CO-REGULATION OF HEAT-SHOCK PROTEIN 90 (HSP90) AND TRANSGLUTAMINASE (TGase) IN *BRACHYPODIUM DISTACHYON* UNDER ABIOTIC STRESS

by
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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2019

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ACKNOWLEDGEMENTS

First of all, I would like to thank my supervisor Assist. Prof. Dr. Bahar Soğutmaz Özdemir for her endless support, guidance and encouragement in my life since the first time we met. I consider myself very lucky to have a mentor who cared me as much as my work.

I would like to thank my co-supervisor Prof. Dr. Dilek Telci for her guidance, patience and useful critiques during this research. I consider myself lucky to have a co-supervisor who trusts me as an intern and accepts working together for this project.

I would like to thank Ayça Ece Nezir and Halime İlhan Sığınç for their guidance in the laboratory and during experiments. I would like to offer my special thanks to Özge Tatlı for her endless support, encouragement, help and for making me hopeful when I'm about to hit the bottom.

I am thankful to all of the members of my two labs, Molecular Cell Biology and Plant Tissue Culture and Genetics groups for making my journey unforgettable.

I want to thank Assist. Prof. Dr. Hüseyin Çimen and YediProt group for offering me a workplace, their help and considering me like one of them.

I am thankful to my dearest friends, Meltem Selen Önal, Duygu Orak, Selen Tiryaki, Ürün Ukan, Beyza Kocaoğlu, Oktay Akyürek, Deniz Uras and Dorukan Gürek for their support and time to cheer me up during my never ending thesis period. A special thanks to Murat Kobaneri for his infinite love, support, patience and encouragement. They are always by my side during this journey.

Finally, I am thankful for my beloved family Şebnem Korkmaz, Ümit Korkmaz and Aysun Korkmaz for their endless support and love during my ups and downs. Without their support, I cannot be who I am today.

ABSTRACT

CO-REGULATION OF HEAT-SHOCK PROTEIN 90 (HSP90) AND TRANSGLUTAMINASES (TGases) IN *BRACHYPODIUM DISTACHYON* UNDER ABIOTIC STRESS

Drought and salinity are two most encountered and severe abiotic stress conditions that cause reduction in crop yield and productivity. Plants continuously encounter stress conditions due to their sessile structure and basic response pathways of plants include heat-shock proteins (HSPs). Main function of HSPs is defined as performing intracellular chaperone role by facilitating protein folding. HSPs can be found in different molecular weights as HSP60, HSP70, HSP90, HSP100 and small heat shock proteins (sHSPs). HSP90 constitutes 1 to 6 percent of the total protein in the cell. HSP90 expression levels are influenced by free or bound polyamines (PAs). Transglutaminase (TGase) is another protein that interacts with PAs and work by means of catalyzation of protein cross-linking and post-translational protein modification as well as free PA incorporation. Both HSP90 and TGase are found to be involved in stress response and their expression levels are affected by stress conditions. In the current study, a monocot model plant *Brachypodium distachyon* (Bd21 line) was exposed to drought (12 days water withholding) and salinity (14 days 320 mM NaCl treatment) stress at their vegetative stage in order to determine whether HSP90 and TGase are co-regulated under abiotic stress. Under drought stress, both HSP90 and TGase transcript levels were increased by approximately 2-fold and 1.11-fold respectively whereas HSP90 protein levels were upregulated by 2-fold. However, TGase protein levels were slightly downregulated. Upon salinity treatment, TGase transcript levels were downregulated by almost 6-folds and also HSP90 transcripts were in a downregulation trend whereas HSP90 and TGase protein levels displayed downregulation by 80 and 82 percent, respectively. Under salinity stress, transcript levels changes of mitochondrial and chloroplastic HSP90 and TGase are parallel together with close downregulation of protein expression levels. Hence, there might be a possible interaction between HSP90 and TGase in *Brachypodium distachyon* under salinity stress. This study highlights the importance of molecular mechanisms behind drought and salinity stress.

ÖZET

ABİYOTİK STRES ALTINDA *BRACHYPODİUM DİSTACHYON*DA HEAT-SHOCK PROTEİN 90 (HSP90) VE TRANSGLUTAMINAZ (TGases) İFADELERİNİN REGÜLASYONU

Kuraklık ve tuz stresi bitkilerin verimliliklerini azaltan en ciddi abiyotik stres türlerindendir. Bitkiler, hareketsiz yapıları nedeniyle sürekli olarak stres koşullarıyla karşılaşırlar ve strese hızlı bir şekilde cevap vermeleri gerekir. Bitkilerin temel tepki yolakları, stres proteinleri olarak da bilinen heat-shock proteinlerini (HSP'ler) içerir. HSP'lerin ana işlevi, protein katlanmasını kolaylaştırarak protein-protein etkileşimlerinde hücre içi şaperon rolünün gerçekleştirilmesi olarak tanımlanmaktadır. HSP'ler 5 farklı moleküler ağırlıkta bulunabilir ve bunlara göre HSP60, HSP70, HSP90, HSP100 ve küçük ısı şok proteinleri (sHSP'ler) olarak isimlendirilirler. HSP90, hücredeki toplam proteinlerin yüzde 1 ile yüzde 6'sını oluşturur ve ekspresyon seviyeleri, serbest veya bağlı form olarak bulunabilen poliaminler (PA)'den etkilenebilir. Transglutaminaz'lar, PA'ler ile etkileşime girebilen bir diğer protein türüdür ve protein çapraz bağlanmasını, translasyon sonrası protein modifikasyonlarını ve PA birleşimini katalize eder. Hem HSP90 hem de TGaz'ların ekspresyon seviyeleri stress koşullarından etkilenmektedir ve stress cevabı oluşturmada etkili oldukları görülmüştür. Bu çalışmada, HSP90 ve TGaz ifadelerinin regülasyonunu anlamak amacıyla, tek çenekli model bir bitki olan *Brachypodium distachyon* (Bd21 hattı) vejetatif evresinde kuraklık (12 gün susuz bırakma) ve tuz (320 mM NaCl uygulaması) stresine maruz bırakılmıştır. Kuraklık stresi altında, HSP90 ve TGaz transkript seviyeleri sırası ile 2-kat ve 1.11-kat artış gösterirken, HSP90 protein seviyesi 2-kat artış göstermiştir. Ancak TGaz protein seviyeleri az bir düşüş göstermiştir. Tuz stresi altında ise TGaz transkript seviyeleri yaklaşık 6-kat düşüş göstermiş, aynı zamanda HSP90 gen seviyelerinde de düşüş trendi görülmüştür. HSP90 ve TGaz protein seviyelerinde ise sırası ile yüzde 80 ve 82 düşüş görülmüştür. Tuz stresi altında, mitokondriyal ve kloroplast HSP90 ve TGaz transkript ve protein seviyelerindeki düşüş paralellik gösterdiğinden dolayı, tuz stresi altında *Brachypodium distachyon* bitkisinde HSP90 ve TGaz etkileşim halinde olabilir. Bu çalışma, kuraklık ve tuz streslerinin ardındaki moleküler mekanizmaları anlamanın önemini vurgulamaktadır.

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°C	Degree centigrade
3D	3-Dimension
Bp	Basepair
Ca^{++}	Calcium
cm	Centimeter
kDa	Kilodalton
Mbp	Megabasepair
µg	Microgram
µg/ml	Microgram per milliliter
µM	Micromolar
ml	Milliliter
mM	Millimolar

ACD	Alpha-crystallin domain
APS	Ammoniumpersulfate
ATP	Adenosinetriphosphate
BSA	Bovine serum albumin
C-terminal	Carboxy-terminal
CAM	Crassulacean acid metabolism
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ClpB-C	Caseinolytic peptidase B protein homolog - cytosolic
ClpB-M	Caseinolytic peptidase B protein homolog - mitochondrial
ClpB-P	Caseinolytic peptidase B protein homolog - plastidial
dH_2O	Distilled water

DNA	Deoxyribonucleic acid
DTT	Diothriuretiol
EC	Electrical conductivity
ECL	Electrochemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and agriculture organization
Gln	Glutamine
Glu	Glutamic acid
HOP	HSP70/HSP90 organizing protein
HSE	Heat shock element
Hsf	Heat shock factor
HSG	Heat shock gene
HSP	Heat shock protein
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
HSP100	Heat shock protein 100
IPCC	Intergovernmental panel on climate change
KCl	Pottasium chloride
MetOH	Methanol
mRNA	Messenger RNA
N-terminal	Amino-terminal
NaCl	Sodium chloride
NBD	Nucleotide binding domain
PA	Polyamine
PIS	Perchloric acid insoluble
PMSF	Phenylmethylsulfonyl fluoride
Put	Putrescine
R groups	Reactive groups
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RWC	Relative water content

SBD	Substrate binding domain
SDS	Sodium dodecyl sulfate
sHSP	Small heat shock protein
Spd	Spermidine
Spm	Spermine
SOS	Salt overly sensitive
TEMED	Tetramethylethylenediamine
TGase	Transglutaminase
TG1	Transglutaminase type 1
TG2	Transglutaminase type 2
TG3	Transglutaminase type 3
TG4	Transglutaminase type 4
TG5	Transglutaminase type 5
TG6	Transglutaminase type 6
TG7	Transglutaminase type 7
TPR	Tetratricopeptide repeat
Tris-HCl	Tris hydrochloride

1. INTRODUCTION

World population is estimated to increase 34 percent and reach 9.1 billion by 2050. According to Food and Agriculture Organization (FAO) (2009), cereal production will need to rise about 1 million tone to supply the food demand for the rising population. Besides exponential growth of the population, climate change is another parameter that worsens the scenario. The atmospheric temperature is predicted to be 4^0C warmer in 2080, leading a doubling in CO_2 concentration [1]. Stress-free land sources compose only the 3.4 percent of the agricultural lands [2]. In parallel with climate change, International Panel of Climate Change (IPCC) predicted that drought and salinity stress, which are the most seen stress factors, will cause approximately 50 percent decrease in the yield of major crops [3,4]. All the information and obstacles stated above implies that "producing more with less" has become a crucial necessity [5].

Due to their sessile structure, plants adapt to the stress conditions by activating and deactivating their molecular mechanisms. Heat-shock proteins (HSPs), also known as stress proteins, are involved in basic molecular response pathways of plants and function as molecular chaperones by facilitating correct protein folding. HSPs are named according to their approximate molecular weights as HSP60, HSP70, HSP90, HSP100 and small HSPs (sHSPs). HSP90 stands out amongst others by consisting almost 1 percent of the total proteins in cell under normal conditions. Under stress conditions, its level can increase up to 6 percent. HSP90 level in cell can be influenced by polyamines (PAs) which are found as free or bound form. PAs are low molecular weight molecules that are naturally found, in all living organisms. They are involved in growth, reproduction, development, adaptation, survival and stress response of plants. The most common plant PAs are Putrescine (Put), Spermine (Spm) and Spermidine (Spd).

Another protein that interacts with PAs is TGase, which can be found in cells of multicellular organisms. The main function of TGase is catalyzing cross-linking and post-translational protein modifications. TGase interaction with PAs is its involvement in PA incorporation. Expression levels of both HSP90 and TGase are affected by stress conditions and each was found to be involved in abiotic stress response. There are no reports that contain both HSP90 and TGase interaction in plants. However, in animal studies, HSP90 is found to be targeted

by TG2 in order to provide cytoprotection in H9c2 (rat embryonic cardiomyoblast-derived) cells. In human fibro sarcoma cells (2fTGH), TG2 and HSP70/HSP90 are found to be interacted via the adaptor protein HOP (HSP70-HSP90 organizing protein) under normal conditions.

In this study, *Brachypodium distachyon*, a monocot model plant, was chosen to understand the HSP90 and TGase co-regulation under drought and salinity stress. *Brachypodium distachyon* is an important model plant that can reflect the mechanisms in other monocot plants, especially economically important cereals and forage grasses. For this purpose, *Brachypodium distachyon* Bd21 line was grown under controlled greenhouse conditions until plants reached their vegetative state. Stress treatments were carried out as 12 days with water withholding and 14 days with 320 mM NaCl application for drought and salinity stress treatments respectively. Expression levels of TGase and HSP90 genes (8 genes of HSP90 distributed in ER, cytoplasm, mitochondria and chloroplast) were determined by qRT-PCR using UBC18 (ubiquitin-conjugating enzyme 18) gene as a reference. The relative expression levels of HSP90 and TGase are determined by western blot with using HSP90 and TGase specific antibodies. Under drought treatment relative HSP90 gene levels showed an almost 2-fold upregulation trend and HSP90 protein levels were upregulated by 2-fold. TGase gene level was changed 1.11-fold and TGase protein levels were 12 percent downregulated. On the other hand, under salinity stress, HSP90 gene levels are found to be approximately 6-fold and HSP90 protein levels are 80 percent downregulated. Transcript level of TGase showed an almost 6-fold downregulation and at TGase protein levels showed 82 percent downregulation. The gene and protein expression levels, together with known mechanisms and PA interactions under drought and salinity stress, have unraveled the possibility of an indirect interaction between HSP90 and TGase in *Brachypodium distachyon*. This study highlights the importance of molecular mechanisms behind drought and salinity stress. Understanding the molecular mechanisms behind osmotic stress will broaden the view for stress resistant crop production.

2. LITERATURE REVIEW

2.1. *BRACHYPODIUM DISTACHYON*

Model organisms allow researchers to understand complex organisms by studying similar but simpler species. At the dawn of 20th century Thomas Hunt Morgan used *Drosophila melanogaster* (fruit fly) to show that genes are located on chromosomes [6]. Ever since *Drosophila* is known as a model organism to study genetics, and the number of model organisms increased. In terms of plants, *Zea mays* (maize) is the first model organism to study kernel pigmentation by Edward M. East and Rollins A. Emerson. About 70 years later, *Arabidopsis thaliana* became a popular model plant because of its small and simple genome size, short lifecycle and capacity to produce many seeds. Also it has been representing the flowering plants in particular the dicotyledons. However, the difference between dicotyledonous and monocotyledonous plants created a demand for a monocotyledonous model plant. *Zea mays* and *Oryza sativa* (rice) were strong candidates for being the monocotyledonous model plant, but both plants have limitations such as having large physical stature long life cycle and a demanding growth conditions [7].

In 2001, a new monocotyledonous plant, *Brachypodium distachyon,* became popular with its small genome size, rapid life cycle and simple growth conditions [8].

The natural habitat of *Brachypodium* is based around Mediterranean region, and north border reaches to Europe and south to Indian subcontinent [9]. *Brachypodium* belongs to Poaceae family with a close relationship with wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and rye (*Secale cereale*) (Figure 2.1) [9]. Besides its small genome, approximately 20 cm short stature, 8-12 weeks lifecycle, self-pollination ability, lacking seed shattering and simple growth conditions are well suited to a model plant [8,10,11]. In addition, highly efficient *Agrobacterium*-mediated transformation methods [12–14], EST libraries and sequences [15], BAC libraries and sequences [16,17], mutagenesis protocols, physical maps, and the complete genome sequence of diploid cytotype (2n=10) make the *Brachypodium* a suitable monocotyledonous model plant [18].

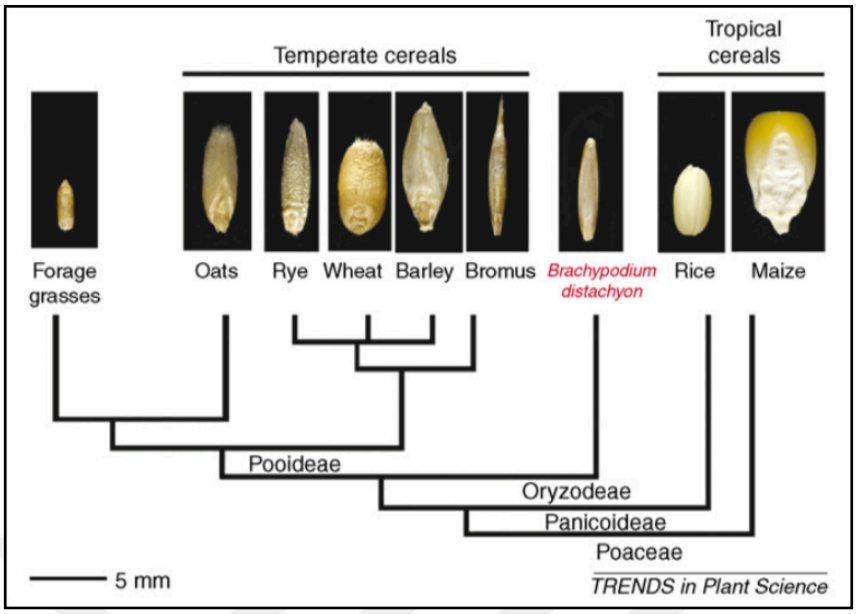


Figure 2.1. Phylogenetic relationship between *Brachypodium distachyon* and other important monocots [9].

272 Mbp genome size that lies between 157 Mbp *Arabidopsis* and 490 Mbp rice, makes *Brachypodium distachyon* (2n=10) the smallest known grass genome [19].

2.2. STRESS FACTORS

All living organisms, including plants, require the best optimal environment to grow and develop. These optimal conditions occur rarely in natural environment. Due to their sessile structure, plants encounter environmental changes much more than other living organisms. Therefore, plants evolved to develop complex mechanisms to give quick response and adapt to environmental changes. Any stimulus that negatively affects plant's natural growth, development and productivity is defined as 'stress factor'. These factors appear in two major groups as biotic and abiotic stress factors (Figure 2.2) [20]. Researches mostly focus on the biological responses and identification of molecules that have role in these responses and tolerance to stress. In the case of stress factor, several interactions and signaling pathways are activated. Kinase reactions, reactive oxygen species (ROS) production and hormone accumulation are the initial molecular responses to stress [21].

