EXPRESSION ANALYSIS OF NAC TYPE TRANSCRIPTION FACTORS ON WHEAT SEEDLINGS UNDER ABIOTIC STRESS CONDITIONS

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ABSTRACT

EXPRESSION ANALYSIS OF NAC TYPE TRANSCRIPTION FACTORS ON WHEAT SEEDLINGS UNDER ABIOTIC STRESS CONDITIONS

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Wheat is the most important grain crop grown in our country providing greatest part of the daily nutritional requirement. Abiotic factors including salinity, drought, cold and heat stresses affect quality and yield of wheat varieties used for the production of both bread and pasta flour.

NAC proteins form one of the widest families of plant specific transcription factors. Members of this family are related with development, defense and abiotic stress responses. *TaNAC69-1* and *TtNAM-B2* genes were isolated from *T.aestivum* and *T.turgidum*, respectively. Then they were cloned into different monocot and dicot expression vectors to be used for further wheat and tobacco genetic transformation studies. To understand effects of salinity, drought, cold and heat stresses on expression profiles of *TaNAC69-1* and *TtNAM-B2* genes, quantitative real time PCR was performed. The time series expression profiles of *TaNAC69-1* show that it was significantly up-regulated following by salt, drought, cold and heat stress treatments.

Except for heat stress, expression of *TtNAMB-2* gene also significantly induced under drought, salt and cold stress conditions.

Microarray analysis also performed to indicate effects of cold and heat stress treatments on global gene expression profiles of wheat. Differentially regulated genes show that temperature changes directly affected a large and complex transcriptional network associated with defense, metabolism and development. Genes involved in cold stress-responsive and different cold acclimation proteins were extremely up-regulated upon exposure to cold stress. Both expression levels of small and large sub-unit heat shock proteins significantly increased following heat stress period. In addition to these protective agents, transcription factors also played a central role to deal with severe effects of low and high temperature conditions.

Keywords: Wheat, NAC Type Transcription Factors, Abiotic Stress, qRT-PCR analysis, Microarray analysis

ÖZ

BUĞDAY FİDELERİNDE ABİYOTİK STRES KOŞULLARI ALTINDA NAC TİPİ TRANSKRİPSİYON FAKTÖRLERİNİN İFADE ANALİZİ

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Buğday günlük besin ihtiyacının büyük bir kısmını sağlayan, ülkemizde yetiştirilen en önemli tahıl ürünüdür. Tuzluluk, kuraklık, soğuk ve sıcak streslerini içeren abiyotik faktörler, hem ekmeklik hem de makarnalık un üretiminde kullanılan buğdayın kalitesini ve verimini etkilemektedir.

NAC proteinleri bitkiye özel transkripsiyon faktörlerinin en geniş ailelerinden birini oluşturmaktadır. Bu ailenin proteinleri gelişim, savunma ve abiyotik stres tepkileriyle ilişkilidir. *TaNAC69-1* ve *TtNAM-B2* genleri sırasıyla *T.aestivum* ve *T.turgidum*' dan izole edilmiştir. Daha sonra, ileride yapılacak olan buğday ve tütün genetik transformasyon çalışmalarında kullanılması için, genler ayrıca farklı tek çenekli ve çift çenekli bitkilere özgü ifade vektörlerine klonlanmıştır. Tuz, kuraklık, soğuk ve sıcak streslerinin, *TaNAC69-1* ve *TtNAM-B2* genlerinin ifade profillerine etkilerini anlamak için, kantitatif gerçek zamanlı PZR yapılmıştır. Zamana bağlı *TaNAC69-1* geninin ifade profili, *TaNAC69-1* geninin ifadesinin tuz, kuraklık, soğuk ve sıcak stres uygulamalarını takiben önemli derecede arttığını göstermiştir. Sıcaklık

stresi dışında, *TtNAM-B2* geninin ifadesi de tuz, kuraklık, ve soğuk stresi durumları altında önemli derecede artmıştır.

Soğuk ve sıcak stres uygulamalarının buğdayın genel gen ifade profili üzerine etkisini göstermek için microarray analizi de yapılmıştır. Farklı düzenlenen genler, sıcaklık değişimlerinin savunma, metabolizma ve büyümeyle ilişkili büyük ve karmaşık bir transkripsiyon ağını direk olarak etkilediğini göstermiştir. Soğuk stresine cevap veren ve farklı soğuk alıştırma proteinlerini kodlayan genlerin, soğuk stresine bağlı olarak aşırı derecede gen ifade seviyeleri artmıştır. Hem küçük hem de büyük alt üniteli ısı şok proteinlerinin gen ifade analizleri sıcaklık stresi periyodunu takiben önemli derecede atrmıştır. Bu koruyucu moleküllere ilaveten, transkripsiyon faktörleri de düşük ve yüksek sıcaklık koşullarının şiddetli etkileriyle mücadelesinde önemli bir rol oynamıştır.

Anahtar Kelimeler: Buğday, NAC Tipi Transkripsiyon Faktörleri, Abiyotik Stres, Kantitatif Gerçek zamanlı PZR Analizi, Mikroarray Analizi.

To my son Mert Can and my wife Pınar

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ABA	Abscisic acid
ABC	ATP-binding cassette
AFLP	Amplified fragment length polymorphisms
AP2/ERF	Apetala 2/ ethylene responsive factor
COR	Cold responsive
DEPC	Diethylpyrocarbonate
DRE	Drought-responsive element
DRE/CRT	Dehydration response element / C-repeat
DREB	Drought responsive element binding
GOEAST	Gene Ontology Enrichment Analysis Software Toolkit
GSH	Glutathione
GSP	Gene Specific Primer
HSP	Heat shock protein
LEA	Late embryogenesis abundant
MAS	Marker assisted selection
MM	Mismatch
MYC	Myelocytomatosis
NAC	NAM, ATAF1, 2 and CUC
PEG	Polyethylene glycol
PM	Perfect match
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphisms
RMA	Robust Multiarray Analysis
ROS	Reactive oxygen species
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SNP	Single nucleotide polymorphisms
TF	Transcription factor

CHAPTER 1

INTRODUCTION

1.1. Characteristics of Wheat Plant

The most important grain crop cultivated in the world is wheat, providing greatest part of the daily nutritional requirement for human diet. Wheat is an annual and a monocotyledonous plant, belonging to the Poaceae family and *Triticum* genus in plant systematics. Members of this family are economically important species including staple food grains, cereal crops, lawn and forage grasses, and bamboo.

It is believed that wheat has originated in Middle East and Southwestern Asia. Recent studies show that the first domestication of wheat is narrowed to a small region of southeastern Turkey (Ozkan *et al*., 2002). Wheat farming had spread to Asia, Europe and North Africa after about 4,000 B.C.

Two wheat species are commonly cultivated throughout the world. The forms of *Triticum aestivum* known as bread wheat are composed of different types of winter and spring cultivars. *Triticum aestivum* is hexaploid (6x), that forms 21 pairs of chromosomes (2n=42) during meiosis. Its genome size is 16 billion base pairs organized into 3 closely related groups of chromosomes, (A, B and D genomes) containing 7 pairs of chromosomes (AABBDD) (Brewer *et al*., 1969). *Triticum aestivum* is obtained from hybridization of tetraploid wheat, *Triticum turgidum*, with the diploid goat grass *Aegilops tauschii* (Akhunov *et al*., 2005). *Triticum turgidum* ssp. durum or pasta wheat includes durum and red durum wheat classes. *Triticum*

durum has about 10 billion base pairs genome size with 28 chromosomes being diploid AABB (Gill and Friebe, 2002).

According to Food and Agricultural Organization of the United Nations (FAO) statistics in 2009, world wheat production was about 685 million tons in 225 million ha of total cultivated area. China, India, United States of America, Russian Federation and Canada are the major wheat producers which provide about 70% of total world production. Turkey is the tenth country with 24 million tons of (nearly 3.5% of total production) wheat production in 2009.

1.2. Plant Abiotic Stresses

The term plant abiotic stress implies numerous stresses including drought, salinity, extreme temperatures (too high or too low), waterlogging, high or low light intensity, metal toxicities, nutrient deficiencies, UV radiation, acidic or alkaline soils. Abiotic stress leads to crop loss worldwide, decreasing average yields of economically important crops by more than 50% (Bray *et al*., 2000). So, these stresses are the most important limiting factors for agricultural productivity, resulting in losses worth hundreds of million dollars each year (Mahajan and Tuteja, 2005).

Plant growth, development and productivity are adversely affected from abiotic stresses, resulting in activation of a series of morphological, physiological, biochemical and molecular changes in plants (Bhatnagar-Mathur *et al*., 2008). Drought, salt, extreme temperatures and combinations of them are the most frequently encountered abiotic stresses for plants. Plants which exposed to abiotic stresses send a signal to induce transcription factors and turns on stress related genes, causing increased levels of metabolites and proteins for protection (Figure 1.1).

1.2.1. Drought and Salt Stresses

Among the abiotic stresses, drought and salinity are the most important ones that decrease agricultural productivity dramatically. Drought and salinity may cause

serious salinization of more than 50% of all arable lands by the year 2050, because of their wide and fast distribution of worldwide (Bray *et al*., 2000). They induce osmotic stress which adversely affects the ion homeostasis and distribution in the cell (Zhu, 2001). Drought, salt and osmotic stress are actually connected each other and trigger formation of oxidative stress resulting in denaturation of functional and structural proteins (Smirnoff, 1998). These environmental stresses induce similar cell signaling pathways and cellular damage (Zhu, 2002). High salt concentration in the soil forms a low water potential zone which makes difficult for the plant to take water and nutrients into cells. So, salt stress cause the formation of water deficit condition in plant and create physiological drought.

Drought stress, also known as water deficit occurs when the water level in the soil is decreased and atmospheric conditions continuously cause water loss by transpiration or evaporation (Jaleel *et al*., 2009). Besides water lack, low temperature and salinity also induce drought stress in plants (Wood, 2005). Drought together with salt stresses brings about loss of water, resulting in stomata closure, limitation of gas exchange and impair in osmotic homeostasis. Consequence of drought stress is enhanced reactive oxygen species (ROS) production in the dehydrated cells. The normal bilayer structure of membrane is disrupted from removal of water. Following disruption, loss of membrane integrity, selectivity, and activity of membrane based enzymes and organelle proteins occur in the lipid bilayer. Besides the cell membrane, cellular metabolism is also affected from water deficit because of increase in concentration of cellular electrolytes in protoplasm (Mahajan and Tuteja 2005). In addition to cellular level, various physiological and biochemical reactions including photosynthesis, respiration, ion uptake, carbohydrate and nutrient metabolism are affected when dehydration occurs. Some morphologic changes in leaf and root tissues are also observed consequences of these physiological and biochemical reactions. While the leaf area is diminished to reduce transpiration, the relative growth of root is enhanced in drought condition to access water source in deeper soil layers. Other drought injury symptoms include development of leaf scorch and premature or abnormal fruit, leaf dropping and thinning of canopy (Bartels & Sunkar, 2005).

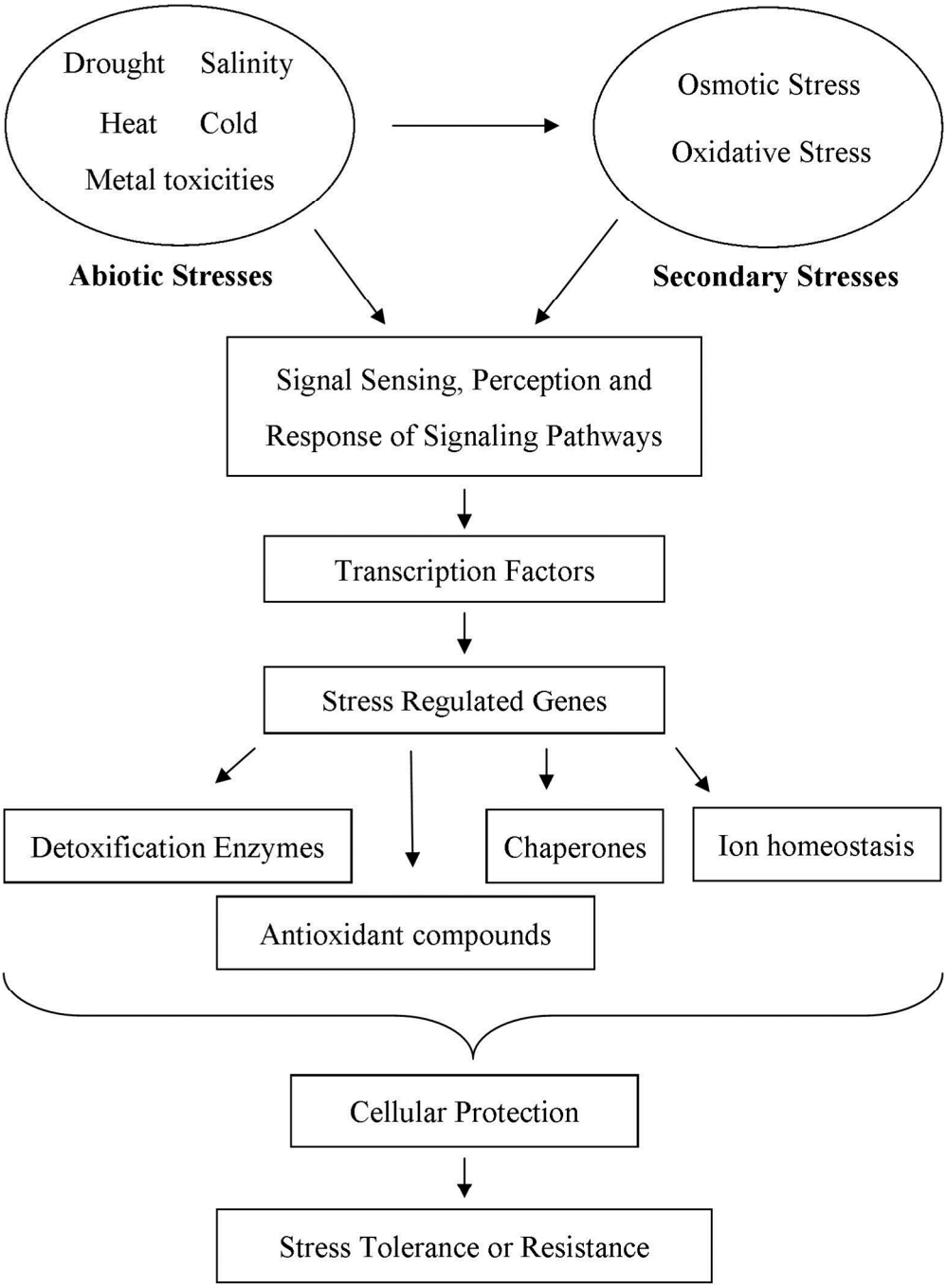


Figure 1.1. Schematic presentation of response of abiotic stress in plants.

The basic mechanism for protection of plants against drought stress is the keeping of turgor during periods of water deficit by adjusting the osmotic pressure of cells. This

can be achieved by two main ways. First one is that ions that increase osmotic pressure in the cell are separated from protoplasm and stored into cellular compartments. Secondly, antioxidant compounds, osmolytes such as proline, glycine betaine, mannitol, trehalose, ononitol and ectoine are produced to readjust cellular osmotic potential (Langridge *et al*., 2006). Other mechanisms to cope with ROS are the production of chemical antioxidants such as ascorbic acid, glutathione and α-tocopherol and enzyme systems such as peroxidases and superoxide dismutase. The damaging electrons originated from ROS are directly removed by chemical antioxidants and scavenged enzymatically by antioxidant enzymes.

Salinity stress is represented as two ways in plants. High concentration of salt can be found in soil which makes it harder for roots to take out water and in the plant cells. More than 800 million hectares of land, accounting for more than 6% of the world's total land area are affected from salt stress (Munns and Tester, 2008). Besides natural salinity from the accumulation of salts over long periods of time, salinity level in the soil has also raised into cultivated agricultural areas due to land clearing or irrigation, which results in availability of high concentration salts in the root zone. Although sodium chloride (NaCl) is the most soluble and abundant form, various types, mainly chlorides of calcium, and magnesium and trace amount of sulfates and carbonates forms are found in the soil (Chhabra, 2005).

Salinity is a soil condition characterized by a high concentration of soluble salts. If the concentration of NaCl exceeds to 40 mM which generates an osmotic pressure of nearly 0.2 MPa, soil can be defined as a saline soils. Plants show different growth responses against salinity so, salinity tolerance is not same for each plants. Among the cereals, rice (*Oryza sativa*) is the most sensitive and barley (*Hordeum vulgare)* is the most tolerant plants. Bread wheat (*Triticum aestivum*) is more tolerant to salt stress than durum wheat (*Triticum turgidum* ssp. *durum*), presented in Figure 1.2. The most tolerant monocot plant species is tall wheatgrass (*Agropyron elongatum*), which is halophytic relative of wheat. The variation of salinity tolerance between dicot and monocot plants is also observed (Munns and Tester, 2008).

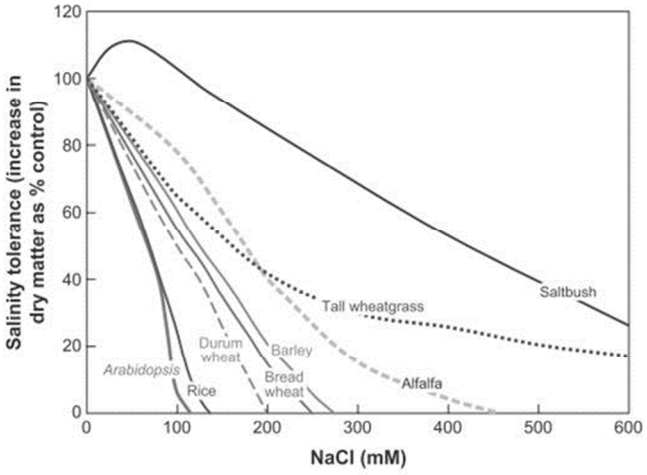


Figure 1.2. Diversity in the salt tolerance of various monocot and dicot plant species (taken from Munns & Tester, 2008).

When the soil salinity increases, the leaf growth rate is slow down because of the osmotic effect of the salt around the roots. Firstly, leaf loses water and turgor changes. Within hours, plant cell adjust its own turgor but cell elongation rates are decreased (Fricke and Peters, 2002). Over days, cell division rate is also reduced, resulting in formation of small and thick leaves. However, growth of root is less affected than leaf growth. When compared to leaf cells, elongation rate of root cells shows fast recovery after exposure to NaCl (Munns, 2002). There is an interaction between stomatal movement and photosynthesis rate under the salt conditions. Stomata movement is affected immediately under the high salt concentration. Consequence of changing turgor in stomata, photosynthesis rates in salt-treated plant leaves are reduced (James *et al*. 2002). When the rate of photosynthesis decreases, increase in the formation of ROS is observed (Foyer and Noctor, 2005), causing activation of enzymes for detoxification of these species. Besides the biochemical reactions, some changes in cellular metabolism also occur under the salt stress. Ionic equilibrium is disrupted because Na^+ ions are taken into cell which facilitates uptake of Cl^- ions down the chemical gradient. High Na^+ ions concentration has a lethal effect on the functioning of some of the enzymes (Niu *et al*., 1995) and causes

formation of nutritional defects due to reduced uptake of phosphorus, potassium, nitrate and calcium ions that are macronutrients for plant growth. Other visible salinity symptoms include reduction in leaf surface, fresh and dry weights of all parts of plant and formation of necrotic zones on leaves.

Plants have evolved three main tolerance mechanisms for salt stress. The first one is tolerance to osmotic stress. Cell expansion in root tips and young leaves enables reduction of osmotic stress, resulting in stomata closure. So, leaf area increases and osmotic stress is diminished. However, greater leaf growth is beneficial only plants have enough soil water. If the water supply in the soil is limited, the soil water is used up before the grain is fully matured. The second mechanism is Na^+ ions exclusion from leaf blades. Na^+ ions are ejected by root cells, so Na^+ ion accumulation in leaves is prevented. The last one is tissue specific salt tolerance. Na^+ and Cl^- ions are stored within the cytoplasm, especially in mesophyll cells in the leaf. So, toxic effects of these ions are avoided at the cellular and intracellular level (Munns and Tester, 2008).

1.2.2. Cold and Heat Stresses

About two thirds of the world's landmass is annually exposed to temperatures below the freezing point and about half of it suffers from temperatures below – 20°C (Beck *et al*., 2004). Therefore, another severe abiotic stress for plants is low temperature stress, which not only limits growth, development and distribution of plants but also cause serious yield losses of number of crop plants (Heino and E Palva, 2004). Low temperature stress includes chilling (below 20˚C), freezing (below 0˚C), and frost (below -20˚C) temperatures. Cold acclimation, also known as cold hardening, is defined as increasing cold temperature tolerance over time. Cold acclimation is actually very complex process which involves biochemical, physiological and gene expression level changes. During the cold acclimation period, hundreds of stress responsive genes regulated again and consequences of this result in production of proteins and metabolites for protection of cell structure and function from low temperature stresses (Kalberer *et al*., 2006). Plant species have different temperature

requirement. Tropical species are chilling-sensitive and affected adversely from higher than the freezing temperature. Chilling tolerant plants such as rye, oats, wheat and barley withstand below zero temperatures, but are severely damaged because of ice formation in the tissues (Heino and E Palva, 2004). However, in a seedling stage these plants require vernalization, which allows them to survive under freezing stress. So, the degree of plant damage depends on species, developmental stage, duration of the stress, the rates of cooling (and rewarming), and whether ice formation occurs intra- or extra-cellularly (Beck *et al*., 2004).

Physiological effects of cold stress on plants are different for chilling and freezing stresses (Figure 1.3). Chilling stress, detrimental to tropic and subtropic plants shows effects of direct result of low temperature. Bio membrane function is primarily affected from chilling stress and its fluidity decreases, resulting in solidification of cell membranes and inactivation or deceleration of membrane-bound ion pumps. Consequently, membrane functions are broken down. Chilling stress also causes slowing down metabolism. If the photosynthetic components are not induced due to slow metabolic activity, light energy cause initiation of oxidative stress (Beck *et al*., 2004). Observed phenotypic symptoms of chilling stress include reduction of leaf enlargement, wilting, chlorosis, necrosis (Mahajan and Tuteja, 2005) and inhibition of plant reproductive development (Wen *et al*., 2002).

Freezing stress cause ice-crystal formation which can be occurred in extracellular space for freezing tolerant plants, or intracellular space for susceptible plants, causing cell death due to disintegration of the cellular membranes (Margesin *et al*., 2007). During the extracellular freezing, ice-crystal formation leads to reduction of the water potential outside the cell. Then free water from the cytoplasm moves through the plasma membrane by osmosis (Figure 1.4). Removed water from a cell is actually affected from initial solute concentration of the cytoplasm and freezing temperature. For example, freezing temperature at -10 ˚C creates in a water potential of – 11.6 MPa. Assuming the initial osmotic potential of cell is -1 MPa, 90% of free water will be removed from the cell. If the initial osmotic potential increases to – 2 MPa due to accumulation of high amount of solutes, in this case only 80% of cellular

water will be moved to outside of the cell at the same freezing temperature (Xin and Browse, 2000). Freeze-induced dehydration occurs as a result of freezing stress. For this reason, freezing, drought and salinity stresses share common features.

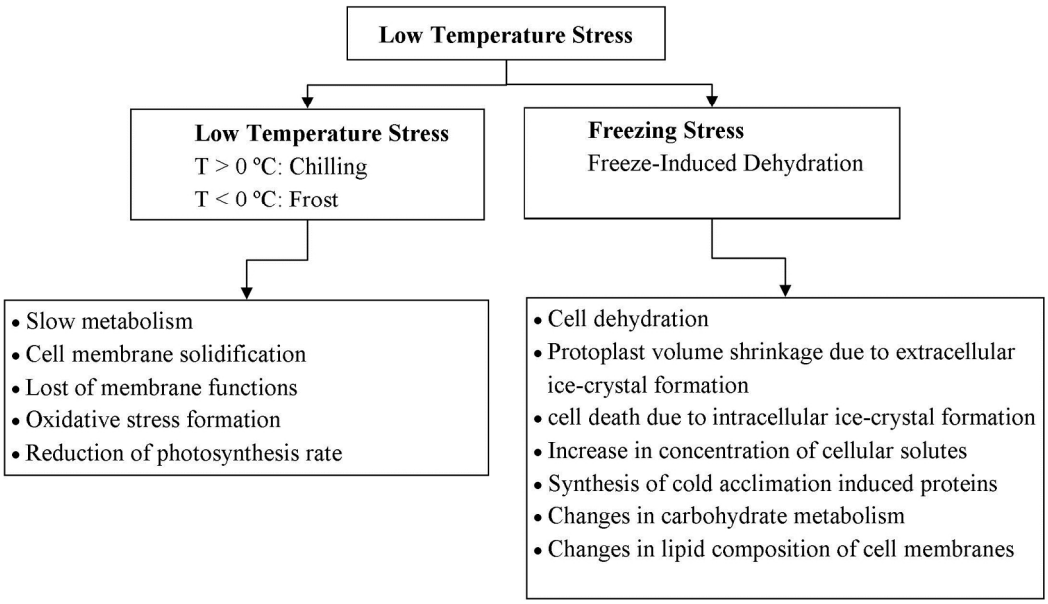


Figure 1.3. The low temperature stress syndrome of plants.

