functional changes in proteins. HSPs sustain proteins in their functional structures, prevent aggregation of non-native proteins, and refold of denatured proteins for cell survival under stress (Wang et al., 2003). In leaf tissue, transcripts of chaperonin genes represented by Ta.1321.1.S1_s_at and Ta.28883.1.A1_at increased in abundance 2.1- and 2.45-fold in response to salt stress. Under salt stress, chaperonin may have a protective role in wheat.

4.4.2 Transcription Factors

Several homeodomain-leucine zipper transcription factor (HDZIP) is thought to be related to environmental adaptation. *Arabidopsis* HD-ZIP genes (*ATH-6, ATHB-7*) were reported to be linked to drought response (Söderman *et al*., 1999; Södermann *et al*., 1996). In leaf tissue, transcript of HDZIP increased in abundance 4-fold under salt stress. In wheat, HDZIP may be involved in salt adaptation in leaf tissue. In addition, it may be upstream of stress-inducible genes.

4.4.3 Transporters

4.4.3.1 Lipid Transport Protein

As mentioned in section 4.3.4.2, Lipid transfer proteins were reported to be induced in salt and drought stresses. In leaf tissue, transcripts of LTP genes were highly induced under salt stress condition. LTPs may participate in formation of cutin that prevents water loss from epidermis, leading to salt tolerance in wheat.

4.4.4 Early Nodulin

As in root tissue, early nodulin genes were also upregulated in leaf tissue. As mentioned before, early nodulin related transcripts were reported to increase in response to salt in wheat root tissue (Mott and Wang, 2007). Expression of early nodulin genes represented by Ta.3341.1.S1_at was induced 24.25 and 14.61 in response to salt and drought stress conditions, respectively. These transcripts may be involved in osmotic stress tolerance in wheat.

4.4.5 Signaling

Phosphatidyl inositol 3- and 4- kinase (PIP5K) synthesizes the formation PIP_2. Mikami *et al* (1998) showed that Arabidopdis *PIP5K* was induced in response to ABA and water stress. In addition, salt stress was shown to increase PIP_2 levels in Arabidopsis (DeWald *et al.*, 2001). In the present study, transcript of gene represented by Ta.2968.1.S1_at and annotated as PIP5K was upregulated 2.68 fold under salt stress. PIP_2, the product of PIP5K, is a signaling molecule in animals. Increase in the expression of PIP5K suggests that wheat may use PIP_2 as a signaling molecule under abiotic stress conditions.

4.4.6 Hormone Biosynthesis

ABA is involved in adaptation of plants to abiotic stresses. It regulates stomatal closure in guard cells and induces the expression of genes encoding enzymes and proteins that play roles in dehydration stress tolerance. Transcript of 9-cis-epoxycarotenoid dioxygenase 2 (NCED) was increased 7.07- and 6.32- fold under salt and drought stress, respectively. This gene is involved in ABA biosynthetic pathway. Increase in the expression level of genes related to ABA biosynthesis was also reported in response to abiotic stresses in previous studies (Xiong and Zhu, 2003 and references therein). It has been shown that expression of some LEA proteins was induced by ABA-dependent pathway. ABA mediated gene expression may take place under salt and drought stress.

4.4.7 Detoxification Enzymes

In leaf tissue, genes annotated as ferritin were highly induced in response to salt stress. In addition, genes annotated as iron transporter decrease in abundance 2.64- and 2.33-folds in response to salt and drought treated leaf tissues. Fenton reaction under salt stress may be prevented by inducing the expression of ferritin and repressing the expression of iron transporters.

Expression of peroxidase and glutathione transferase genes was also upregulated under salt stress, indicating protection against oxidative stress.

4.5 Summary of Microarray Data

Hypothetical model of wheat response to drought- and salt stress conditions is proposed (Figure 4.1). Model represents hypothetical signaling pathways. This model was mostly derived from *Arabidopsis* gene expression studies.

In leaf tissue, expressions of several genes reported previously as stress-related genes were induced in response to drought and salt stress conditions. Cloning of these genes and characterization of them provide more information about the stress mechanisms in wheat.