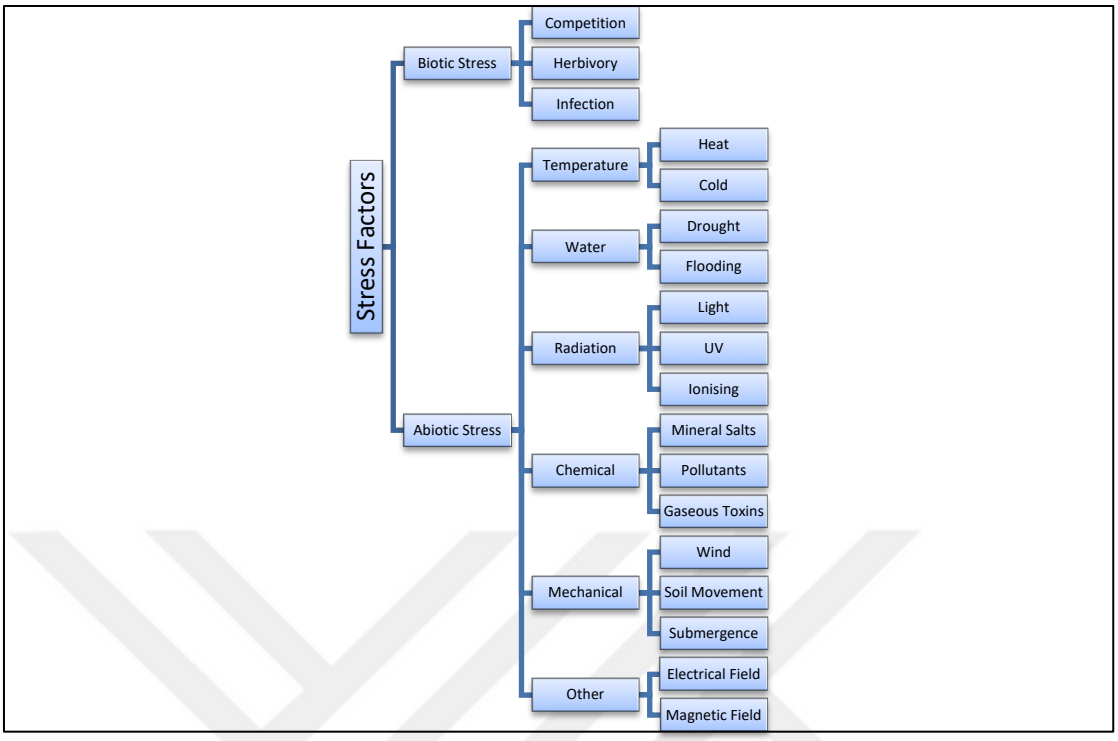


Figure 2.2. Environmental stress factors (modified from Tatlı *et al.,* 2017) [22].

2.2.1. Biotic Stress

'Biotic stress' is the stress type that occurs due to the attack of living organisms. Bacteria, fungi, viruses, parasites, insects and weeds are the main causes of biotic stress. The damage done by those living organisms are very similar to each other; therefore, diagnosis is difficult even with detailed observation. Biotic stressors cause a decrease in plant yield [23].

2.2.2. Abiotic Stress

The term 'abiotic stress' refers to stressors as non-living organisms that arise from environmental changes. Drought, salinity, heavy metals, freezing, flooding, light intensity, changes in nutrients and pollution are some of the abiotic stress factors. With global warming, abiotic stress factors are predicted to increase significantly in near future according to Intergovernmental Panel on Climate Change (IPCC) [4]. Therefore, the priority of plant sciences is to understand the molecular mechanisms of abiotic stress response [21,24]. Abiotic stress may appear due to various factors, so it was predicted that there should have

been one strong cellular molecule required for plant response to each stress. According to the previous reports, reactive oxygen species (ROS) was produced in different forms and locations in general response to all types of abiotic stress [25–30]. ROS is highly toxic; however, it also serves as the inducer of gene expression leading either programmed cell death or production of scavenging proteins. In order to preserve cellular functions, detoxification and production of ROS must be in balance and kept under control [21,31] .

2.2.2.1. Drought Stress

Improving the yield is the greatest goal of agriculture. However, increasing atmospheric temperature reduces the available water in soil while plants continue to lose water by transpiration and evaporation causing drought stress. Water deficit or drought stress is one of the biggest issues against sustainable food production by causing a reduction in plant growth and yield [32]. Characterization of drought stress is provided by water content reduction, diminishment of leaf water potential and turgor loss, stomatal closure, cell enlargement and reduction in growth [33]. There is a complex cellular activity upon drought stress. The major role of this network belongs to protein expression. Reprogramming in transcription is the first step of drought stress response and it continues with physiological changes such as stomatal closure, osmolite and antioxidant synthesis [34–36].

2.2.2.2. Salinity Stress

High salinity has also direct effect on the decrease of plant yield. Due to high salinization, arable lands will dramatically decrease in near future [37]. Especially in irrigated areas, salt stress negatively affects agriculture worldwide [38]. According to FAO reports (2000), 831 million hectares of soil is salt-affected [39]. Factors that are responsible for the soil salinity can be divided into two main groups as primary and secondary salinization. Primary salinity is caused by natural causes such as rock weathering, sea water intrusion, wind-blown salt laden sand and impeded drainage while secondary salinity arise from human activities like fertilizer overuse, natural plant cover removal and poor quality ground water use for irrigation [37]. Sodic and saline soils are the two types of salt-effected soils. The discrimination between the two is the sodic soils contains exchangeable sodium at excess

concentrations while in saline soils all ion levels are high [40]. The main reason of the salinity stress is the excess amount of sodium (Na^+) and chloride (Cl^-) ions in soil that causes a decrease in osmotic potential of plants, thus the roots cannot reach the available water [37]. High saline ions in soil diminishes germination, plant growth, reproduction capacity, photosynthesis, respiration, cell membrane properties, balance of the nutrients, enzymatic and metabolic activities, homeostasis and ROS production in early developmental stages of plant, but at severe conditions salt stress leads to plant death [41–44]. In order to control ion homeostasis and Na+ exclusion, salt overly sensitive (SOS) pathway a well-defined signaling pathway, is required to be activated. The SOS signaling pathway is known to be the key mechanism for ion homeostasis and Na^+ exclusion. [45].

Salinity stress protein and stress-associated proteins are two groups of proteins that are induced by salinity stress. Stress-associated proteins are distinguished from salinity stress proteins by their accumulation in all kinds of abiotic stress whereas salinity stress proteins accumulate only due to salinity stress [46]. Protein accumulation play a critical role in osmotic adjustment [47]. Proteins that are increased in expression under salinity stress might be present with low expression in the absence of stress stimulus or have *de novo* synthesis in response to stress.

2.3. HEAT-SHOCK PROTEINS (HSPs)

2.3.1. Protein Folding

The structure of the protein is determining the protein function. A newly synthesized protein, found as amino acids in a polypeptide chain, is called primary structure. The secondary structure shows a folded polypeptide chain due to an interaction between backbone atoms. α-helices and β-pleated sheets, which occur by hydrogen bonding between carbonyl O and amino H atoms, are the most common versions of secondary structures. In the next level, R groups of polypeptide chains form disulfide bonds and hydrophobic interactions and the total chain becomes three-dimensional, known as the tertiary structure. The proteins that need multiple polypeptide chains, called subunits, need quaternary structure to reach their functional (native) forms. Quaternary structure occurs when different polypeptide chains bind together and become a functional protein. The whole process of reaching the final 3D

protein structure is known as protein folding or protein self-assembly and the main reason is to lower its free energy. Self-assembly can be occur in two different ways: some polypeptide chains can fold by themselves however appropriate molecular chaperone must support the assembly of the rest [48,49]. In order to pass through membranes to organelles or to the extracellular matrix (ECM), most proteins stay unfolded to an almost linear conformation and such proteins refold into their native configurations once membrane transfer is complete [48].

2.3.1.1. Strict Self-Assembly

Some polypeptide chains do not need any assistance to reach their native form. They have the ability to assemble themselves correctly in cell and the only requirement is the primary structure itself. This type of assembly is known as strict self-assembly [49].

2.3.1.2. Assisted Self-Assembly

In contrast to strict self-assembly, appropriate molecular chaperone is needed in addition to the primary structure for the assembly of some proteins. Existence of a molecular chaperone prevents misassembly either by creating an energy barrier for incorrect folding or reducing the activation energy of the correct folding pathway [49].

2.3.2. Molecular Chaperones

Molecular chaperones are defined as the proteins that can interact with, stabilize or help unfolded or aggregated proteins to reach their functional form –native state- but not present in the final conformation [50]. There are several different classes of molecular chaperones that form cooperative networks and pathways. Molecular chaperone families are mostly known as heat-shock proteins (HSPs) or stress proteins. HSPs are expressed in “normal” conditions but are upregulated under stress conditions which proteins leaning on to form aggregates [50].

2.3.3. Heat-shock Proteins (HSPs)

Basic molecular response pathways of plants include heat-shock proteins (HSPs) which also known as stress proteins or stress-induced proteins [51,52]. The first HSP was identified in *Drosophila melanogaster* after heat exposure [53] and named as HSP by Tissieres *et al*. (1974) [54]. All HSPs contain a characteristic carboxylic terminal called heat-shock domain. Under "normal" conditions, expression of HSPs is tightly controlled while almost all stress types trigger *HSP* gene expression. HSPs are encoded by a heat-shock gene (*hsg*) when a plant encounters a stress condition. Regulation of *hsg* gene is controlled by cytoplasmic heat stress transcription factors (Hsfs) that are present as monomers in an inactive state, called as heat stress transcription factors (Hsfs) [55,56]. One leucine amino acid at C-terminal and three N-terminals are shared among all Hsfs. Under stress conditions, monomeric Hsfs are transported into the nucleus to form trimers and become active [57]. In trimer form of Hsfs binds to the promoter of heat shock element (HSE), which consist of alternating units of 5º-nGAAn-3º in the promoter region. At least Hsfs are required to bind to HSE [58,59]. Binding of Hsfs to HSG leads to transcription of HSP mRNA and then its translation to HSP. At the end of post-translational modifications following the translation, functional HSPs are formed (Figure 2.3) [59,60].

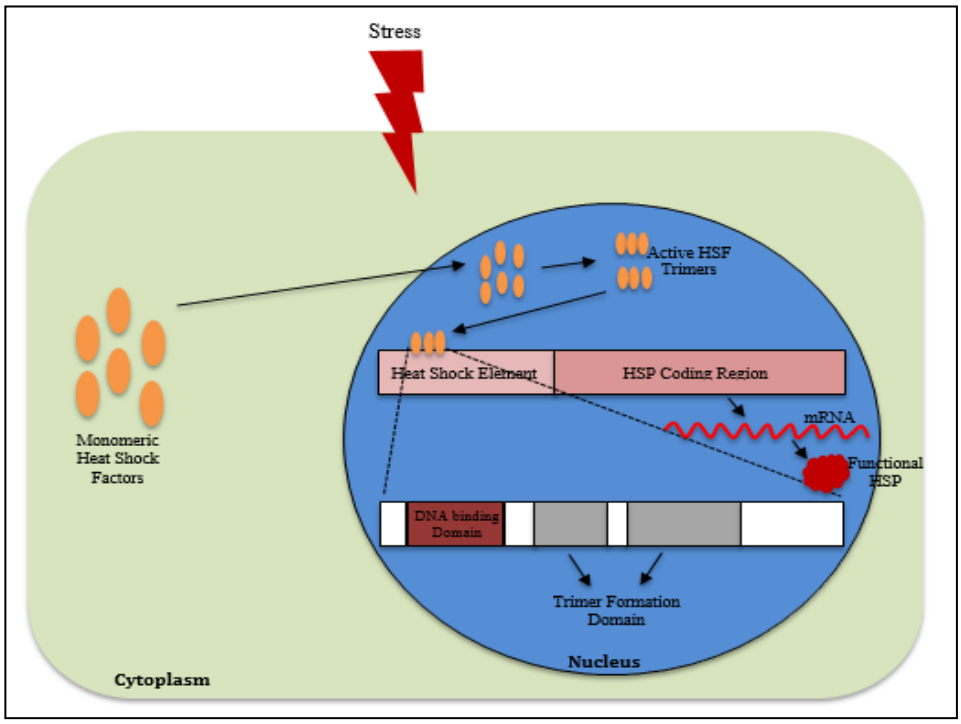


Figure 2.3. Expression of functional HSP (modified from Hasanuzzaman *et al.,* 2013) [40].

Protecting cells from damage during stress conditions and facilitating survival during the recovery phase are the important roles of HSPs. However, the main function of HSPs is to act as an intracellular chaperone role in the protein-protein interactions to facilitate protein folding. In case of heat stress, HSPs participate in protein folding in order to inhibit irreversible protein aggregation. However, in non-thermal stress, since the protein unfolding is not the primary response, the protection role of HSPs could be through an alternative way [61,62]. Regulation of the protein folding, localization and degradation are defined as general roles of HSPs in all multicellular organisms [52,56,63–65]. In the evolutionary, physiological and ecological importance of HSPs, it can be concluded that HSP is ubiquitously expressed under natural conditions. Also, their expression is dependent on the stress factors and expression levels are correlated with the strength of the stimulus. Although *hsp* genes exists in all living organisms, their expression patterns are varied [63].

As molecular chaperones, the main functions of HSPs are defined as inducing refolding of denatured proteins, participating in the final folding of the *de novo* synthesized proteins and reducing the protein aggregation [66].

In 1990, HSPs in plants were divided into 5 groups based on their approximate molecular weights as HSP60, HSP70, HSP90, HSP100 and small heat shock proteins (sHSPs) [67–69]. However, this classification was revised in 2010, and amino acid sequence homology and function became the criteria for HSP family organization [52].

2.3.3.1. HSP60

The first HSP60 was found in *Mycobacterium leprae* [70], but ever since it has been sequenced from various species including bacteria, mitochondria and chloroplast of eukaryotes [71]. HSP60 is defined as a conserved group of proteins since they are found both in eukaryotic and prokaryotic cells with homology at amino acid level. However, there is no trace of close HSP60 homologues in eukaryotic cytosol [71]. HSP60 is encoded by nuclear DNA, although, its synthesis takes place in the cytoplasm and it is targeted to mitochondria [72]. Similar to other types of HSPs, HSP60 is expressed under "normal" conditions at low expression level, but its expression level is increased under stress [51]. All HSPs are associated with heat stress, however all other stress types affect their expression [73–75].

The main role of HSP60 is defined as prevention of protein aggregation by direct interaction after the transcription of mitochondria and chloroplast transported proteins [75–77]. Also, HSP60 is involved in assisting the folding of plastid proteins such as ribulose biphosphate carboxylase-oxygenase (RUBISCO), which is the most abundant enzyme in plants. In addition, its ability of fixing CO_2 makes RUBISCO the key enzyme in photosynthesis [78]. Although the main function of HSP60 is to provide the appropriate folding of polypeptide chains into oligomeric structures in an ATP-dependent manner, it was found that HSP60 was also involved in the transmission of plant viruses in aphids [79].

2.3.3.2. HSP70

HSP70 consists of three main domains namely nucleotide binding domain (NBD), substrate binding domain (SBD) and C-terminal lid. The conserved ~44-kD NBD locates at the N-terminal and works as ATPase domain. The ~18-kD SBD serves as a dock for the hydrophobic regions of the proteins. The variable ~10-kD C-terminal lid assists the holding of client proteins at SBD [80]. HSP70 has two forms as constitutively expressed and stress-inducible forms. Stress-inducible form promotes the correct folding of polypeptide chains through binding and releasing in an ATP-dependent manner. The main function of HSP70 is to prevent the accumulation of newly synthesized proteins during their transfer to final location [81]. Also, HSP70 family proteins are found to be involved in correct folding of nascent proteins, protein translocation across membranes and targeting proteins towards degradation [50]. HSP70 interacts with HSP90 with the help of the adaptor protein p60/HOP, which provides the substrate transfer from HSP70 to HSP90 [82].

2.3.3.3. HSP90

HSP90 can be found mainly in cytoplasm. They can also be present in chloroplast, endoplasmic reticulum (ER) and mitochondria, although this is rare. Both in eukaryotic and prokaryotic cells, HSP90 is abundant and constitute almost 1 percent of the total proteins in the cell. In addition, under heat stress, HSP90 level is increased to approximately 6 percent of the total proteins. They are involved in plant development, disease resistance and stress response by acting as molecular chaperones. Also, canalization, assimilation and phenotype

alterations are under direct influence of HSP90. Expression level of HSP90 is induced by temperature change, salinity and heavy metal stress factors.

HSP90 share similar N- and C- terminal domains with other HSPs that contain ATP binding and dimerization domain, respectively. Substrate binding domain is located between these two domains in the middle (Figure 2.4) [83].

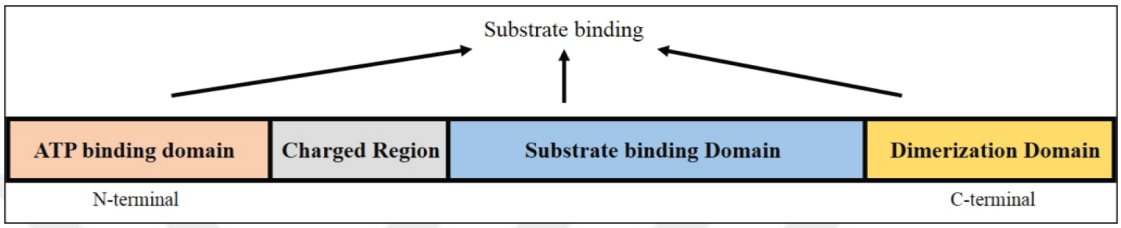


Figure 2.4. Structural model of HSP90 (modified from Xu, et al., 2012) [83].

ATP is required for HSP90 to function [84,85]. N-terminal domain of the HSP90 serves as an ATP/ADP binding domain. In the presence of ATP, conformation of HSP90 changes to a closed –active– conformation and it participates in signal transduction and protein folding (Figure 2.5). On the other hand, if ATP is not present, HSP90 dimers stay in the open conformation known as inactive state. ATP binds to N-terminus of the HSP90 and acts as a lid that keeps substrate protein inside HSP90 dimers. Closed conformation allows substrate binding domain to interact with the N-terminal resulting in a twisted conformation that allows ATP hydrolization [86,87]. Dimerization is occurred in C-terminus of the HSP90. Both C- and N-terminus have substrate binding properties. Some natural substrates, e.g. HSP90 inhibitor geldanamycin, bind to N-terminal domain of HSP90 whereas tetratricopeptide repeat (TPR) domain containing substrates bind to the pentapeptide domain (MEEVD) which is located at C-terminal domain [84,88]. The middle substrate binding domain is the key player of substrate-HSP90 binding. It not only serves as a primary substrate-binding site but also as a sensor for the ATP-γ phosphate with its catalytic ring to interact with it. The participation of middle domain affects the HSP90 function [89,90]. Between the N-terminal domain and middle substrate binding domain, there is approximately 50 amino acid long residues acting as charge zone that is not crucial for the HSP90 function, but it is involved in covalent interactions. In the presence of ATP, it assists the middle substrate binding domain and N-terminal domain to retain the HSP90 conformation [91].