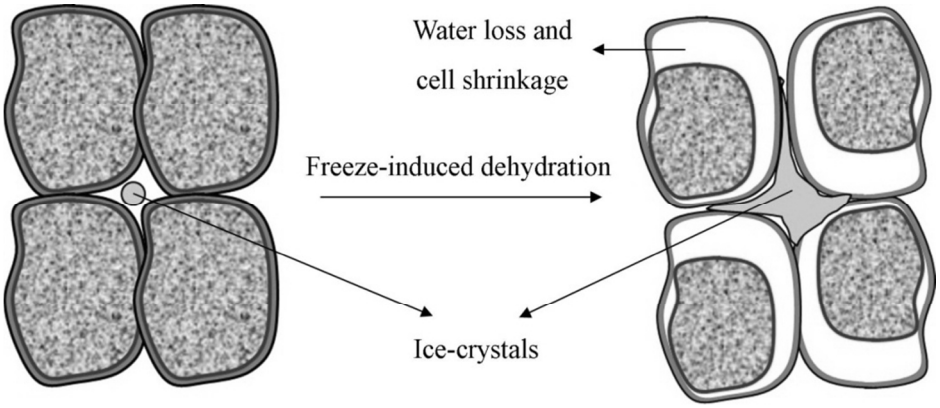


Figure 1.4. Freezing stress cause ice-crystal formation in extracellular space, leading to freeze-induced dehydration.

During the low temperature stress condition, some physiological and biochemical changes occur to increase tolerance against chilling or freezing stresses. The most important modification is related with membrane. Level of unsaturated fatty acids is raised, when compared to chilling-resistant and sensitive plants and it has been shown that the ratio of unsaturated fatty acids increases during acclimation to cold temperature (Palta *et al*., 1993). As a result of lipid alternation, membranes remain fluid at lower temperature conditions (Figure 1.5). Decrease in the amount of unsaturated fatty acids during low temperature stress cause inactivation of membrane-localized enzymes. So, another cold tolerance mechanism is the ability of keeping saturation of fatty acids in membrane lipids (Szalontai *et al*., 2003). Reduction of growth and water content in tissues, accumulation of osmolytes, soluble sugars, proteins and increase in abscisic acid (ABA) and antioxidant levels are the structural and biochemical properties that facilitate enhancing plant freezing stress tolerance. Besides physiological and biochemical changes, activation of cold-induced genes play crucial role for freezing tolerance during cold acclimation period. Different types of proteins with chaperon and membrane stabilizer function are synthesized in the cytosol. These proteins are classified as groups of late-embryogenesis-abundant proteins (LEA proteins), heat shock proteins (HSPs) and other transcription factors (Margesin *et al*., 2007).

Heat stress also known as high temperature stress is described as increase in temperature above the threshold level for a certain time period which causes irreversibly to plant growth and development (Wahid *et al*., 2007). Transient elevation of temperature, usually 10–15°C above is generally considered as heat stressor heat shock. Actually, the effect of heat stress depends on intensity of heat temperature, exposed time period and rate of increase in temperature. Worldwide crop production is seriously threated by heat stress derived from high ambient temperatures. Gaseous emissions from human activities and greenhouse gases cause rise in world's average ambient temperatures. The worldwide mean temperature will rise to 0.3°C per decade (Jones *et al*., 1999). So, according to a report of the Intergovernmental Panel on Climatic Change (IPCC), global mean temperature will reach to nearly 1 and 3°C above the present value by years 2025 and 2100,

respectively. Rising mean annual temperatures leads to global warming and results in alternation of geographical distribution and growing season of agricultural crops. Cellular structure and organization are seriously damaged at very high temperatures. Protein denaturation and aggregation, and increased fluidity of membrane lipids are the direct effects of high temperatures causing cell death within minutes. Heat stress increases the speed of the kinetic energy rate and movement of molecules across membranes. So, chemical bonds within molecules of biological membranes are relaxed. Denaturation of proteins and an increase in unsaturated fatty acids cause more fluid lipid bilayer in biological membranes (Wahid *et al*., 2007) (Figure 1.5). Plants can survive at moderately high temperatures, but indirect heat injuries occur because of inactivation of enzymes found in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation, loss of membrane integrity and changing microtubule organization. All these injuries finally initiate starvation, growth inhibition, and result in production of toxic compounds and reactive oxygen species. Heat stress significantly influences on pre and post-harvesting periods in which some injuries including scorching and sunburns on leaves, branches and stems, leaf senescence and abscission, shoot and root growth inhibition, fruit discoloration and damage, and reduced yield occur (Vollenweider and Günthardt-Goerg, 2006). Besides these morphological symptoms before harvesting, during anthesis and grain filling stages, kernel growth is reduced at prolonged high temperature conditions leading to losses in kernel density and weight in spring wheat (Guilioni *et al*., 2003) and maize (Wilhelm *et al*., 1988). Atomical changes occurred under heat stress conditions are generally similar to those under drought stress. Cell size reduction, closure of stomata, increased stomatal density and greater xylem vessels of both root and shoot are the most widely observed damages under high ambient temperature (Banon, 2004).

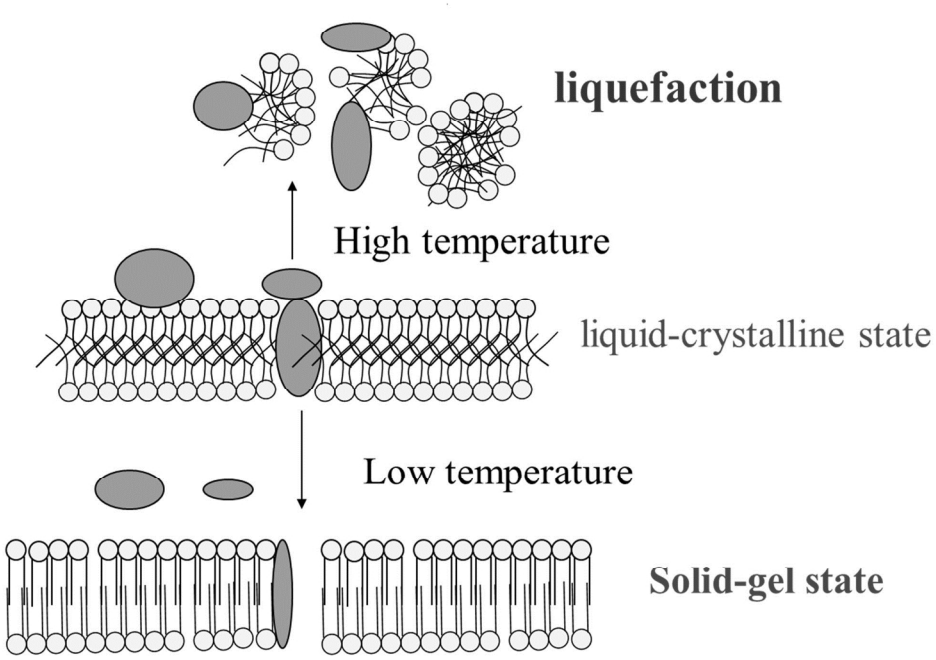


Figure 1.5. Membrane structure and fluidity at low-high temperature conditions.

When compared to other stresses, fluctuation in ambient temperature occurs more likely and rapidly. Because of their sessile nature, plants are exposed to wide variety of temperature shifts both diurnally and seasonally. Therefore, they must adapt to temperature stress quickly and efficiently (Krishna, 2004). The most important response of heat stress is the production of heat shock proteins (HSPs), which functions as molecular chaperones. They bind to other proteins which have unstable structure and control the cellular protein quality (Boston *et al*., 1996). They are grouped into families according to their molecular weight. For example, molecular weight of HSP 100 ranges between 100 and 104 and different HSP families including HSP 90, 70, 60 and small HSPs have different functions. HSPs are not only one component of the response to high temperature stress, but also they are included in different pathways which help to plants to tolerate heat. The chaperones

are involved in many processes such as protein folding and transportation across membranes, regulation of protein degradation and prevention of irreversible protein aggregation. The last action is considered for important to survive at high temperature stress and explain how these proteins are expressed by high temperature (Jenks and Hasegawa, 2005). A common adaptive mechanism for abiotic stresses, including salinity, water deficit and extreme temperatures is accumulation of certain organic compounds such as sugars and sugar alcohols (polyols), proline, ammonium and phonium compounds (Sakamoto and Murata, 2002). Abscisic acid (ABA) and ethylene (C_2H_4) known as stress hormones, act as signal molecules which involves in the regulation some physiological processes. Under the high temperature conditions, their levels increase to regulate gene expression in response to stress (Wahid *et al*., 2007). So, heat stress causes accumulation of osmolytes, secondary metabolites and plant hormones to cope with stress condition.

1.2.3. NAC Type Transcription Factors in Plants

Plants are constantly threatened by biotic and abiotic stresses and have evolved complex response mechanisms including genes encoding important metabolic proteins or regulatory proteins known as transcription factors and protein kinases (Xia *et al*., 2010). Response of transcription factors to environmental stimuli is initiated by a signaling cascade. Then they bind to specific regulatory sites upstream of interested genes in a regulatory network by direct physical interaction or in combination with other proteins. Among plant transcription factors, members of NAC, WRKY, C_2H_2 type zinc finger, AP2/EREBP, and MYB families have been well characterized in terms of their regulatory roles to response of plant stresses (Guo *et al*., 2004; Buchanan-Wollaston *et al*., 2005; Agarwal *et al*., 2010). The NAC-type transcription factors constitute one of the largest families and have involved in diverse roles in plant development and in the recognition of environmental stimuli coming from biotic and abiotic factors (Olsen *et al*., 2005). Protein of this family contains NAC domain which was originally characterized from consensus sequences from petunia NAM and Arabidopsis ATAF1, ATAF2 and CUC2 (Aida *et al*., 1997). The NAC family has 149 and 106 predicted members in

Oryza sativa and *Arabidopsis thaliana* genomes, respectively (Gong *et al*., 2004; Xiong *et al*., 2005).

In recent years, the gene expression analysis of NAC-type transcription factors has been well studied in various plant species under the different abiotic stress conditions. In recombinant inbred wheat lines, quantitative trait locus was mapped on chromosome arm 6BS. The complete sequencing of this region (DQ871219) revealed five genes high-resolution genetic map which was used for determination of the linkage between these genes and the *Gpc-B1* locus. Quantitative PCR results show that expression levels of three *TtNAM* genes were low in flag leaves before anthesis, but their levels significantly are raised to grain maturity. Transcripts were also detected in green spikes and peduncles (Uauy *et al*., 2006).

The *NAC* gene, *CarNAC3* was isolated from cDNA libraries constructed from the PEG-treated and -nontreated seedling leaves of chickpea. Organ-specific expression was observed for *CarNAC3* gene. Its expression was largely dependent on leaf age. *CarNAC3* showed differential expression patterns during seed development and germination, and could be significantly induced by drought stress, abscisic acid (ABA), ethephon (Et) and indole-3-acetic acid (IAA), but was inhibited by N-6-benzyl-adenine (6-BA) (Peng *et al*., 2009).

A 31 unigenes containing the complete open reading frames of GmNAC proteins were defined and cloned from soybean. *GmNAC* genes are differentially expressed in different organs, which mean that they have various functions during plant growth and development. In addition to dehydration stress, the responses of *GmNAC* genes to other stresses such as high salinity, cold and with abscisic acid hormone treatments were screened by quantitative real-time PCR analysis (Tran *et al*., 2009)

A six full-length (*GhNAC1–GhNAC6*), intact putative transcription factors were isolated from *Gossypium hirsutum* L. All *GhNAC* genes were highly expressed in leaves while they had little to no expression in stems, roots and 7-day-post-anthesis fibers. According to real-time quantitative RT-PCR results, the genes showed

different expression patterns under drought, high salt, cold and/or ABA conditions (Meng *et al*., 2009).

A rice NAC gene, *ONAC045*, was functionally characterized in response to abiotic stress conditions. Expression analysis of *ONAC045* revealed that drought, high salt, and low temperature stresses, and abscisic acid (ABA) treatment altered gene expression level in wheat leaves. Also, overexpression of *ONAC045* gene in transgenic rice, which showed enhanced tolerance to drought and salt treatments, supports the expression studies (Zheng *et al*., 2009).

A novel wheat *NAC* gene, designated as *TaNAC4* was isolated using *in silico* cloning. *TaNAC4* shows high homology with rice *OsNAC4* gene, whose predicted protein is composed of 308 amino acid residues. The expression level of *TaNAC4* gene differs in different tissues of wheat seedling and higher level of gene expression was observed in roots rather than leaves and stem. Abiotic stresses coming from high salinity, wounding, and low-temperature also caused induction of *TaNAC4* gene expression. They concluded that this novel *TaNAC4* gene serves as a transcriptional activator included in wheat response to biotic and abiotic stresses (Xia *et al*., 2010).

1.3. Biotechnological Approaches for Abiotic Stress Tolerance in Wheat

Wheat is most important and widely grown cereal grain crop in the world, and cultivated on 17% of worldwide lands. About 40% of the world population is nourished by wheat products which provide 20% of total food calories and protein in human diet (Gupta *et al*., 2008). Hexaploid type is the most grown wheat type, accounting for 95% of total wheat production. Remaining of 5% is tetraploid (durum) which is used for pasta and biscuit manufacturing. A human need against the wheat products grows increasingly. In order to meet this request, wheat production should be raised to an annual rate of 2%, without any additional land (Patnaik, 2001). It is also predicted that average wheat production will be 760 and 900 million tons in 2020 and 2050, respectively (Rosegrant *et al*., 2001). Recently, studies associated with genetic improvement in wheat have therefore concentrated

on increasing the grain yield, quality characteristics and minimizing crop loss because of biotic and abiotic stress conditions. Some breeding and biotechnological techniques are discussed.

1.3.1. Marker Assisted Selection in Wheat Breeding

There are different marker types including morphological, biochemical and molecular markers. Conventionally, plant breeding relies on morphological and phenotypic markers to identify agronomic visible traits. Biochemical markers depend on proteins. With the advent of new molecular biology tools, it is possible to analyze plant gene structure and function. Molecular markers are widely used for identification plant genome organization and construction of genetic linkage maps to identify traits (Patnaik, 2001). They differ from usual genes because they do not contain any information. They are determinable DNA sequences which are found as constant landmarks at specific locations of the genome. The most important advantages of molecular markers are unaffected from any environmental changes and can be obtained at any time periods of plant life cycle. Molecular markers are classified into different groups such as, restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), microsatellites and single nucleotide polymorphisms (SNPs) (Guimarães *et al*., 2007). Marker assisted selection (MAS) allows fast identification of resistance genes and their corresponding molecular markers and this facilitates the evolvement of new plant varieties by lessening the time.

Selection of wheat varieties with desired characters is difficult process and understanding of these characters and relationships of the germplasm is still very limited. In conventional breeding, varieties are selected according to morphological features. Conventional breeding is not able to discriminate polygenic characters. This difficulty is accomplished by using molecular marker assisted selection which provides selection for individual trait of interest (William *et al*., 2007). Initial studies using molecular markers in wheat based on RFLP maps providing easy detection of desirable genes. The usage of RFLP in wheat is restricted by the limited number of

polymorphisms observed among wheat lines and the high cost for screening in breeding situations (Patnaik, 2001). Despite of these limitations, RFLP have been broadly utilized for detection of agronomic traits including determination of seed storage protein loci map (Dubcovsky *et al*., 1997), loci related with flour color (Langridge, 1998), vernalization and frost resistance genes (Galiba *et al*., 1995) resistance to pre-harvest budding markers (Anderson *et al*., 1993), quantitative trait loci (QTLs) controlling tissue culture response (Amer *et al*., 1997). RFLP markers are also used for identification of pest and pathogens resistance markers such as nematode resistance (Lagudah *et al*., 1997), wheat spindle streak mosaic virus (Khan *et al*., 2000), resistance against powdery mildew (Speer *et al*., 1995).

With the coming of polymerase chain reaction (PCR) technique, random amplified polymorphic DNA (RAPD) become more convenient and effective technique which makes easy to screen markers in translocation lines (Devos and Gale, 1992). RAPD analysis is alternative technique to RFLP analysis, and used for identification of markers linked to a single trait within near isogenic lines. He *et al*., (1992) developed a detection method via combination of RAPD and DGGE (denaturing gradient gel electrophoresis) for pedigree analysis and fingerprinting of wheat cultivars. Sequence characterized amplified region (SCAR) markers are the products of transformed RAPD markers and show the simple banding pattern compared to RAPD markers. SCAR markers attached to resistance genes against fungal pathogens have been identified by combining of RAPD and RFLP methods (Liu *et al*., 1999). For detection of desirable agronomic trait and disease resistance markers, utilization of Sequence characterized amplified region (SCAR), Sequence tagged sites (STS) and Differential display reverse transcriptase PCR (DDRT-PCR) markers based on RAPD and PCR techniques, have been increased in recent years. They are used for development of effective breeding programs for wheat cultivars.

Another PCR based marker is simple sequence repeats also known as microsatellites which are more reliable molecular markers for the identification and differentiation of genotypes within a species (Patnaik, 2001). Because of their codominant inheritance, high level of polymorphism and repeatability and easy handling,

microsatellite markers are highly practical for various applications in wheat breeding program (William *et al*., 2007). In future, large scale sequencing of gene rich regions provide simultaneous and sequential selection of agronomically important genes, development of perfect markers and agronomically important traits to be used in MAS (Lange and Whittaker, 2001).

1.3.2. Tissue Culture and Genetic Transformation Studies in Wheat

Although conventional breeding techniques including crossing, back crossing and selection allows introduction of desirable traits in wheat cultivars, it is time consuming and difficult to follow newly developed pathogenic micro-organisms and pests for selection of markers (Patnaik, 2001). Besides these disadvantages, conventional breeding enables transferring a set of genes from the donor to the recipient, not a defined single gene. So, through breeding techniques desired characters with undesired characters are passed on recipient. With the advent of biotechnology, *in vitro* technologies contribute the development of new cultivars with only wanted character. Biotechnology involves the usage of biological processes, organisms or systems to manufacture products for the beneficial use. Plant or agricultural biotechnology is the branch of biotechnology and involves discovery, delivery, integration and expression of foreign gene into plant cells, which are then regenerated to whole transgenic plant into tissue culture environment. Plant biotechnology has number of tools and elements of conventional plant breeding, plant tissue culture, plant physiology, and molecular biology techniques. So, through the plant biotechnology applications, traditional plant breeding is complemented by reducing the time.

To produce the fertile transgenic wheat plant, three processes namely, transformation, regeneration and screening-selection should be achieved. Firstly, gene of interest should be stably integrated into the genome using reliable gene delivery technique and expressed into plant cells. Secondly, complete whole wheat plant should be regenerated into *in vitro* culture using totipotency feature of plant cells (Jones, 2005). Finally, efficient screening and selection methods should be

applied to obtain fertile and healthy wheat plants. Wheat tissue culture is the foundation and in most cases the bottle-neck step for successful wheat transformation. Different types of wheat explants and transformation techniques including *Agrobacterium*-mediated and direct gene transfer methods have been used. Wheat is recalcitrant species for tissue culture studies. Although a number of tissues have been studied for *in vitro* regeneration of wheat plants, only two wheat tissues, immature inflorescence and scutellum of immature zygotic embryos (Jones, 2005) have been routinely used for making transgenic wheat plant. *In vitro* wheat tissue culture is affected not only different types of explant (Redway *et al*., 1990; Baric, 2003) but also used cultivars genotype (Özgen *et al*., 1998; Raziuddin *et al*., 2010) tissue culture media composition (Filippov *et al*., 2006; Ren *et al*., 2010). Immature scutellum is the most widely used tissue type for wheat transformation including both particle bombardment and *Agrobacterium*-mediated transformation (Pastori *et al*., 2001). As an alternative explant source for wheat transformation is immature inflorescences of which isolation is easier than immature scutellum (Kavas *et al*., 2008). However, tissue culture response of immature inflorescence shows high genotype dependence when compared to immature scutellum (Rasco-Gaunt and Barcelo, 1999).

Successful wheat improvement program using genetic engineering tools depends mainly on the delivery, integration and expression of gene cassette into regenerable explants. Expression cassette also known as gene cassette contains suitable promoter and terminator, scorable and/or selectable marker genes and defined functional foreign gene. The introduction of reporter gene into recipient plant cells or intact tissues provides valuable information related with expression pattern. Monitoring of reporter gene expression is easy and integration of reporter gene into the host genome shows the whether a gene of interest is taken up or not. Reporter genes are also utilized for testing promoter activity in different tissues or conditions or gene functions. The most widely used reporter gene in plant molecular biology studies is *uidA* or *gus* gene from *Escherichia coli*, encoding the enzyme β-glucuronidase. The product of *gus* gene, break downs β-glucuronide compounds, resulting in blue spot formation which is simply detected by histochemical procedure (Jefferson *et al*.,

1987). Because of its easy and rapid applications, this scorable marker is chosen in wheat transformation (Vasil *et al*., 1993; Weeks *et al*., 1993; Becker *et al*., 1994). Although it is extremely useful for optimization of different parameters in genetic transformation studies, transgenic living cells or tissues are destroyed, so putative transgenic plants are lost. To overcome limitation of *gus* gene, green fluorescent protein (*gfp*) and firefly luciferase genes are utilized. They encode for anthocyanin biosynthesis which enables easy visualization of them in living cells. In wheat genetic transformation studies, *gfp* and luciferase genes are also usually preferred by different groups (Klöti *et al*., 1993; McCormac *et al*., 1998).

Development of efficient selection method is one of the most important steps for production of transgenic plants. The selection strategy is based on expression of a specific gene that produces an enzyme providing resistance to a cytotoxic substance often an antibiotic or a herbicide. In wheat transformation, *bar* (bialaphos resistance gene) and *ppt* (herbicide resistance) genes are mostly utilized selection genes which encode enzymes called as phosphinothricin acetyl transferase (pat) and 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), respectively. They provide tolerance to glyphosate and glufosinate ammonium herbicides (Patnaik, 2001). As an alternative to these herbicide tolerant genes, antibiotic resistant markers are also employed for selection of putative transgenic wheat explants. *nptII* gene (neomycin phosphotransferase II) and *hpt* gene (hygromycin phosphotransferase) are antibiotic selectable marker genes widely used in wheat transformation (Goodwin *et al*., 2005). In recent years, mannose-6-phosphate isomerase, encoded by *manA* gene from *E. coli*, has been characterized as a positive selection marker. After transformation, only transgenic tissues are able to grow on selection medium supplemented with mannose as a sole carbon source. So, transformants are selected according to growth in selection medium (Hansen and Wright, 1999).

There are many genetic transformation methods including electroporation, microinjection, polyethylene glycol uptake, but the most used ones for wheat transformation are particle or microprojectile bombardment (also called as biolistics) and *Agrobacterium* mediated. Principle of the particle bombardment is based on

adsorption of gold or tungsten coated plasmid or linear naked DNA into recipient plant cells using acceleration device which provides high velocity and vacuum (Sanford, 1988; Sanford *et al*., 1993; Jones, 2005). Particle bombardment method has several parameters that should be optimized to deliver coated plasmid DNA into target tissue efficiently. Despite of relatively genotype independent technique, microprojectile type, size and quantity, coated material and plasmid DNA quantity should be standardized. It should be also considered acceleration device parameters including pushing force, helium pressure and target distance. All of these parameters can influence the efficiency of DNA delivery (Altpeter *et al*., 1996; Harwood *et al*., 2000). Although particle bombardment method has been generally selected for wheat genetic transformation, it produces multiple transgene copies integration into genome which results in difficulties in subsequent analysis. So, this has forced to development and utilization of *Agrobacterium tumefaciens* in wheat transformation. *A. tumefaci*ens is a soil pathogen causing crown call disease via transferring small fragment of its DNA (T-DNA) into its host plant cells. Using genetic engineering tools, tumor-inducing plasmid DNA (also known as Ti plasmid) was modified and commonly used in genetic transformation studies (Hoekema *et al*., 1983). Vector systems in *Agrobacterium*-mediated transformation method are composed of two plasmids. The first one has a multiple cloning site flanked by T-border sequences in which origin of replication for easy maintenance in *E. coli*, and marker gene or expression cassette are found. Another plasmid is disarmed Ti plasmid without tumor-inducing genes but keeping the *vir* genes whose products facilitate DNA transfer to the plant cell (Jones, 2005). For easy characterization of transgenic wheat plant at the molecular level, fewer transgene copies and no rearrangements are desirable features. Using particle bombardment method in wheat transformation, the frequency of single copy is very low and un-rearranged insertions are rare. Therefore, although cereals are not natural hosts for *Agrobacterium* species, some investigations have made on strains, plasmids, selection systems, wheat genotypes and media compositions (Cheng *et al*., 2004). In recent years, *Agrobacterium*-mediated transformation has been mostly preferred method for wheat genetic transformation because of introducing larger segments of DNA with minimal

rearrangement and with fewer copies of inserted transgenes at higher efficiencies and at lower cost (Shibata and Liu, 2000; Hu *et al*., 2003).

Transferring resistance to crop plants by traditional breeding methods is difficult process because of complexity of stress tolerance traits. As an alternative method to traditional approaches, genetic engineering tools enable direct introduction of genes encoding biochemical pathways or endpoints of signaling pathways. So, products of these genes cause protection against environmental stresses. Mannitol is osmolyte which adjusts the cellular osmotic potential under the stress conditions. Its accumulation in plant cells under the osmotic stress increases stress tolerance. *mtlD* gene encoding mannitol-1-phosphate dehydrogenase was transformed into wheat plants. Accumulated mannitol in transgenic wheat plants provided water and salinity stress tolerance (Abebe *et al*., 2003). Wheat plants transformed with *HVA1* gene which is late embryogenesis abundant (LEA) protein gene showed tolerance against water deficit stress (Sivamani *et al*., 2000). Increased salinity tolerance in wheat was achieved by expressing a vacuolar Na^+/H^+ antiporter gene and reported in many studies (Xue, 2004; Wu *et al*., 2005; Huang *et al*., 2006). In addition to development of abiotic stress resistant transgenic wheat plants, many agronomically important genes related with resistance to biotic stress, improved bread-making and nutritional qualities, increased yield have been introduced into wheat have been also introduced into wheat (Vasil, 2007).