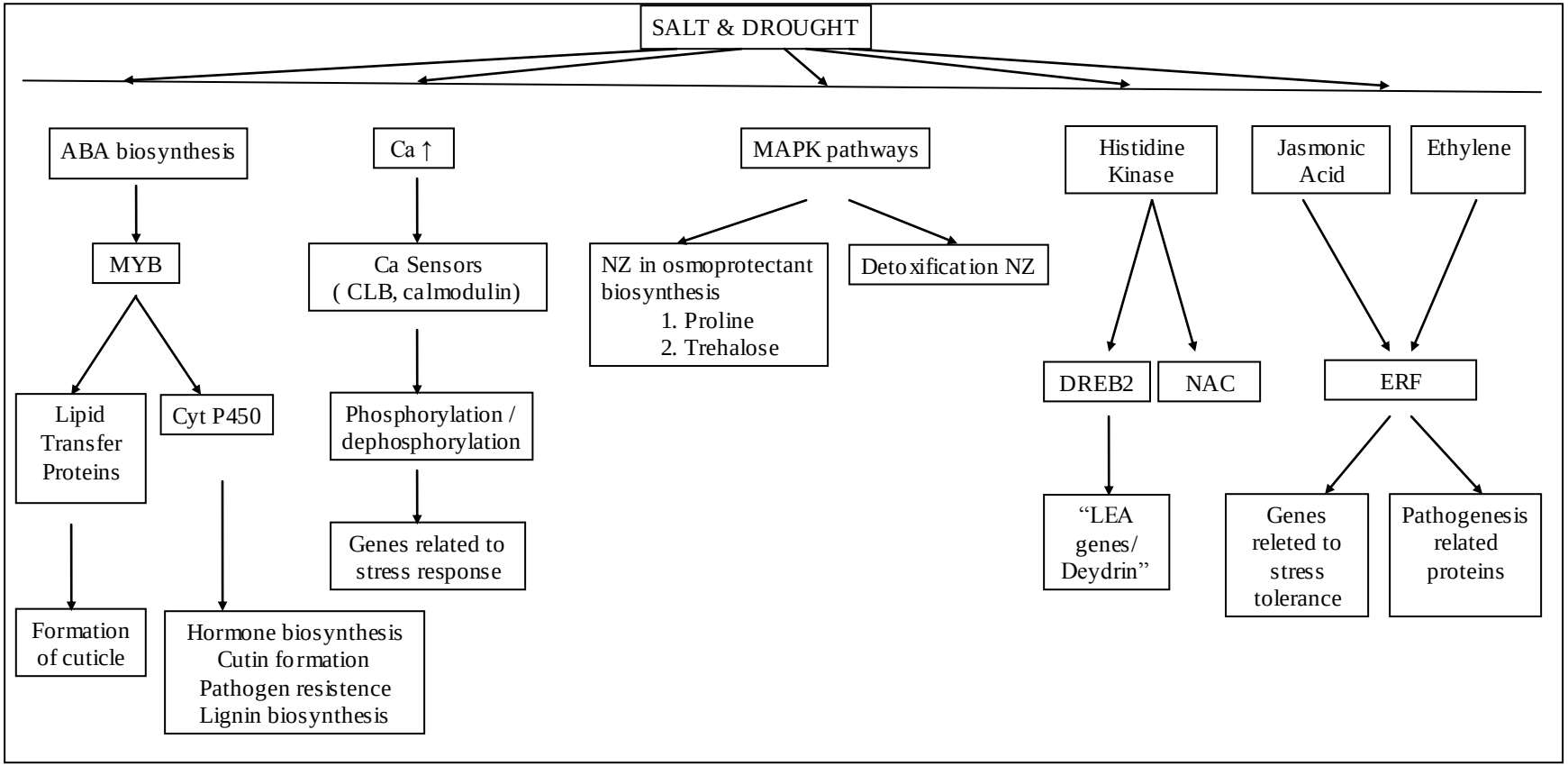


Figure 4.1 Hypothetical model of wheat response to drought- and salt stress conditions

CHAPTER 5

CONCLUSION

The aim of this study was to clone and examine the expression of wheat *TPS* as well as to analyze gene expression profiles of 10-days old seedlings in response to drought and salt stress treatments.