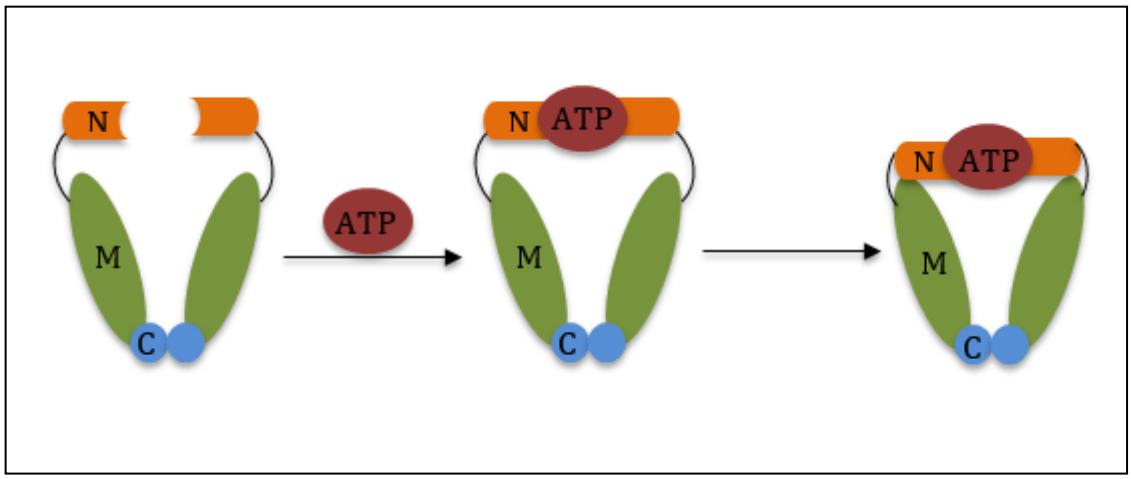


Figure 2.5. Molecular clamp of HSP90 (modified from Xu et al., 2012) [83].

Mainly three groups of proteins interact with HSP90 including co-chaperones (accessory proteins), regulators and substrates (client proteins). Co-chaperones are the main players in the regulation of HSP90's ATP enzymatic activity, and mediate HSP90 and substrate interaction. Co-chaperone and HSP90 interaction depends on the TPR domain of co-chaperone and pentapeptide (MEEVD) domain of HSP90 C-terminus. In case of handling the client proteins, co-chaperones provide the main assistance to HSP90.

The function of HSP90 is regulated by phosphorylation. Casein kinase II is the main player of phosphorylation and rephosphorylation of HSP90 *in vitro*. Although it is known that HSP90 should be phosphorylated for its action, the critical phosphorylation sites and *in vivo* phosphorylation mechanisms are still needs to be elucidated.

Client proteins and substrates of HSP90 are defined as the proteins that need HSP90 for their correct folding. HSP90 is involved in many mechanisms in cell, thereby the list of HSP90 substrates is large and still growing [92].

In *Arabidopsis thaliana*, seven HSP90 isoforms exist as HSP90.1, HSP90.2, HSP90.3, HSP90.4, HSP90.5, HSP90.6 and HSP90.7. First four isoforms are closely related and localized in cytosol. HSP90.5 is localized in chloroplast, HSP90.6 in mitochondria and HSP90.7 in ER [93]. The inhibition of all seven At-HSP90s was resulted in epinastic cotyledons, alterations in rosette and leaf symmetry, and abnormal root hair growth [94]. Chloroplastic HSP90.5 was related with red-light-response and chloroplast development [95–97] where HSP90.7 was found to be mainly expressed in shoot meristems [98].

In case of pathogen attack, Avirulence (Avr) proteins of pathogens are recognized by Resistance (R) proteins and activate the plant immune mechanism. An active resistance pathway induces the hypersensitive response and leads to the programmed cell death at the infection point [64,99]. HSP90, RAR1 (required for *Mla12* resistance) and SGT1 (suppressor of G2 allele kinetochore protein) together form a complex to provide stability for R proteins, which plays a key role in its activity of R proteins [100]. In *Nicotiana benthamiana*, HSP90 interacted with RAR1 and TIR-NB-LRR to protect the plant from tobacco mosaic virus (TMV) [101]. Also, in wheat and barley, HSP90-SGT1-RAR1 complex was found to be necessary for Lr21-mediated leaf rust and Mla13-mediated powdery mildew resistance, respectively [102–104].

Although the mechanisms are not clear, HSP90 is found to be involved in plant abiotic stress response pathway [105]. In *Arabidopsis thaliana*, HSP90.2 was suppressed HSF to prevent the transcription of heat-induced genes. However, under heat stress, cytosolic HSP90.2 was inactivated and HSFs induced the expression of HSPs [106]. Also, under drought stress HSP90.2, HSP90.5 and HSP90.7 were overexpressed leading to a reduced the plant tolerance in *Arabidopsis thaliana* [107]. In *Oryza sativa*, HSP90 gene was found to be overexpressed under salinity treatment [105]. Under multiple stress conditions, such as heat combined with drought stress, delayed HSP901a expression was detected in *Vitis vinifera* L. [108]. There is an insufficient amount of research on HSP90 in enhancing abiotic stress resistance, although it is known that HSP90 has an important role in abiotic stress response [109].

2.3.3.4. HSP100

HSP100 can be found in cytoplasm, chloroplast and mitochondria as ClpB-C, ClpB-P and ClpB-M, respectively [110]. HSP100s are also named as Clp due to the ability of HSP100 to associate with caseinolytic protease (serine protease) that degrades casein. HSP100 proteins are divided into two groups based on their ATPase domains. Class I HSP100s have two ATPase domains while Class II HSP100s have one ATPase domain [111]. HSP100s are involved in the unfolding of proteins, untangling of aggregates and the assembly of protein complexes; however, the unique function of HSP100 is defined as the resolubilization of aggregated proteins by reactivating them and aiding the degradation of irreversibly damaged proteins [75,111,112]. Also, cytoplasmic HSP100 is necessary for the high heat tolerance

while it is not required for the germination and growth of plant under normal conditions [113].

2.3.3.5. sHSPs

Structurally, all small HSPs share an 80-100 amino acid containing alpha-crystallin domain (ACD) that is located in the C-terminal [114]. Functionally, sHSPs interact with partially folded and denatured substrates proteins to prevent irreversible unfolding and incorrect protein aggregation instead of involving in the refolding of non-native proteins [115]. Also, sHSPs are involved in the degradation of the unsuitably folded proteins. They have an important role in membrane quality control, thus they are potentially involved in membrane integrity maintenance especially under stress [116]. Unlike other HSPs, activity of sHSPs is independent of ATP [117]. Studies on six divergent *Anthophyta* species including C3, C4, CAM, monocot and dicot plants showed that there was a link between sHSP accumulation and thermotolerance under heat stress [118].

2.4. TRANSGLUTAMINASES (TGases)

2.4.1. Polyamines (PAs)

Polyamines (PAs) can naturally be found in all living organisms. They are low molecular weight polycationic aliphatic amines and perform crucial functions in the plant growth, reproduction, development, adaptation, survival, and biotic and abiotic stress response. Putrescine (Put), Spermine (Spm) and Spermidine (Spd) are the most common plant polyamines PAs that can be found both in free form and bound form, conjugated with phenolics. The modulation of both forms is related to stress protection [119,120]. PAs are required in cell-cycle for cytokinesis and protection of DNA from hypoxia [121–123]. PAs have an affinity to bind negatively charged molecules in their nature. As structure, PAs have two amino terminals that can be involved in the bridge formation between endoglutamyl residues of proteins. Spm and Spd are synthesized from methionine and ornithine where Put is derived from arginine [124].

PAs tend to accumulate under stress stimuli such as salinity [125], drought [126], light [127] and nutrient deficiency [128,129]. Under drought and salinity stress, activities of PAs and their related enzymes were significantly increased [130]. Lin and Kao (1995) suggested that PAs play a role in salinity tolerance in rice by detecting an increase in Spd, decrease in Put levels and increased accumulation of Spd, Put and Spm in response to salt stress [131]. In addition to role of PAs in stress response, they have a crucial role in protein synthesis and biomembrane stabilization [35].

2.4.2. Transglutaminases (TGases)

Transglutaminases (TGases) are Ca^{++} dependent enzyme family of pleiotropic enzymes that can be found both in extracellular and intracellular milleu. TGases catalyze a reaction between an acyl acceptor on a glutamyl residue and an amine donor on a lysyl residue of the same or another protein as well as reaction between an acyl acceptor on a glutamyl residue of a protein and a free primary or polyamine.

In addition to amination and protein cross-linking, TGases also catalyze post-translational protein modifications by lysine acylation, esterification, deamidation and isopeptide cleavage (Figure 2.6) [132–134]. The first TGase was identified from guinea pig liver extract and later it was identified as tissue transglutaminase based on its low-molecular-weight primary amine to protein incorporation catalyzing ability [135]. Since then TGases have been identified in several organisms including mammals and plants [136].

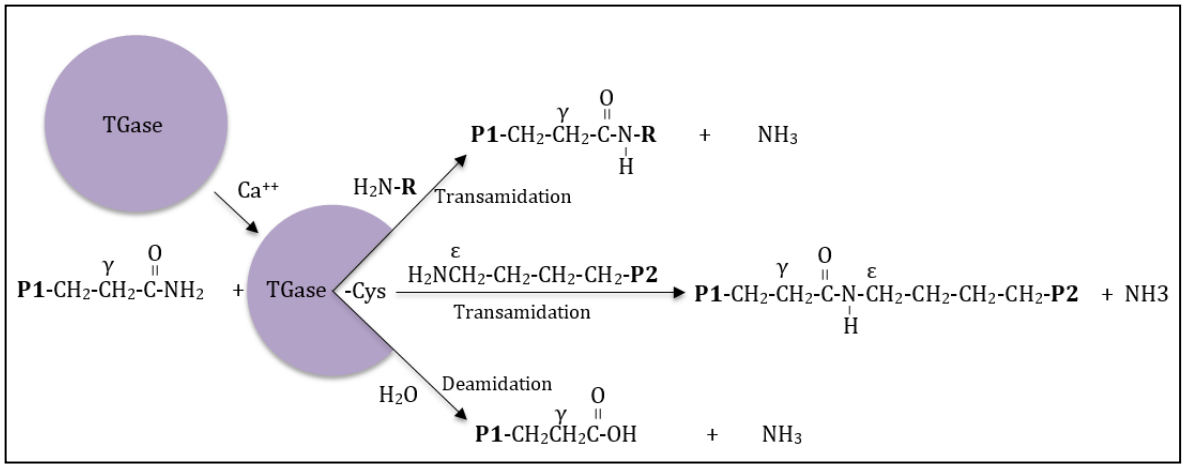


Figure 2.6. Basic roles of transglutaminases (modified from Eckert et al., 2014) [134].

2.4.2.1. Mammalian TGases

In humans, nine TGase genes are identified as TG1 (Keratinocyte TGase), TG2 (Tissue TGase), TG3 (Epidermal TGase), TG4 (Prostate TGase), TG5, TG6, TG7, Factor XIIIa and Erythrocyte membrane protein band 4.2 (Band 4.2). First eight TGases are catalytically active enzymes whereas Band 4.2 is inactive. Also, Band 4.2 is distinguished from other TGase family proteins since they do not share the identical amino acid sequence at the active site with other TGases [136].

The expression sites of TG1 are upper digestive tract, lower female genital tract and epithelia of the skin. The catalytic activity of TG1 is activated by Ca^{++} levels, increased tazarotene-induced gene 3 (TIG3) and proteolytic cleavage [133,137,138]. Ubiquitously expressed tissue transglutaminase, TG2, is the most studied TGase and can be found in cytosol, nucleus, mitochondria and on the plasma membrane [132]. Besides its role in transamidation reactions, TG2 also have GTPase, ATPase, protein kinase and protein disulphide isomerase (PDI) activity. Epidermal TGase, TG3, can be found in hair follicles, epidermis and brain. Main function of TG3 is catalyzing the crosslinking of keratin intermediate filaments and trichohyalin. It is also involved in cell envelope formation [139]. Prostate TGase, TG4, is found in the seminal plasma, prostate glands and prostatic fluids [136,140,141]. The expression site of TG5 is in skeletal muscles, epithelial barrier lining and foreskin keratinocytes. Ca^{++} promotes crosslinking ability of TG5 whereas ATP and GTP inhibit the activity [142]. TG6 and TG7 are expressed in the testes, lungs and brain [143]. Plasma TGase, in other words Factor XIIIa, is present in plasma, platelets, heart, astrocytes, macrophages, osteoblasts, chondrocytes, synovial fluid, placenta, eyes and dermal dendritic cells. It catalyzes the crosslinking of fibrin molecules in Ca^{++}-dependent manner and is involved in the blood coagulation cascade [144]. The only member of TGase family without catalytic function is Erythrocyte Membrane Protein Band 4.2 (Band 4.2), which is an important member of the erythrocyte membrane cytoskeleton. It can be found in erythrocytes, bone marrow, fetal liver and spleen and is involved in membrane maintenance and cell stability regulation [145].

2.4.2.2. Plant TGases

Although TGases are well studied in mammals, there is still insufficient information about their roles in plants. However, plant TGases are found to be expressed in chloroplast, mitochondria, cytoplasm and cell wall. Functionally, plant TGase is related to flowering, cell wall formation, cell cycle, photoprotection, apical and seedling growth, differentiation, stress response and programmed cell death [146].

The first plant TGase was isolated and characterized at the molecular level in *Arabidopsis thaliana*. Its activity was overlapped with animal TGases and shared typical TGase catalytic domain Cys-His-Asp triad. *AtPng1p* gene was found to encode the first plant TGase at low level, but ubiquitously [147]. Further studies reported plant TGases to share typical animal TGase properties like products of catalysis [148], presence of Cys in the active site [147] and its activation/inhibition with Ca^{++} [149]. In addition plant TGases was showed to be recognized by animal TGase antibodies [147] and inhibited by animal TGase inhibitors [149].

In the parenchyma tissues of *Helianthus tuberosus* tubers, TGase activity was detected in cell division through PA conjugation [150]. The TGase activity was gradually increasing from G1 to S phase [151].TGase activity was detected in shoots and roots of pea, broad bean, barley and wheat. Leaf tissues showed a lower TGase activity when compared with the roots at the same developmental stage. Also, TGase activity was high during development and early growth phase in the roots, however TGase activity was decreased in mature organs [152]. A Ca^{++}-dependent TGase in pollen tubes of apple were found to be expressed both intra- and extracellularly. Intracellular TGase was found in stroma, thylakoids, cytosol and cell wall. Incorporation of polyamines into cytoskeletal proteins, actin and tubulin, catalyzed by the TGase activity [153].

Recently, Zhong *et al.* (2019) reported that TGases were involved in the regulation of photosynthetic gene transcription through PA accumulation under salinity stress in cucumber (*Cucumis sativus* L.). According to their work, TGase transcript levels were lower in young leaves and plant development affected TGase transcript levels. Although all tissues contain TGase, its levels are tissue-specific. They were found to be highly expressed in leaves and flowers while in roots and stems TGase was minimally expressed. The

overexpression of TGase was resulted in high biomass, photosynthesis rate, PA accumulation, grana number and size. The photosynthesis-related genes' transcription was induced by the TGase overexpression under salinity treatment. In addition, TGase was found to be localized in cell wall and chloroplast, near grana [154]. Same group also reported that the TGase was important for the photosynthesis regulation in tomato (*Solanum lycopersicum* L. cv Ailsa Craig). Although total RUBISCO activity was not changed significantly in WT, transgenic plants with TGase overexpression and *tgase* mutant with 10 bp loss generated using CRISPR/Cas9 genome editing technology, initial RUBISCO activity and its activation state was found to be directly correlated with endogenous TGase activity. Also in *tgase* mutants, Calvin-cycle related genes were found to be downregulated leading to a decrease in the photosynthetic capacity [155].

2.5. THE INTERACTION BETWEEN HSP90 AND TGases

In plants and animals, the interaction between HSP90 and TGase is still unknown. However, in animals, there are a few studies on HSP90 and TG2 interaction. According to the study of Altuntas *et al.* (2015), under normal conditions, TG2 interacted with HSP70/HSP90 complex via the adaptor protein HOP (HSP70-HSP90 organizing protein) (Figure 2.7) [156]. It was also reported that in order to maintain cytoprotection, HSP90 was targeted by TG2 in H9c2 (Rat embryonic cardiomyoblast-derived) cells [157]. In another study, TG2 and its binding partner HSP70 interaction was found to be necessary for cell migration, [158]. On the other hand, in plants, HSP70 and HSP90 multichaperone complex was shown to be involved in the conservation of cellular protein homeostasis [159]. The interaction of animal TG2 and HSP90 interaction is necessary for the cell and homeostasis (1); TG2 and HSP70 are binding partners and besides HSP70/HSP90 multichaperone complex is required for the protein homeostasis conservation (2). With the consideration of the plant TGase and animal TG2 are alike (3) and in plants both TGase and HSP90 are involved in stress response (4), the possibility of interaction between HSP90 and TGase in plants is need to be determined. Therefore, in this study, the co-regulation of HSP90 and TGase under abiotic stress was aimed to be investigated in the model monocot plant, *Brachypodium distachyon*.

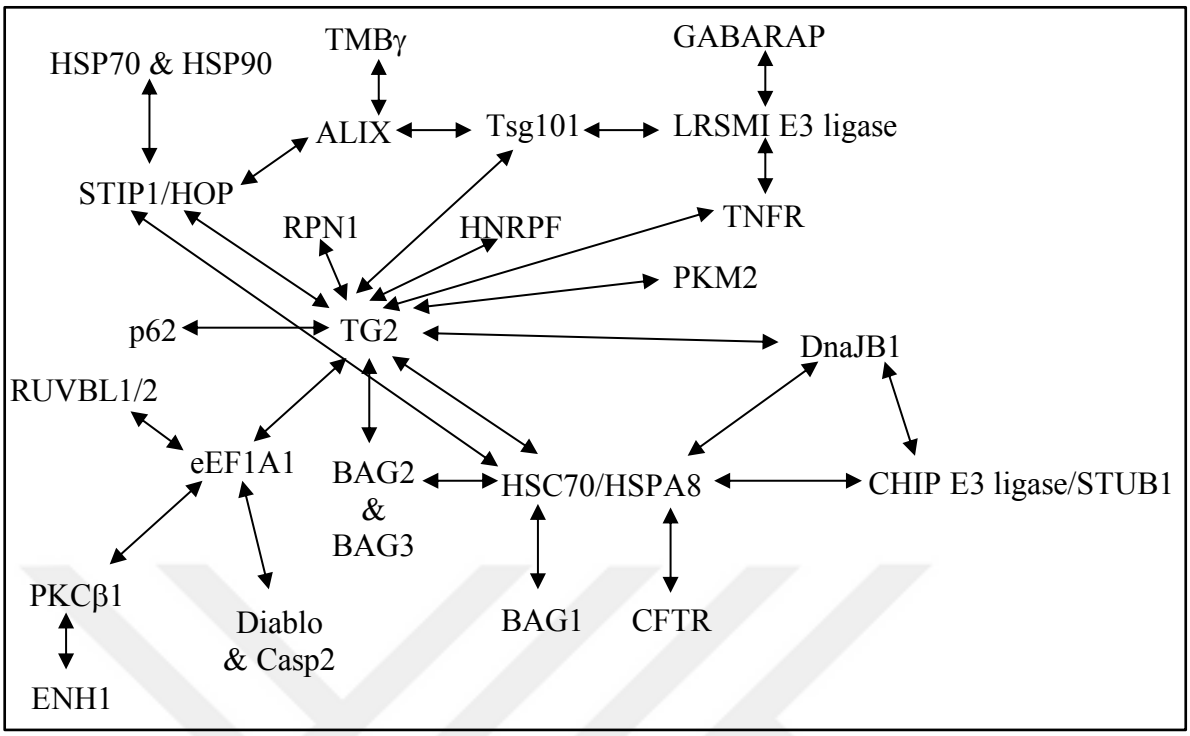


Figure 2.7. Representation of TG2 interacting proteins (modified from Altuntas *et al.*, 2015) [156].