1.3.3. Genomic Approaches for Wheat Improvement

Until now, plant molecular biology techniques offer analysis of single gene. On the advent of omics technologies, this situation has changed, which enables the analysis of whole genome of organisms. Genome organization, expression analysis and interaction between other genes have been studied on a genome-wide scale simultaneously. Genes are organized within the genome and collection, analysis and organization of this information for the biological systems are described as genomics (Campos-De Quıroz, 2002). Genomics provides powerful device for comprehension of the molecular backbone of phenotypic variation, hastening gene cloning and

marker assisted selection, and for enhancing the efficiency of using genetic diversity (Eversole, 2010). So, plant genomics actually allows the understanding of the genetic architecture of plant genomes. The tools of plant genomics produce comprehensive datasets related with changes in gene expression, protein profiles, and metabolites (Fleury *et al*., 2010).

The development of molecular markers causes for construction of complete genetic maps of economically important plant species. A genetic map shows position and distance between molecular markers on chromosomes. Genetic distance is measured by centiMorgans (cM), providing determination of frequency of recombination events or crossovers between markers (Figure 1.6.a). As a result of linage of markers gives graphical display of the organization of markers along chromosomes (Collard *et al*., 2005). A whole-genome linkage map of plants has been created from F2 population, backcross population; recombinant inbred (RI) population, or doubled haploid (DH) population (Lehmensiek *et al*., 2010). Selection of parents is critical point because parents should carry sufficient genetic polymorphism and lack co-ancestral genome regions (Young, 1996). The development of a genetic linkage map is composed of 4 main steps: (i) manufacturing of a mapping population; (ii) polymorphism assessment; (iii) genotyping the population with polymorphic markers and (iv) linkage analysis (Lehmensiek *et al*., 2010). Genetic maps provide understanding of organization of plant genome and identification of Quantitative Trait Loci (QTL) and Marker Assisted Selection (MAS). Most important agronomic traits are plant height and quality which exhibit a continuous distribution rather than discrete classes. These traits are controlled by several loci. Combinations of different alleles at these loci produce different phenotypes.

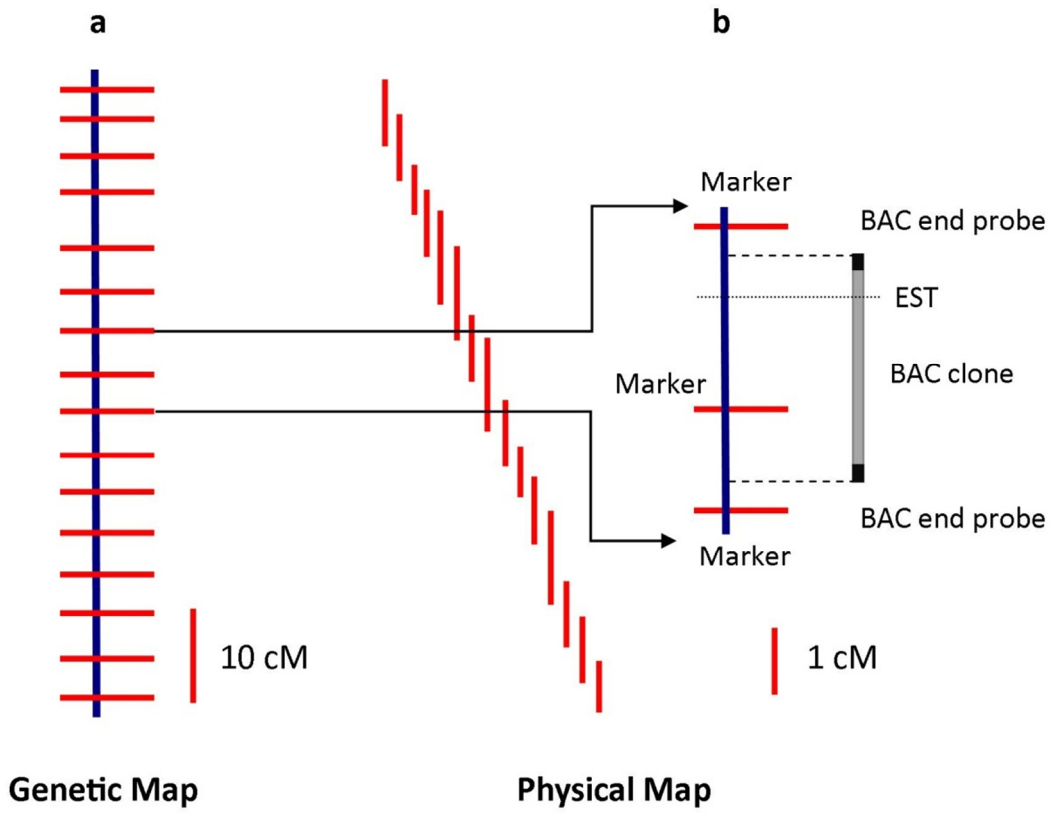


Figure 1.6. Maps used in plant genetics. **a)** Genetic and physical maps of a hypothetical chromosome. Horizontal lines on the genetic map represent loci targeted by a molecular marker; vertical lines represent overlapping BAC clones. **b)** Alignment of genetic and physical maps using BAC ends sequence (dashed lines), ESTs (dotted line).

Although genetic maps provide information for localization of markers along the chromosomes, they are still not enough to identify gene regions. Generally, about 30 to 100 or even more genes can be found in 1 cM intervals (Figure 1.6.b). So, there is a large gap between molecular markers. Physical maps play crucial role for joining of these gaps. It is based on covering of distance between the available markers using large insert genomic library (YAC, BAC or PCA). Initially, physical maps in

plants are constructed using YACs (Yeast Artificial Chromosomes). Because of chimerism and stability difficulties, bacterial artificial chromosomes (BACs) and P1-derived artificial chromosomes (PAC) have been chosen for mapping studies. BAC vectors carry 80 to 200 kb inserts and are able to select with traditional plasmid selection methods such as an antibiotic resistance gene. Manipulation of BACs clones is easier than YACs. When the BAC library prepared, clones are assembled into contigs using matching probabilities. Combination of physical and genetic maps cause the formation of continuity from phenotype to genotype. Physical maps combine gaps and resolution of maps is increased by the help of genetic maps (Campos-De Quiroz, 2002).

Genetic and physical maps complement each other and provide a key layer of genomic information. However, sequencing of genetic data is ultimate level of genetic information. For large sequencing projects, two main techniques, namely shot-gun and clone-by-clone approaches have been widely used. In the sot-gun method, the entire genome is firstly cut into small fragments using restriction enzymes or mechanically shearing. Then, these fragments are sequenced individually. Finally, the sequences of these fragments are then reassembled into their original order, based on overlaps, ultimately yielding the complete sequence (Figure 1.7.a). In clone-by-clone method, genome is mapped according to genetic and physical maps or both of them. Then, genome is divided into fragments whose rough map is figured for each of these fragments. Fragments are also separated into smaller bits, with plenty of overlap between each of the bits which are finally sequenced and re-assembled according to minimal tiling paths (Figure 1.7.b). The assembly process in clone-by-clone sequencing is simpler and less expensive than shot-gun sequencing.

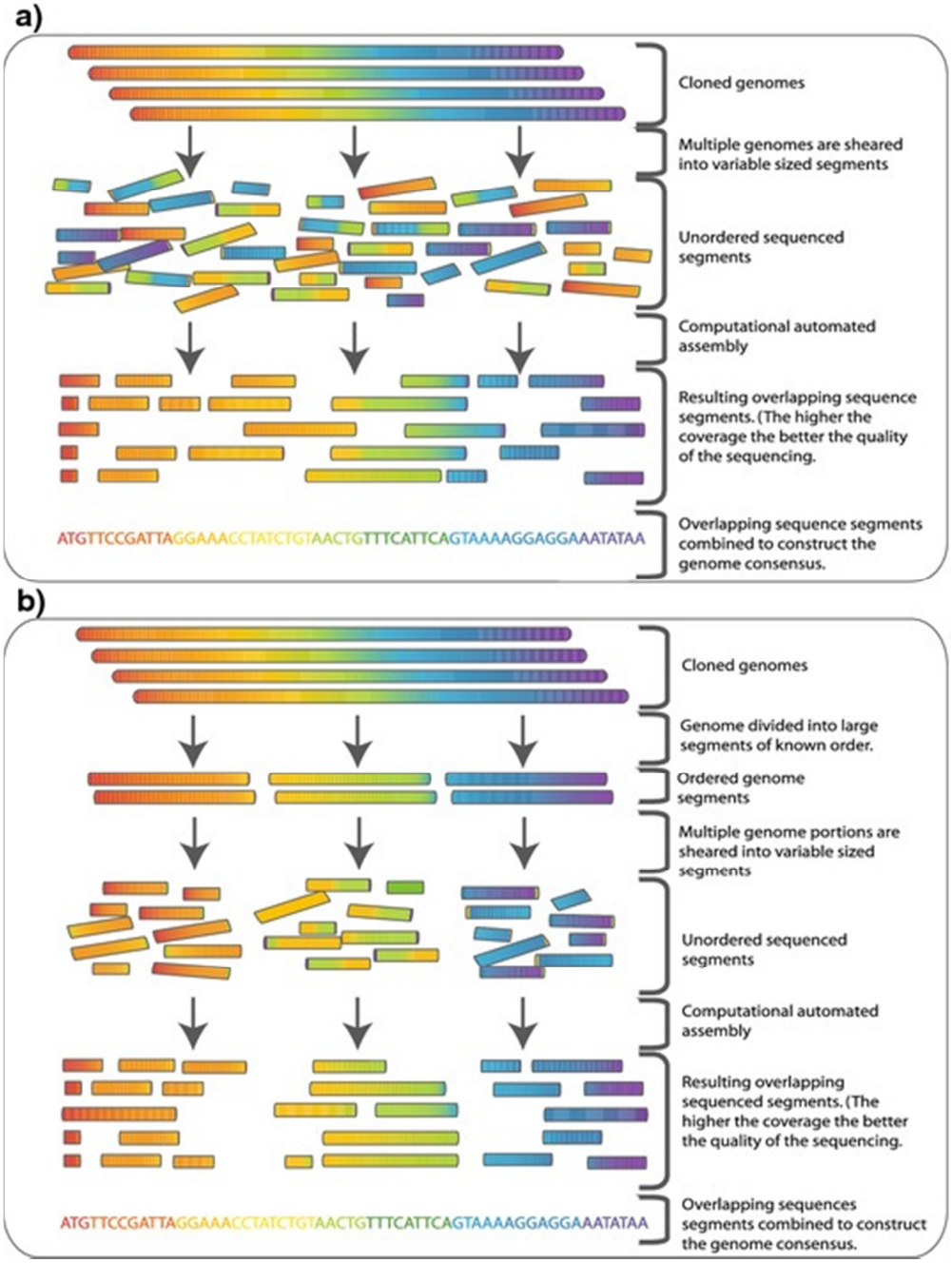


Figure 1.7. Overview of whole genome sequencing techniques. **a)** Whole genome shotgun sequencing: Genome is cut into small sized fragments which are subsequently sequenced in both directions followed by cloning. Then sequences are assembled and resulting in formation of whole genome sequence. **b)** Clone-by-clone sequencing: The genome is broken into a series of large equal segments of known order which are then subject to shotgun sequencing (taken from Commins et al., 2009).

The size of plant genome which shows the variations in different plant species is large and complex because of gene regions, repetitive sequences, polyploidy and duplication events and transposable elements. Among the agricultural crops, common bread wheat (*Triticum aestivum*) has the largest genome at 16,000 Mb. Wheat genome is larger about five, eight and forty times than human, maize and rice, respectively (Gill *et al*., 2004). It has a complex structure, composed of three independent genomes (A, B, D) and repetitive DNA regions (>80%). A wide range of genomic resources are available for wheat plant, including: (i) genome-wide genetic and physical maps (including expressed sequence tag (EST) maps and molecular function maps); (ii) a large number of known quantitative trait loci (QTL) (iii) about 1.07 million wheat ESTs (dbEST, December 2010; http://www.ncbi.nlm. nih.gov/dbEST/dbEST_summary.html) (iv) BAC libraries for each of the three sub-genomes (v) identified gene-rich regions (GRRs) on chromosome six launched by ″International Wheat Genome Sequencing Consortium″ (IWGSC; http://www.wheatgenome.org) (vi) availability of BLAST-searchable wheat genomic sequences (http://www.cerealsdb. uk.net/search_reads.htm) (Kumar, 2011). The sequence decoding has been done with the aid of advanced sequencing technology designed by 454 Life Sciences, USA, which enables reading of the DNA sequences hundred times faster than human genome sequencing. This technique provides scientists for finishing whole wheat genome sequencing in about one year compared to 13 years in case of the human genome sequencing which has five times shorter genome sequence.

International Wheat Genome Sequence Consortium (IWSGC) was established by a group of plant scientists, breeders, and growers dedicated to sequencing the wheat genome to enhance the structure and function of the wheat genome. The aim of the IWGSC is to accelerate wheat improvement by developing DNA-based tools and products through the creation of a physical map anchored to the genetic map and ultimately by obtaining a complete sequence of the hexaploid bread wheat genome. To achieve this vision, the IWGSC establishes strategic plans with short- and mid-term goals (Figure 1.8), facilitates and coordinates research projects, develops and supports the design of research proposals, provides a framework for the

establishment of common guidelines, protocols, and resources, and organizes scientific meetings and workshops (IWGSC; http://www.wheatgenome.org).

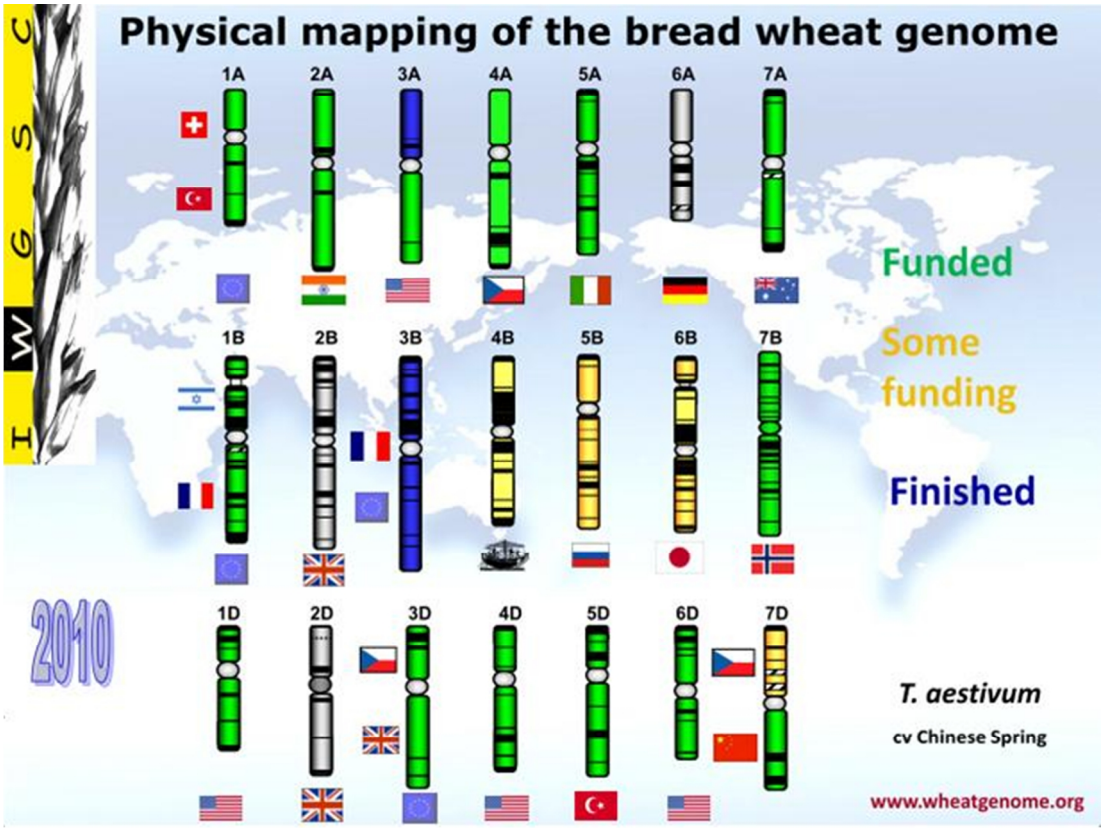


Figure 1.8. Progress of wheat genome sequencing and situation of physical mapping of the bread wheat genome last modified in 2010 (taken from IWGSC; http://www.wheatgenome.org).

Because of the massive genome size of wheat, whole genome sequencing and analysis of the genetic code in wheat has delayed. Chinese spring wheat, in which repetition of same gene occurs around six times, is used for sequencing (Eversole, 2010). The future prospect of wheat genome sequencing project is the identification of genes related with agronomically important traits such as drought, salinity, yield

improvement, and nutritional quality. It will also provide information for identification of useful markers which increase the speed and efficiency of plant breeding.

1.4. Gene Cloning Strategies

Gene or DNA cloning, basic technique in genetic engineering, is used for getting a large number of copies of specific DNA fragment, recovering large quantity of protein produced by the concerned gene, production of genomic DNA and cDNA libraries and DNA sequencing studies. Polymerase chain reaction (PCR) is a technique which reproduces DNA fragments in large quantities. In molecular cloning studies, gene of interest or DNA fragment can be copied from both genomic DNA and complementary DNA (cDNA). When the genomic DNA is selected for cloning of gene of interest, regulatory sequences and introns are also multiplied, which will create a problem for further gene expression studies. For example, if the gene of interest is cloned from eukaryotic organism and wanted to be expressed in prokaryotic organism, protein is not produced in prokaryotic organism due to lack of mRNA processing (splicing) in prokaryotes. To accomplish this situation, cDNA is generally used. cDNA is made of mRNA using reverse transcriptase enzyme which converts the mRNA molecule to cDNA molecule. It does not contain any intron regions and mostly selected as initial genetic material for cloning and further expression studies. In recent year, bases of DNA fragments or concerned gene are artificially synthesized using chemical agents. Gene cloning into plasmid vectors involves several steps. Digestion of DNA and plasmid vector with same restriction enzyme is initial step of cloning. Then, ligation of digested products is done using ligation enzymes. Finally, ligation product is transformed into competent *E.coli* cells via different transformation methods and selection and screening of desired recombinant is performed. Gene cloning steps are summarized in Figure 1.9.

1.4.1. Sticky-End Cloning

Among the gene cloning methods, sticky-ended cloning is the most efficient and commonly used method (Conze *et al*., 2009). Generally, to produce sticky or complementary ends for sticky-end DNA cloning, insert DNA and vector are separately cut with same restriction endonuclease enzymes. Then, insert DNA is ligated into plasmid vector by DNA ligase. Although this method works efficiently, shortage of suitable restriction sites either in plasmid vector or in insert DNA limits the usage of this method (An *et al*., 2010).

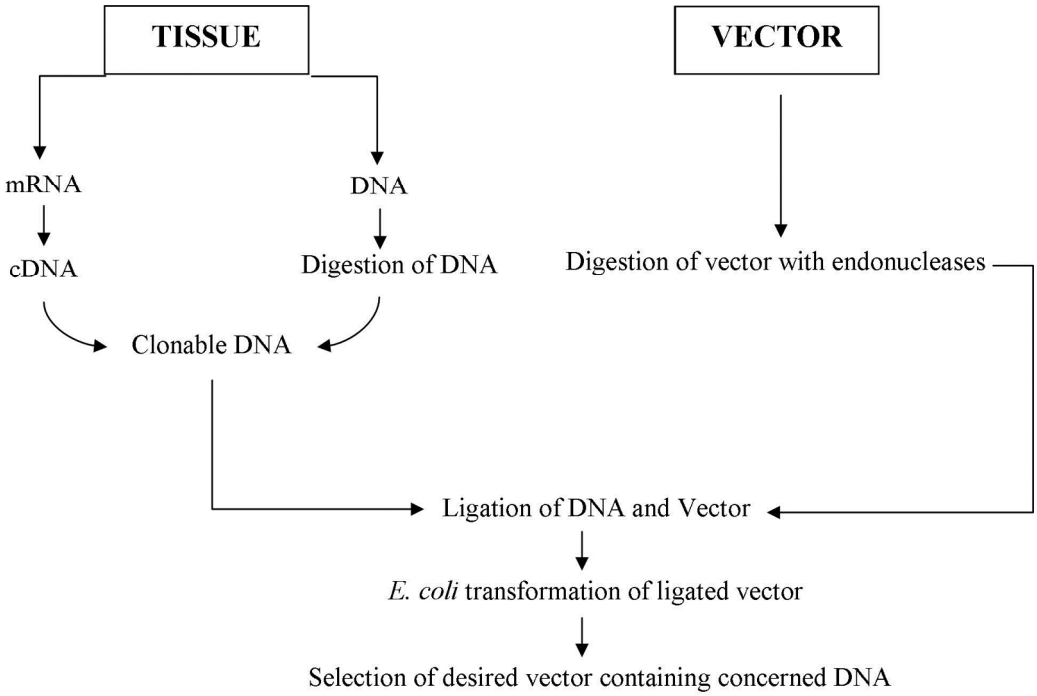


Figure 1.9. Gene cloning steps into plasmid vector.

1.4.2. Blunt-End Cloning

Blunt-end DNA cloning is another cloning technique which introduces the inserting of blunt-ended DNA or 5'-end-phosphorylated PCR product into a linearized and blunt-ended vector (Upcroft *et al*., 1987). The limitations of blunt-end cloning are none-directional ligation and self-ligation of vector. Therefore, compared to stick-end cloning, it is less efficient. Because of these disadvantages, blunt-end cloning should not be preferred for cloning of very large DNA fragments or constructing of comprehensive mutant libraries (An *et al*., 2010). Generally, ligation reactions are affected from different parameters including temperature, components of buffer, ratio of DNA insert and plasmid vector. To obtain optimal ligation condition for blunt-ended DNA ligation, several studies were done (Liu and Schwartz, 1992; Costa and Weiner, 1994). However, the efficiency of ligation reaction is still low and a significant improvement should be very necessary.

1.4.3. TA Cloning

TA cloning is achieved by *Taq* DNA polymerase which has non-template-dependent terminal transferase activity which helps adding of a single deoxyadenosine (A) to the 3′ ends of PCR products. As a result, the PCR product can be directly cloned into a linearized T-vector that has single base 3'-T overhangs on each end (Zhou *et al*., 1995). Despite of widely used, the major drawback of cloning is not directional, because overhanging T and A bases are flanked each sites of linearized vector. So, DNA or PCR product is inserted in linearized T-vector for both orientations. There are some commercial vectors for TA cloning. Invitrogen (UK) produces TA cloning vectors that have Topoisomerase I enzyme. TA cloning vector contains specific sites (CCCTT) for binding of Topoisomerase I enzyme which removes the phosphodiester backbone in one strand (Shuman, 1991). This process causes production of energy that is conserved by formation of a covalent bond between the 3′ phosphate of the cleaved strand and a tyrosyl residue (Tyr-274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can be attacked by the 5′ hydroxyl of the

original cleaved strand and causes removing of Topoisomerase I enzyme from the cleaved strand (Shuman, 1994) (Figure 1.10)

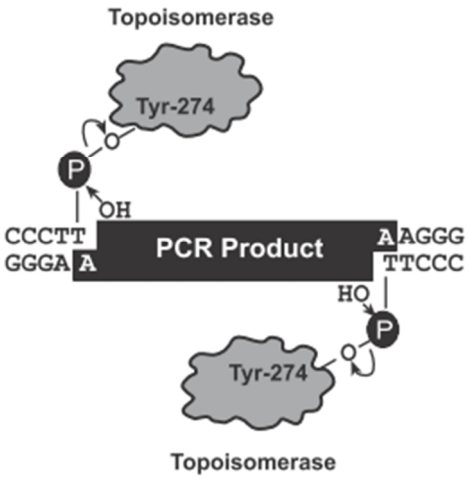


Figure 1.10. Topoisomerase activity of Invitrogen TA cloning vector (Taken from Invitrogen TA Cloning® Kit Manual).

1.4.4. Gateway Cloning

Gateway® Technology, developed by Invitrogen, is a universal cloning method which uses the site-specific recombination properties of bacteriophage lambda (Landy, 1989). This cloning method allows transferring of DNA fragment into different cloning vectors without using restriction endonucleases and ligase. Orientation of DNA insert and open reading frame are maintained because of the site-specific recombination properties of Gateway® Technology. Once DNA insert has entered the Gateway system, it can be transferred other Gateway vectors which provides functional analysis, protein expression, and cloning and subcloning of DNA segments. Gateway compatible vectors contain specific recombination sites

including attL, attR or attB, attP. Two recombination reactions, namely LR and BP reactions are catalyzed by LR and BP recombinases (clonases), respectively. Firstly, gene of interest is inserted into entry clone whose recombination sites are flanked between attL sites. Then, attL sites are cut to form sticky ends by the Gateway clonase. The sticky ends of the destination vector, which contains attR restriction sites, are matched with sticky ends of gene of interest which contains attL sites. This Gateway reaction is called LR reaction and mediated by LR clonase mix containing the recombination proteins, Int, IHF and Xis (Figure 1.10.a). So, *in vitro* version of the excision reaction is achieved. The second Gateway reaction is BP reaction which is the reverse of the LR reaction. In this case, concerned DNA fragment flanked by attB sites is inserted into another destination vector containing attP sites, which is mediated by BP clonase mix, Int, IHF (Figure 1.10.b). So, *in vitro* version of integration reactions is performed (Barampuram and Zhang, 2011).