Wheat *TPS* gene, *TaTPS*, was cloned by RACE. Based on BLASTP analysis, the putative TaTPS showed great similarity with other TPS from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Zea mays*, *Selaginella lepidophylla*. Like other plant *TPS* genes, *TaTPS* contains "family GT-20" TPS domain at N-terminal and TPP domain at C-terminal. It can be conclude that this cDNA may encode functional wheat TPS protein.

In the present study, expression of *TaTPS* increased in root tissue in response to drought stress. Since transgenic plants accumulating trehalose showed enhanced tolerance to drought stress, increase in *TPS* expression in drought stressed root may be indicative stress tolerance. In leaf tissue, both salt and drought treatments repressed the expression of this gene. Decreased in abundance of *TaTPS* transcript in leaf tissue may be the result of tightly regulation of levels of T6P that has a role in plant growth and development.

Second part of this thesis consists of microarray profiling of drought- and salt-treated wheat roots and leaves using Wheat GeneChip (Affymetrix). Based on Venn diagram analysis, many genes were found to be induced by both treatments, which was indicative of a cross talk between salt and drought stresses. Many candidate drought- and salt-inducible genes were identified in both tissues. Among them, LEA, LTPs,

ENODs, histidine kinase, Transcription Factors (NAC, ERF, HSF, DREB, MYB), 1-pyrroline-5-carboxylate synthetase, glutathione-S-transferase, Ferittin, pathogenesis related genes, NCED, heat shock proteins, and ABC transporters were previously shown to be induced in response to salt and drought stress conditions using microarray. HKT genes were found to be repressed under salt stress. This result is meaningful since HKTs are low affinity Na^+ transporters. The decrease in the abundance of these transcripts under salinity may be indicative of improvement of plant salt tolerance.

In the future, following studies can be carried out:

1. Functionality of wheat TPS gene: Functionality of putative wheat TPS gene will be determined by complementation of yeast mutant.
2. Expression of TPS gene under salt and drought stress conditions: In this study, 24 hours drought and salt stress was applied to wheat. Examination of TPS expression at multi time points will probably give more information about the regulation of this gene.
3. Transgenic plants overexpressing wheat TPS: By transgenic wheat overexpressing TPS provide information about TPS function in wheat. In addition, fusion wheat TPS-TPP gene will be used to engineer agronomically important crops, which may lead to abiotic stress tolerant plants.
4. Validation of microarray: Real time quantitative RT-PCR analysis will be needed to validate microarray data.
5. Transgenic approach: In order to understand the functions of candidate genes found in this study, gain-of-function and loss-of-function genetic approaches will be needed. For instance, overexpression of transcripton factors (NAC, MYB, ERF, DREB) provide the information about their stress related roles as well as their downstream genes. Understanding function of candidate genes is important for the improvement of stress tolerance of agriculturally important crops by gene manipulation.

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APPENDIX A

Chemicals/Kits

Chemicals /Kits	Suppliers
Polyethylene glycol (PEG) 6000	Applichem
Diethyl pyrocarbonate (DEPC)	Applichem
β-mercaptophenol	Merck
Water saturated phenol	Applichem
Sarcosyl	Sigma
Sodium chloride	Applichem
Sodium citrate	Applichem
Guanidine thiocyanate	Sigma
Isopropanol	Merck
Chloroform	Applichem
Molecular Grade Alcohol	Applichem
Sodium Acetate	Applichem
Deoxyribonuclease I	Fermentas
GeneRuler DNA Ladder	Fermentas
RevertAid First Strand cDNA Synthesis Kit	Fermentas
Taq DNA polymerase	Fermentas
phenol-chloroform-isoamylalcohol (25:24:1)	Applichem
SMART RACE cDNA Amplification Kit	Clontech
streptavidin-phycoerythrin	Invitrogen
biotinylated anti-streptavidin antibody	Sigma
GeneChip(R) IVT Labeling Kit	Affymetrix
GeneChip(R) One-Cycle cDNA Synthesis Kit	Affymetrix
GeneChip(R) Sample Cleanup Module	Affymetrix

GeneChip(R) Poly-A RNA Control Kit	Affymetrix
GeneChip(R) Hybridization Control Kit	Affymetrix
Tween-20	Merck
herring sperm DNA	Promega
DMSO	Merck
Wheat GeneChip	Affymetrix
bovine serum albumin	Invitrogen

APPENDIX B

Hoagland's Solution (Plant Nutrient Solution)