2.6. AIM OF THE STUDY

The objective of this study is to investigate a possible association between Heat-shock protein 90 (HSP90) and Transglutaminase (TGase) under salinity and drought stress in model plant *Brachypodium distachyon*.

Drought (12-days of water withdrawal) and salinity (14-days of 320 mM salt treatment) stress were applied separately to *Brachypodium distachyon* (Bd21 line) plants at their vegetative stage in greenhouse conditions. qRT-PCR analysis with one TGase and 8 different HSP90 gene specific primers and western blotting with HSP90 and TGase specific antibodies were performed using sampled leaf tissues to investigate whether the expression of HSP90 and TGase are co-regulated under abiotic stress conditions.

3. MATERIALS

3.1. CHEMICALS AND CONSUMABLES

10X PCR Buffer (Thermo Fisher Scientific, USA), 2-mercaptoethanol (Merck, Germany), 96-well plates (SPL Life Sciences, Korea), Acetone (vWR, USA), Acrylamide/bisacrylamide (Merck, Germany), Agarose (Sigma, USA), Ammonium Acetate (Merck, Germany), Ammonium persulfate (Sigma, USA), anti-mouse secondary antibody (Santa-Cruz, USA), Bovine Serum Albumin (Biosera, France), Bradford Reagent (Thermo Scientific, USA), Bromophenol Blue (PanReac, Spain), CHAPS (Serva, Germany), Dithiothreitol (Merck, Germany), dNTP (Thermo Fisher Scientific, USA), Ethidium Bromide (Fisher Scientific, Germany), Ethylenediamine tetra acetic acid (Merck, Germany), Falcon 15 ml (Axygen, Germany), Falcon 50 ml (Sarstedt, Germany), GangNam Prestained Protein Ladder (iNtRON Biotechnology, USA), GeneRuler DNA Ladder Mix (Thermo Scientific, USA), Glycerol (Sigma, USA), Glycine (Bioshop, Canada), Goat anti-rabbit IgG (H&L) HRP conjugated (Agrisera, Norway), Isopropanol Alcohol (Merck, Germany), Liquid Nitrogen Tank (Isotherm, Germany), Methanol (Merck, Germany), micropipettes 2ul, 20ul, 200ul, 1000ul (Gilson, USA), Mortar and Pestle (Isolab, Germany), non-fat dry milk (Pınar, Turkey), Petri Dishes 90 mm (Isolab, Germany), Phenol Solution (Sigma, USA), Phosphate Buffered Saline (Lonza, Switzerland), PMSF (Merck, Germany), Pottasium chloride (Merck, USA), Rabbit anti-HSP90-1 Primary Ab (Agrisera, Norway), SYBR Green (Bio-Rad, USA), Sodium chloride (Sigma, USA), Sodium dodecyl sulfate (Sigma, USA), Sucrose (Bioshop, Canada), Taq Polymerase (Thermo Fisher Scientific, USA), Tetramethylethylenediamine (Sigma, USA), TGM2 CUB7402 (Thermo Fisher Scientific, USA), Tris-Base (Sigma, USA), Tris-HCl (Sigma, USA), Tween 20 (Merck, Germany), Urea (Merck, Germany)

3.2. EQUIPMENTS

+4^{0}C Refrigerator (Arçelik, Turkey), -20^{0}C Freezer (Arçelik, Turkey), Agarose Gel Electrophoresis System (BioRad, USA), Centrifuge (Hettich Zentrifügen, Germany), CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA), Chemidoc (BioRad, USA), Chlorophyll meter (Konica Minolta, Japan), Conductivity meter (EcoSense, USA), Heat Block (INOVIA Technology, Switzerland), Power Supply (Cleaver Scientific, UK), Shaker (Sartorius, Germany), Spectrophotometer (Thermo Scientific, USA), Thermocycler (Bio-Rad, USA), Ultracentrifuge (Eppendorf, Germany), Ultrafreezer (Thermo Scientific, USA), UV Transilluminator (Fisher Scientific, USA), Western Blot module (BioRad, USA), ZD-07 4 in 1 Soil Survey Instrument (Gain Express, Hong Kong)

3.3. KITS

Plant/Fungi Total RNA Purification Kit (Norgen, Canada), RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA)

3.4. PLANT MATERIAL

Brachypodium distachyon seeds (accession Bd21) were provided from Joint Genome Institute (DOE Joint Genome Institute, Walnut Creek, California) collection.

4. METHODS

4.1. PLANT GROWTH CONDITIONS

Brachypodium seeds were planted between wetted filter paper in a petri dish and kept for 5-7 days at 4^0C in dark. After cold treatment, petri dishes were kept in day light for 3-5 days at room temperature until germination. The seeds that are germinated are planted into peat-soil mixture in viols until the third leaf emergence. Then, plantlets are transferred to plastic pots containing peat-soil mixture as 3 plants/pot and grow under controlled environment in the greenhouse (16/8hours light/dark photoperiod, 22-25^0C, 60-70 percent relative humidity, 320 µmol m^{-2} s^{-1} photosynthetic photon flux that provided by fluorescent lamps at canopy height). For basal fertilization, 200 mg/kg $Ca(NO_3)_2$, 100 mg /kg KH_2PO_4, 20 mg/kg K_2SO_4, 5 mg/kg Fe-EDTA and 2.5 mg/kg $ZnSO_4$ were applied to soil in every 20 days.

4.2. STRESS TREATMENT AND SAMPLE COLLECTION

Drought and salinity treatments were applied when *Brachypodium* plants reached their vegetative stage. Before drought treatment, each day soil moist levels were checked with soil survey instrument (ZD-07 4 in 1 soil survey instrument). When soil moisture level dropped below 30 percent (Table 4.1), drought stress treatment was applied with water withholding for 12 days. Besides, control group was watered each day with totally 70 ml dH_2O in two doses (35 ml in the morning and 35 ml afternoon).

Table 4.1. Soil moisture levels according to soil survey instrument indicators.

Soil Survey Instrument Indicator	Moisture Range Percentage	Stress Level
Wet +	70-80	Well-Watered
Wet	60-70	Light Drought
Nor	50-60	Moderate Drought
Dry	35-50	Severe Drought
Dry +	25-30	Extreme Drought

For salinity treatment, 320 mM NaCl solution was applied as 70 ml each day in two doses for 14 days whereas salinity control group was continued to be watered with 70 ml dH_2O likewise the drought control group.

Table 4.2. Experimental set-up for drought and salinity stress treatments under greenhouse conditions.

Stress Treatments			
Drought Control	**Drought**	**Salinity Control**	**Salinity**
5 pots/treatment x 3 plants/pot = 60 plants			
During sampling, 3 plants in each pot were pooled			

At the end of stress treatments, all plants were harvested by cutting the leaves from their petioles and the samples were immediately freezed in liquid nitrogen. *Brachypodium* leaf samples from each pot were pooled before freezing (Table 4.2) and collected samples were stored at -80^0C until usage.

4.3. ELECTRICAL CONDUCTIVITY MEASUREMENTS

Measurements of electrical conductivity (EC) were done in order to estimate the soil salinity. 10 gr of soil was weighted and mixed with 25 ml ddH_2O in a falcon tube. Tubes were incubated on a shaker for 30 mins. Mixed soil and ddH_2O were filtered through filter paper. EC of the flow through was measured by using Conductivity Meter (EcoSense, USA) according to the manufacturer's instructions.

4.4. RELATIVE WATER CONTENT MEASUREMENTS

Relative water content (RWC) was measured by using the procedure of Barrs and Weatherly (1962) in order to evaluate the leaf water uptake capacity at the end of stress treatment [160]. Stressed and unstressed *Brachypodium* leaves were weighted to record the fresh weight (FW) at the end of 12^{th} day for drought and 14^{th} day for salinity stress. Weighted leaves were put in a petri dish with dH_2O and sealed. After 4 hours in dark incubation, leaves were dried between filter papers and then weighted to obtain turgor weight (TW). Hydrated leaves were

put in filter paper envelopes and incubated at 80^0C in the oven for 24 hours. After the leaves were completely dried, they were weighted to obtain the dry weight (DW).

$$\mathbf{RWC}\ (\%) = \frac{\mathbf{FW-DW}}{\mathbf{(TW-DW)}}\mathbf{x100} \quad (4.1)$$

Aforestated equation was used to calculate the RWC of treated and untreated (control) *Brachypodium* plants.

4.5. CHLOROPHYLL CONTENT ANALYSIS

Leaf chlorophyll content of stress treated and control plants were measured daily throughout the stress application by using SPAD-502Plus chlorophyll meter. The same leaf of a plant, even the marked area, was placed on emitting window of the chlorophyll meter and infrared and red radiation transmittance passing through the leaf is measured as SPAD value. The given SPAD value was used to calculate the leaf chlorophyll content by Uddling's Equation [161]:

$$\mathbf{Chl\ Content}\ \left(\frac{\mu g}{cm^2}\right) = \mathbf{5.99xe^{0.0493xSV}} \quad (4.2)$$

4.6. RNA ISOLATION

In order to check mRNA levels of TGase and HSP90, total RNA isolation was performed from frozen samples of *Brachypodium distachyon* leaves by using Norgen Plant/Fungi Total RNA Purification Kit. All steps were done according to manufacturer's instructions on ice. 50 mg of harvested Brachypodium leaf tissue was suspended in liquid nitrogen using mortar and pestle and powdered samples was transferred into 2 ml microcentrifuge tube. 600 µl of Lysis Buffer C was added into tube immediately, vortexed and incubated at 55^0C for 5 mins. The lysate then transferred into Filter Column and centrifuged at 20000xg for 2 mins. The clear flowthrough was transferred into a microcentrigude tube and equal volume of EtOH (96 percent) was added and vortexed. 600 µl of lysate was transferred to Spin Column and centrifuged at 3500xg for 1 min followed by an additional min at 20000xg. 400 µl of Wash

Solution A was added into column and centrifuged at 20000xg for 1 min in order to wash the column. The washing step was repeated twice more and the column was placed into 1.7 ml Elution tube. 50 µl of Elution Solution A was added into column and centrifuged at 20000xg for 2 mins. The flowthrough containing isolated RNA was then stored at -80^{0}C until use.

4.7. cDNA SYNTHESIS

Isolated RNAs were converted to cDNA by using RevertAid First Strand cDNA Synthesis Kit (Thermoscientific) according to manufacturer's instructions on ice. 5 ng of isolated total RNA was transferred to a PCR tube. 1 µl of oligo $(dT)_{18}$ primer, 4 µl of 5X Reaction Buffer, 1 µl of RiboLock RNase Inhibitor (20 U/µl), 2 µl of 10 mM dNTP Mix and 1 µl of RevertAid M-MuLV Reverse Transcriptase (200 U/µl) was added into PCR tube. The reaction mixture was then finalized to 20 µl with nuclease-free H_2O. For cDNA amplification PCR tubes were incubated at 42^{0}C for 60 mins followed by the termination step at 70^{0}C for 5 mins. Synthesized cDNA samples were stored at -80^{0}C until use.

4.8. GENE SPECIFIC PRIMERS

Primers for the HSP90 genes were obtained from the study of Zhang *et al.,* (2017) [162]. However, there was no information for the sequence for *Brachypodium distachyon* TGase gene in the literature. In order to design primers for TGase, *Brachypodium distachyon* genome was BLAST with *Zea mays* and *Oryza sativa*. The reason for using *Zea mays* and *Oryza sativa* genome was their known TGase gene sequences; and therewithal both plants are relatives of *Brachypodium distachyon*. Two primer sequences were chosen from highly conserved TGase sequences of *Zea mays*, *Oryza sativa* and *Brachypodium distachyon*. Primer-BLAST tool was used to design primers for the highly conserved TGase sequence by considering the optimal primer attributes as no self-complementarity, maintaining 40-60 percent GC content and G or C residue locating at the 3' end. Since both of the designed TGase primer pairs gave PCR products, the gene expression studies were conducted only with one of the primer pairs. As an internal control, constitutively expressed ubiquitin-conjugating enzyme 18 (UBC18) was used for normalization [22,163]. All the primers used

in this study are listed in Table 4.3.

Table 4.3. Gene names, primer sequences and product lengths used in the study.

Gene	Primer Sequence (5' to 3')
UBC18	**F:** GGAGGCACCTCAGGTCATTT **R:** ATAGCGGTCATTGTCTTGCG
TGase	**F:** AGGCAGAAAAGCTCATCAGGG **R:** AGGTGGGTGCGGATCATTTG
Bradi5g02307	**F:** ACCCCATCTACCTCTGGACC **R:** GGCTCACCTCCTTCACCTTC
Bradi3g39620	**F:** CTGACAAGGCCACAAACACG **R:** AGCCAACACCAAACTGACCA
Bradi3g39590	**F:** CTGAGGAGGGCAAGGTTGAG **R:** AGTCGTTGGTCAGGCTCTTG
Bradi3g39630	**F:** ACGAACACACTCACGCTCAT **R:** AGCCAACACCAAACTGACCA
Bradi1g30130	**F:** TACCAGACGGCTCTCATGGA **R:** CGCCTTTGTGGTCTCCTTCT
Bradi4g06037	**F:** TCCCCGTGTGTTCTAGTTGC **R:** TGGACTGTGCCCTCATCAAC
Bradi4g32941	**F:** GTGGGACTTGGGGAAGAAGG **R:** GCCTGCCAAACGTCCAAAAT
Bradi3g38897	**F:** AGACCGCTCTGATCTCCAGT **R:** AGACCTACCCCATCTCCCAC

4.9. PCR AMPLIFICATION AND AGAROSE GEL ELECTROPHORESIS

In order to verify the annealing temperatures of the primers and expected PCR product, PCR amplification was done by using Taq Polymerase, dNTP, MgCl2, 10X PCR Buffer and cDNA as DNA template. PCR cycles for HSP90 genes and UBC18 were set according to the study of Zhang *et al.,* (2017) [162] and Tatlı *et al.,* (2017) [22] respectively. The PCR conditions for HSP90 gene primers are as follows: 95^0C for 3 mins, 40 cycles of 95^0C for 20

secs, 61^{0}C for 15 secs, followed by 72^{0}C for 10 secs. The PCR conditions for UBC18 gene primers are 95^{0}C for 15 mins, 36 cycles of 94^{0}C 15 mins, 61^{0}C for 30 secs and followed by 72^{0}C for 30 secs. For TGase primers gradient PCR was run and cycles were set as stated in Table 4.4. In order to visualize PCR products 1.5 percent agarose gel containing 0.75 gr of Agarose, 50 ml of TAE buffer and 1 µl of EtBr, was run at 110 V until the bands of DNA Ladder Mix were separated. PCR products were visualized using UV Transilluminator and GelDoc System.

Table 4.4. Gradient PCR cycles used for amplification of TGase gene.

PCR Cycles		
95^{0}C	3 min	
95^{0}C	20 sec	40 cycles
55-61^{0}C	15 sec	
72^{0}C	10 sec	
72^{0}C	5 min	
4^{0}C	∞	

4.10. QUANTITATIVE REAL TIME PCR (qRT-PCR) ANALYSIS

In order to determine mRNA levels of HSP90 and TGase genes qRT-PCR was done according to Tatlı *et al.* (2017) [22]. Each well of the qRT-PCR plate contains 12,5 µl 2X SYBR green supermix, 0.3 µM primer, 100 ng cDNA and RNase-free H_2O up to 25 µl. Amplifications were done using the conditions as 95^{0}C for 15 mins, 36 cycles of 94^{0}C for 15 mins, 55^{0}C for 30 secs (for UBC18 and TGase genes) and 61^{0}C for (HSP90 genes) followed by 72^{0}C for 30 secs. All qRT-PCR reactions were performed as technical and biological triplicates, thus final C_t values were the means of nine replicates. Bio-Rad CFX Manager Software was used for normalization to UBC18 gene and $\Delta\Delta C_t$ method was used to calculate fold change differences of HSP90 and TGase genes under drought and salinity stress.

4.11. TOTAL PROTEIN EXTRACTION AND QUANTIFICATION

Total proteins from young leaves of *Brachypodium distachyon* were extracted according to the study of Faurobert *et al.* (2006) [164]. Harvested young leaves were grounded by using liquid nitrogen. The grounded tissue was suspended in extraction buffer including 50 mM EDTA, 500 mM Tris-HCl, 700 mM sucrose, and 100 mM KCl. 2 percent 2-mercaptoethanol and 1mM PMSF and incubated on ice for 10 minutes. After incubation, Tris-buffered phenol was added and incubated further at room temperature for 10 minutes. Tris-buffered phenol was used for the separation of phenolic phase and interphase of the sample. After phases are separated, the phenolic phase, top phase, was transferred in a new tube and back-extracted with using extraction buffer. Phase separation was done by using centrifugation and the top phase was collected in a new tube. Precipitation solution, which contained 0.1 M ammonium acetate and cold methanol, was added to collected phenolic phase. The sample was incubated overnight at -20^{0}C. After the incubation samples were centrifuged in order to obtain a protein pellet. Pellets were washed with precipitation solution and lastly with cooled acetone. To remove the acetone, pellets were dried under laminar flow. The dried pellets were suspended in Rehydration buffer containing 8M urea, 2 percent CHAPS and 50 mM DTT for further analysis. Quantification of the total protein was done by Bradford protein assay as described in Bradford (1976) [165]. Absorbance values of 0.25, 0.5, 0.75, 1, 1.5, 2 µg/µl concentrated BSA (bovine serum albumin), as standard, and unknown proteins were determined by spectrophotometer at 595 nm. Obtained BSA standard absorbance values were used to plot standard curve and the obtained equation was used to calculate protein concentrations.