The large number of entry vectors and Gateway destination vectors are available designed for different purposes. Two types of selection systems including a positive (antibiotic resistance) and a negative (the cytotoxic *ccdB* gene) systems are mainly used for selection of final construct. Because of lethal gene, negative selection system does not permit the growth of *E.coli* (Magnani *et al*., 2006).

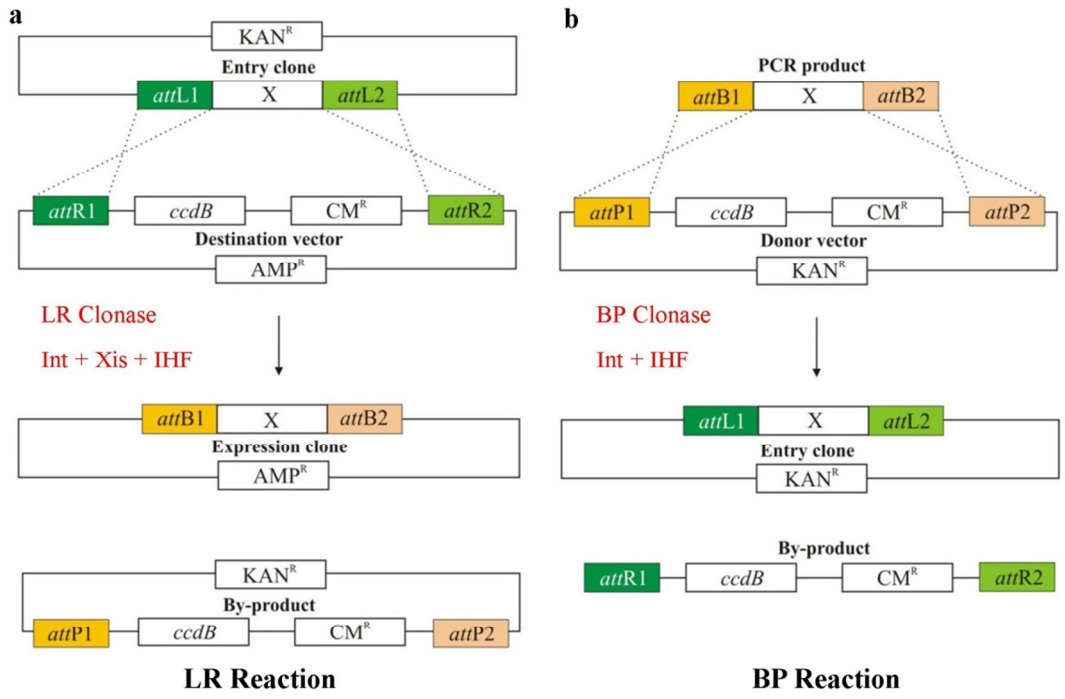


Figure 1.11. Diagram of gateway cloning technology. **a)** Gateway LR reaction of an entry clone containing a DNA fragment shown as X. **b)** BP reaction of DNA fragment flanked by attB sites. (Adapted from Magnani *et al*., 2006).

1.5. Plant Gene Expression Analysis

The main step in any functional genomics study is the analysis of gene expression. Genomics provides analysis of the expression of thousands of gene simultaneously; resulting in creation of big picture of changes occurring in the transcriptome across different conditions. Gene expression analysis studies can be divided into two categories: closed and open systems. Only well characterized genes are analyzed in closed system which is strictly depends on knowledge of interested genome. If no information is available for a specific gene or genome, it cannot be assessed in closed systems. The most common methods used in closed systems are quantitative real time polymerase chain reaction (qRT-PCR) and microarrays. In contrast to closed systems, no prior comprehensive knowledge of the transcriptome is required

in open systems, so the field of discovery is open. Closed and open systems are complementary each other. When the novel gene or novel orthologs of known genes have been discovered, they can also be used in closed systems. The common feature of these two systems is that the output of analysis results in gene lists, which need a well-planned strategy for annotation and classification by functional role of hierarchies (Green *et al*., 2001).

1.5.1. Quantitative Real-time PCR

The quantitative real-time, fluorescence-based reverse transcription polymerase chain reaction (qRT-PCR) is a technology which enables precisely detection of mRNA level for quantitative data analysis in molecular medicine, biotechnology, microbiology and diagnostics. qRT-PCR is composed of three steps including; (1) reverse transcription reaction in which mRNA is converted to cDNA (2) cDNA amplification and (3) the detection and quantification of end-products in real time (Nolan *et al*., 2006).

When compared to end-point or known as conventional PCR, real time PCR generates significantly reliable data for the quantification of nucleic acids and shows extremely wide dynamic range (more than eight orders of magnitude) (Wilhelm and Pingoud, 2003). To understand limitation of end-point PCR, it is better to explain PCR phases which are classified as an exponential, linear and plateau phases (Figure 1.12). In the exponential phase PCR product is accumulated at every cycle (assuming 100% reaction efficiency). Linear phase indicates higher variability and all the components of PCR reaction is consumed up; resulting in decrease in reaction rate. In the plateau phase, reaction has stopped completely and no PCR products are made. For end-point-PCR, this phase is detection phase in the gel. The conventional PCR reactions produce copies of a DNA template in an exponential fashion. Accumulation of inhibitors and pyrophosphate molecules and limitation of reagents cause termination of generating template at an exponential rate. So, reaction conditions are not same in exponential phase; resulting in manufacturing of more or less product in the plateau phase. So, this is the most important limitation of end-

point conventional PCR whose results are unreliable for mRNA quantification (Ginzinger, 2002). Real time PCR has ability to measure the PCR products at a point in which the reaction is still in the exponential range. Exponential phase of real time PCR makes it possible to guess the determination of initial amount of template.

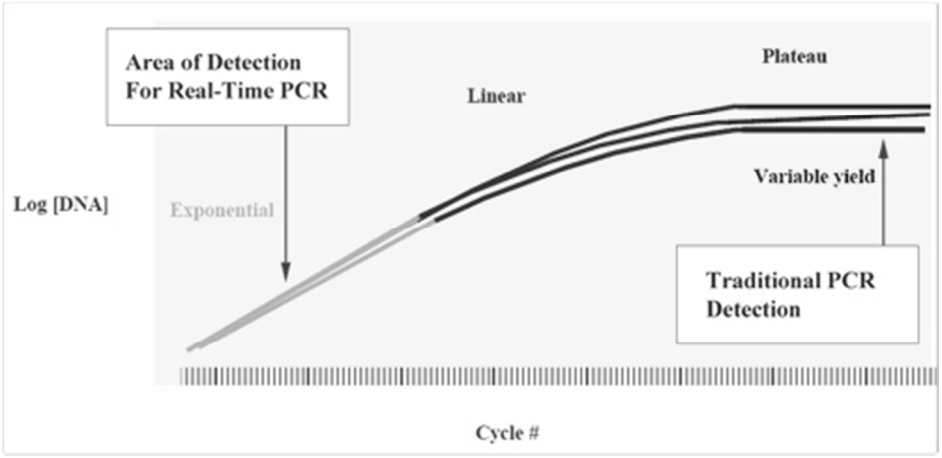


Figure 1.12. PCR phases and detection points of amplification in Real-Time PCR and Traditional PCR (Adapted from Applied Biosystems Manual).

The real-time polymerase chain reaction uses fluorescent reporter dyes which enable combination of DNA amplification and detection steps in a single tube format. During the each step of real-time polymerase chain reaction, fluorescent signal is recorded and the increase in fluorescent signal is equivalent to the amount of DNA synthesized. The threshold cycle (Ct) value is the cycle number which can be defined as raising the first fluorescence above a defined background fluorescence (Bustin, 2005). There is a correlation between Ct values and amount of starting template, which shows the fact that the lower Ct value, the more abundant the initial starting material. The real time PCR provides accurate quantification of target molecules over a wide dynamic range, while retaining the sensitivity and specificity

of conventional end-point PCR assays. Figure 1.12 also shows that three replicate samples with same amount of initial DNA reflect different quantities at the plateau phase. Traditional PCR is not able to detect variations at the plateau phase. However, Real time PCR takes precise measurements during the exponential phase, where the replicate samples are amplifying exponentially.

1.5.2. Microarray Analysis

Microarray technology is based on hybridization of DNA and/or RNA. The basic idea behind DNA microarray technology is immobilization of known DNA sequences referred to as probes in micrometer-sized spots on a solid surface (microarray) and hybridization of a complementary sequence of the DNA or a target. Fluorescently labeled samples facilitate fluorescent detection of the presence or absence of a particular target or gene in the sample. The intensity of the fluorescence coming from hybridization of labeled samples and targets or the sequence similarity of probes and targets is detected by using laser scanning and fluorescence detection devices such as CCD cameras. Different target hybridization patterns are read on the microarray and the results quantitatively analyzed (Micro and Heller, 2006). DNA microarray is one such technology that enables analysis of on thousands of genes simultaneously. So, applications of DNA microarray are usually directed at gene expression analysis or screening samples for single nucleotide polymorphisms (SNPs). Besides these biologically related analyses, DNA microarray technology is used for pharmacogenomics research, infectious and genetic disease and cancer diagnostics, and forensic and genetic identification purposes.

Two types of microarray platforms, namely the oligonucleotide microarray and cDNA microarray are commonly selected for gene expression studies. The most widely used oligonucleotide microarray is Affymetrix GeneChip® microarrays. Through this technique, highly ordered DNA oligomers on the chip are achieved with precise construction. The sequences of the probes are designed such that non-specific binding is diminished. Very low expression levels are also detected by this sensitive technique (Hazen *et al*.,2003). Affymetrix GeneChip Arrays are produced

as a single array caged in a sealed cartridge. In the GeneChip system, probes are designed using as little as 200 to 300 bases of gene, cDNA or EST sequence and manufactured by light-directed chemical synthesis process. For designing of eukaryotic probe sequences 3` end of mRNA molecules, less susceptible to degradation are selected (Lipshutz *et al*., 1999). Probes are represented on the chip by 11-20 unique oligomer, consisting of a 25-mer perfect match (PM) probe and a 25-mer mismatch (MM) probe, which has mismatched base at the central position (i.e. base 13). The MM probes serves as a control for non-specific or semi-specific hybridization. The fluorescence intensity of MM probes is subtracted from the PM probe signal. So, MM probes allow discrimination between real signals and those due to non-specific or semi-specific hybridization.

Several microarray and macroarray platforms have been generated for analysis of global gene expression profiles of cereals under the biotic and abiotic stresses (Langridge *et al*., 2006). Transcriptome analysis of biotic stress response of wheat plant using different types of microarray platforms has been reported in many studies. Table 1.1 shows some recent reports associated with analysis of global expression profiles of different wheat tissues under the aphid and fungal inoculation.

During the cold acclimation period, expression of various numbers of genes has altered. cDNA microarray with 5740 feature was constructed to compare changes in gene expression in cold-tolerant winter wheat and less tolerant spring cultivar. Over 450 genes were differentially regulated as a result of cold treatment between cold-tolerant and less tolerant cultivars. A 130 of them are related with signaling or regulatory gene candidates including, transcription factors, protein kinases, ubiquitin ligases and GTP, RNA and calcium binding proteins. The expression level changes occur in all periods of cold acclimation for both cultivars. Especially, 90% of all genes with increases in transcript levels were observed during the first day (Gulick *et al*., 2005).

Table 1.1. Transcriptome analysis of aphid and fungi inoculation as a biotic stress response of different wheat tissues.

Organisms	Caused disease	Tissue	Microarray Platform Types	References
Diuraphis noxia	Russian wheat aphid (RWA)	Wheat leaves	cDNA microarray	(Botha *et al*., 2006)
Mycosphaerella graminicola	Leaf blotch disease	Wheat leaves	cDNA microarray	(Keon *et al*., 2007)
Fusarium graminearum	Fusarium head blight (FHB)	Wheat spikes	cDNA microarray	(Golkari *et al*., 2007)
Rust and mock	Rust diseases	Wheat flag leaves	The GeneChip (Affymetrix)	(Hulbert *et al*., 2007)

Drought stress causes alternations in gene expression levels in plants. To identify genes related with wheat adaptation to water deficit, differentially expressed genes in wheat plants exposed to a drought stress were isolated using a technique called suppressive subtractive hybridization. A set of 300 unique ESTs was used to microarray analysis to indicate differential gene expression between leaves of well-watered wheat plants and plants subjected to water deficit stress. About half of these ESTs were significantly up-regulated or down-regulated under water deficit stress. To confirm the microarray results, northern blot analysis was also performed using a subset of genes from the microarray experiment (Way *et al*., 2005).

High water use efficiency known as transpiration efficiency (TE) in wheat is a desirable physiological characteristics, which improve grain development under water-limited environments. Microarray analysis with 16,000 unique wheat ESTs was performed to identify genes which are differentially expressed between high TE and low TE wheat progeny lines. Ninety-three genes of which one fifth were markedly responsive to drought stress were defined between these progeny lines.

Expression levels of several potential growth-related regulatory genes are higher in the high TE lines than the low TE lines. Quantitative RT-PCR was also used for accuracy of microarray analysis. The expression levels of 11 of the 18 genes were positively correlated with the high TE trait. The characterization of genes associate with high TE trait facilitates the selection for genotypes with higher TE using molecular markers or quantitative trait loci (QTLs) (Xue *et al*., 2006).

Affymetrix GeneChip® Wheat Arrays were used to identify differentially expressed genes in roots and leaves of five wheat germplasm lines under the salt stress. Dehydrin, LEA, lipid transfer proteins, cytochrome P450 genes are up-regulated in leaf tissues. On the other hand, expression level of nodulin, wound induced proteins, methyltransferases genes increased and genes encoding peroxidases are down-regulated in roots (Mott and Wang, 2007).

For determination of salt-responsive genes in wheat, global expression analysis of transcripts was carried out using oligo-DNA microarrays containing 32,000 unique wheat ESTs. About 6,000 genes alternation in expression profiles with more than two fold change and 12 groups are created according to correlations in expression patterns. Genes associated with transcription factor, transcription-regulator activity, and DNA-binding functions were found in early response groups. However, genes related with transferase and transporter activity were classified into late response groups. So, this suggested that multiple signal transduction pathways play crucial role for responding to salinity in wheat (Kawaura *et al*., 2008).

In another study, roots of hard red spring wheat were exposed to drought stress to examine changes in gene expression pattern. A 394 distinct transcripts were identified, which show at least 1.5 fold change between water-limited and control roots. Following the water limitation, 190 transcripts were up-regulated and 204 transcripts were down-regulated. Among them, multiple putative glucanases and class III peroxidases were defined (Mohammadi *et al*., 2008).

1.6. Aim of the Study

Environmental stresses including drought, salinity and extreme temperature cause adverse effects on the growth and development of plants; resulting in crop loss and reduce average yields. A series of morphological, physiological, biochemical and molecular changes occur in plants as a result of these abiotic stresses. In the post-genome era, abiotic stress response of plants is mainly characterized by regulation of transcription factors and stress-related genes. Identification, characterization and expression profiling of them have become an important tool to understand response of plants against severe environmental changes and to develop stress resistant plants. The plant-specific NAC transcription factors play crucial roles in plant development and abiotic and biotic stress responses. *TaNAC69-1* and *TtNAM-B2* genes were isolated from *T.aestivum* and *T.turgidum*, respectively. Then they were cloned into different monocot and dicot expression vectors to be used for further wheat and tobacco genetic transformation studies. Monitoring transcript level changes of NAC type transcription factors may serve as a potential target for improving plant tolerance to abiotic stress. So, the first aim of this study is determination of time series expression profiles of *TaNAC69-1* and *TtNAMB-2* genes under drought, salt, cold and heat stresses using Real-Time PCR.

Microarray technology is a powerful tool for identification genes induced by environmental stresses and provides analysis of whole genome expression profiles in response to environmental signals. Another aim of this thesis is to explore effects of cold and heat stresses on global gene expression profiles using Wheat GeneChip (Affymetrix) and analysis of transcriptome profile changes in wheat seedlings.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material

A winter wheat cultivar, *Triticum turgidum* spp.durum cv. Kızıltan-91, and a summer-sown wheat cultivar, *Triticum aestivum* L. cv. Yüreğir-89 were used throughout this study. The two wheat cultivars seeds were provided by Turkish Ministry of Agriculture and Rural Affairs, Central Research Institute for Field Crops, Ankara.

2.1.2. Bacterial Strains and Plasmids

In this study, *Eschericia coli* DH5α cells were utilized as competent cells in the cloning studies. Two different commercial vectors, pJET1.2/blunt (Fermentas, Cornaredo, Italy) and pENTR™/D-TOPO® (Paisely, UK) were used as a cloning vectors. pAHC25 which was kindly provided by Dr. Alan H. Christensen, and pIPKb002 from Leibniz Institute of Plant Genetics and Crop Plant Research (Germany), were used for monocot transformation vectors. pORE-E3 (provided by Dr. Dwayne Hegedus) containing tobacco constitutive promoter was designed as dicot transformation vector. pIPKb002 and pORE-E3 transformation vectors including gene of interest were transferred into *Agrobacterium tumefaciens* strains AGL1 and EHA105, respectively.

2.1.3. Bacterial Growth Media

E.coli strains were grown in Luria-Bertani (LB) Broth (Appendix A) supplemented with appropriate antibiotics. SOC medium (Appendix A) which is richer than LB Broth was used after transformation to aid fast recovery of competent *E.coli* cells. For *Agrobacterium tumefaciens* strains, Yeast extract broth (YEB) media (Appendix A) was used with antibiotics depending on bacterial selection marker on binary vectors.

2.1.4. Chemicals, Kits, and Restriction Endonucleases

Antibiotics (kanamycin, ampicillin, streptomycin, and spectinomycin) and all other chemicals for preparation of solutions were supplied from Merck, Sigma, Applichem and *Phyto*Technology Laboratories. Total plant RNA was extracted using both RNeasy Plant Mini Kit Qiagen (Valencia, CA, USA) and Trizol reagent from Invitrogen (Paisely, UK). cDNAs were synthesized from RNA samples according to the manual of RevertAid™ First Strand cDNA Synthesis Kit from Fermentas (Cornaredo, Italy). The isolated fragments were cloned in both PCR Cloning Kit, CloneJET™ PCR Cloning Kit, from Fermentas, and pENTR™/D-TOPO®, from Invitrogen. Cloned fragments were sequenced and compared with NCBI database using the BLAST program. Primer designing studies were carried out using Vector NTI Advance 10 software (Invitrogen, Paisely, UK). Qiagen RT-PCR Kits and GeneChip Wheat Genome Arrays from Affymetrix (Santa Clara, CA, USA) were used in gene expression studies. Restriction enzymes and DNA modifying enzymes used for cloning studies were from New England Biolabs (NEB, Ipswich, MA, USA) and Fermentas (Cornaredo, Italy).

2.2. Molecular Genetic Methods

2.2.1. Growth of Plants

The seeds of wheat cultivars, Kızıltan-91 and Yüreğir-89 were surface sterilized by immersion in 20% (v/v) sodium hypochlorite for 20 minutes, then rinsed with three times of distilled water. Seeds were germinated in plastic pots and irrigated with ½ strength Hoagland`s solution (Appendix B) in a growth chamber with 16 h light period at 25 °C for 14 days. Salt stress treatment was performed to increase *TaNAC69-1* and *TtNAMB-2* genes expression levels. So, after the 14$^{th}$ day of growth, salt stress was initiated with ½ strength Hoagland`s solution containing 200 mM of sodium chloride and kept for 5 days. The leaf tissues from control and stressed plants were collected and immediately frozen in liquid nitrogen for RNA isolation.

2.2.2. Primer Design for Cloning Studies

Two types of gene specific primers for each gene (Table 2.1) were designed based on cloning vectors used in cloning reactions of *TaNAC69-1* and *TtNAMB-2* genes in different expression vectors. Specificity of designed primers was checked in NCBI/Primer-BLAST web tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

Table 2.1. Gene specific primers designed for different cloning vectors.

Genes	**pJet1.2/blunt**	**pENTR™/D-TOPO®**
NAC69	5`-GCCATCTTCTCCTCCTCCTC-3` 3`-TTCTTATCGGTCGGTCGGTC-5`	5`-CACCATGCCAATGGGCAGCAGC-3` 3`-TCACATGTGCAGCTGCTGGCTG-5`
NAMB2	5`-ATGGGCAGCTCGGACTCATC-3` 3`-TCAGGGATTCCAGTTCACGC-5`	5`CACCATGGGCAGCTCGGACTCATC3` 3`-TCAGGGATTCCAGTTCACGC-5`

2.2.3. Total RNA Isolation

Total RNA was extracted according to the Qiagen RNeasy Plant Mini Kit procedure and a modified procedure from Chomczynski and Sacchi, (1987) using Invitrogen TRIzol reganet. RNeasy technology simplifies total RNA isolation by combining the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-membrane purification (Figure 2.1.a). In the TRIzol method, chloroform treatment and isopropanol precipitation were performed (Figure 2.1.b).

RNA quality and quantity was determined photometrically by using a single beam spectrophotometer, AlphaSpect μL Spectrophotometer (AlphaInnotech Inc., USA). The absorbance of a diluted RNA sample is measured at 260nm (A_{260}) and 280nm (A_{280}). An A_{260} reading of 1.0 is equivalent to ~40 μg/ml single-stranded RNA. The A_{260}/A_{280} ratio is used to assess RNA purity. An A_{260}/A_{280} ratio of 1.8 - 2.1 is indicative of highly purified RNA. Quality of the total RNA was also compared by gel electrophoresis. RNase-free 1% agarose gels solution was prepared and RNA samples were loaded into the gels and run for 40 minutes at 100V. Sharpness of 28S bands and 18S of bands show integrity of RNA samples.

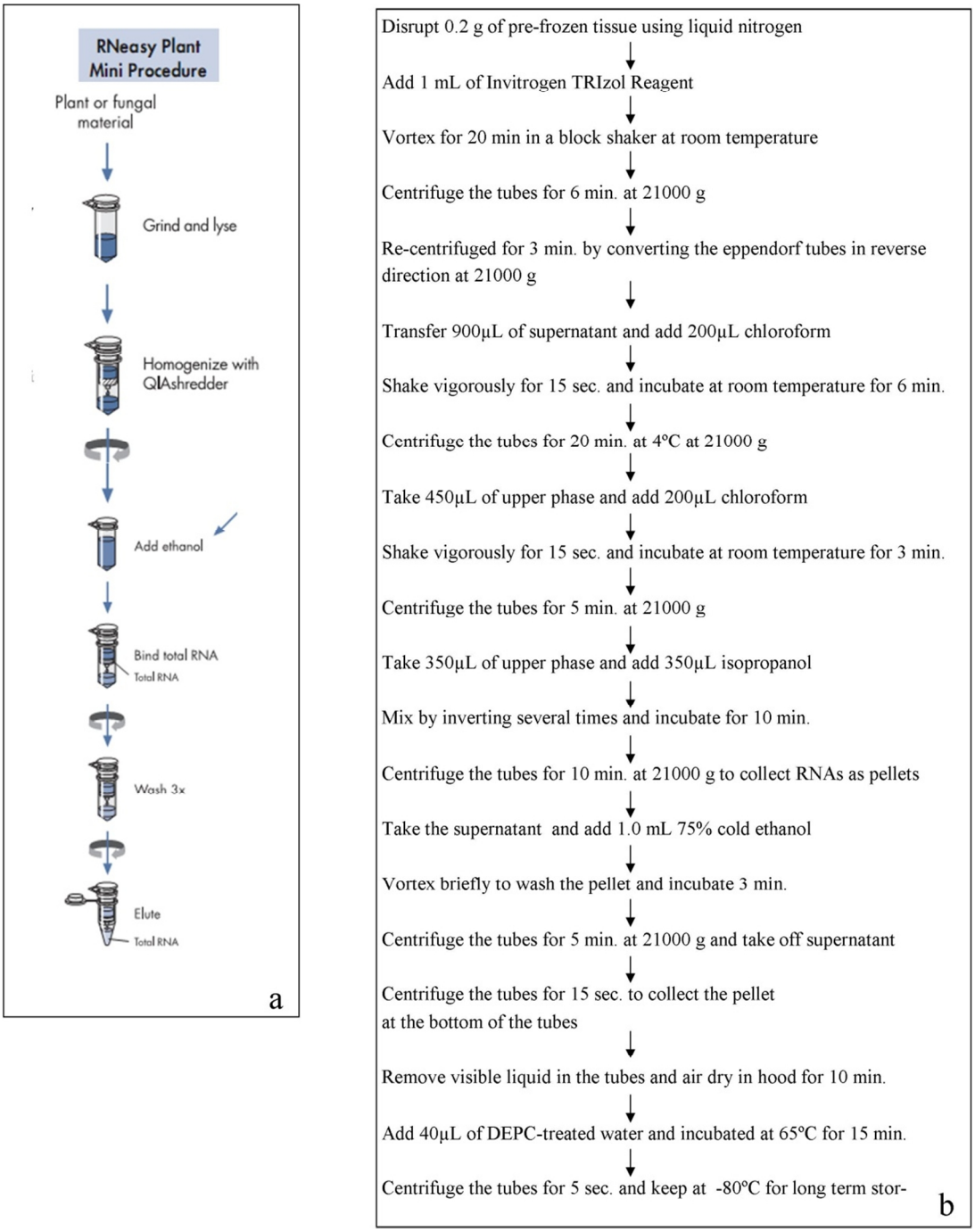


Figure 2.1. Total RNA isolation methods, a) the Qiagen RNeasy Plant Mini Kit (taken from its manual) procedure b) modified procedure from Chomczynski (1993) using Invitrogen TRIzol reagent.