Component	Stock Solution	mL Stock Solution/1L
2M KNO_3	202g/L	2.5
2M $Ca(NO_3)_2 \times 4H_2O$	236g/0.5L	2.5
FeEDTA	15g/L	1.5
2M $MgSO_4 \times 7H_2O$	493g/L	1
1M NH_4NO_3	80g/L	1
1M KH_2PO_4	136g/L	0.5
Minors:		1
H_3BO_3	2.86g/L	
$MnCl_2 \times 4H_2O$	1.81g/L	
$ZnSO_4 \times 7H_2O$	0.22g/L	
$CuSO_4$	0.051g/L	
$Na_2MoO_4 \times 2H_2O$	0.09g/L	

1) Make up stock solutions and store in separate bottles with appropriate label.
2) Add each component to 800mL deionized water then fill to 1L.
3) After the solution is mixed, it is ready to water plants.

APPENDIX C

The Preparation of Trizol Reagent

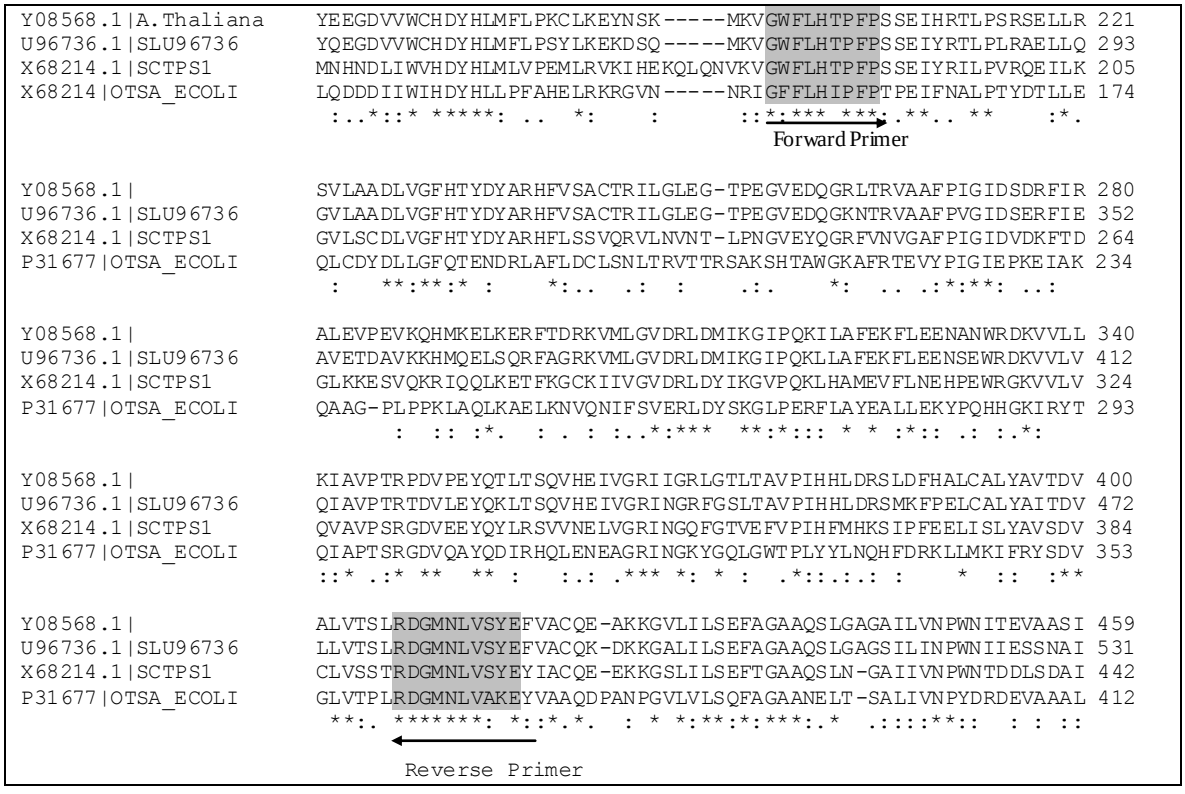
1. Prepare 100mM Sodium citrate at pH 7.0.
2. Prepare 2M Potassium acetate at pH 4.8.
3. Prepare the following solution in a DEPC-treated flask:

- Weigh out 23.7 grams of guanidine thiocyanate,
- Add 12.5 mL of 100mM sodium cirate (pH: 7.0),
- Add 715 μL of 35% sarcosyl,
- Add 340 μL of β-mercaptoethanol,
- Bring the volume to 50mL with DEPC-treated distilled water,

Autoclave the solution after mixing and dissolving all solid materials. After autoclaving, add 50 mL of water-saturated phenol and 10 mL of 2M potassium acetate (pH: 4.8) onto the solution.