4.12. WESTERN BLOT ANALYSIS

Detections of the expression difference for HSP90 and Transglutaminase (TGase) in plant extracts were checked by Western blot analysis according to Yang and Mahmood (2012) [166]. Isolated proteins from the control and stress groups leaves were denatured with 5X Laemmli buffer, containing 50 percent (v/v) glycerol, 20 percent (v/v) 1.5 M Tris-HCl (pH=6.8), 20 percent (v/v) SDS, 25 percent (v/v) 2-mercaptoethanol and 5 percent (v/v) bromophenol blue at 95^{0}C for 5 mins. 12 percent separating polyacrylamide gel was prepared with 1.7 ml of dH_2O, 1.25 ml of 1.5 M Tris/0.4 percent SDS (pH=8.8), 2 ml of 30 percent acrylamide/bisacrylamide, 50 µl of 10 percent APS and 5 µl TEMED while 4 percent

stacking polyacrylamide gel was prepared with 1.25 ml of 0.5M Tris/0.4 percent SDS (pH=6.8), 500 µl of 30 percent acrylamide/bisacrylamide, 3.25 ml of dH_2O, 40 µl of 10 percent APS and 10 µl of TEMED. First, separating gel mixture was poured between casting gel glasses. To avoid its contact with air isopropanol was poured on it. After the polymerization of separating gel, isopropanol was discarded and stacking gel was poured onto separating gel. 1 mm thick 10-well comb was carefully placed into stacking gel after the polymerization carefully removed and gel plate was placed in western blot modules electrophoresis apparatus. In order to perform separation in gel, running buffer, containing 25 mM Tris-base (pH=8.5), 192 mM glycine, 0.1 percent (w/v) SDS, and dH_2O was used in the tank. Denatured protein samples and protein ladder were loaded to wells and run for 70V in stacking gel and at 90 V at separating gel, at room temperature until the last band of the ladder reached at the bottom of the gel casting glasses. SDS gel was used for blotting proteins to the membrane. A blotting sponge pad, 3 layers of whatman paper, 0.45 nm pored nitrocellulose membrane, SDS gel, 3 layers of whatman paper and blotting sponge pad were placed to prepare so-called blotting sandwich and placed into the blotting cassette which further placed into western modules tank and filled with ice-cold transfer buffer. Transfer buffer for blotting composed of 192 mM glycine, 25 mM Tris-base (pH=8.3) and 20 percent (v/v) MetOH. Transfer of the proteins to membrane was conducted at 4^0C, 16 V for 16 hours. After blotting, membranes were incubated in 5 percent fat-free milk solution in TBST buffer (9 percent NaCl, 1M Tris-HCl (pH=7.4) and 0.5 percent Tween-20), for 2 hours for the blocking, which was used in order to overcome non-specific antibody binding. HSP90 protein was detected by incubating with rabbit anti-HSP90-1 primary antibody (Agrisera Antibodies, Sweden, Product NO: AS08346) for 16 hours at 4^oC and goat anti-rabbit IgG secondary antibody (Agrisera Antibodies, Sweden, Product NO: AS09602) for 2 hours at room temperature. On the other hand, TGase protein was detected by incubating with TGM2 CUB primary antibody (Invitrogen) for 16 hours at 4^oC and anti-mouse secondary antibody (Santa-Cruz) for 2 hours at room temperature. Antibodies were diluted according to manufaturers' instructions in TBST buffer. Following the secondary antibody incubation, membranes were washed three times with 1X TBST for 10 minutes and lastly with PBS buffer. Images of the blots were taken with ECL solution (BioRad) under ChemiDoc XRS+Gel Imaging System for 20 mins.

4.13. STATISTICAL ANALYSIS

The results are expressed as mean ± SE (n=4 for western blot analysis, and n=9 for qPCR analysis). Statistical analysis of the results was performed with Student's t-test by using MS Excel 2007 for physiological and gene expression studies, and GraphPad Prism 8 for western blot analysis. For transcriptional expression, further analysis was conducted with multiple t-test where the p value is 0.05 and Volcano Plot constructed with $-\log_{10}$(p value) against $\log_2$(fold change) via GraphPad Prism 8.

5. RESULTS

5.1. MORPHOLOGICAL CHANGES UNDER DROUGHT AND SALINITY STRESS

Drought treatment was carried with water withheld at the vegetative stage of *Brachypodium distachyon* for 12 days. During drought-treatment, green color of leaves were turned to brownish and leaves were rolled towards the midrib whereas the control plants were phenotypically healthy with their dark green leaves. Morphological changes caused through drought-treatment are shown in the images of drought-treated and untreated (control) *Brachypodium* plants that represent the four time points as 1st, 4th, 8th and 12th day of drought treatment (Table 5.1). Salinity treatment was applied with 320 mM NaCl solution at the vegetative stage of *Brachypodium distachyon* for 14 days. During salinity-treatment, leaf blade became gritty. Morphological changes caused by salinity-treatment are shown in the images of salinity-treated and –untreated (control) *Brachypodium* plants that represent the four time points as 1st, 4th, 8th, 14th day of salinity treatment (Table 5.2).

Table 5.1. Representative images of *Brachypodium distachyon* plants at 1st, 4th, 8th and 12th days of drought treatment (3 plants/pot).

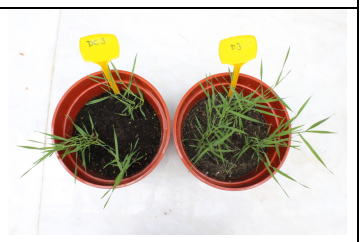
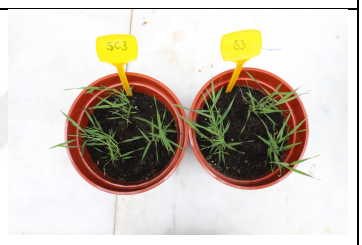
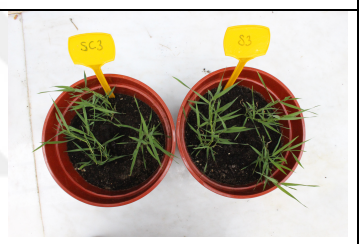
	Drought Control	**Drought Treatment**	**Drought Control vs Drought Treatment**
Day 1			
Day 4			
Day 8			
Day 12			

Table 5.2. Representative images of *Brachypodium distachyon* plants at 1st, 4th, 8th and 14th day of salinity treatment (3 plants/pot).

	Salinity Control	**Salinity Treatment**	**Salinity Control vs Salinity Treatment**
Day 1			
Day 4			
Day 8			
Day 14			

5.2. RELATIVE WATER CONTENT ANALYSIS

As a physiological measurement, relative water content (RWC) indicates the water status and osmotic regulation of the leaf and it is used to estimate the leaf water retention potential. The water holding capacity of the leaves was measured at the end of both stress treatments at full turgidity, and also deficit of water was measured. The values obtained from three different water statuses (fresh weight, turgor weight, dry weight) of leaves were used to estimate the relative water content of *Brachypodium distachyon* plants under both drought and salinity stress.

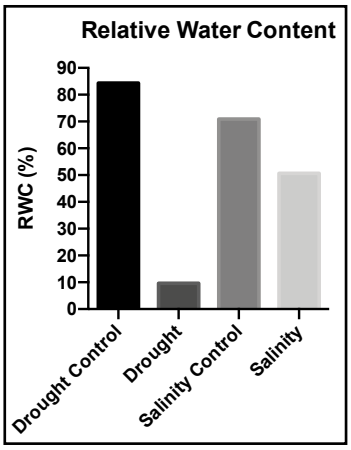


Figure 5.1. Relative water content analysis of drought control, drought-treated, salinity control and salinity-treated *Brachypodium distachyon* plants.

When stress treated plants were compared with their control groups, RWC decreased approximately 90 percent upon drought-treatment in *Brachypodium distachyon* plants at the end of 12 days and salinity treatment caused approximately 20 percent decrease in RWC at the end of 14 days (Figure 5.1).

5.3. ELECTRICAL CONDUCTIVITY OF SOIL

Since salinity-treatment with NaCl changes the EC of the soil, electrical conductivity (EC) values were used as an indicator of salinity treatment. As shown in Figure 5.2, EC values of salinity-treated *Brachypodium distachyon*'s soil was found to be 10.4 mS with a 4.1-fold increase when compared to the control group ($p=0.0003$).

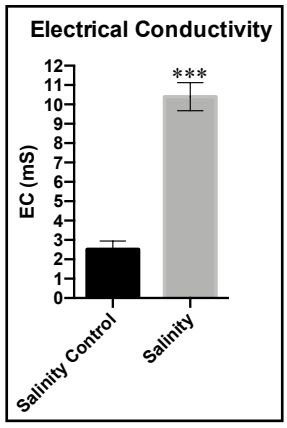


Figure 5.2. Electrical conductivity of the soil belonging to salinity control and salinity-treated *Brachypodium distachyon* plants. Each value represents the mean ± SE of 3 replicates. *Asteriks* shows significance at * p≤0.05, ** p≤0.01, *** p≤0.001.

5.4. CHLOROPHYLL CONTENT ANALYSIS

Chlorophyll contents of *Brachypodium distachyon* leaves were determined during drought and salinity-treatment as an indicator of physiological response of the plants to stress treatment. Under drought-treatment, chlorophyll content was decreased in the first day. It reached its maximum point at day 10 and dramatically decreased afterwards (Figure 5.3).

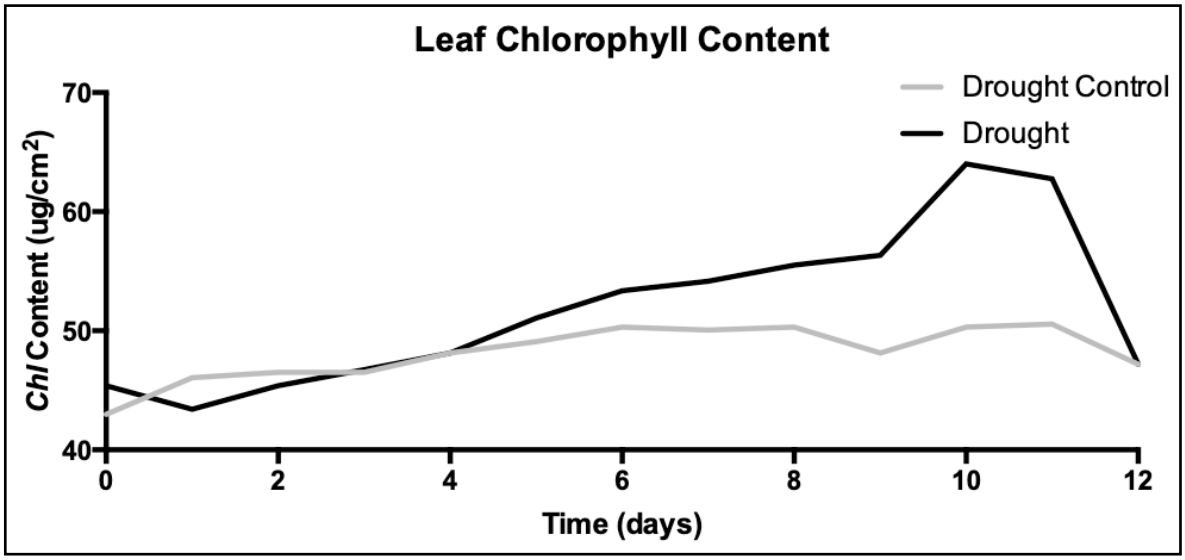


Figure 5.3. Chlorophyll content of drought control and drought-treated *Brachypodium distachyon* plants. Each value represents the mean ± SE of 3 replicates.

Upon salinity-treatment, chlorophyll content was decreased at first day. It reached its maximum point at day 6 and minimum level at day 12. Although, chlorophyll content was in a decreasing trend after day 10, it kept fluctuating during salinity treatment (Figure 5.4).

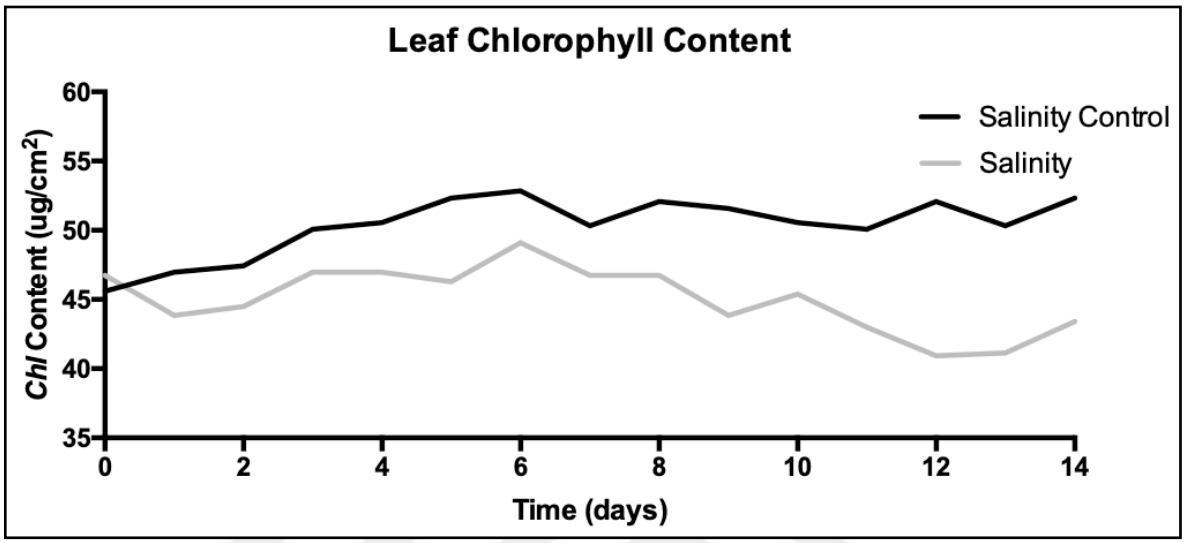


Figure 5.4. Chlorophyll content of salinity control and salinity-treated *Brachypodium distachyon* plants. Each value represents the mean ± SE of 3 replicates.

5.5. OPTIMIZATION OF PCR CONDITIONS

The PCR conditions and annealing temperature of the HSP90 gene primers were verified by conventional PCR and the expected PCR products were visualized by agarose gel electrophoresis in order to obtain optimal results in qRT-PCR. With all HSP90 gene primers (Bradi5g02307, Bradi3g39620, Bradi3g39590, Bradi3g39630, Bradi1g30130, Bradi4g06370, Bradi4g32941, Bradi3g38897) the expected PCR product range of 150-200 bp was achieved at 65^0C annealing temperature (Figure 5.5 and Figure 5.6).

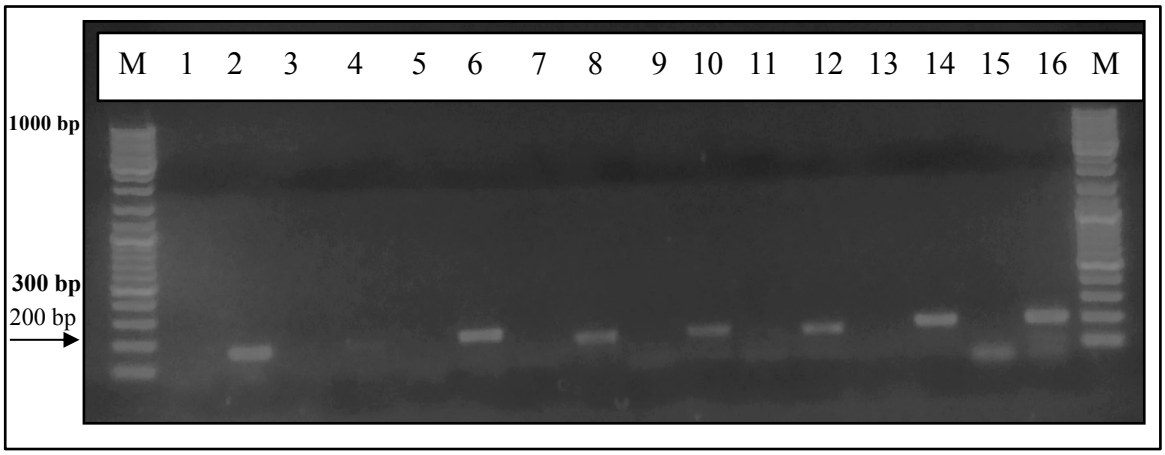


Figure 5.5. PCR products of HSP90 genes run in EtBr stained 1.5 percent agarose gel. Notes: Arrow shows the expected HSP90 genes' PCR products. Lane M: Molecular marker GeneRuler™ DNA Ladder Mix; 1: negative control for 02307; 2: 02307; 5: negative control for 39590; 6: 39590; 7: negative control for 39630; 8: 39630; 9: negative control for 30130; 10: 30130; 11: negative control for 06370; 12: 06370; 13: negative control for 32941; 14: 32941; 15: negative control for 38897; 16: 38897.

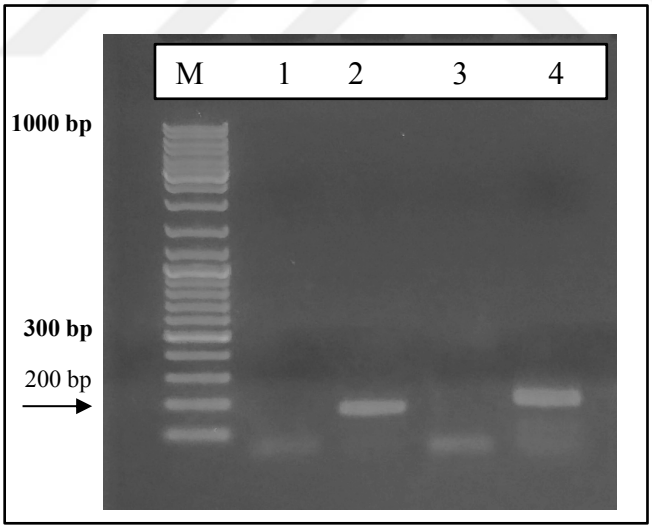


Figure 5.6. PCR products of HSP90 genes run in EtBr stained 1.5 percent agarose gel. Notes: Arrow shows the expected PCR products of HSP90 genes. Lane M: Molecular marker GeneRuler™ DNA Ladder Mix; 1: negative control for 39620; 2: 39620; 3: negative control for 38897; 4: 38897.

In order to optimize the PCR amplification conditions of TGase primer, gradient PCR was applied with annealing temperature ranging between 61-55^{0}C. The intensity of the expected

100 bp PCR product obtained at annealing temperature of 55^0C (Figure 5.7).