2.2.4. Agarose Gel Electrophoresis

RNA, plasmid DNA molecules and PCR products throughout the study were visualized on agarose gel which was performed according to Maniatis *et al*., 1989. Different gel concentrations (0.8-1%) were prepared in 1X TAE electrophoresis buffer solutions. For 1% agarose gel, 1 g of agarose was melted completely in 100 ml of 1X TAE buffer by heating. Then the solution was left to cool around 50°C and 5 µl of ethidium bromide added to the gel solution. The gel was poured into the electrophoresis tray and solidified at room temperature. The electrophoresis tank was filled with 1X TAE buffer. The samples to be loaded mixed with 6X loading buffer were applied into the wells. Together with the samples, commercial DNA size markers such as Fermentas SM0311 or SM0331 were loaded into separate wells to determine approximate size of samples. Then the electrophoresis tank was connected to a power supply and run under constant voltage of 90-100 volts. The gel was visualized under UV transilluminator and photographed at UVP gel imaging system.

2.2.5. cDNA Synthesis

cDNAs were synthesized from RNA samples according to the manual of RevertAid™ First Strand cDNA Synthesis Kits from Fermentas. 2.5 µg of total RNA samples, Oligo dT primers and DEPC-treated water were added to reaction mixture and incubated at 70°C for 5 minutes. Then, 5x Reaction Buffer, dNTP and RiboLock™ RNase Inhibitor were added into PCR tubes. Mixture was incubated at 37ºC for 5 minutes in PCR machine. Total volume was rolled up to 20 µL by addition of RevertAid™ M-MuLV Reverse Transcriptase. Then, the compounds were mixed gently and spin down for 5 seconds in a micro centrifuge. PCR tubes were incubated at 42ºC for 60 minutes and 70ºC for 10 minutes in PCR machine.

2.2.6. PCR Amplification

TaNAC69-1 and *TtNAM-B2* genes were amplified using wheat cDNA samples. *Pfu* DNA Polymerase (Fermentas, Cornaredo, Italy) which is highly thermo stable

polymerase was used for amplification of genes for all of the cloning studies. The enzyme is obtained from the archaeum *Pyrococcus furiosus* and has proofreading activity which provides amplification of an error free *TaNAC69-1* and *TtNAM-B2* genes. Before starting PCR amplification of genes using *Pfu* DNA Polymerase, some PCR components such as primer, cDNA, and $MgCl_2$ concentrations and annealing temperature should be optimized using *Taq* DNA polymerase. Each variable should be tested independently for optimization experiments. To investigate the effects of four PCR components at three different concentration levels, 81 independent experiments should be performed. Taguchi methods are widely used to eliminate these extensive experimental investigations. Using this method, only nine reactions are enough to see effect of each component under the same experiment condition (Cobb and Clarkson, 1994). Tagushi arrays show the effects and interactions of components simultaneously (Appendix C).

2.2.7. Isolation of DNA Fragments and Plasmid from Agarose Gel

PCR products, restriction enzyme digested DNAs and plasmid DNAs were loaded on 0.8% agarose gel and visualized under the UV light which should be minimized to protect DNA from UV exposure. Desired DNA fragment bands were excised from the gel. The volume of the sliced gel was determined by its weigh and placed into eppendorf tube. The Qiagen QIAquick^R Gel Extraction Kit was used for isolation of DNA fragment from gel according to the manufacturer's manual.

2.2.8. Cloning of DNA Fragments into Cloning and Expression Vectors

Different cloning strategies were performed to clone *TaNAC69-1* and *TtNAMB-2* genes into monocot and dicot expression vectors. The first approach contains using restriction enzyme treatment of both genes and vectors. PCR products of *TaNAC69-1* and *TtNAM-B2* genes generated with *Pfu* DNA polymerase were cloned into blunt sides of pJET1.2/blunt cloning vector (Figure 2.2).

For sticky-end cloning, pJET1.2/blunt vector containing *TaNAC69-1* and *TtNAM-B2* genes and pORE-E3 binary vector were treated with *Not*I and *Cla*I restriction enzymes. So, genes and linear form of pORE-E3 binary vector carried *Not*I and *Cla*I restriction enzyme sites on 5` and 3`ends, respectively. Through the ligation reaction, they were combined each other. For blunt-end cloning, pORE-E3 binary vector carried *TaNAC69-1* and *TtNAM-B2* genes and pAHC25 monocot expression vector were cut with *Sma*I and *Sac*I restriction enzymes. However only 5` end of genes cut with *Sma*I enzyme produced blunt end. 3` end of both genes and pAHC25 vector were also shifted from sticky end to blunt end. Finally, ligation reaction was performed to ligate genes into pAHC25 vector. The second approach contains TOPO® Cloning and Gateway® Cloning strategies. For TOPO® Cloning, *TaNAC69-1* and *TtNAM-B2* genes were firstly cloned into pENTR™/D-TOPO vector. It enables directional cloning of double-strand DNA using TOPO-charged oligonucleotides by adding 3` overhang to the incoming DNA (Cheng and Shuman, 2000). In this system, forward primer contains CACC which provides directionally cloning of PCR product into pENTR™/D-TOPO vector which has GTGG overhang. So, PCR product is stabilized in the correct orientation (Figure 2.3).

Gateway cloning is based on homolog recombination of appropriate Gateway-compatible recombination sites (e.g. attL1 and attL2). It involves a two-step process including cloning of genes into an entry vector and LR reaction between the entry clone and the destination vector. Through the LR reaction between attL and attR sites, *TaNAC69-1* and *TtNAM-B2* genes were transferred into homolog recombination sites of pIPKb002 monocot expression vector.

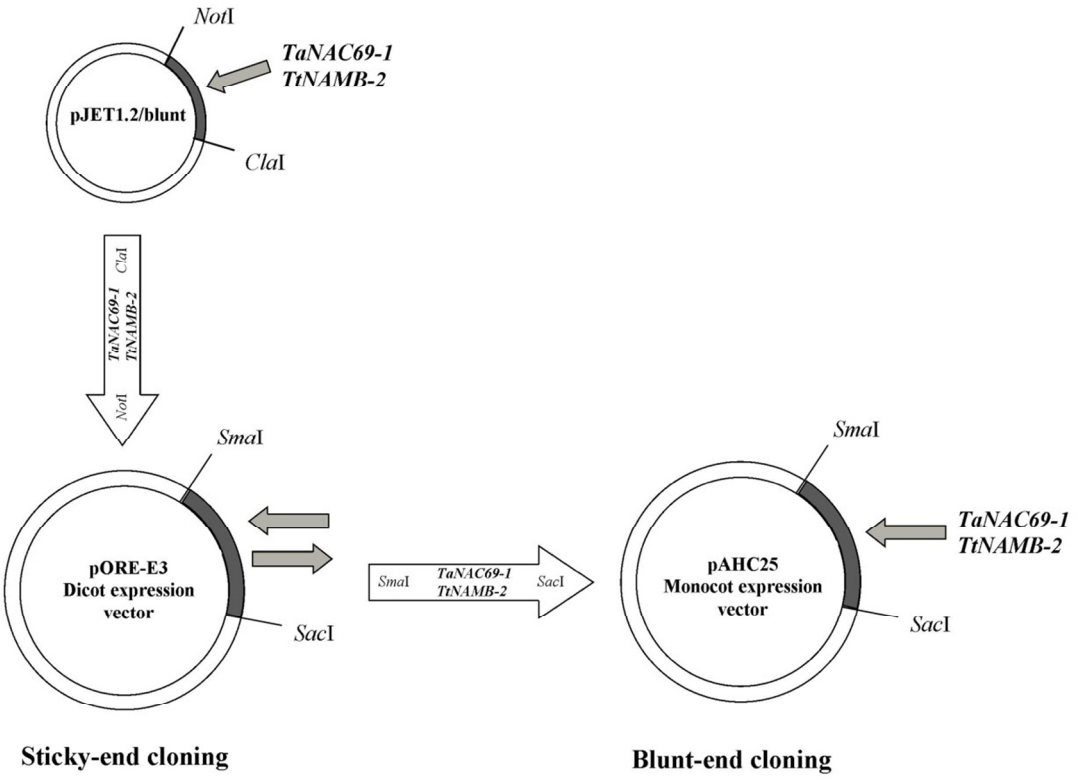


Figure 2.2. Presentations of sticky and blunt-end cloning of *TaNAC69-1* and *TtNAM-B2* genes into expression vectors.

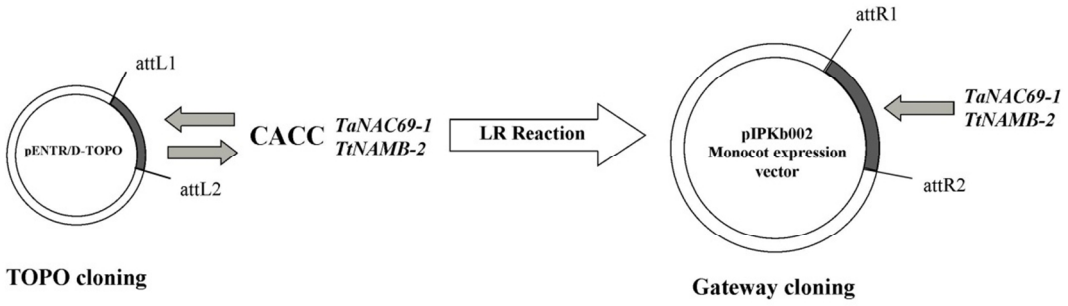


Figure 2.3. Presentations of TOPO® Cloning and Gateway® cloning strategies for cloning of *TaNAC69-1* and *TtNAM-B2* genes.

2.2.9. Transformation Studies for Bacterial Cells

E.coli and *A.tumefaciens* cells should be prepared as a competent form for transformation with the vectors.

2.2.9.1. Preparation of Competant *E.coli* cells

E.coli DH5α strain was streaked on LB agar plate and incubated for 1 day at 37°C. Then single colony of DH5α strain was inoculated into 5 ml of LB medium and grown at 37°C overnight. From this initial culture, subculture was made by diluting 1:100 into 250 ml of LB medium and grown until reach to log phase (OD_{600}= 0.6-0.7). The culture was firstly incubated on ice for 15 minutes. From this point, the cells should be maintained on ice. The cells were centrifuged at 3500g for 10 minutes at 4ºC in a prechilled rotor. The supernatant was discarded and added 0.4 volume (100 ml for 250 ml culture) transformation buffer I (TfbI) (Appendix D). After resuspension of cells with TfbI, the cells were incubated on ice for 15 minutes. The cells were lastly centrifuged at 3500g for 10 minutes at 4ºC and discarded supernatant. The second resuspension was made with 0.04 volumes (10 ml for 250 ml culture) of transformation buffer II (TfbII) (Appendix D). The cells were finally dispensed 100-µl aliquots of cells into eppendorf tubes. They were freezed in liquid nitrogen, and stored at -80ºC.

2.2.9.2. Preparation of Competant *A. tumefaciens* cells

A. tumefaciens EHA105 and AGL1 strains were streaked on LB agar plates and incubated for 2 days at 28°C. Then single colony from each strain was transferred to 5 ml of LB medium and grown at 28°C overnight. These fresh saturated cultures of *A. tumefaciens* strains, EHA105 and AGL1 were separately inoculated 500 ml of LB medium in the late afternoon. The cultures were incubated at 28ºC with vigorous agitation and harvested the following morning. When the cells have reached log phase (OD_{550}=0.5-0.8), the culture was chilled by gently swirling in an ice-water bath. The cells were centrifuged at 4000g for 10 minutes at 4ºC in a prechilled rotor.

The supernatant was discarded and added 10 ml of ice-cold H_2O. The cells were gently resuspended with a wide-bore pipette until no clumps remain. The suspension volume was adjusted to 500 ml with ice-cold H_2O. The cells were centrifuged at two times and resuspended sequentially in a final volume of 250 and 50 ml of ice-cold H_2O. The cells were lastly centrifuged and resuspended in 5 ml of 10% (v/v) ice-cold sterile glycerol and dispensed 50-µl aliquots of cells into eppendorf tubes. They were freezed in liquid nitrogen, and stored at -80ºC. This protocol was adapted from Shen and Forde (1989).

2.2.10. Transformation of Bacteria with Plasmids

The cloning and expression vectors used throughout this study were transferred to competent *E.coli* cells via the heat shock method. The expression vectors with *TaNAC69-1* and *TtNAM-B2* genes used in cloning studies were transformed to competent *A.tumefaciens* cells via electroporation method.

2.2.10.1. Transformation of *E.coli*

Ligation products or plasmids were added into 100 µl of competent *E.coli* cells which thawed on ice. This cell and DNA mixture was incubated on ice for 30 minutes for cold treatment. Then a heat shock at 42ºC for 90 seconds was applied to facilitate the entrance of ligation products or plasmids into the cells. The mixture was immediately placed on ice and incubated for 5 minutes. Then liquid SOC medium was added on bacteria cell suspension and grown at 37ºC for 60 minutes. Finally, the bacteria was plated on pre-warmed LB medium with appropriate antibiotics and incubated overnight at 37°C.

2.2.10.2. Transformation of *A. tumefaciens*

Agrobacterium competent cells of EHA105 and AGL1 were thawed on ice. 1 µg DNA was added to the cells and mixed together on ice. Cell mixture was incubated on ice for 3 minutes. The mixture was transferred to a prechilled 0.1 cm

electroporation cuvette. MicroPulser Electroporation (Bio-Rad) was set to "Agr" mode for electroporation of *Agrobacterium* cells. After the pulse, 1 ml of LB was added to the cuvette, and immediately transferred the bacterial suspension to a 15-ml culture tube. The cells were incubated for 4 hours at 28ºC with gentle agitation. The cells were collected by centrifugation at 3000g for 10 minutes and spread on an LB agar plate containing the appropriate antibiotic. Plates were incubated for 3-4 days at 28ºC. When the colonies have grown, colony PCR was performed to verify the presence of plasmid DNA. Glycerol stocks of the appropriate clones were made, and stored at -80ºC.

2.2.11. Colony PCR

To analyze the presence and orientation of the DNA insert into recombinant clones, some methods including colony PCR, restriction analysis and sequencing were performed depending on the purpose. The colony PCR method was firstly applied to detection of recombinants. The following protocol was used for colony screening by PCR (Table 2.2.). PCR master mix was prepared and dispensed into 20 µl of the mix into the PCR tubes on ice. Individual colony was picked and resuspended in 20 µl of the PCR master mix. PCR was performed as; 95°C, 3 min; 94°C, 30 s, 55°C, 30 s, 72°C 1 min/kb; 25 cycles. The PCR products were analyzed on an agarose gel. Also, restriction analysis was performed using appropriate restriction endonuclease enzymes. Plasmid DNA was isolated from an overnight bacterial culture and cut with restriction endonucleases which found on the map of cloning vector. If the colony carries right orientation of the DNA insert, plasmid was sequenced with forward and reverse sequencing primers.

2.2.12. Plasmid Isolation and Manipulation

Plasmids were isolated from transformed *E.coli* and *A.tumefaciens* to verify the recombinant bacteria cells. Miniprep plasmid DNA isolation was performed by using The GeneJET™ Plasmid Miniprep Kit (Fermentas) according to the manufacturer's manual. Purity and integrity of the plasmid DNA were confirmed by a single beam

spectrophotometer, AlphaSpect µL Spectrophotometer (AlphaInnotech Inc., USA) and gel electrophoresis, respectively. The isolated plasmids were used for restriction enzyme digestion to show presence of desired foreign genes.

Table 2.2. Colony PCR ingredients for detection of recombinants.

Component	**Amount**
10X Taq buffer	2.0 µl
dNTP mix, 2 mM each	2.0 µl
$MgCl_2$, 25 mM	1.2 µl
Forward sequencing primer (pJET1.2 or M13) or Forward gene specific primer, 10 µM	0.4 µl
Reverse sequencing primer (pJET1.2 or M13) or Reverse gene specific primer,10 µM	0.4 µl
Water, nuclease-free	13.9 µl
Taq DNA polymerase 5 u/µl	0.1 µl
Total volume	20.0 µl

2.2.13. DNA Sequencing

The plasmids with desired gene were sent to RefGen Gen Araştırmalar ve Biyoteknoloji Ltd. Şti. (Ankara, Turkey) for 5` and 3` sequencing using suitable primers. Sequences were compared with NCBI database using the BLAST program. Sequence similarities were analyzed using the Geneious software (Biomatters Ltd, New Zealand). Relationships between sequences were determined using the Clustal W method with PAM 250 residue weight table (Thompson *et al*., 1994).

2.2.14. Restriction Enzyme Digestions

Restriction enzymes from Fermentas (Cornaredo, Italy) were used for all restriction enzyme digestion reactions. Double digestion reactions were preformed according to DoubleDigest™ web tool (http://www.fermentas.com/en/tools/doubledigest) which provides selection of recommended buffer for double digestion reaction. Plasmid DNA (~200 ng) containing inserted fragment were digested with 1 µl of restriction enzymes (10 U) in a reaction mixture including appropriate enzyme buffers in 1X final concentration. Reaction mixture was roll up to 20 µl of final volume with nuclease-free water and incubated at 37° C for overnight for complete digestion.

2.2.15. Ligation of DNA Fragments into Plasmids

Rapid DNA Ligation Kit from Fermentas (Cornaredo, Italy) was used for ligation reactions. Concentration of the plasmid and insert DNA was determined by using AlphaSpect µL spectrophotometer. For successful ligation reaction, ratio of plasmid and insert DNA is crucial and should be adjusted to 1:4. So, ligation reaction mixture contains 1:4 ratio of plasmid and inserts DNA, T4 DNA Ligase (5u/µl), 5X Rapid Ligation Buffer and nuclease-free water. The ligation mixture was incubated at 22°C for 1 hour. Ligation reaction mixture was transformed into the competent *E.coli* cells. Colonies which grown overnight at 37°C were randomly selected for colony PCR to show the presence of the desired gene. Colonies that carried desired gene was grown in liquid culture to isolate plasmid. Further analysis including restriction enzyme digestion and sequencing were performed using isolated plasmid. The overview of the whole cloning steps was shown in Figure 2.4.

2.3. Conventional Cloning

2.3.1. PCR Amplification of *TaNAC69-1* and *TtNAM-B2* genes with Tagushi Method

TaNAC69-1 and *TtNAM-B2* had been cloned prior to this study (Uauy *et al*., 2006). mRNA and protein complete sequences of these genes (Appendix E) were obtained in FASTA format from NCBI. *TaNAC69-1* and *TtNAM-B2* gene sequences were aligned by Vector NTI Advance 10 software (Invitrogen, Paisely, UK) to design gene specific primers. Tagushi method was applied to *TaNAC69-1* and *TtNAM-B2* genes for amplification. Compared PCR components for both genes include different concentration of primers, cDNA, $MgCl_2$ and annealing temperature. Tagushi PCR ingredients for *TaNAC69-1* and *TtNAM-B2* are given in Table 2.3.

Table 2.3. Tagushi PCR ingredients for *TaNAC69-1* and *TtNAM-B2* genes.

		A	**B**	**C**
	Primer µM	0.4	0.6	0.8
	Annealing Tem.°C	58	60	62
	$MgCl_2$ mM	1	1.5	2
	cDNA µL	3	4	5
Reaction	**Primer**	**Annealing Tem.**	**$MgCl_2$**	**cDNA**
1	0.4	58	1	3
2	0.4	60	1.5	4
3	0.4	62	2	5
4	0.6	58	1.5	5
5	0.6	60	2	3
6	0.6	62	1	4
7	0.8	58	2	4
8	0.8	60	1	5
9	0.8	62	1.5	3

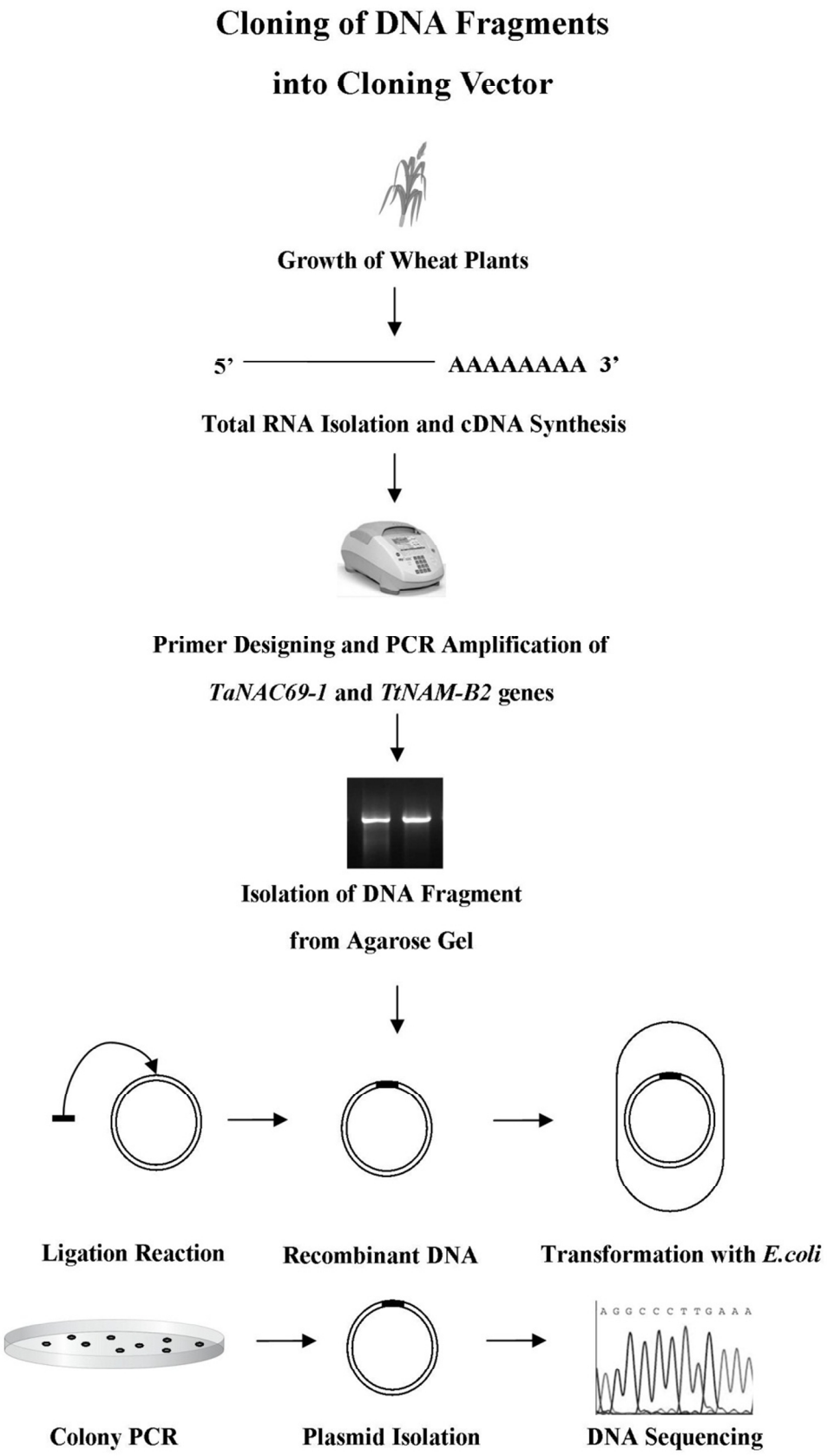


Figure 2.4. The overview of the whole cloning steps.

2.3.2. Cloning of *TaNAC69-1* and *TtNAM-B2* genes into Cloning Vector

The CloneJET™ PCR Cloning Kit (Fermentas, Cornaredo, Italy) was used for cloning of PCR products of *TaNAC69-1* and *TtNAM-B2* genes generated with *Pfu* DNA polymerase. Cloning vector pJET1.2/blunt (Figure 2.5) contains novel positive selection site which includes a lethal gene, *eco47IR*. When DNA insert was ligated into the cloning site, this lethal gene was disrupted. So, only cells with recombinant plasmids are able to survive under the selection condition. 2x Reaction Buffer, PCR product and pJET1.2/blunt cloning vector were added into PCR tubes and the mixture was rolled up to 19 µL by addition of nuclease-free water. Finally T4 DNA Ligase was added into tube and incubated at 22ºC for 30 minutes. Following the incubation, other steps were formerly described in section 2.2.15.

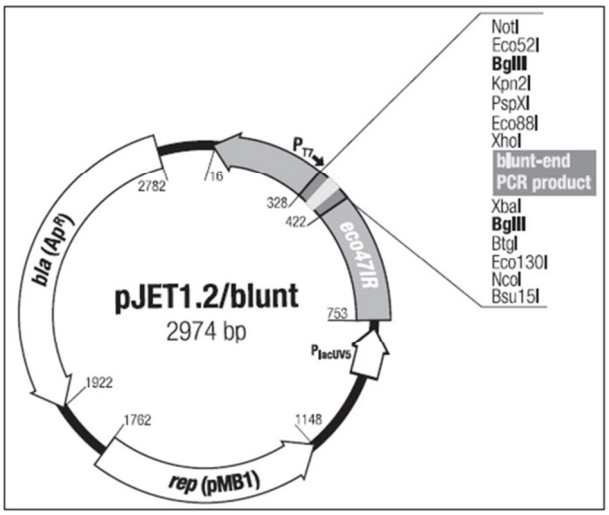


Figure 2.5. The map of the pJET1.2/blunt cloning vector (taken from CloneJET™ PCR cloning kit manual).