APPENDIX D

Degenerate Primer Design



APPENDIX E

Internal Conservative Fragment

CTCNGNATTACGCACTCTGCATCACGCTCGGAGCTGCTTCGCTCCGTGCT
CTGCGCTGATTTAGTCGGATTTCATACATACGACTATGCAAGGCATTTCG
TGAGCGCATGTACCAGAATACTCGGACTCGAGGGTACCCCTGAAGGTGT
GGAGGACCAGGGNAAGTTAACACGGGTTGCAGCGTTTCCTATTGGGATA
GACTCTGATCGTTTCAAAAGGGCGTTGGACATTGATGCAGCAAAAAGAC
ATGTCAATGAATTGAAACAGCGATTTGCGGGACGGAAGGTAATGCTTGG
TGTTGATCGACTTGACATGATCAAAGGAATTCCCCAAAAGATTTTGGCCT
TTGAAAAGTTTCTTGAGGAAAACCCTGAATGGATTGATAAAGTGGTTCTA
CTTCAAATTGCTGTGCCAACTAGAACTGACGTCCCTGAGTATCAGAAGCT
TACAAGCCAAGTGCATGAAATTGTTGGGCCCATAAATGGACGATTTGGA
ACATTGTCTGCTGTTCCCATTCATCATCTGGATCGATCTCTTGATTTCCAT
GCCTTGTGTGCTCTTTATGCAgTCACTGATGTGGCTCTTGTAACATCACTG
AGCGACGGAATGAACCTTGAANGCTACNAAANCTT

APPENDIX F

TBLASTX Results

```
>gi|1865676|emb|Y08568.1|ATTPS  A.thaliana mRNA for trehalose-6-phosphate synthase
Length=2970

 Score =  363 bits (786),  Expect = 8e-98
 Identities = 153/177 (86%), Positives = 161/177 (90%), Gaps = 0/177 (0%)
 Frame = +3/+1

Query  3     LRSVLCADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGKLTRVAAFPIGIDSDRFK  182
             LRSVL ADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQG+LTRVAAFPIGIDSDRF
Sbjct  889   LRSVLAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGRLTRVAAFPIGIDSDRFI  1068

Query  183   RALDIDAAKRHVNELKQRFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPEWIDKVRL  362
             RAL++   K+H+ ELK+RF  RKVMLGVDRLDMIKGIPQKILAFEKFLEEN  W DKV L
Sbjct  1069  RALEVPEVKQHMKELKERFTDRKVMLGVDRLDMIKGIPQKILAFEKFLEENANWRDKVVL  1248

Query  363   LQIAVPTRTDVPEYQKLTSQVHEIVGRINGRFGTLSAVPIHHLDRSLDFHALCALYA  533
             L+IAVPTR DVPEYQ LTSQVHEIVGRI GR GTL+AVPIHHLDRSLDFHALCALYA
Sbjct  1249  LKIAVPTRPDVPEYQTLTSQVHEIVGRIIGRLGTLTAVPIHHLDRSLDFHALCALYA  1419


>gi|4100324|gb|SELAGINELLA LEPIDOPHYLLA  Selaginella lepidophylla trehalose-6-
phosphate synthase mRNA,
complete cds
Length=3223

 Score =  356 bits (772),  Expect = 7e-96
 Identities = 149/177 (84%), Positives = 161/177 (90%), Gaps = 0/177 (0%)
 Frame = +3/+3

Query  3     LRSVLCADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGKLTRVAAFPIGIDSDRFK  182
             L+ VL ADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGK TRVAAFP+GIDS+RF
Sbjct  984   LQGVLAADLVGFHTYDYARHFVSACTRILGLEGTPEGVEDQGKNTRVAAFPVGIDSERFI  1163

Query  183   RALDIDAAKRHVNELKQRFAGRKVMLGVDRLDMIKGIPQKILAFEKFLEENPEWIDKVRL  362
              A++ DA K+H+ EL QRFAGRKVMLGVDRLDMIKGIPQK+LAFEKFLEEN EW DKV L
Sbjct  1164  EAVETDAVKKHMQELSQRFAGRKVMLGVDRLDMIKGIPQKLLAFEKFLEENSEWRDKVVL  1343

Query  363   LQIAVPTRTDVPEYQKLTSQVHEIVGRINGRFGTLSAVPIHHLDRSLDFHALCALYA  533
             +QIAVPTRTDV EYQKLTSQVHEIVGRINGRFG+L+AVPIHHLDRS+ F  LCALYA
Sbjct  1344  VQIAVPTRTDVLEYQKLTSQVHEIVGRINGRFGSLTAVPIHHLDRSMKFPELCALYA  1514
```