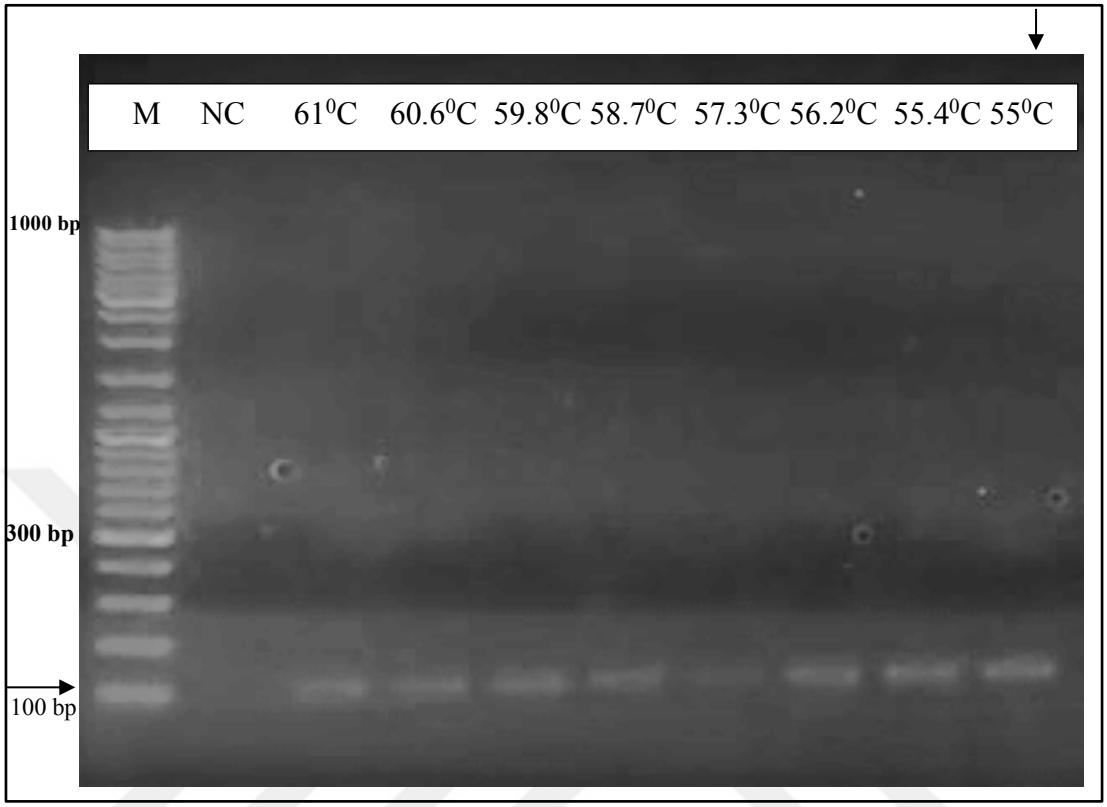


Figure 5.7. Gradient PCR products of TGase gene run in EtBr stained 1.5 percent agarose gel. Notes: Arrow at the left indicates the expected PCR product of TGase gene at different annealing temperatures. Arrow at the top shows the optimal Tm. Lane M: Molecular marker- GeneRuler™ DNA Ladder Mix.

5.6. TRANSCRIPTIONAL ANALYSIS OF HSP90 GENES UNDER DROUGHT AND SALINITY STRESS

In order to determine the transcript levels of HSP90 under drought and salinity stress HSP90 gene primers that are specific to the cytoplasmic (Bradi5g02307, Bradi3g39620, Bradi3g39590, Bradi3g39630), ER (Bradi1g30130), mitochondrial (Bradi4g06370) and chloroplastic (Bradi4g32941 and Bradi3g38897) HSP90 were used in qRT-PCR [162]. As shown in Figure 5.8 and Figure 5.9, under drought stress transcript levels of all HSP90 genes were mostly found to be upregulated by almost 2.02-fold in Bradi5g02307, 2.06-fold in Bradi3g39590, 1.77-fold in Bradi3g39630, 2.23-fold in Bradi4g06370, 2.22-fold in Bradi4g32941 and 1.45-fold in Bradi3g38897 except Bradi1g30130 gene was

downregulated 1.2-fold (Figure 5.8E) when compared to drought control group. The upregulations of Bradi5g02037, Bradi3g39590, Bradi4g06370 and Bradi4g32941 were found to be statistically significant. On the other hand, all HSP90 genes were significantly downregulated under salinity stress. Thus, Bradi5g02307 (5-fold), Bradi3g39620 (2.7-fold), Bradi3g39590 (7.14-fold), Bradi3g39630 (7.14-fold), Bradi1g30130 (1.96-fold), Bradi4g06370 (5.88-fold), Bradi4g32941 (5.88-fold) and Bradi3g38897 (5.88-fold) showed downregulation in the gene expression (Figure 5.10 and Figure 5.11) downregulated under salinity treatment.

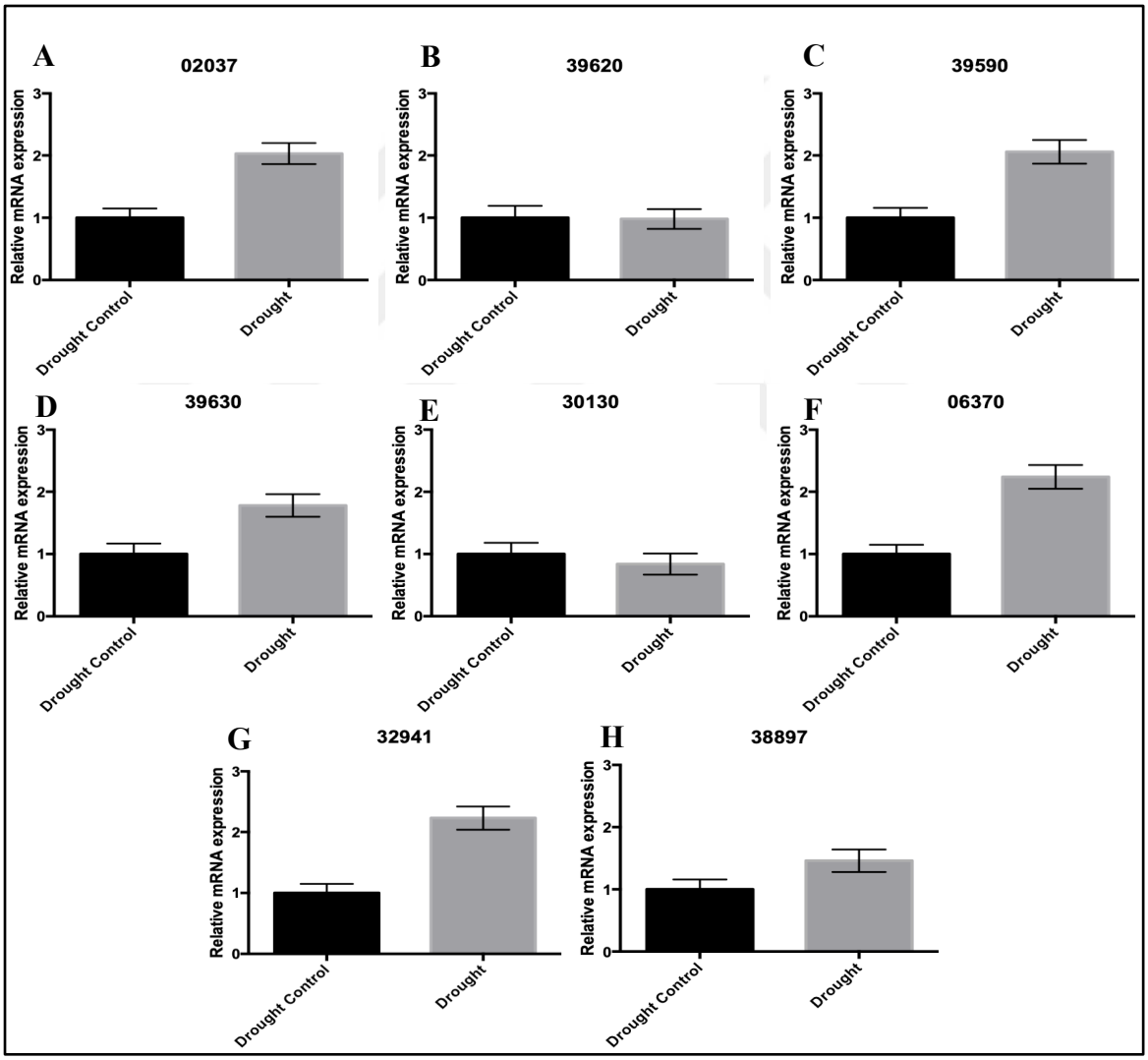


Figure 5.8. Transcript levels of HSP90 genes (Bradi5g02307, Bradi3g39620, Bradi3g39590, Bradi3g39630, Bradi1g30130, Bradi4g06370, Bradi4g32941 and Bradi3g38897) under drought stress against UBC18 gene. Each value represents the mean ± SE of 9 replicates.

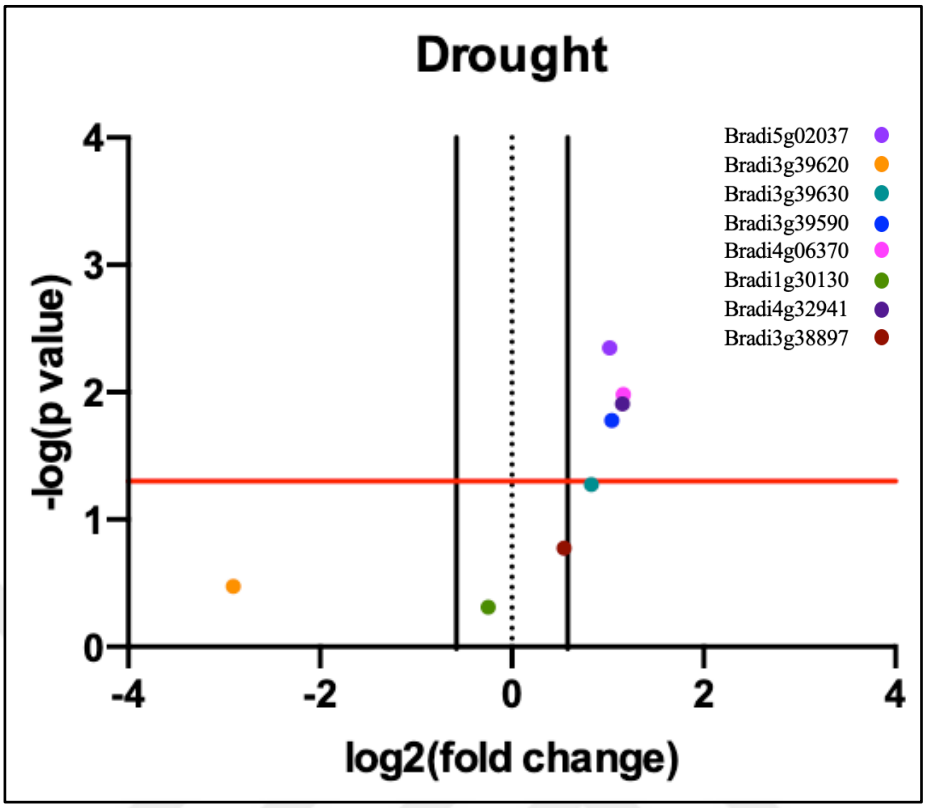


Figure 5.9. Gene expression differences and significance levels of HSP90 transcripts under drought stress represented as volcano plot. Upper left area indicates the significantly downregulated genes, lower left indicates downregulated genes, upper right indicates significantly upregulated genes and lower right indicates upregulated genes. The red line indicates the -log(0.05)=1.301. The straight horizontal black lines indicate the 1.5-fold change threshold. Dot colors represents the distributions of HSP90 transcripts.

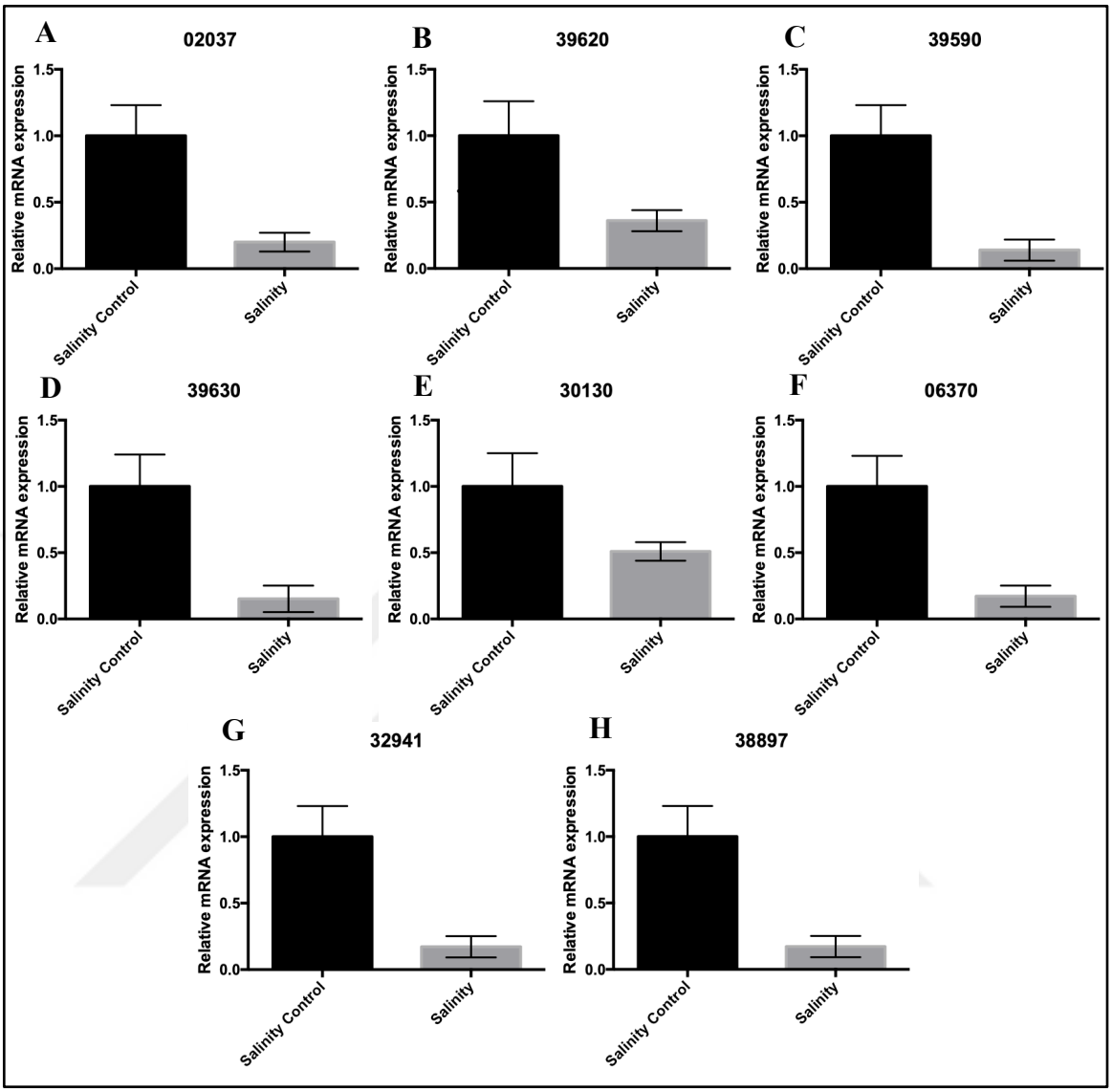


Figure 5.10. Transcript levels of HSP90 genes (Bradi5g02307, Bradi3g39620, Bradi3g39590, Bradi3g39630, Bradi1g30130, Bradi4g06370, Bradi4g32941, Bradi3g38897) under salinity stress against UBC18 gene. Each value represents the mean ± SE of 9 replicates.

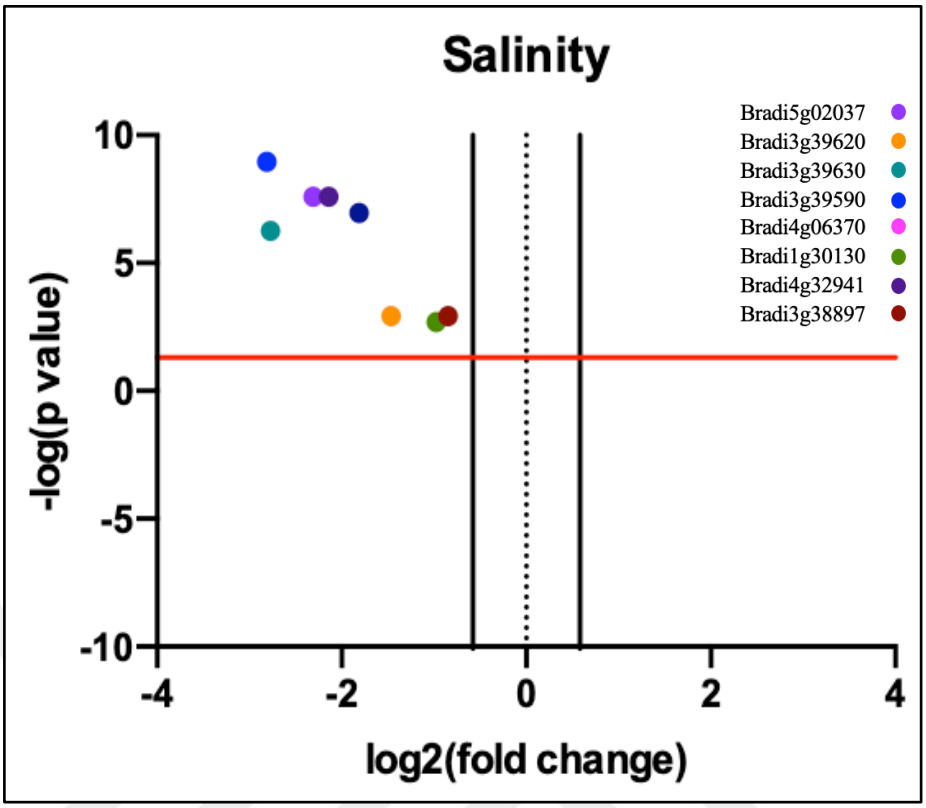


Figure 5.11. Gene expression differences and significance levels of HSP90 transcripts under salinity stress represented as volcano plot. Upper left area indicates the significantly downregulated genes, lower left indicates downregulated genes, upper right indicates significantly upregulated genes and lower right indicates upregulated genes. The red line indicates the -log(0.05)=1.301. The straight horizontal black lines indicate the 1.5-fold change threshold. Dot colors represents the distributions of HSP90 transcripts.

5.7. TRANSCRIPTIONAL ANALYSIS OF TGase GENE UNDER DROUGHT AND SALINITY STRESS

In order to determine the gene expression levels of TGase under drought and salinity stress, designed TGase gene primer pair was used in qRT-PCR. Transcript level of TGase gene was slightly changed by 1.11-fold under drought stress whereas under salinity stress it was downregulated when compared to their control groups (Figure 5.12 and Figure 5.13).

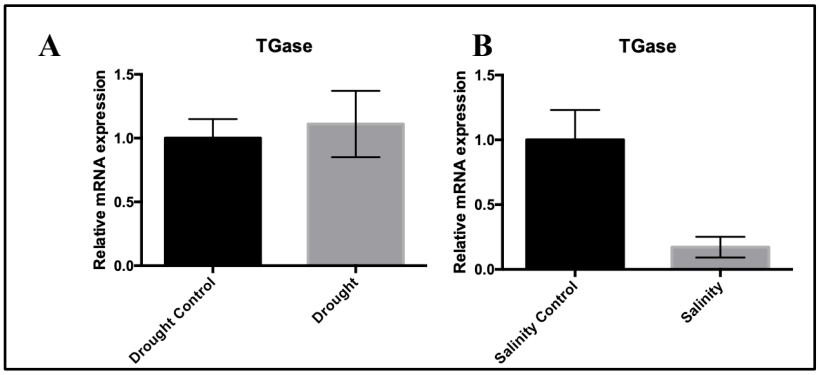


Figure 5.12. Transcript levels of TGase gene under **(A)** drought and **(B)** salinity stress against UBC18 gene. Each value represents the mean ± SE of 9 replicates.