2.3.3. Cloning of *TaNAC69-1* and *TtNAM-B2* genes into Dicot Binary Vector

pORE series of binary vectors are the new generation binary vectors which provide many useful features for improvement of plant transformation (Ref). The pORE series has three basic types including open, reporter and expression series and are abbreviated as pORE-O, pORE-R and pORE-E, respectively. Open and reporter series are used for general plant transformation and promoter analysis, respectively. Expression series used in this study were designed for constitutive expression of transgenes. The sets compose of different combinations of promoters including P_{HPL}: *Arabidopsis thaliana* hydroperoxide lyase promoter, $P_{ENTCUP2}$: Tobacco cryptic constitutive promoter and P_{TAPADH}: *Triticum aestivum* lipid transfer protein promoter, various selectable markers (nptII and pat) and reporter genes (*gusA* and *smgfp*) (Figure 2.6.a.).

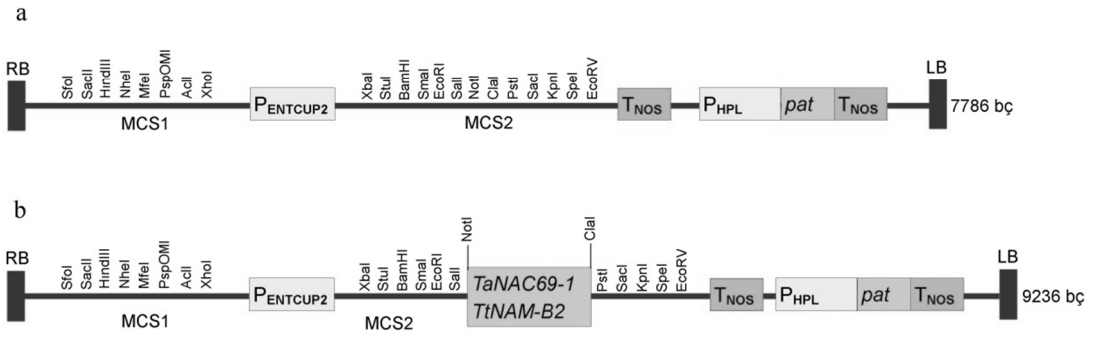


Figure 2.6. Map of expression series of pORE-E3 binary vector. **b)** After insertion of *TaNAC69-1* and *TtNAM-B2* genes into pORE-E3 binary vector.

To transfer *TaNAC69-1* and *TtNAM-B2* genes from pJET1.2/blunt cloning vector to binary vector pORE-E3, both vector cut with *Cla*I and *Not*I restriction enzymes to form same sticky ends. pORE-E3 binary vector became a linear plasmid form and *TaNAC69-1* and *TtNAM-B2* genes with suitable sticky ends for binary vector were released from cloning vector. Ligation of binary vector with *TaNAC69-1* and *TtNAM-B2* genes was achieved using T4 DNA ligase (Figure 2.6.b.). Ligation product was transferred into competent *E.coli*. Recombinant plasmids were checked and transformed into *A. tumefaciens* strain, EHA105 via electroporation.

2.3.4. Cloning of *TaNAC69-1* and *TtNAM-B2* genes into Monocot Expression Vector

pAHC25 designed as monocot expression vector contains both a selectable marker *bar* (*hpt*) gene and a scorable marker *GUS (uidA)* gene, each under the transcriptional control of a separate Ubi-1 promoter (Figure 2.7.a)

GUS gene was removed from pAHC25 and replaced by *TaNAC69-1* (Figure 2.7.b) or *TtNAM-B2* (Figure 2.7.c) genes using blunt-end cloning method. This vector is about 9700 bp size which can be tolerable for particle bombardment. After insertion of *TaNAC69-1* or *TtNAM-B2* genes into pAHC25 vector, plasmid size would be increased to about 11000 bp. As a result, particle bombardment efficiency would be decreased. Because of the nearly same size of *GUS* gene and *TaNAC69-1* or *TtNAM-B2* genes, plasmid size does not change after removing of *GUS* gene and insertion of desired genes into pAHC25. *GUS* gene can be removed from pAHC25 vector using *Sma*I and *Sac*I restriction enzymes. Although *Sma*I restriction enzyme produces blunt end, after digestion with *Sac*I, sticky end is produced. For blunt-end cloning, 5` and 3` ends should contain blunt end. So, sticky end was changed to blunt end using T4 DNA Polymerase that is ability to create blunt ends.

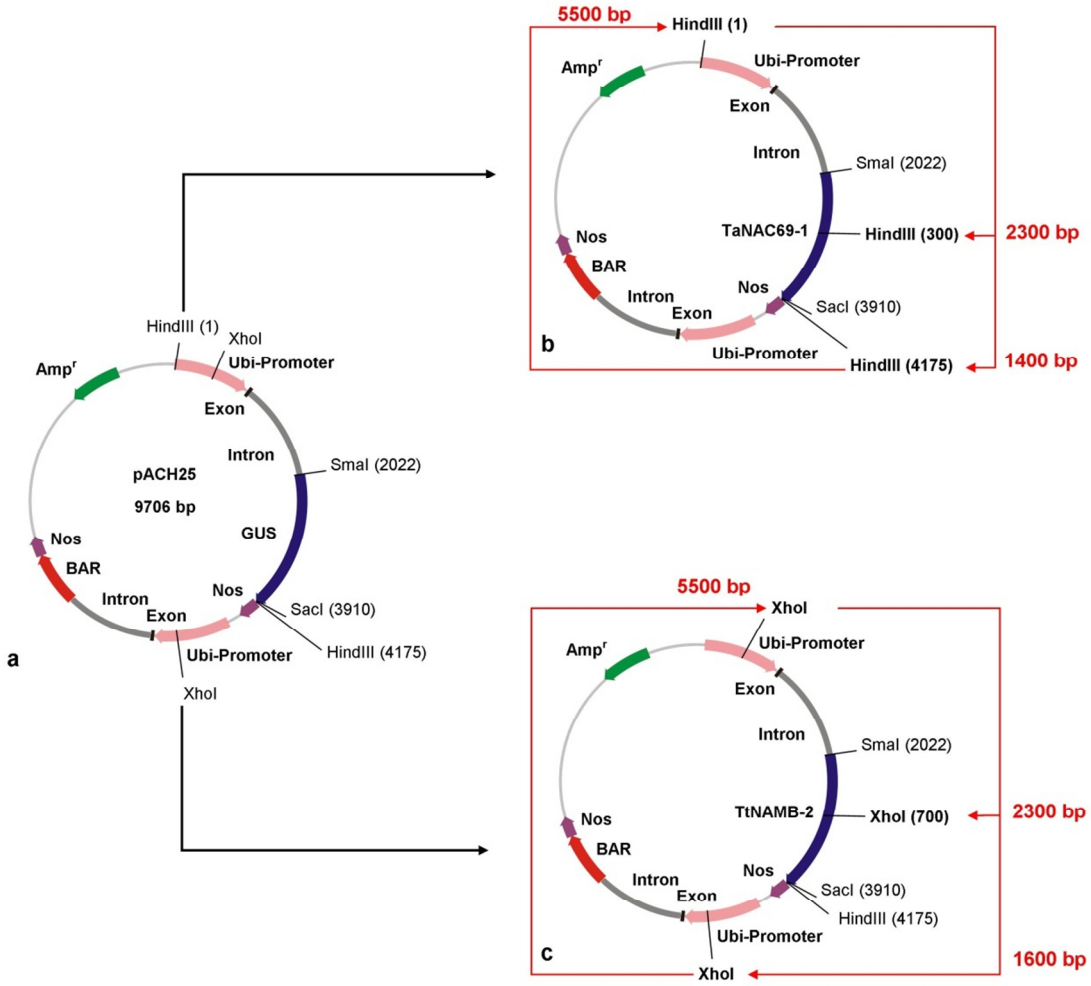


Figure 2.7. Map of pACH25, monocot expression vector. After removing of *GUS* gene from pACH25 replaced by **b)** TaNAC69-1 and **c)** TtNAM-B2.

The T4 DNA Polymerase, a template-dependent DNA polymerase, catalyzes 5' to 3' synthesis from primed single-stranded DNA. The enzyme has a 3' to 5' exonuclease activity, but lacks 5'to 3' exonuclease activity (Figure 2.8).

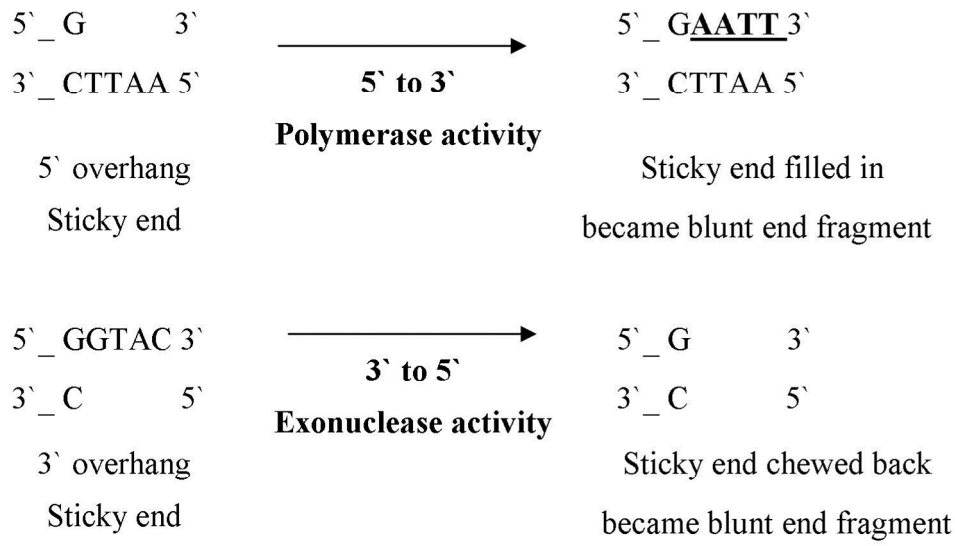


Figure 2.8. Action mechanism of T4 DNA Polymerase.

T4 DNA polymerase (Fermentas, Cornaredo, Italy) treatment of pAHC25 vector was performed according to producer`s manual. 5X reaction buffer, double digested linear pACH25 (used *Sma*I and *Sac*I restriction enzymes) vector, dNTP and T4 DNA polymerase was added into PCR tubes. The total volume was rolled up to 20 µL by addition of nuclease-free water. Then, the compounds were mixed gently and spin down for 5 seconds in a microcentrifuge. PCR tubes were incubated at 11ºC for 30 minutes and 75ºC for 10 minutes in PCR machine. *TaNAC69-1* or *TtNAM-B2* genes were ligated to blunt ends of pAHC25 vector using T4 DNA ligase. Ligation products were transferred into competent *E.coli*.

Because of the blunt-end cloning, DNA fragments can be inserted into reverse orientation. So, orientation of insert DNA into vector should be checked using restriction mapping which was the most used technique for determination of insert DNA orientation in recombinant plasmid. Restriction mapping is a technique in which digestion of plasmid with a series of restriction enzymes and then separation of the fragmented DNAs is achieved by agarose gel electrophoresis. The distance

between all restriction sites can be calculated by looking the profiles of fragments which are manufactured by the restriction enzyme digestion. In this way, information about the position of an unknown piece of DNA can be obtained. For confirmation of *TaNAC69-1* gene orientation in pAHC25 vector, *Hind*III restriction enzyme was selected because of presence of its restriction site in both *TaNAC69-1* gene and pAHC25 vector. *TaNAC69-1* gene contains *Hind*III restriction site at a position of 300bp. pAHC25 vector involves two *Hind*III restriction sites (Figure 2.7.b). As a result, three pieces of DNA fragment were obtained. pAHC25 vector was also digested with *Xho*I restriction enzyme to confirm *TtNAM-B2* gene orientation in the vector. *Xho*I restriction sites are found both *TtNAM-B2* gene and vector. *TtNAM-B2* gene contains *Xho*I restriction site at a position of 700bp. pAHC25 vector involves two *Xho*I restriction sites within the Ubi-promoter. (Figure 2.7.c). As a result of digestion reaction with *Xho*I, three pieces of DNA fragment were obtained.

2.4. Gateway® Cloning Technology

2.4.1. PCR Amplification of *TaNAC69-1* Gene with *Pfu* Taq Polymerase

PCR amplification of *TaNAC69-1* gene with *TaNAC69-1* forward and reverse primers using *Pfu* DNA Polymerase was performed to obtain *TaNAC69-1* gene. cDNA was used as a template DNA. PCR conditions and program used for PCR amplification are given in Table 2.4 and Table 2.5, respectively.

2.4.2. Cloning of *TaNAC69-1* Gene into Entry vector

The pENTR™ Directional TOPO® Cloning Kit (Paisely, UK) was used for cloning of purified PCR products of *TaNAC69-1* gene generated with *Pfu* DNA polymerase. TOPO®Cloning strategy allows directionally cloning of blunt-end PCR product into cloning vector, pENTR™/D-TOPO®. This cloning vector has attL1 and attL2 sites which provide site-specific recombination for Gateway® compatible destination vectors (Figure 2.9). Salt solution, PCR product and pENTR™/D-TOPO® cloning vector were added into PCR tube and gently mixed. Ligation mixture was incubated

at 22ºC for 30 minutes and directly used for bacterial transformation. To analyze the presence the DNA insert into recombinant clones, firstly colony PCR was performed using gene specific primers then recombinant plasmids were sequenced with M13 forward and reverse primers. Finally, *TaNAC69-1* gene found in entry vector was transferred into destination vector via site-specific recombination between entry and destination vectors.

Table 2.4. Optimized PCR condition for amplification of *TaNAC69-1* gene.

Reagents	**[stock]**	**Unit**	**[final]**	**µl**
GSP primer I	10	µM	0.5	1.25
GSP primer II	10	µM	0.5	1.25
dNTPs	2.5	mM	0.2	2
Buffer-$MgSO^4$	10	X	1	2.5
Pfu DNA pol	2.5	units/µl	0.025	0.25
DMSO	100	%	5	1.25
DNA/water	5	ng/µl	-	3
$MgSO_4$	25		1.5	1.5
dH_2O				12
Total				25

Table 2.5. PCR cycling conditions for amplification of *TaNAC69-1* gene.

PCR Steps		**Temperature**	**Time**	**Cycle**
Initial denaturation		95 °C	3 min.	1
Amplification	Denaturation	94 °C	20 sec.	35
	Annealing	56 °C	20 sec.	
	Extension	72 °C	2 min.	
Final Extension		72 °C	10 min.	1

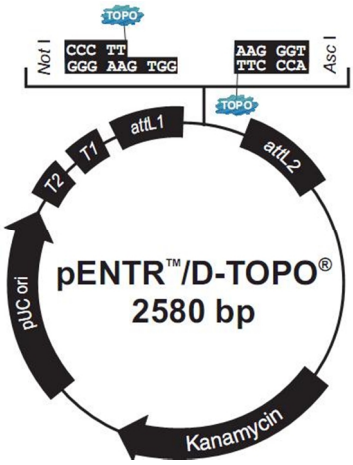


Figure 2.9. Map of entry vector pENTR™/D-TOPO® (taken from pENTR™ Directional TOPO® Cloning Kit).

2.4.3. Cloning of *TaNAC69-1* Gene into pIPKb002

pIPKb002 which is Gateway-based binary destination vector was developed by Himmelbach *et al*., (2007) for foreign gene overexpression in cereals. pIPKb002 contains suitable recombination sites (attR1 and attR2) for LR reaction, integrated downstream of maize ubiquitin 1 promoter. This destination vector also includes toxin gene providing negative selection for bacteria and hygromycin resistance gene for plant selection. Gateway® LR Clonase™ II enzyme mix (Paisely, UK) was used for LR reaction between entry vector, pENTR™/D-TOPO® with *TaNAC69-1* gene and binary destination vector, pIPKb002. Site-specific recombination occurs between attL and an attR recombination sites. As a result of LR reaction, attB-containing expression clone is generated (Figure 2.10). Gateway® LR Clonase™ II enzyme mix is a combination of enzymes including the bacteriophage lambda recombination proteins Integrase (Int) and Excisionase (Xis), the *E. coli*-encoded protein Integration Host Factor (IHF) and buffer formulation. Entry vector containing *TaNAC69-1* gene, binary destination vector, TE buffer and LR Clonase™ II enzyme mix were added into PCR tubes. Ligation mixture was incubated at 25ºC

for 60 minutes. To terminate the reaction, 1 µl of the Proteinase K solution was added into reaction mixture and incubated at 37°C for 10 minutes. Then the ligation mixture was directly used for bacterial transformation. To analyze the presence of the attB-containing expression clones of *TaNAC69-1* gene, colony PCR was performed using gene and destination vector specific primers. Recombinant plasmids were transformed into *A. tumefaciens* strain, AGL1 via electroporation.

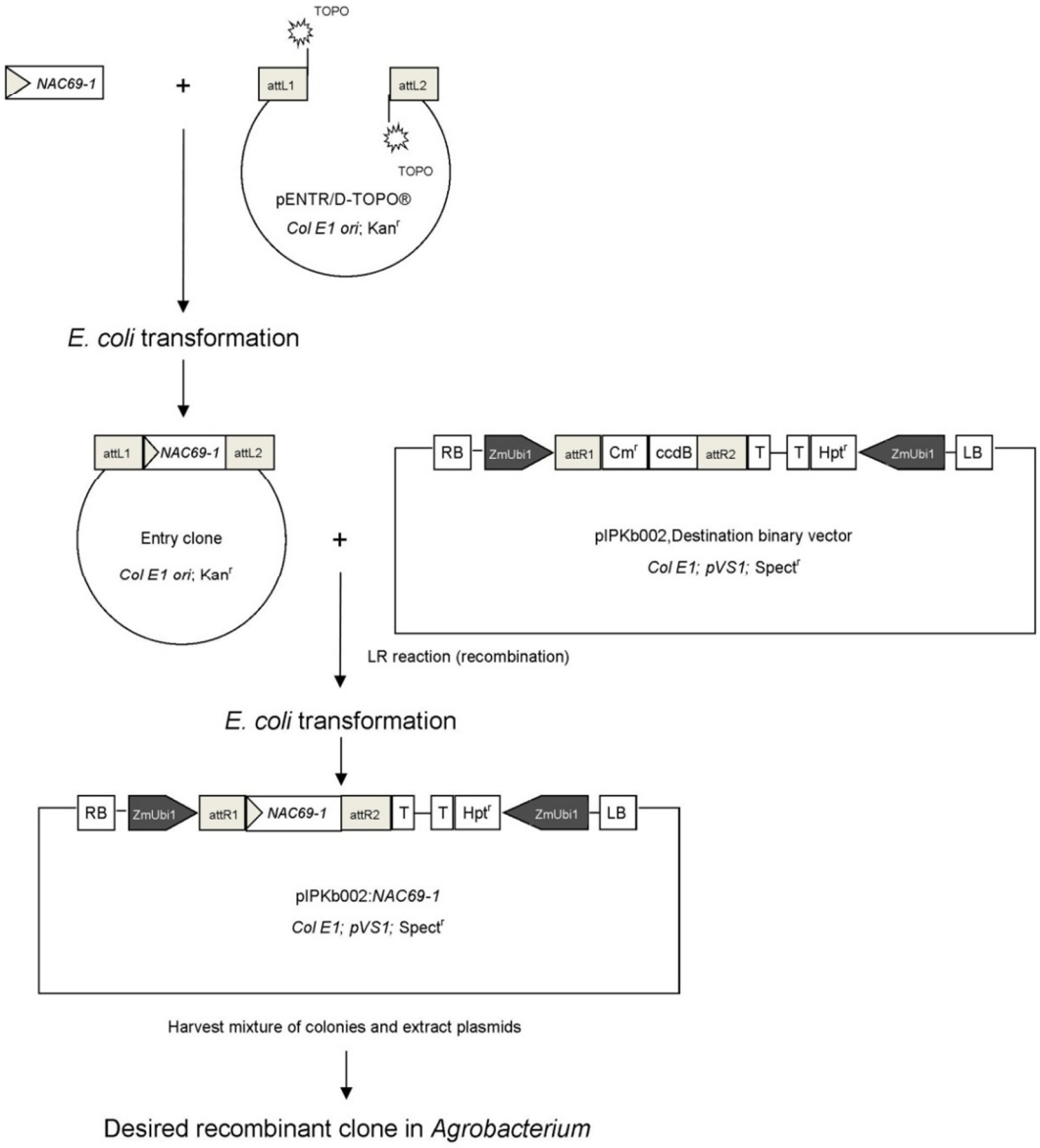


Figure 2.10. LR reaction between entry vector and destination binary vector.

2.5. Gene Expression Studies

2.5.1. Growth of Plants & Stress Applications

Kızıltan-91 (*Triticum turgidum* spp.durum.) winter-sown, resistant to drought and cold, wheat cultivar was used for gene expression studies including quantitative PCR (qPCR) and microarray. The seeds were obtained from Tarla Bitkileri Merkez Araştırma Enstitüsü, Ankara. Seeds were surface sterilized with 10% NaOCl for 10 minutes and then washed with distilled water for three times. Then, they were transferred to plastic container filled with agar for support and grown in hydroponic culture containing ½ strength Hoagland's Solution for 10 days in a plant growth chamber at 24 ± 2ºC with 16 hours light and 8 hours dark photoperiod at a light intensity of 1000 lux.

All stress treatments were initiated at 10^{th} day of normal growth. For drought and salt stresses, 20% polyethylene glycol 6000 (PEG-6000) and 250mM of sodium chloride (NaCl) were added into ½ strength Hoagland's Solution, respectively. Both treated (stress) and non-treated (control) plants were kept in the growth chamber at same growth conditions. Plants were removed from the growth chamber for sample collection at 0^{th}, 1^{st}, 3^{rd}, 6^{th}, 12^{th} and 24^{th} hours, and on 2^{nd}, 3^{rd}, 5^{th} and 7^{th} days of stress application. For cold and heat stresses, plants were removed from the growth chamber (24ºC) and transferred to 4ºC and 40ºC adjusted growth chamber for cold and heat stress applications, respectively. Leaf samples from treated (stress) and non-treated (control) plants were taken at 0^{th}, 1^{st}, 3^{rd}, 6^{th}, 12^{th}, 24^{th} and 48^{th} hours of stress application. The leaf tissues from three biological replicates were collected and immediately frozen in liquid nitrogen for RNA isolation.

2.5.2. Total RNA Isolation & Characterization

Total RNA was extracted according to the modified procedure from Chomczynski (1993) using Invitrogen TRIzol reganet. Quant-iT™ RiboGreen® RNA reagent (Invitrogen) which is an ultra-sensitive fluorescent nucleic acid stain was used for

quantitating RNA in solution. Three different dilutions from RNA samples were prepared and added to RiboGreen® RNA reagent. After 5 minutes incubation at room temperature, the fluorescence of the samples was measured with NanoDrop 3300 Fluorospectrometer (Thermo Scientific). RNA concentration of the samples was determined from the standard curve generated in RNA Standard Curves. Quality of the total RNA was also compared by gel electrophoresis. RNase-free 1% agarose gels solution was prepared and RNA samples were loaded into the gels and run for 40 minutes at 100V. Sharpness of 28S bands and 18S of bands show integrity of RNA samples.

DNAs in RNA samples were removed using Dnase I (Fermentas) that digests single- and double-stranded DNA, according to the manufacturer's manual (Table 2.6). Following reaction ends, precipitation protocol was immediately started.

Table 2.6. Conditions for DNase I treatment for RNA samples.

Components	**Amount**
RNA	1 µg
10X Reaction Buffer with $MgCl_2$	1 µl
DNase I	1 µl (1u)
DEPC-treated water	to 10 µl
Incubate at 37ºC for 30 min.	
50 mM EDTA	1 µl
Incubate at 65ºC for 10 min.	

After DNase treatment, precipitation with sodium acetate was carried out to concentrate RNA samples. Total volumes of RNA samples in eppendorf tubes were calculated and sodium acetate solution (3M, pH=5.2) was added into the tubes to adjust final concentration of sodium acetate equal to 0.3 M. Required volume of

100% cold ethanol was added into the tubes so as to make the final concentration of ethanol equal to 70%. After inverting the tubes several times, they were incubated at -20ºC overnight. Then, the tubes were centrifuged for 30 minutes at 4ºC at 21000 g and supernatant was removed carefully. To wash RNA pellet, 1 mL of 70% cold ethanol was added into the tubes and centrifuged for 5 minutes at room temperature at 21000 g. Any visible liquid was removed and then the tubes were air dried for 10 minutes. Finally, 20µL of DEPC treated water was added to dissolve RNAs and the tubes were kept at 65°C for 5 minutes. RNA samples were kept at -80°C for long term storage.

Bioanalyzer is a microfluidics-based platform which produces short and precision analysis for DNA, RNA and protein samples. The Agilent 2100 Bioanalyzer was used for quantification and quality control of RNA samples to check fragmented aRNA samples in microarray study.