APPENDIX G

cDNA Sequence of Wheat TPS

>gi|206600898|gb|**FJ167677.1**| Triticum aestivum trehalose-6-P synthase (TPS) mRNA, partial cds

ATGAAGCAGCGCCTCCTCGTCGTGGCCAACCGCCTCCCCGTCTCCGCCAATCGCCGCGGCGAGGATCAGTGGTCCC
TGGAGATCAGCGCCGGTGGCCTCGTCAGCGCGCTCCTCGGTGTGAAAGATGTCGACGCGAAGTGGATCGGCTGGGC
CGGTGTGAATGTCCCCGACGAGGTCGGCCAGCAGGCTCTCACCAATGCACTCGCCGAGAAGAGATGCATACCAGTC
TTCCTGGACGAGGAGATCGTGCACCAGTACTACAACGGCTACTGCAACAACATACTGTGGCCGCTCTTCCACTACC
TCGGGCTGCCGCAGGAGGACAGGCTGGCAACCACCCGGAACTTCGAGTCGCAGTTCGACGCGTACAAGCGGGCCAA
CCAGATGTTTGCTGATGTCGTCTACCAGCACTACCAGGAAGGGGATGTGATCTGGTGCCATGACTACCACCTCATG
TTCCTGCCCAGGTGCCTCAAGGAGCATGACATCAACATGAAGGTCGGGTGGTTCCTGCACACGCCCTTCCCTTCCT
CGGAGATTTACCGCACTCTGCCATCACGCTCGGAGCTGCTTCGCTCCGTGCTCTGCGCTGATTTAGTCGGATTTCA
TACATACGACTATGCAAGGCATTTCGTGAGCGCATGTACCAGAATACTCGGACTCGAGGGTACCCCTGAAGGTGTG
GAGGACCAGGGAAAGTTAACGCGGGTTGCAGCGTTTCCTATTGGGATAGACTCTGATCGTTTCAAAAGGGCGTTGG
ACATTGACGCAGCAAAAAGACATGTCAATGAACTGAAACAGCGATTTGCGGGACGGAAGGTAATGCTTGGTGTTGA
TCGACTTGACATGATCAAAGGAATTCCCCAAAAGATTTTGGCCTTTGAAAAGTTTCTTGAGGAAAACCCTGAATGG
ATTGATAAAGTGGTTCTACTTCAAATTGCTGTGCCAACTAGAACTGACGTCCCTGAGTATCAGAAGCTTACAAGCC
AAGTGCATGAAATTGTTGGGCGCATAAATGGACGATTTGGAACATTGTCTGCTGTTCCTATTCATCATCTGGATCG
ATCTCTTGATTTCCATGCCTTGTGTGCTCTTTATGCAGTCACTGATGTGGCTCTTGTAACATCACTGAGGGATGGC
ATGAATCTTGTAAGCTACGAATATGTTGCATGCCAGGGATCAAAAAAAGGAGTTCTGATATTGAGTGAGTTTGCCG
GTGCAGCACAATCGCTTGGTGCTGGTGCCATTCTTGTAAATCCCTGGAATATTACAGAAGTTGCAGACTCAATAAA
ACATGCTTTGACAATGACATCTGATGAGAGAGAGAAGCGGCACAGGCATAACTACGCGCATGTAACAACTCATACC
GCCCAAGATTGGGCTGAAACTTTTGTATGTGAGCTAAACGATACAGTTGCTGAAGCTCTGATGAGAACAAGACAAG
TTCCCCCTGACCTTCCTAGTCGAACGGCCATCCAGCAATATCTGCAGTCAAAAAACCGTTTGCTCATATTGGGTTT
CAATTCAACATTGACCGAGCCAGTTGAATCCTCTGGGAGACGGGGCGGTGATCAAGTCAAGGAGATGGAACTCAAG
TTGCATCCTGACTTAAAGGGTCCTTTGAGAGCCCTCTGCGAGGACGAGAGCACTACGGTTATCGTTCTCAGCGGAA
GCGACAGGAGTGTTCTTGATGAAAATTTCGGAGAATTTAACTTGTGGCTGGCAGCAGAGCATGGGATGTTCTTACG