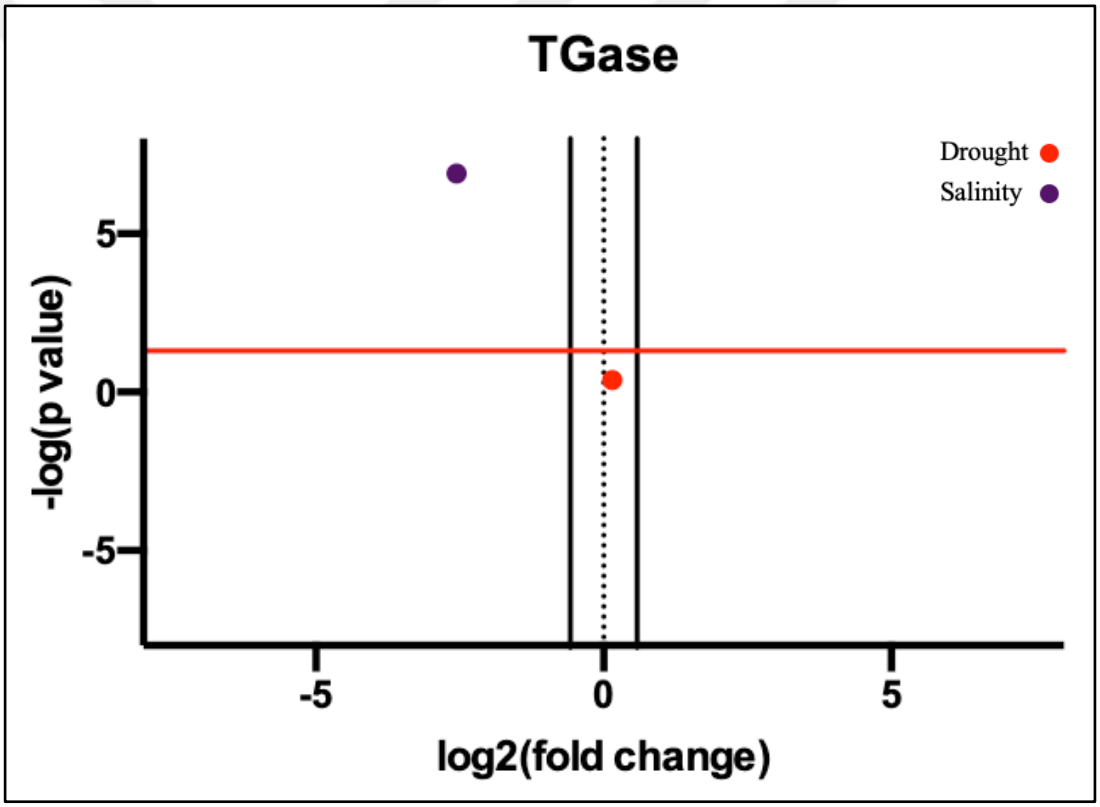


Figure 5.13. Gene expression differences and significance levels of TGase transcripts under drought and salinity stress represented as volcano plot. Upper left area indicates the significantly downregulated genes, lower left indicates downregulated genes, upper right indicates significantly upregulated genes and lower right indicates upregulated genes. The red line indicates the -log(0.05)=1.301. The straight horizontal black lines indicates the 1.5-fold change threshold. Dot colors represents the stress type.

5.8. HSP90 PROTEIN EXPRESSION UNDER DROUGHT AND SALINITY STRESS

In order to see expression difference of HSP90 protein under drought and salinity treatment western blot was performed using anti-HSP90-1 primary antibody (Agrisera Antibodies, Sweden) and goat anti-rabbit IgG secondary antibody (Agrisera Antibodies, Sweden). The protein level of HSP90 was upregulated under drought while a downregulation in HSP90 level was evident under salinity treatment in *Brachypodium distachyon* when compared to control samples of drought and salinity. As shown in Figure 5.14, Figure 5.15 and Figure A1, relative HSP90 protein expression was significantly upregulated approximately by 2-folds under drought stress when compared to its control along with a p value of 0.013.

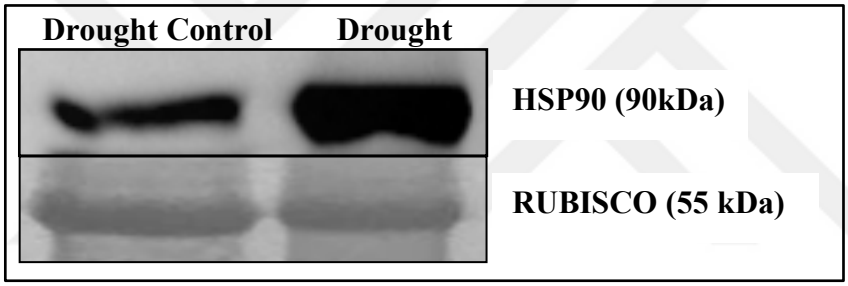


Figure 5.14. Effect of drought treatment on HSP90 protein levels. Representative western blot (of four independent experiments performed) reflecting total HSP90 protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

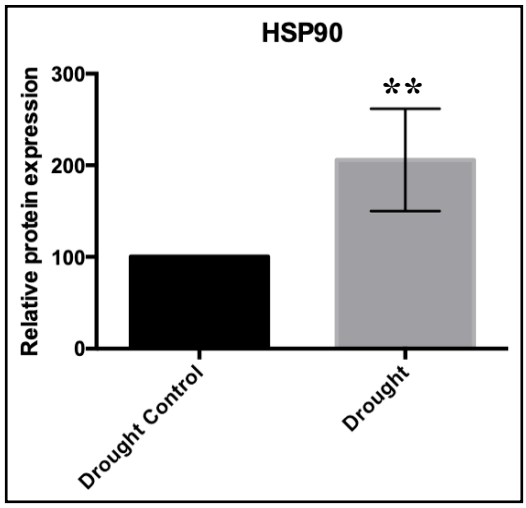


Figure 5.15. Relative HSP90 protein expression against RUBISCO under drought stress. Each value represents the mean ± SE of 4 replicates.. *Asteriks* shows significance at * p≤0.05, ** p≤0.01, *** p≤0.001.

Under salinity stress, relative HSP90 protein expression was significantly downregulated by 80 percent along with a p value of 0.005 when compared to its control (Figure 5.16, Figure 5.17 and Figure A2).

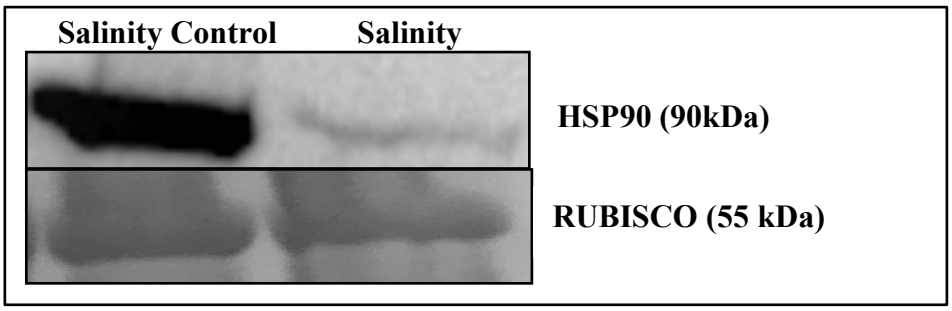


Figure 5.16. Effect of salinity treatment on HSP90 protein levels. Representative western blot (of three independent experiments performed) reflecting total HSP90 protein level obtained from salinity-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

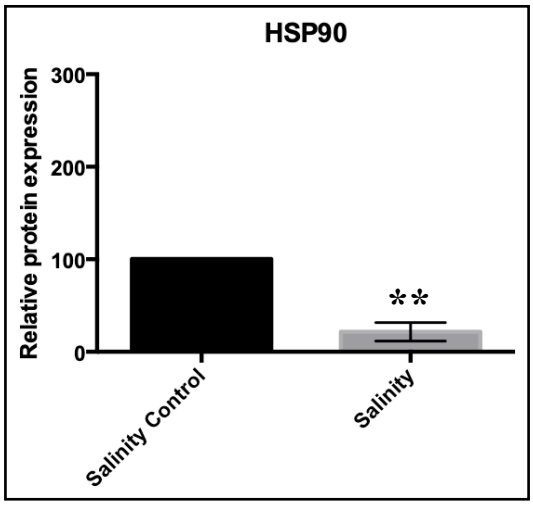


Figure 5.17. Relative HSP90 protein expression against RUBISCO under salinity stress. Each value represents the mean ± SE of 4 replicates. *Asteriks* shows significance at * p≤0.05, ** p≤0.01, *** p≤0.001.

5.9. TGase PROTEIN EXPRESSION UNDER DROUGHT AND SALINITY STRESS

In order to see changes in TGase protein levels under drought and salinity treatment western blot was performed using TGM2 CUB primary antibody (Thermo Fisher Scientific, USA) and anti-mouse secondary antibody (Santa-Cruz, USA). RUBISCO was used as a loading control for the normalization of the data. TGase protein was found to be downregulated under drought and salinity treatment in *Brachypodium distachyon* when compared to their control groups (Figure 5.18 and Figure 5.20). As shown in Figure 5.18, Figure 5.19 and Figure A3, under drought-treatment TGase protein expression was downregulated approximately 12 percent albeit this decrease was found not be statistically significant (p=0.41).

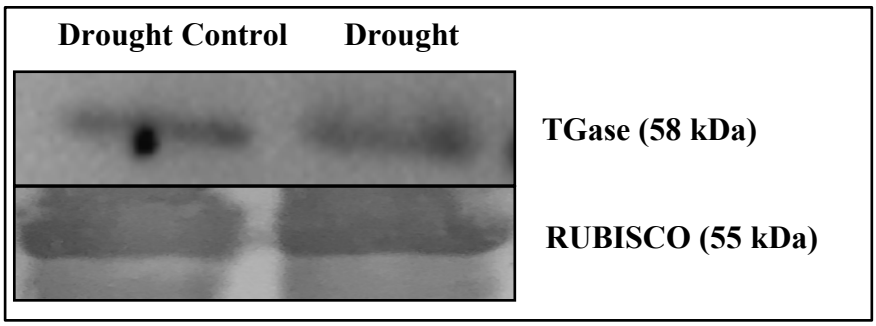


Figure 5.18. Effect of drought treatment on TGase protein levels. Representative western blot (of four independent experiments performed) reflecting total TGase protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

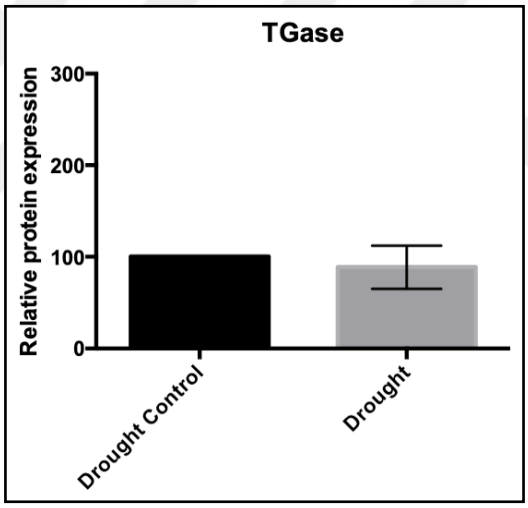


Figure 5.19. Relative TGase protein expression against RUBISCO under drought stress. Each value represents the mean ± SE of 4 replicates. *Asteriks* shows significance at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

As shown in Figure 5.20, Figure 5.21 and Figure A4, under salinity treatment, TGase protein expression was significantly downregulated almost by 82 percent when compared to its control along with a p value of 0.001.

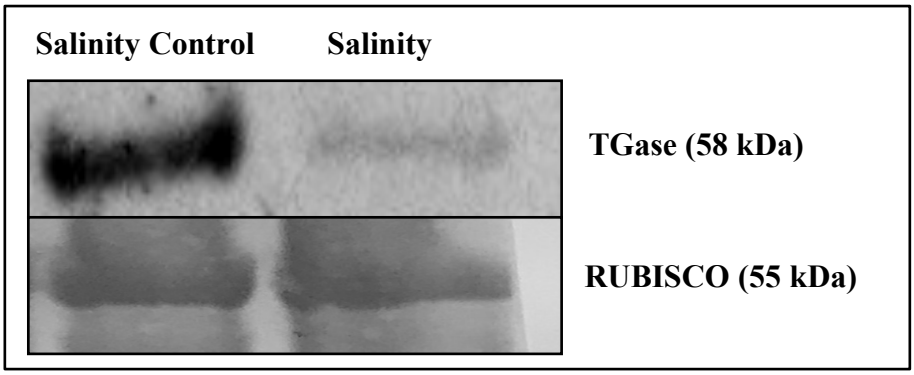


Figure 5.20. Effect of salinity treatment on TGase protein levels. Representative western blot (of four independent experiments performed) reflecting total TGase protein level obtained from salinity-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

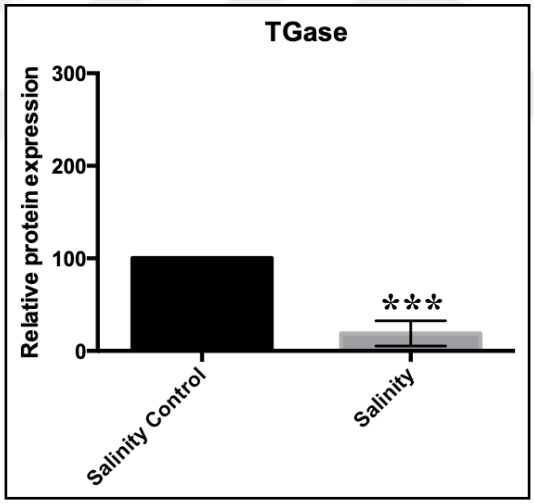


Figure 5.21. Relative TGase protein expression against RUBISCO under salinity stress. Each value represents the mean ± SE of 4 replicates. *Asteriks* shows significance at * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

6. DISCUSSION

The sessile structure of plants makes them the most stress-confronted organisms. As a result of the increasing atmospheric temperature, available water in soil is keep decreasing. Plants transpiration and evaporation leads plants to lose water as well. When these factors comes together drought stress is occurred [32]. On the other hand, in salinity stress, natural and human caused salinization comes together with water loss in the soil and causes changes in the ion balance osmotic potential. Water uptake by the root depends on the osmotic pressure, and because of its reduction, plants cannot take enough water from the soil [37]. Upon encountering with stress factor, plants go in a shock, which results in tolerance reduction, called alarm phase. In acclimation phase, plants try to deal with the stress by changing its cellular metabolism, leading an increase in tolerance level. If the stress stimulus continues, plants enter exhaustion phase. At this last phase, stress-induced homeostasis maintenance fails. If stress stimulus ends before the critical point, plants enter into the recovery phase, or else stress leads to death of the plant [167]. Plants give similar responses to drought and salinity stress since both stress factors share almost the same cellular mechanisms. The major role of cellular activities behind stress response belongs to protein expression, and transcriptional reprogramming is the first step. Basic stress response pathways depend on heat-shock proteins (HSPs), also known as stress proteins. The expression of HSP90 is affected by abiotic stress factors [168]. Also, the cross-linking enzyme TGase, which is well studied in animals, is involved in abiotic stress response pathways [169]. Plant TGases share same catalytic domain, immunorecognition sites and inhibitors with animal TG2 [147]. Further reports claimed that in order to maintain cytoprotection HSP90 was targeted by TG2 in H9c2 (Rat embryonic cardiomyoblast-derived) cells [157]. Also, under normal conditions, TG2 interaction with HSP70/HSP90 complex via the adaptor protein HOP (HSP70-HSP90 organizing protein) was elucidated [156]. As accumulating evidence suggests an interplay between TG2 and HSP90 in cells exposed to stress conditions, in this study, the objective was to understand whether HSP90 and TGase was co-regulated under drought and salinity stress in *Brachypodium distachyon*.

For this purpose, *Brachypodium distachyon* plants were grown and treated with 12 days of drought and 14 days of salinity stress. During drought-treatment, smaller plant stature, occurrence of brownish color in leaves, midrib rolled and flimsy leaves were observed.

Impaired mitosis caused by drought leads to elongated and expanded cells. Therefore, size of plant stature and leaf blade was reduced (Table 5.1) [170,171]. Also, cell membranes lose their sustainability and toughness under water deficit resulting in flimsy leaves [172]. Environmental stresses have direct effect on photosynthesis by damaging crucial photosynthesis elements, and chlorophyll content shows the photosynthetic capacity of the plant [173,174]. Chlorophyll degradation and pigment photo-oxidation lead to decrease in total chlorophyll content (Figure 5.3) and therefore the green color turned into brownish color in leaves (Table 5.1) [47,175]. Relative water content (RWC) indicates the water retention of the plant [175]. In response to drought stress, RWC was dramatically decreased in *Brachypodium distachyon* (Figure 5.1).

In salinity-treatment, plant stature and leaf blade size was reduced (Table 5.2) due to low osmotic potential, nutritional and ion imbalance [124,176]. As in the drought stress, decrease in the total chlorophyll content was observed (Figure 5.4) due to chlorophyll degradation. In response to salinity stress, RWC was reduced when compared to salinity control group (Figure 5.1). Another indicator for salinity-treatment is electrical conductivity of the soil. If the EC value of soil in the root zone is higher than 4 mS/cm, soil is defined as a saline soil [42,177]. In this study, soils of salinity control groups EC values were ranged between 2.20 mS/cm to 3.00 mS/cm where salinity-treated groups EC values were between 9.65 mS/cm and 11.10 mS/cm. This indicated that salinity treatment was sufficient enough to cause salinity stress on plants.