2.5.3. qRT-PCR and Measurement of *TaNAC69-1* and *TtNAMB-2* Genes Expression Level Changes

TaNAC69-1 and *TtNAMB-2* genes expression level changes under the abiotic stresses including salt, drought, cold and heat stresses were measured on Corbett Rotor-Gene™ 6000 using One-step RT-PCR method (Qiagen, Valencia, CA, USA). One-step RT-PCR method provides cDNA synthesis and PCR reactions to occur in a single tube. Total RNAs of the leaf samples of control and stressed wheat plants were used for qRT-PCR experiments. The amounts of RNA in each qRT-PCR reaction were adjusted to 100 ng/µl using NanoDrop 3300 Fluorospectrometer. TaNAC forward primer 5`_ACTACCAGCTGCCTCCCGAAAACC_3` and TaNAC reverse primer 5'_GCCGTAGTCATCTACGCGCGCC_3` and TtNAM forward primer 5`_AACAGGAGCAGAAATGTCGGCAAC_3` and TtNAM reverse primer 5`_GGATGACATGCTGTTGATGGTAGG_3` were designed for qRT-PCR reactions of *TaNAC69-1* and *TtNAMB-2* genes, respectively. qRT-PCR conditions and program used for amplification of *TaNAC69-1* and *TtNAMB-2* genes fragment are given in Table 2.7 and Table 2.8, respectively.

Table 2.7. Optimized qRT-PCR condition for amplification of *TaNAC69-1* and *TtNAMB-2* genes fragment.

Reagents	**[stock]**	**Unit**	**[final]**	**µl**
TaNAC/ TtNAM forward primers	10	µM	0.5	1
TaNAC/ TtNAM reverse primers	10	µM	0.5	1
RT-PCR Master Mix	2	X	1	10
RT Mix	1		0.01	0.2
RNA	100	ng/µl		1
dH_2O				6.8
Total				20

Table 2.8. qRT-PCR cycling conditions for amplification of *TaNAC69-1* and *TtNAMB-2* genes fragment.

PCR Steps		**Temperature**	**Time**	**Cycle**
Reverse Transcription		50 °C	30 min.	1
Initial Denaturation		95 °C	15 min.	1
Amplification	Denaturation	94 °C	15 sec.	45
	Annealing	56 °C	30 sec.	
	Extension	72 °C	30 sec.	
Melt Curve		50-90 °C		1

To analyze *TaNAC69-1* and *TtNAMB-2* genes expression level changes in qRT-PCR, absolute quantification method was applied using external standards of plasmid DNA. *TaNAC69-1* and *TtNAMB-2* genes fragment were amplified in PCR using gene specific forward and reverse primers and cloned into pENTR™/D-TOPO®

cloning vector. The threshold cycle (C_T value) was plotted versus the log10 of dilution series of 6 different concentrations (10^7 and 10^2 copies/ µl) of the standard to generate standard curve. The slope of a standard curve indicated the efficiency of the qRT-PCR. After determination of plasmid DNA concentration, the copy number of standard DNA molecules was calculated using the following formula:

(X g/µl DNA / [plasmid length in basepairs x 660]) x 6.022 x 10^{23} = Y molecules/µl

No-template control (NTC) reactions which contained all qPCR components except the template was performed in each run of qRT-PCR. DNA contamination in the master mix and during the preparation steps of qRT-PCR was checked by amplifications in the NTC reaction.

qRT-PCR was performed in triple technical replicates for each RNA samples coming from three biological replicates. The dilution series and *TaNAC69-1* and *TtNAMB-2* genes fragment were amplified in separate wells. The C_T values of standards were determined. C_T values of RNA samples obtained from control and stressed wheat plant were compared with the standard curve to determine the amount of *TaNAC69-1* and *TtNAMB-2* genes fragment in the samples.

2.5.4. Microarray Analysis of Wheat Seedlings under Cold and Heat Stresses

Microarray analysis was performed to compare the gene expression profiles of wheat seedlings under the cold and heat stresses. According to qRT-PCR results of samples exposed to cold and heat stresses, 12^{th} hours of stress applications were selected for microarray analysis. Samples taken from control wheat plant at 12^{th} hours were used as a control samples to compare the gene expression profiles under stress conditions. The number of biological replicates was three for each treatment, so a total of 9 GeneChip® Wheat Genome Array (Affymetrix) was used for microarray study. The integrity and concentrations of DNase treated RNA samples were checked using Agilent 2100 Bioanalyzer and NanoDrop 3300 Fluorospectrometer, respectively.

Affymetrix GeneChip® 3‟ IVT Express Kit was used for preparation of RNA samples for hybridization.

Before starting reverse transcription reaction, diluted Poly-A RNA controls were added into all RNA samples to monitor the labeling process. First-strand cDNA synthesis was achieved using 0.5 µg total RNA with T7 oligo (dT) primers. Then, single-stranded cDNA was converted to second-strand cDNA which was used as a template for transcription reaction. DNA polymerase and RNase H were enzymes that degraded RNA and synthesized second-strand cDNA, respectively. aRNA (amplified RNA) was produced from the double-stranded cDNA via in vitro transcription. aRNA was labeled with biotin-conjugated nucleotides in IVT labeling reaction. To remove unreacted NTPs, enzymes, and salts, the labeled aRNA was purified to increase stability and quantified by measuring its absorbance at 260 nm using NanoDrop 3300 Fluorospectrometer. 15 µg of biotin-labeled aRNA was fragmented before hybridization with arrays. The fragmented aRNA was verified using both 1% agarose gel and Agilent 2100 Bioanalyzer. Hybridization cocktail consisting of 12.5 µg fragmented and labeled aRNA, control oligonucleotide B2, hybridization controls, hybridization mix and 10% DMSO was rolled up to 250 µl and loaded on wheat genome arrays. The wheat arrays were hybridized in Affymetrix Hybridization Oven 640 at 45 °C and 60 rpm for 16 hours with the hybridization cocktail (Table 2.9).

Wheat arrays were washed in Fluidics Station 450 (Affymetrix) and stained with streptavidin-phycoerythrin (Invitrogen) and biotinylated antistreptavidin antibody (Sigma), according to the protocol for Affymetrix 49 format wheat array. Wheat arrays were finally scanned using GeneChip Scanner 3000 (Affymetrix). GeneChip®Operating Software 1.4 was used for extraction of microarray data from scanned GeneChip images. Hybridization reaction, scanning and preliminary data analysis were performed at METU Central Laboratory.

Table 2.9. RNA amplification procedure using GeneChip 3' IVT Express Kit.

Microarray Steps	Temperature	Time
First-strand cDNA synthesis	42 °C	2 hours
Second-strand cDNA synthesis	16 °C	1 hour
	65 °C	10 min.
IVT Labeling	40 °C	16 hours
aRNA Purification and Fragmentation		
Hybridization with wheat arrays	45 °C	16 hours
Array Washing, Staining and Data Analysis		

Analysis of microarray data from all hybridizations and array normalization were done using GeneSpringGX 11.0 (Agilent) software. The raw data, imported as .CEL files, were normalized per chip using Robust Multi-array Analysis (RMA) that is model normalization for multiple arrays.

Differentially expressed genes were defined by filtering on expression levels and increasing to fold changes over 2. One-way ANOVA was done for determination of significantly different regulated genes at p value below 0.05. Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) is a web-based tool kit for the analysis of Gene Ontology enrichment (Zheng & Wang, 2008). Using the Affymetrix GeneChip® platform within the GOEAST package, probe-set IDs from Affymetrix microarrays datasets were analyzed. The analysis was performed using the statistical method "Hypergeometric" with the significance level of FDR set at 0.01. The significantly different probe sets were annotated using Harvest: Wheat (version 1.54) (http://harvest.ucr.edu). Differentially regulated genes under the control and cold, heat stress conditions were visualized in the pathways using MapMan 3.5.0 Beta Software with Taes_AFFY_0709 mapping file.

CHAPTER 3

RESULTS AND DISCUSSION

NAC type plant specific transcription factors are related with development, defense and abiotic stress responses. For further wheat and tobacco genetic transformation studies, *TaNAC69-1* and *TtNAM-B2* genes were isolated from *T.aestivum* and *T.turgidum*, respectively and cloned into different monocot and dicot expression vectors. To understand effects of abiotic stresses including salinity, drought, cold and heat stresses on expression profiles of *TaNAC69-1* and *TtNAM-B2* genes, quantitative real time PCR was performed. Microarray analysis also carried out to indicate effects of cold and heat stress treatments on global gene expression profiles of wheat plant.

3.1. Cloning of NAC Type Transcription Factors

3.1.1. Cloning Strategies

To increase gene expression level of *TaNAC69-1* and *TtNAM-B2* genes, both cultivars, Yüreğir-89 and Kızıltan-91, were grown under salt stress condition. Total RNA was isolated from leaves of both cultivars using Qiagen RNeasy Plant Mini Kit and TRIzol reagent. RNAs were reverse transcribed for cDNA synthesis which was used for cloning of *TaNAC69-1* and *TtNAM-B2* genes.

RNA quality and quantity was determined photometrically by using a single beam spectrophotometer, AlphaSpect μL Spectrophotometer as explained in section 2.2.3.

Concentration and purity of RNAs obtained from both cultivars using Qiagen RNeasy Plant Mini Kit was shown in Table 3.1.

Table 3.1. Quantity and purity of RNA samples using Qiagen RNeasy Plant Mini Kit.

Cultivars	**Sample #**	**A_{260}**	**Ratio $A_{260/280}$**	**ng/uL**
Kızıltan-91	1	5.42	2.29	216.6
	2	5.19	2.28	207.7
	3	7.81	2.25	312.3
Yüreğir-89	1	8.63	2.13	345.2
	2	3.92	2.30	156.8
	3	5.25	2.27	209.8

The quality of RNA samples was also verified by %1 agarose gel electrophoresis (Figure 3.1). Observation of 28S rRNA and 18S rRNA bands in the gels clarified the integrity of RNA samples. DNA contamination was easily detected by checking bands in the wells or any smear formation above the rRNA bands (Farrell, 2005). As shown in Figure 3.1, there is a visible DNA contamination which is easily observed in second sample of Kızıltan-91 and first and third samples of Yüreğir-89. The results of agarose gel electrophoresis were consistent with the spectrophotometric results. Therefore, RNA samples were also isolated using TRIzol reagent.

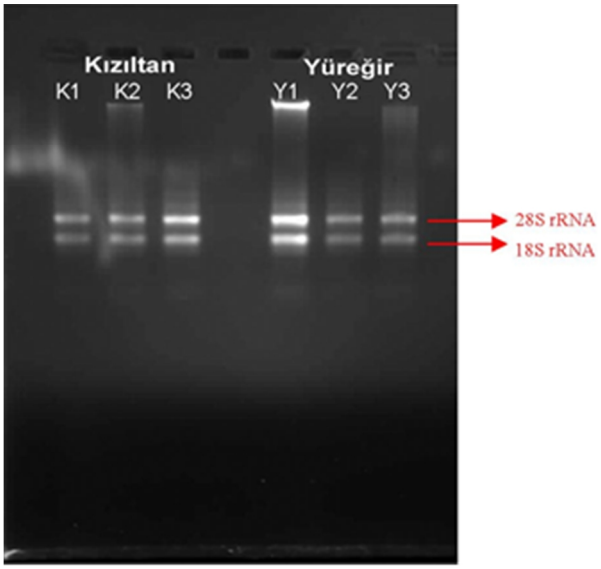


Figure 3.1. Agarose gel electrophoresis results of total RNA samples taken from Kızıltan-91 and Yüreğir-89. RNAs were isolated using Qiagen RNeasy Plant Mini Kit and run in RNase-free 1% agarose gel with 1X TAE solution for 40 minutes at 100V.

To obtain high quality RNA samples for further studies, RNA was also isolated using TRIzol method. Quantity and purity of RNA from both cultivars using TRIzol method was indicated in Table 3.2.

Table 3.2. Quantity and purity of RNA samples using TRIzol reagent.

Cultivars	**Sample #**	**A_{260}**	**Ratio $A_{260/280}$**	**ng/uL**
Kızıltan-91	K1	18.90	1.9	755.9
	K2	20	1.85	803.7
	K3	19.12	1.9	764.13
Yüreğir-89	Y1	11.54	2.08	461.5
	Y2	19.48	1.81	779
	Y3	18.66	1.84	746.4

Figure 3.2 shows the quality of RNA samples obtained from using TRIzol method. 28S, 18S and 5S ribosomal bands were clearly observed in %1 agarose gel electrophoresis. DNA contamination was eliminated from RNA samples and these results were compatible with the spectrophotometric results. Quantity and purity of RNA obtained from TRIzol method was higher than Qiagen RNeasy Plant Mini Kit.

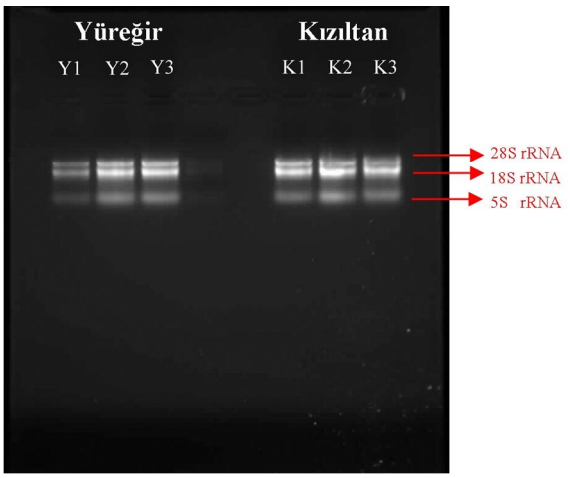


Figure 3.2. Agarose gel electrophoresis results of total RNA samples taken from Kızıltan-91 and Yüreğir-89. RNAs were isolated using Qiagen RNeasy Plant Mini Kit and run in RNase-free 1% agarose gel with 1X TAE solution for 40 minutes at 100V.

cDNA obtained from wheat cultivars of Kızıltan-91 and Yüreğir-89 was used as a template DNA for amplification of the coding sequence with 3` and 5` untranslated regions (UTRs) primers of *TaNAC69-1* (1450 bp) and *TtNAM-B2* (1500 bp) genes. The amplification of both genes was done using Tagushi PCR with gene specific primer pairs as described in the section 2.2.6. As a result of Tagushi reactions, amplification of the *TtNAM-B2* gene was achieved in the all of the nine reactions. However, *TaNAC69-1* gene amplicons were observed in only third and seventh reaction. The amplified fragments belonging to these genes were observed on agarose gel and shown in Figure 3.3.

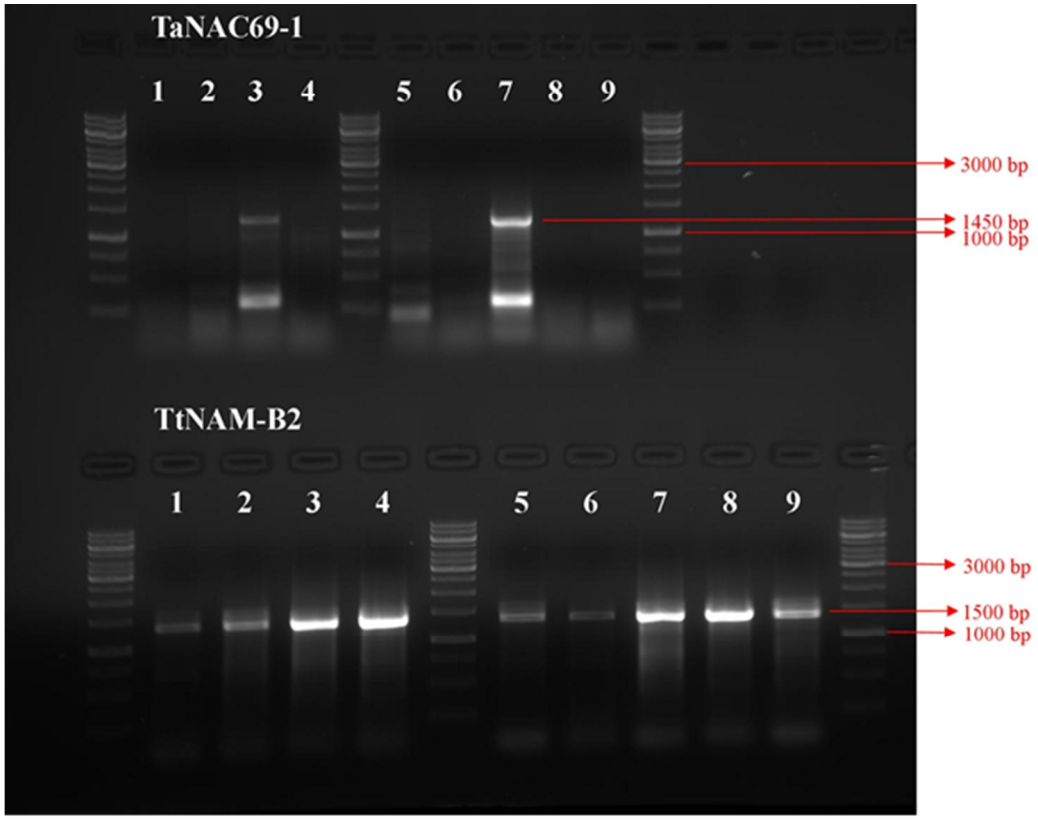


Figure 3.3. Agarose gel electrophoresis results of PCR amplification of *TaNAC69-1* and *TtNAM-B2* with Tagushi method. Amplicon length of *TaNAC69-1* and *TtNAM-B2* genes were 1450 bp and 1500 bp, respectively. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V. Each lane was loaded with 10µL of PCR product and 1 kp DNA Marker (Fermentas, SM0311).

For Gateway cloning, same cDNA from both wheat cultivars was used for amplification of open reading frame (ORF) of *TaNAC69-1* (1060 bp) gene. The amplification of *TaNAC69-1* gene was done using *Pfu* DNA polymerase as described in the section 2.4.1. Gene product of *TaNAC69-1* gene was visualized on agarose gel shown in Figure 3.4.

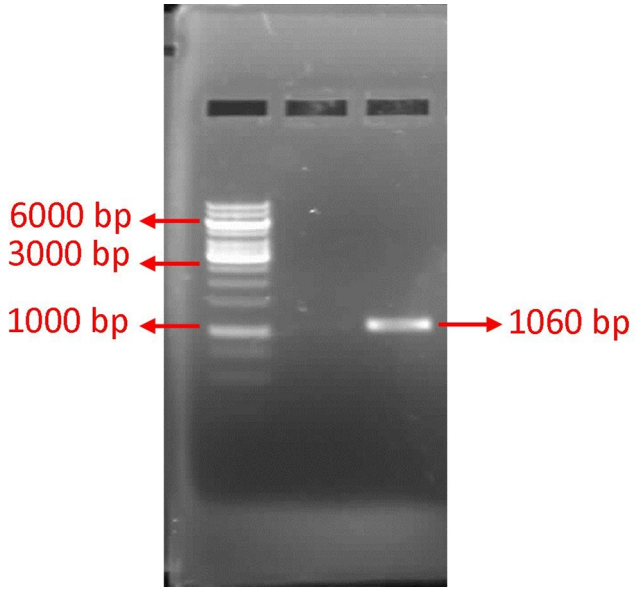


Figure 3.4. Agarose gel electrophoresis results of PCR amplification of *TaNAC69-1*. Amplicon length of *TaNAC69-1* gene was 1060 bp. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

3.1.2. Characterization of Cloned Genes

After obtaining full length of *TaNAC69-1* and *TtNAM-B2* gene amplicons, they were eluted from gel. *TaNAC69-1-TtNAM-B2* genes with 3` and 5` UTRs and ORF of *TaNAC69-1* gene were cloned into cloning vectors, pJET1.2/blunt cloning vector and pENTR™/D-TOPO® cloning vector, respectively. pJET1.2/blunt cloning vector and pENTR™/D-TOPO® cloning vector were used for conventional cloning and Gateway cloning studies, respectively. In order to show each gene insertions into cloning vectors, firstly colony PCR and then restriction digestion of cloning vectors were performed. Colony PCR of *TaNAC69-1-TtNAM-B2* genes with 3` and 5` UTRs was performed using gene specific primers. Colony PCR conditions was previously described in the section 2.2.11. The amplification products that belong to *TaNAC69-1* and *TtNAM-B2* genes were visualized on a 1% agarose gel and indicated in Figure 3.5.

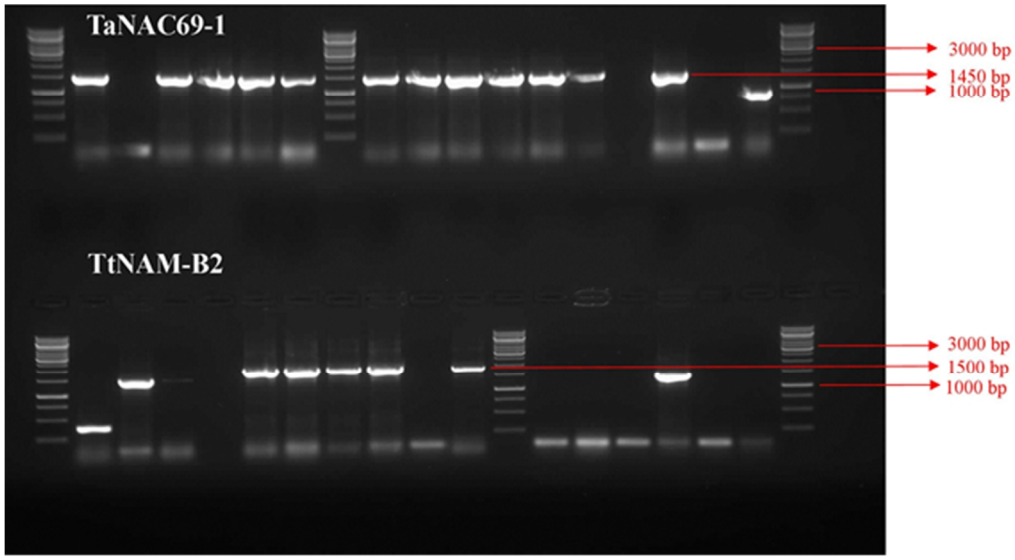


Figure 3.5. Agarose gel electrophoresis results of colony PCR amplification of *TaNAC69-1* and *TtNAM-B2*. Amplicon length is about 1450 bp for *TaNAC69-1* and 1500 bp for *TtNAM-B2*. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

pJET1.2/blunt cloning vector contains *Bgl*II sites that flank the vector to simplify excision of the cloned PCR product. Restriction digestion with *Bgl*II was performed on the pJET1.2/blunt cloning vector to show integration of *TaNAC69-1* and *TtNAM-B2* genes with 3` and 5` UTRs. Empty vectors and inserts were visualized in Figure 3.5.

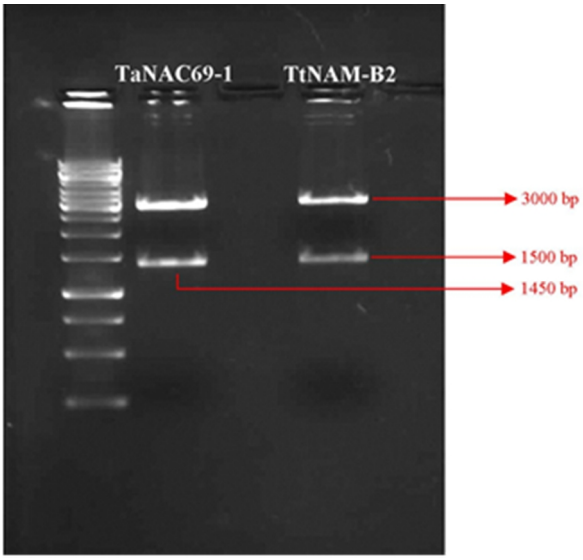


Figure 3.6. Agarose gel electrophoresis results of pJET1.2/blunt cloning vector containing *TaNAC69-1* and *TtNAM-B2* genes with 3` and 5` UTRs cut with *Bgl*II for. Amplicon length is same with corresponding genes. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

pENTR™/D-TOPO® was also checked by colony PCR using gene specific primers to indicate ORF of *TaNAC69-1* gene insert. From the nine of five colonies containing cloning vector carried gene insert and indicated in Figure 3.7.

Sequence analyses of the promising colonies were performed using M13 primers. Nucleotide blast from NCBI webpage (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used for sequence comparison. According to NCBI results, *TaNAC69-1* gene insert fully matched with previously known sequence of *NAC* gene (Uauy *et al*., 2006) (Genebank accesion number: AY625682). *TtNAM-B2* gene insert was also identical to *NAM* gene (Uauy *et al*., 2006) (Genebank accesion number: DQ869676). Sequence analysis of positive colonies showed that an error free full-length *TaNAC69-1* and *TtNAM-B2* genes were successfully cloned into cloning vector and used for construction of different plant transformation vectors.

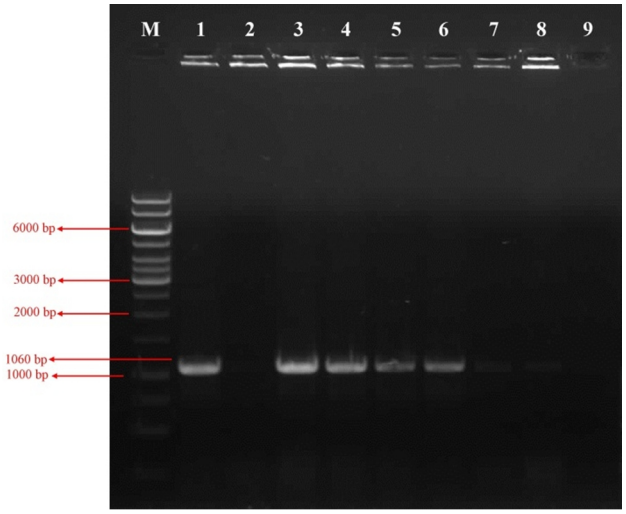


Figure 3.7. Agarose gel electrophoresis results of colony PCR for ORF of *TaNAC69-1* gene insert whose length is 1060 bp. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

Figure 3.8. NCBI sequence comparison of *TaNAC69-1* and *TtNAM-B2* genes.