CCCAACTGATGGAGAATGGATGACAACAATGCCTGAGCATCTGAACATGGATTGGGTCGACAGTGCAAAGCATGTT
TTTGAGTACTTCACAGAAAGAACCCCAAGATCTCATTTTGAACATCGTGAAACATCATTTGTGTGGAATTACAAGT
ATGCCGATGTTGAGTTTGGGAGGCTCCAAGCAAGAGATATGCTGCAGCACTTGTGGACCGGTCCAATCTCAAATGC
AGCTGTGGATGTTGTTCAAGGGAGCCGTTCAGTTGAAGTTCGCTCTGTTGGAGTTACAAAGGGTGCTGCAATTGAT
CGTATTCTAGGAGAGATAGTTCACAGCAAAAGCATGGTTACTCCGATTGACTATGTGCTATGCATAGGCCACTTCC
TAGGAAAGGACGAAGACATCTATGTGTTTTTTGACCCTGAATACCCTTCTGAGCCAAAAGTGAAACCGGACGGTGC
GTCGGTATCCGTCGACAGGAGGCAGAACGGGCGGCCATCAAACGGCCGGAGCAACTCGAGGAACTCGCAGGCGAGG
ACACAAAAGCCTCAGGTCGCGCCGCCGCCTCCGGAGAGGTCATCGTCGTCATCCGACCACAGCACCGCAAACAACA
ACAGCCACCACGACTGGCGCGAAGGGTCGTCGGTCCTCGACCTCAACGGCGACAACTACTTCTCCTGCGCGGTCGG
GAGGAAGCGCTCCAACGCCCGTTACCTGCTCAACTCGTCGGAGGACGTCGTCTCATTCCTTAAGGAGATGGCGGAG
TCGACGACGCCCCGCGCCGGTGGCCTCCCGCCCGGCGCTGCCGCGGACTACATGTTCTTGGATAGGCAGTAGACCA
AGTTTGCCTCTGCCAATCAGAATCACCCGGGCCGCCCCGCCCCGCCCCGCCGGAGAAGAAGAGTTAACAACAGCCC
TGAATAACATCGGTGTTCCCTGTACGCGCGCCGGAGTAGCCGCTGTCGCTGGCCCGGCGCGGCGTGGTGTACAATC
CTGTACAAAGTATGACTGAGCTCCTGGTTTTACTGTTACTGCGAGAGGAGACTGACGCTCTGTGACCGTGAGTGAT
CCCGAGAGTTTGTGCTAGGCGGAATTGATGGATATTTGTTATTATGTGATGTGATGACTAATCTCAGTATTGACTG
TCTATCTCTGGTTCAAAAAAAAAAAAAAAAAAAAAAAAA

APPENDIX G

GeneChip Probe Sets Related to Trehalose Biosynthesis Genes

Trehalose-6-phosphate phosphatase

Probe ID	e-value	Functional Annotation	Matching Organism
Ta.10237.1.S1_at	1.00E-126	Trehalose-6-phosphate phosphatase	*Oryza sativa*
Ta.10237.2.S1_at	2.00E-47	Trehalose-6-phosphate phosphatase	*Oryza sativa*
Ta.5372.1.S1_at	1.00E-156	Trehalose-6-phosphate phosphatase	*Oryza sativa*
Ta.6144.1.S1_a_at	1.00E-153	trehalose-phosphatase B	*Oryza sativa*
Ta.6144.2.S1_a_at	1.00E-153	trehalose-phosphatase B	*Oryza sativa*
TaAffx.113062.1.S1_at	4.00E-47	Trehalose-phosphatase family	*Oryza sativa*
TaAffx.112809.1.S1_at	7.00E-37	Trehalose-phosphatase	*Oryza sativa*
TaAffx.58849.1.S1_at	1.00E-113	Ramosa 3	*Zea mays*