Proteins induced by salinity stress are divided into two groups as stress associated proteins and salinity stress proteins. The stress associated proteins accumulate not only due to salinity stress but also other abiotic stress factors where salinity stress proteins accumulate only in response to salinity stress [46]. In both prokaryotic and eukaryotic cells, Heat-shock protein 90 (HSP90) consists of approximately 1 percent of the total proteins in normal conditions, and in case of heat stress HSP90 increases to almost 6 percent of total protein [178,179]. HSP90s are present mainly in cytoplasm and rarely found in endoplasmic reticulum (ER), mitochondria and chloroplast in eukaryotes [83,180]. In this study, expression difference of HSP90 transcript and protein levels under drought and salinity-treatments in *Brachypodium distachyon* was detected by qRT-PCR and immunoblotting. Plants that are treated with 12 days of drought were harvested at the critical point of the stress and analyzed for all HSP90 transcript levels. qPCR results showed upregulation of HSP90 transcripts in the range of

1.45- to 2.23-fold except for Bradi3g39620 (chloroplast distributed) and Bradi1g30130 (ER distributed) that were downregulated by 1.02-fold and 1.19-fold, respectively (Figure 5.8 and Figure 5.9). Under salinity treatment, HSP90 transcripts were downregulated in the range of 1.96- to 7.14-fold (Figure 5.10 and Figure 5.11). According to the Zhang et al. (2017), HSP90 genes were divided into 3 phylogenetic groups by using MUSCLE program and MCMC (Markov Chain Monte Carlo) based on Bayesian interference. According to subcellular localization analysis, group 1 HSP90 genes were mainly distributed in nucleus (20 percent) and cytoplasm (80 percent). Group 2 genes were mostly distributed in ER (78 percent) and Group 3 genes were distributed in chloroplast (40 percent) and mitochondria (45 percent) [162]. The genes and their corresponding distributions investigated in this study are as follows: Bradi5g02370, Bradi3g39620, Bradi3g39590 and Bradi3g39630 in cytoplasm, Bradi1g30130 in ER, Bradi4g06370 in mitochondria, Bradi4g32941 and Bradi3g38897 in chloroplast. The provided gene localizations are verified by using BLAST with known *Arabidopsis thaliana*, *Triticum aestivum* and *Oryza sativa* HSP90 gene sequences. The two chloroplastic HSP90 transcripts were found to be segmental duplications, in addition, although primer sequence and phylogenetic location was stated, there were no expression results in 12-24-48hour drought and salinity treatment [162]. On the other hand, upon 12 days drought and 14 days salinity treatment, Bradi4g32941 expression levels were found to be upregulated by 2.22-fold and downregulated by 5.88-fold, respectively (Figure 5.8 and Figure 5.10). It can be suggested that Bradi4g32941 might be involved in the late response mechanism instead of early response to osmotic stress. According to the study of Xu *et al*. (2013), *Glycine max* HSP90 transcripts, which were localized in chloroplast and mitochondria were in a fluctuating trend under 24 hours of NaCl treatment. In dicot model plant *Arabidopsis thaliana*, the overexpression of HSP90.5 and HSP90.7 genes were resulted in higher salinity sensitivity. Furthermore, overexpression of HSP90 genes in *Arabidopsis thaliana* interrupted the Ca^{++} -binding protein homeostasis and Ca^{++} signaling pathway interruption. Thus, it was suggested that HSP90 was regulated by one of the Ca^{++}-dependent proteins [181].

At protein level, HSP90 was found to be upregulated by 2-folds (Figure 5.14 and Figure 5.15). According to a study of Ashoub *et al*. (2013), HSP90 protein is also upregulated in drought-treated barley (*Hordeum vulgare* L.) leaves [182]. In addition, stomatal closure, which is one of the primary effect of water deficit, was found to be dependent on HSP90 and

HSP70 [33,183]. Environmental stresses have an impact on protein conformation as an increase in misfolding rate. Those misfolded and denatured proteins lose their ability to function normally and tend to form aggregates. In order to prevent this aggregates caused by drought treatment, levels of molecular chaperones was observed to increase in rice [184]. In contrast, in *Brachypodium distachyon*, HSP90 protein levels were downregulated almost 80 percent when compared to its control group after 14 days of salinity-treatment (Figure 5.16 and Figure 5.17). In four different *Solanum lycopersicum* L. genotypes, HSP90 protein were found to be downregulated under salinity [185]. Also, in *Puccinellia tenuiflora* (Turcz.), a poaceae family member, HSP90 protein expression levels were found to be downregulated under salinity stress [186]. Another two different cultivars, Penncros and Penn-A4, monocot plant *Agrostis stolonifera* showed a downregulation in HSP90 protein level under salinity stress [187]. All these studies are confirming the 80 percent downregulation of HSP90 protein expression under salinity stress in *Brachypodium distachyon*.

In order to detect whether changes in the expression levels of HSP90 genes in response to abiotic stress was in parallel with TGase, levels of TGase transcript and protein under drought and salinity-treatment in *Brachypodium distachyon* were detected by real-time PCR and immunoblotting, respectively. At the transcript level, TGase showed a 1.11-fold change under drought and 5.88-fold downregulation under salinity stress (Figure 5.12). Total TGase protein levels upon drought-treatment were reduced by approximately 12 percent (Figure 5.18 and Figure 5.19) whereas upon salinity-treatment 82 percent downregulation was observed (Figure 5.20 and Figure 5.21).

Although drought and salinity control groups of *Brachypodium distachyon*s were grown under the same conditions, there was a difference between HSP90 and TGase protein levels in stress control groups. The main reason for this comes from the duration of stress treatments. Drought-treatment lasts 12 days where salinity-treatment lasts 14 days. So, after drought control samples were harvested, salinity control samples were grown for 2 more days until the end of salinity treatment. Within those two days, *Brachypodium distachyon* plants differ in their growth stage, and therefore, proteome profile might have been changed as well. According to a study of Lilley *et al.* (1998), TGase activity was found to be higher in the roots, and also TGase activity levels were depending on the growth stage and found to be decreased with development of barley and wheat, which are close relative of *Brachypodium distachyon* [152]. Interestingly, in a recent study of Zhong *et al.* (2019),

TGase activity was found to be higher in leaves rather than roots and increased during development of tomato [154], suggesting that expression level of TGase was species-dependent and each plant species had its own expression profile. The results of TGase gene expression levels under salinity stress showed a correlation with TGase protein; however under drought stress, protein level showed a decrease of 12 percent where at transcript level it showed a change of 1.11-fold. The reason for this inconsistency is probably the localization of TGase protein. As in mammalian TGases, plant TGases also can be found extracellularly [188]. So, the TGase protein level within cell was decreased where the TGase transcript level was increased.

TGases have roles in free PA to PIS-bound-PA (perchloric acid insoluble bound PA) transformation. According to the study of Liu *et al.* (2003), free spermidine, free spermine and PIS-bound-putrescine increased the osmotic tolerance of wheat [189]. Thus, the decrease in TGase protein level might have resulted in the increase of free Spm and Spd as a response to drought and salinity treatment. As a close relative, *Brachypodium distachyon* might share the same mechanism in response to osmotic stress. The study of Garcia-Jimenez et al. (2007) showed that the Spm, Spd and Put levels increased because of the reduced TGase activity under salinity treatment in *Grateloupia doryphora* [190]. HSP expression could be effected by PAs either directly or indirectly. Indirect effect of PAs to HSP expression is due to the free-Put accumulation. The membrane integrity might decrease in response to excessive accumulation of free-Put. The loss of membrane integrity leads to damages in membrane-perception and signal transduction of environmental stimulus; by this way, expression level of HSP-coding genes is affected [191]. The direct effect of PAs on HSP levels is at protein synthesis level. In many organisms, PAs are found to be involved in protein synthesis by stabilizing the nucleic acids molecular structure, promoting the ribosomal subunit association and affecting the nascent polypeptide chains elongation [192]. Imbalanced PA level due to environmental stress might lead to the opposite effect of PAs on protein synthesis [191]. In rice, the increase in PA accumulation was reported under salinity stress [130]. According to a recent study of Toumi *et al.* (2019), the upregulation in PA levels leaded to a downregulation of HSP90 transcript and protein levels [193]. Also, in wheat, the increase in free spermine and spermidine was found to be related to osmotic tolerance through stomatal closure, and conserving the (free-SPD+free-Spm)/(free-Put) ratio as free-Put levels were found to be disadvantageous [189]. As TGases are the responsible proteins

for the PA incorporation, decreased levels of TGase might be related to conservation of free PA form in order to provide salinity tolerance.

Under drought stress, changes in TGase transcript were not found to follow a parallel trend with that of any HSP90 transcript. However, under salinity stress, regulation of TGase transcript level and the mitochondrial and chloroplastic HSP90 transcript levels were correlated with 5.88-fold downregulation. It can be suggested that there might be no interaction between HSP90 and TGase under drought stress while under salinity stress transcript levels of HSP90 and TGase might be co-regulated. At protein level, changes in the levels of proteins of interests were not in association under drought stress. On the other hand, under salinity stress HSP90 and TGase were downregulated by 80 and 82 percent, respectively. Although drought and salinity stress response pathways are alike, there is an important difference between them. Salinity stress causes an imbalance in soil ions. The main reason for this difference comes from the stress characteristics. Drought stress is related to water and can be classified as osmotic stress type. However, salinity stress has an effect on water status as well as ion balance of the soil. Thus, it is related to both osmotic (as a secondary effect) and chemical stress factor. Differences in protein and transcript levels of chloroplastic and mitochondrial HSP90 were parallel with TGase transcript and protein level differences. Also, one of the localizations of TGase is the chloroplasts [194,195]. Thus, HSP90 and TGase interaction might be required for chloroplast-related stress response.

7. CONCLUSION AND FUTURE PROSPECTS

The co-regulation of HSP90 and TGase in response to drought and salinity treatment in the monocot model plant *Brachypodium distachyon* was studied.

Under drought stress, HSP90 transcript levels were found to be upregulated and TGase transcript level was slightly changed by 1.11-fold. At protein level, HSP90 protein expression was found to be upregulated by approximately 2-folds while TGase protein expression was downregulated by 12 percent.

Under salinity stress, HSP90 transcripts were downregulated where TGase transcript level was downregulated by 5.88-fold. HSP90 protein expression level was found to be downregulated by 80 percent. On the other hand, TGase protein expression was found to be downregulated by almost 82 percent.

According to the transcript and protein levels, known animal and plant pathways and PA interactions under osmotic stress, there is no evidence about HSP90 and TGase co-regulation under drought stress. Under salinity stress, transcript levels changes of mitochondrial and chloroplastic HSP90 and TGase are together parallel with close downregulation of protein expression levels. Hence, there might be an interaction between HSP90 and TGase in *Brachypodium distachyon* under salinity stress.

In the future work, detecting the localizations of both HSP90 and TGase under drought and salinity stress will give further information about their interaction. Also, detecting the PA levels under salinity stress by HPLC analysis will reveal whether the interaction between HSP90 and TGase is dependent on PA mechanisms. This study can be used in understanding the molecular pathways behind the drought and salinity stress responses and may create a milestone in developing stress tolerant plants.

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APPENDIX A: WESTERN BLOT REPLICATES REPRESENTING THE EFFECT OF DROUGHT AND SALINITY TREATMENTS ON HSP90 AND TGase.

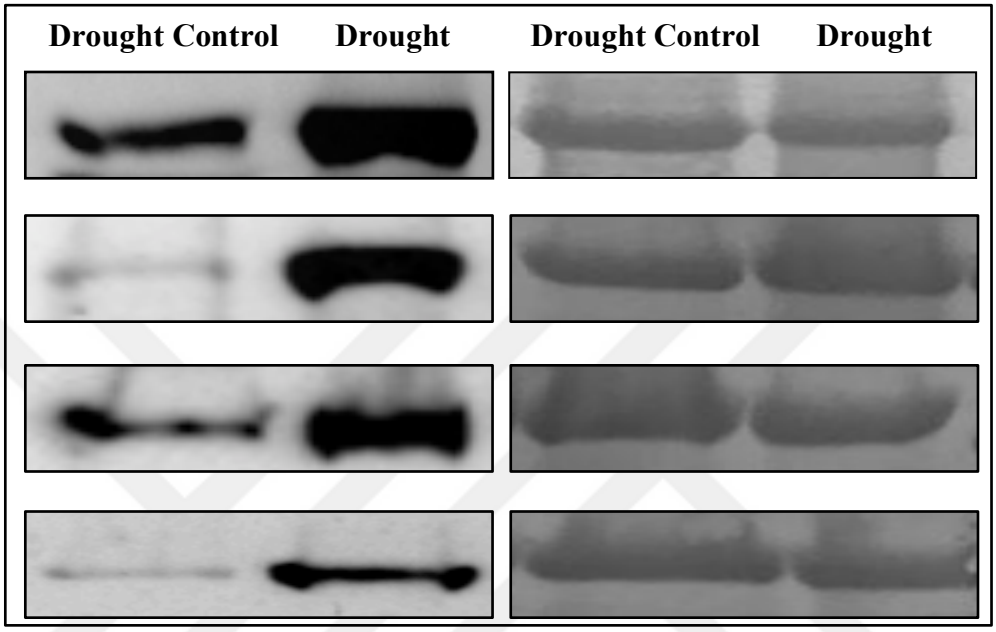

Figure A.1. Effect of drought treatment on HSP90 protein levels. Western blots reflecting total HSP90 protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

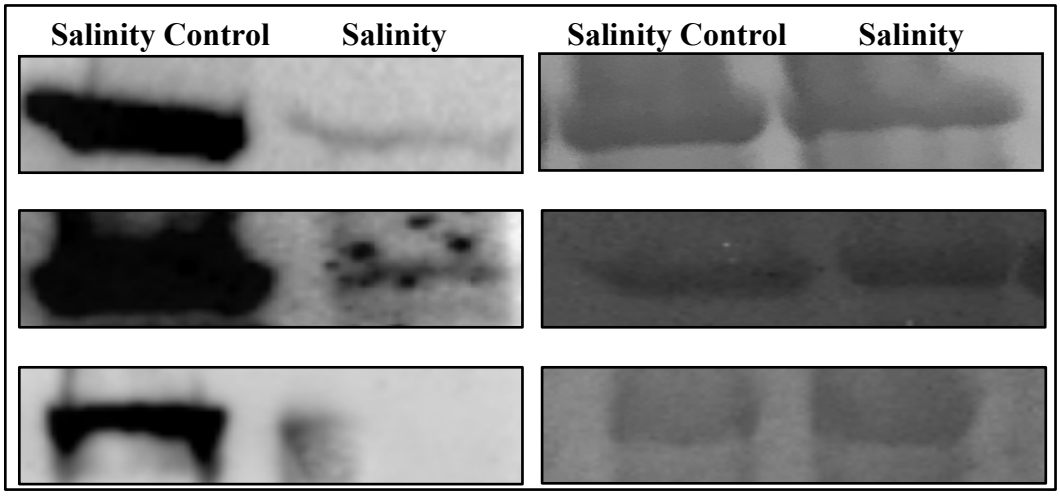

Figure A.2. Effect of salinity treatment on HSP90 protein levels. Western blots reflecting total HSP90 protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

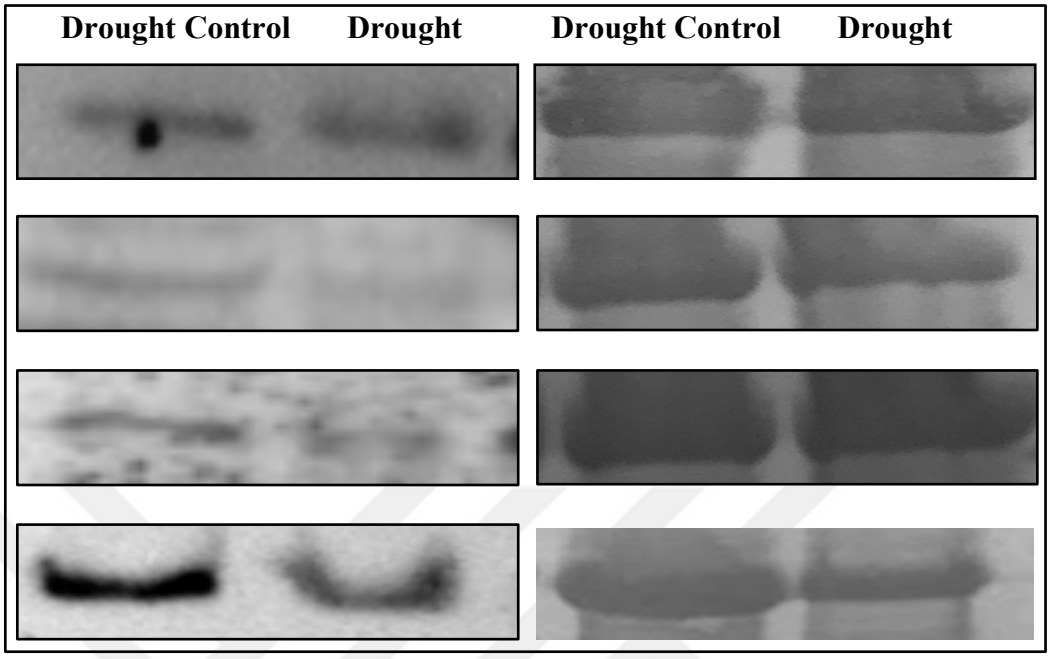

Figure A.3. Effect of drought treatment on TGase protein levels. Western blots reflecting total HSP90 protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.

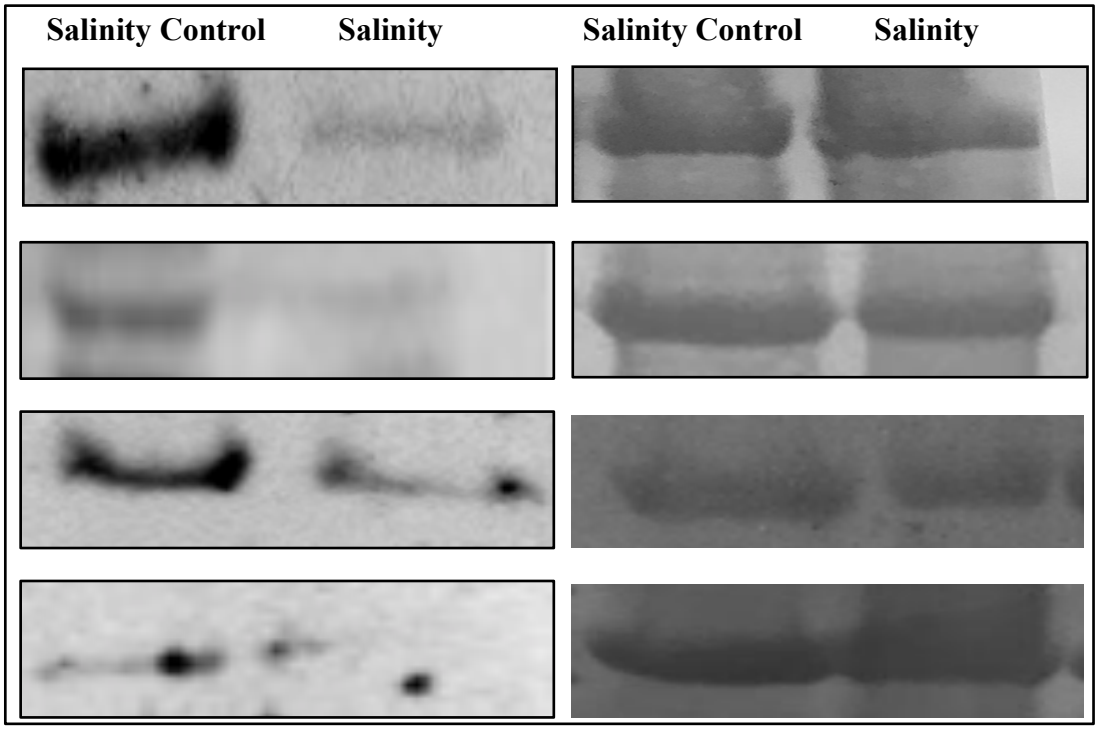

Figure A.4. Effect of salinity treatment on TGase protein levels. Western blots reflecting total HSP90 protein level obtained from drought-treated *Brachypodium distachyon* plant leaves, RUBISCO was used as loading control.