Trehalose-6-phosphate synthase/phosphatase

Probe ID	e-value	Functional Annotation	Matching Organism
Ta.20649.1.S1_a_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.20649.1.S1_x_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.20649.2.S1_a_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.20649.3.S1_x_at	4.00E-33	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.30416.1.S1_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.30416.2.S1_a_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.30416.2.S1_at	1.00E-40	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.30416.3.S1_a_at	0	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
Ta.30416.3.S1_x_at	4.00E-33	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
TaAffx.110156.1.S1_at	2.00E-49	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
TaAffx.35347.1.S1_at	6.00E-38	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*
TaAffx.6695.1.S1_at	1.00E-35	Trehalose-6-phosphate synthase/phosphatase	*Oryza sativa*

Trehalose-6-phosphate Synthase

Probe ID	e-value	Functional Annotation	Matching Organism
Ta.14558.1.S1_at	0	Trehalose-phosphate synthase	*Oryza sativa*
Ta.25334.1.A1_at	9.00E-51	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6470.1.S1_at	0	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6470.1.S1_x_at	0	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6470.2.S1_a_at	0	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6470.2.S1_x_at	9.00E-37	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6534.2.S1_at	1.00E-146	Trehalose-6-phosphate synthase	*Musa balbisiana*
Ta.26810.1.A1_at	2.00E-40	Trehalose-6-phosphate synthase	*Oryza sativa*
TaAffx.37036.1.S1_at	4.00E-69	Trehalose-6-phosphate synthase	*Musa balbisiana*
TaAffx.51134.1.S1_at	4.00E-54	Trehalose-6-phosphate synthase	*Oryza sativa*
Ta.6534.1.S1_at	1.00E-151	Trehalose-phosphate synthase	*Oryza sativa*
Ta.6534.1.S1_s_at	1.00E-151	Trehalose-phosphate synthase	*Oryza sativa*
TaAffx.128418.75.S1_at	5.00E-30	Trehalose-phosphate synthase	*Oryza sativa*

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Gençsoy Ünsal, Beray
Nationality: Turkish (TC)
Date and Place of Birth: 27 November 1976, Trabzon
Marital Status: Married
email: berayunsal@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Biochemistry Department	2002
BS	METU Biology Department	1999

WORK EXPERIENCE

Year	Place	Enrollment
2001-2006 November	METU Informatic Institute	Research Assistant

PUBLICATIONS

Thesis

"Biochemical Characterization of Trehalase from Wheat Suspension." Master of Science Thesis. Biochemistry METU 2002.

Conference and Workshop Publications

ÜNSAL Beray Gençsoy, Hamamcı H., Öktem, Yücel M. "Buğday Trehaloz-6-Fosfat (TPS) Geninin Belirlenmesi" 15. Ulusal Biyoteknoloji Kongresi Antalya/TÜRKİYE (2007) pp:93-95

Beray Gençsoy Ünsal, Mehmet Tufan Öz, Remziye Yılmaz, Haluk Hamamcı, Hüseyin Avni Öktem, Meral Yücel "Transcription Profiling of Wheat Seedlings (Triticum aestivum L.) under Salt and Drought Stress Conditions" International Workshop on Nanobiotechnology and Genome Technologies October 31-November 3 2007 Antalya, TURKEY Abstract Book 39

Gençsoy Ünsal,B., Yücel,M., Hamamcı,H. and Öktem,H.A., *Triticum aestivum* trehalose-6-P synthase (TPS) mRNA gene, gene bank accession no FJ167677

PROJECTS

- **TOVAG 106O139**

"Isolation of Trehalose-6-phosphate Synthase Gen From Wheat and Examination of the Gene Expression Under Drought and Salt Stresses" Prof. Dr. Meral YÜCEL, Prof. Dr. Haluk HAMAMCI, Prof. Dr. Hüseyin Avni ÖKTEM, Beray Gençsoy ÜNSAL (2006-2008)