3.1.3. Vector Construction

TaNAC69-1 and *TtNAM-B2* genes with 3` and 5` UTRs from pJET1.2/blunt cloning vector were transferred into dicot binary vector, pORE-E3. After digestion of both plasmids with *Cla*I and *Not*I restriction enzymes, pORE-E3 binary vector became a linear plasmid form and *TaNAC69-1* and *TtNAM-B2* genes had sticky ends that are compatible with pORE-E3 binary vector (Figure 3.9.). Ligation of binary vector with *TaNAC69-1* and *TtNAM-B2* genes was achieved using T4 DNA ligase. Ligation products belonging to both genes firstly were transferred into competent *E.coli* and then *Agrobacterium tumefaciens* strain EHA105. Colony PCR was performed to check positive colonies into *A. tumefaciens* strain EHA105, which contains *TaNAC69-1* and *TtNAM-B2* genes with 3` and 5` UTRs (Figure 3.10).

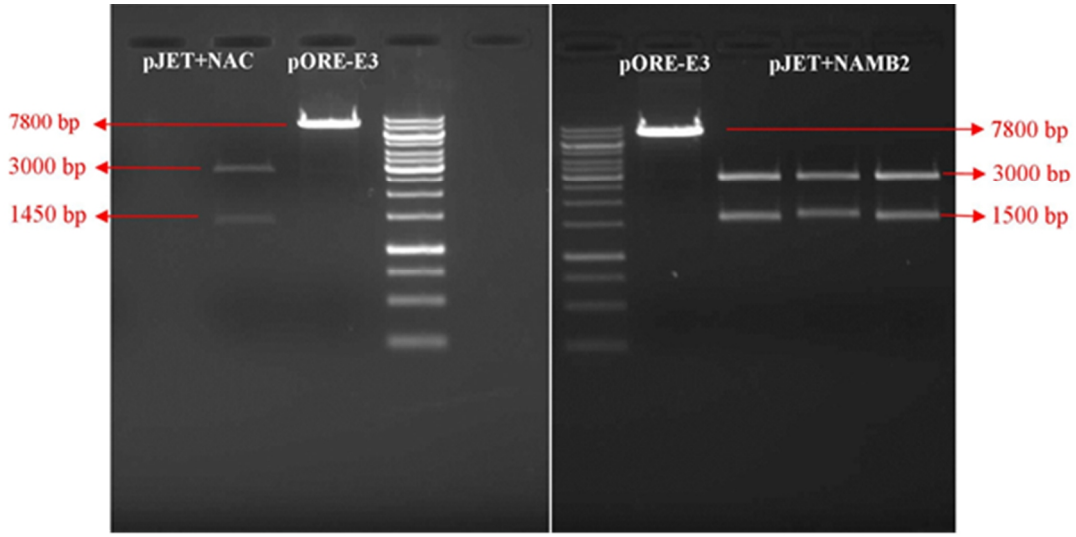


Figure 3.9. Agarose gel electrophoresis results of pJET1.2/blunt cloning vector and pORE-E3 binary vector cut with *Cla*I and *Not*I. *TaNAC69-1* and *TtNAM-B2* gene inserts were excised from cloning vector with sticky ends. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

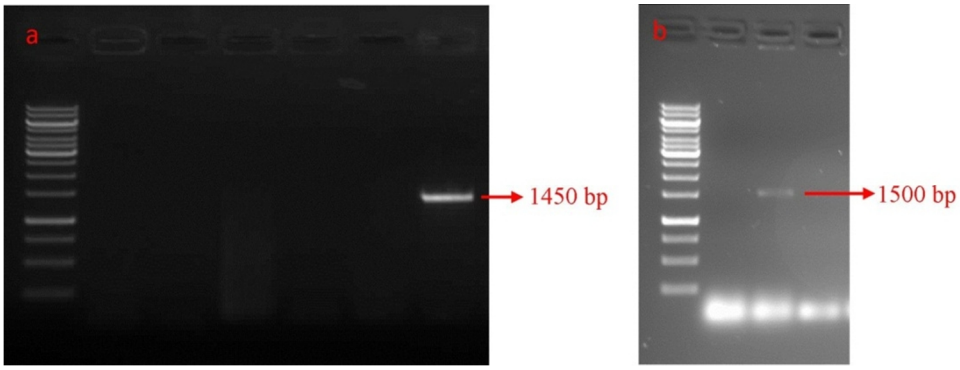


Figure 3.10. Agarose gel electrophoresis results of colony PCR of **a)** *TaNAC69-1* and **b)** *TtNAM-B2* genes found in *A. tumefaciens* strain EHA105. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

TaNAC69-1 and *TtNAM-B2* genes with 3` and 5` UTRs from pORE-E3 dicot expression vector were transferred into monocot expression vector, pACH25. pACH25 (Christensen and Quail, 1996) was designed as a monocot expression vector for particle bombardment transformation. The general features of pACH25 are given in Figure 3.11.a. It contains both a selectable marker *bar* gene (*hpt*) and a scorable marker *GUS* gene (*uidA*), each under the transcriptional control of a separate Ubi-1 promoter. *GUS* gene was removed from pACH plasmid using restriction enzymes, *Sma*I and *Sac*I (Figure 3.11.b.). Digested linear plasmid was recovered from the gel and treated with T4 DNA polymerase. Then treated pACH25 was ligated to *TaNAC69-1* and *TtNAM-B2* genes, separately. Ligation products were transferred into competent *E.coli*. To confirm position of genes into pACH25, restriction mapping was used.

For confirmation of *TaNAC69-1* gene position in pACH25 vector, *Hind*III restriction enzyme was selected because its restriction site is found in both *TaNAC69-1* gene and pACH25 vector. *TaNAC69-1* gene contains *Hind*III restriction site at a position of 300bp. pACH25 vector involves two *Hind*III restriction sites (Figure 3.12.). So, three DNA fragments were obtained after the *Hind*III enzyme digestion of positive colonies.

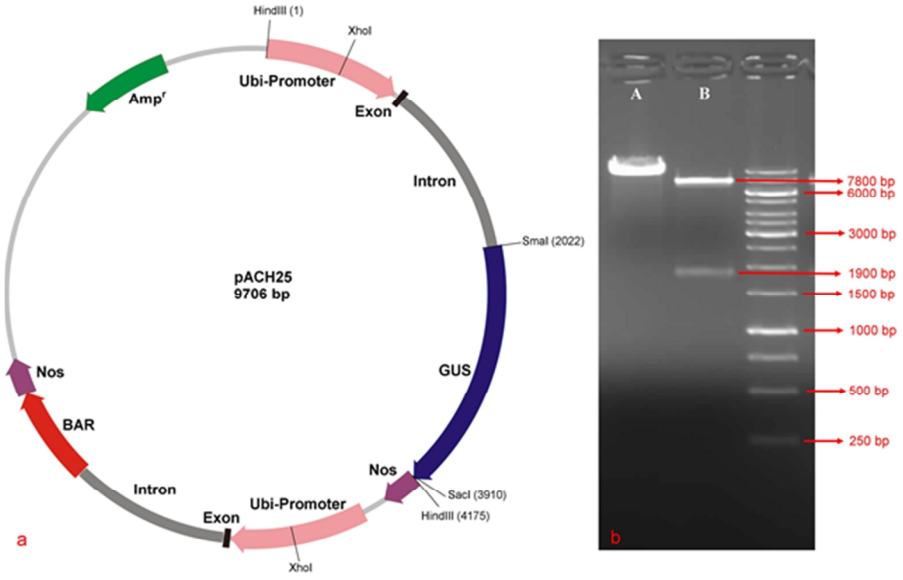


Figure 3.11. a) Map of pACH25, monocot expression vector. **b)** Agarose gel electrophoresis results of pACH25 monocot expression vector cut with **A:** *Sac*I and **B:** *Sac*I and *Sma*I double digestion to remove GUS gene. Length of GUS gene and linear pACH25 without GUS gene are about 1900 bp and 7800 bp, respectively.

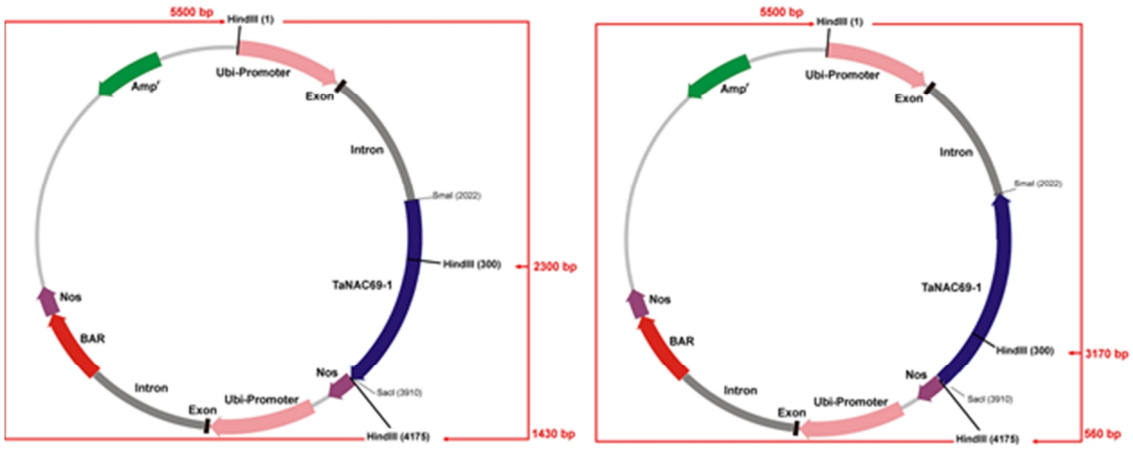


Figure 3.12. Position of *Hind*III restriction sites in pACH25 vector containing *TaNAC69-1* gene. **a)** Right insertion of *TaNAC69-1* gene within the vector. **b)** Opposite direction of *TaNAC69-1* gene within the vector. Red arrows indicate DNA fragment lengths.

According to position of *TaNAC69-1* gene in the vector, different fragment sizes were formed after *Hind*III digestion of pACH25 vector. If *TaNAC69-1* gene was inserted at right position in the vector, 2300 bp, 1430 bp and 5500 bp fragments would be obtained. If the *TaNAC69-1* gene is found at reverse direction in the vector, in this case 3170 bp, 560 bp and 5500 bp DNA fragments would be produced. If the vector is ligated without gene, 5500 bp and 2300 bp DNA pieces were observed in gel (Figure 3.13.).

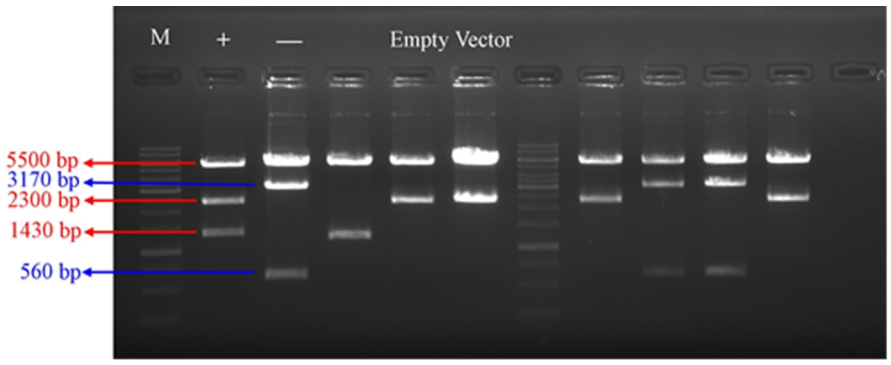


Figure 3.13. Agarose gel electrophoresis results of pACH25 vector containing *TaNAC69-1* gene digested with *Hind*III. Red arrows indicate DNA fragments that belong to insertion of *TaNAC69-1* gene at right direction. Blue arrows show DNA bands of *TaNAC69-1* gene at reverse direction. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

pACH25 vector was also digested with *Xho*I restriction enzyme to confirm position of *TtNAM-B2* gene in the vector. *Xho*I restriction sites are found both *TtNAM-B2* gene and vector. *TtNAM-B2* gene contains *Xho*I restriction site at a position of 700bp. pACH25 vector involves two *Xho*I restriction sites within the Ubi-promoter. (Figure 3.14.). So, three DNA fragments were obtained after the *XhoI* enzyme digestion of positive colonies.

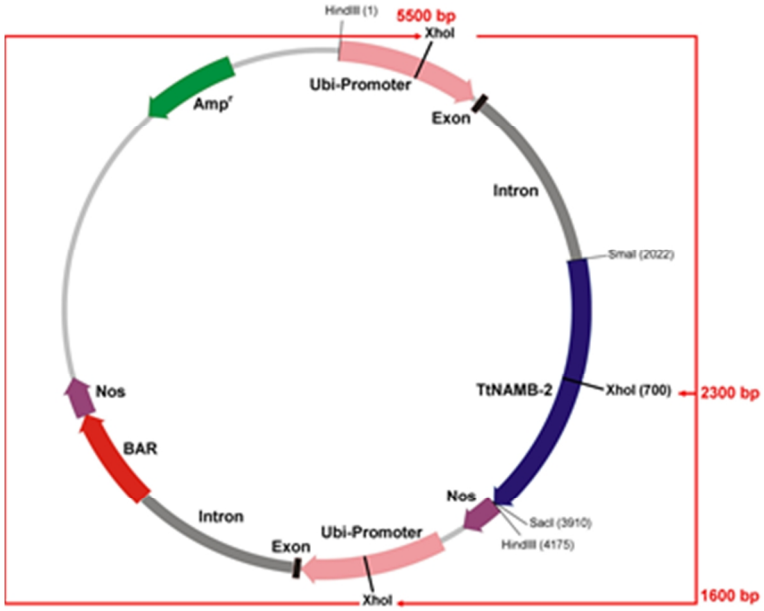


Figure 3.14. Position of *Xho*I restriction sites in pACH25 vector containing *TtNAM-B2* gene. Three *Xho*I restriction sites are found in vector which resulting formation of three DNA fragments. Red arrows indicate DNA fragment lenghts

After digestion of pACH25 vector containing *TtNAM-B2* gene with *Xho*I restriction enzyme, 2300 bp, 1000 bp, 600 bp and 5500 bp DNA fragments were observed. It was expected that 1600 bp fragment should be a single band but it probably contains another restriction site not indicated in the map. So, four DNA fragments were visualized after restriction digestion of pACH25 vector containing *TtNAM-B2* gene at right position. Self ligation of pACH25 vector was also observed. (Figure 3.15.).

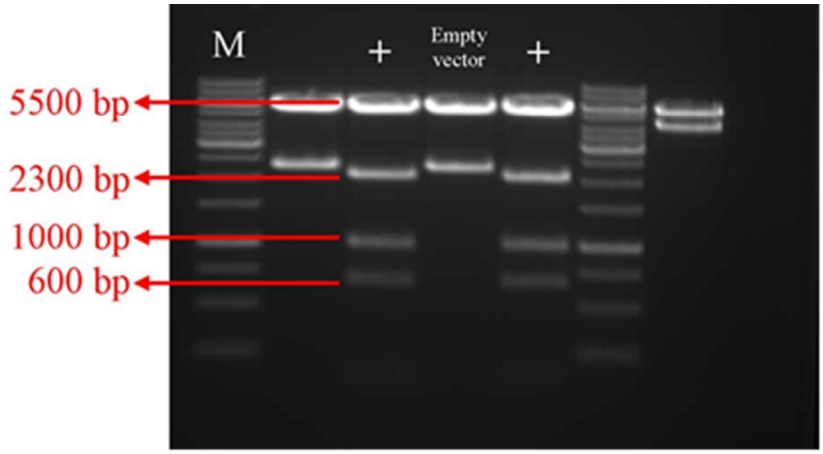


Figure 3.15. Agarose gel electrophoresis results of pACH25 vector containing *TtNAM-B2* gene digested with *Xho*I. Red arrows indicate DNA fragments that belong to insertion of *TtNAM-B2* gene at right direction. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

Gateway compatible pIPKb002 vector (Himmelbach *et al*., 2007) was designed as binary destination vector for *Agrobacterium*-mediated transformation of cereals. ORF of *TaNAC69-1* gene from pENTR™/D-TOPO® cloning vector was transferred into monocot expression vector, pIPKb002 which contains suitable recombination sites (attR1 and attR2) for Gateway cloning. Through the LR reaction, site-specific recombination occurred and an attB-containing expression clone of ORF of *TaNAC69-1* gene was formed. To analyze the presence of *TaNAC69-1* gene, colony PCR was performed using both gene specific primers and *hygromycin* gene primers (Figure 3.16). From the positive colonies, plasmid was isolated and transformed into *A. tumefaciens* strain AGL1, which was also checked by colony PCR using gene specific primers (Figure 3.17).

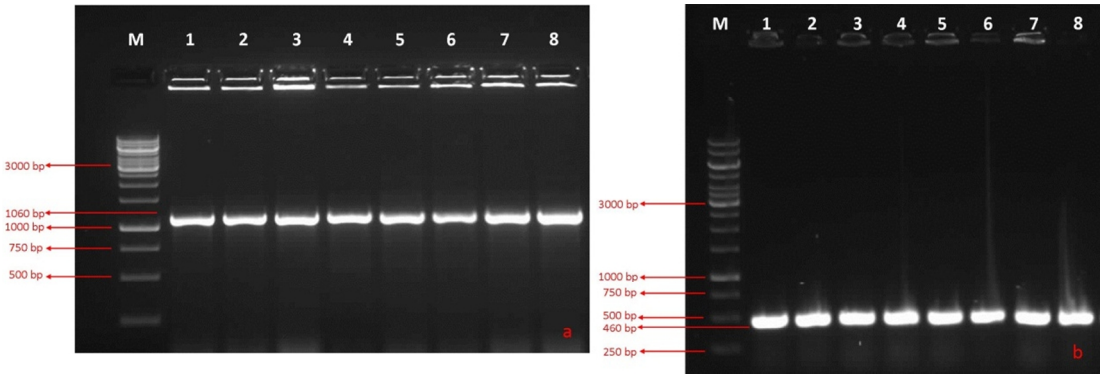


Figure 3.16. Agarose gel electrophoresis results of colony PCR using **a)** *TaNAC69-1* specific primers and **b)** *hygromycin* specific primers. Aplicon sizes were 1060 and 460 for *TaNAC69-1* gene and *hygromycin* gene, respectively. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

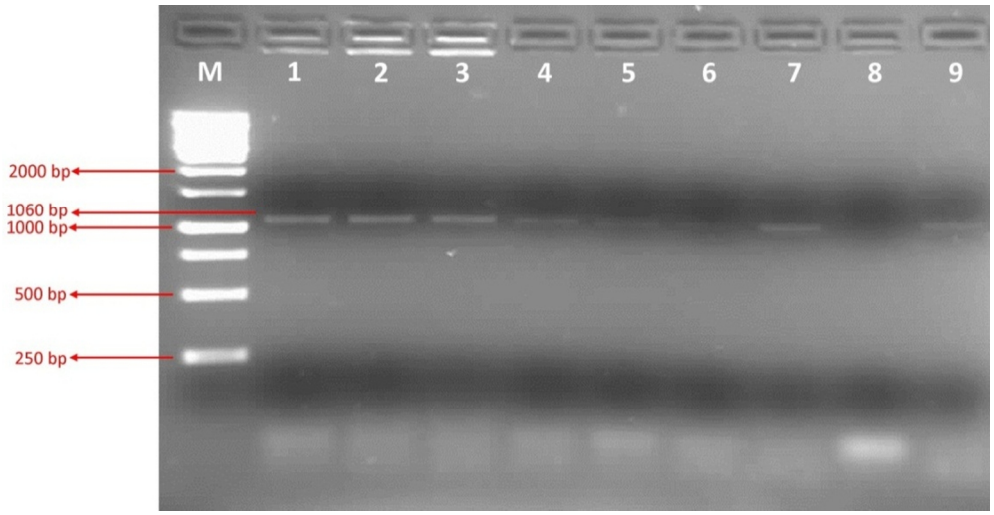


Figure 3.17. Agarose gel electrophoresis results of colony PCR of ORF of *TaNAC69-1* gene found in *A. tumefaciens* strain, AGL1. 1% agarose gel in 1X TAE solution was run for 60 minutes at 100V.

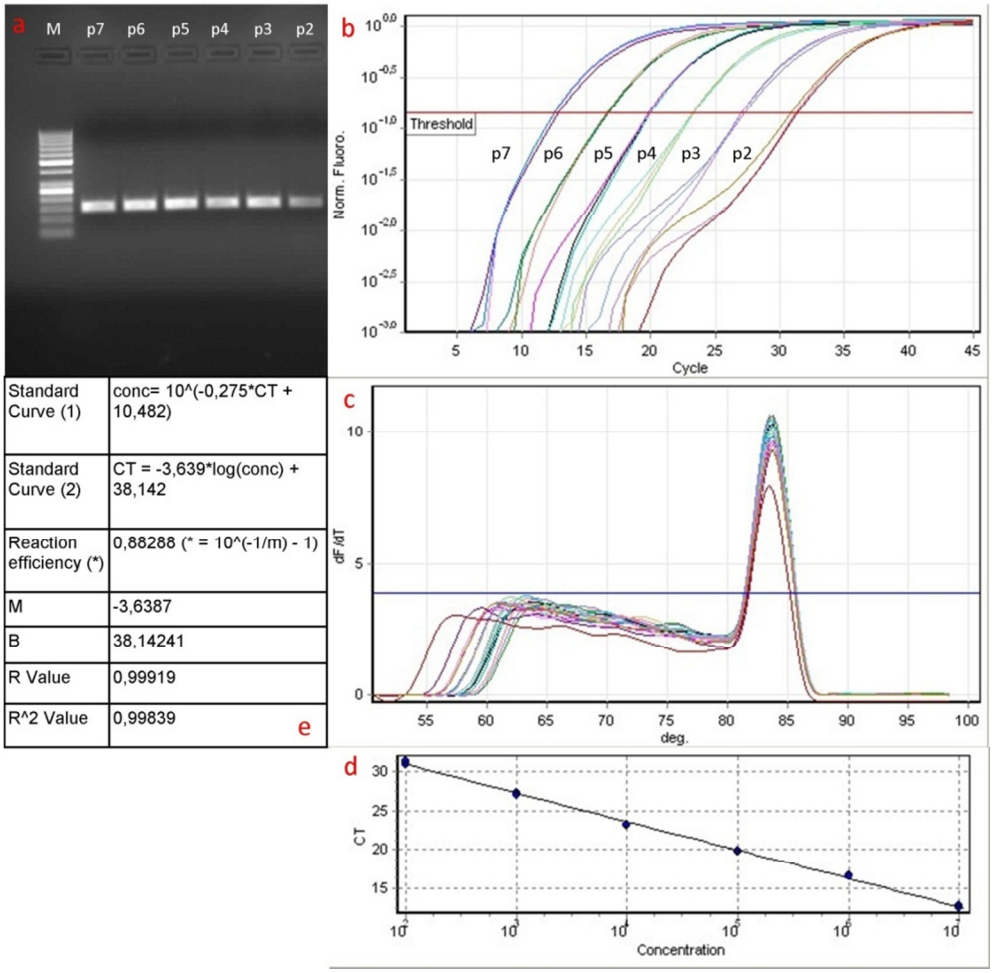
3.2. Expression Analysis of NAC Type Transcription Factors

To elucidate the expression patterns of NAC type transcription factor genes, *TaNAC69-1* and *TtNAM-B2*, under the salt, drought, cold and heat stress conditions, qRT-PCR was performed using RNA samples taken from leaves of wheat cultivar, Kızıltan-91 at the different time periods. Also, whole wheat genome expression profiles under the cold and heat stresses were determined using microarray technique.

3.2.1. Quantitative Real Time-PCR Analysis

TaNAC69-1 and *TtNAM-B2* gene expression profiles in wheat were monitored under salt, drought, cold and heat stresses at different time periods including 1st, 3rd, 6th, 12th and 24th and 48th hours. Data analysis of all of the qRT-PCR studies was performed according to absolute quantification method. Two different standard curves were generated for *TaNAC69-1* and *TtNAM-B2* genes expression analysis.

For construction of standard curve of *TaNAC69-1*, 146 bp small gene fragment was firstly cloned into pENTR™/D-TOPO® cloning vector. Then, the copy number of plasmid DNA molecules was calculated as described in section 2.5.3. Finally, six different amounts of the standard were prepared and run in firstly conventional PCR then qRT-PCR (Figure 3.18.a and b). Amplification of plasmid dilutions generated no primer-dimers or non-specific products. So, qRT-PCR conditions were optimized according to results of conventional PCR. For SYBR Green based amplicon detection, it is important to run a melting or dissociation curve following the real time PCR. This is due to the fact that SYBR Green has ability to bind and detect any double stranded DNA including primer dimers, contaminating DNA, and PCR product from misannealed primer. By viewing a melting curve, only desired amplicon should be detected. Melt curve analysis of all plasmid dilutions showed similar melt temperatures, with one narrow peak, indicating amplification of only *TaNAC69-1* gene fragment (Figure 3.18.c).

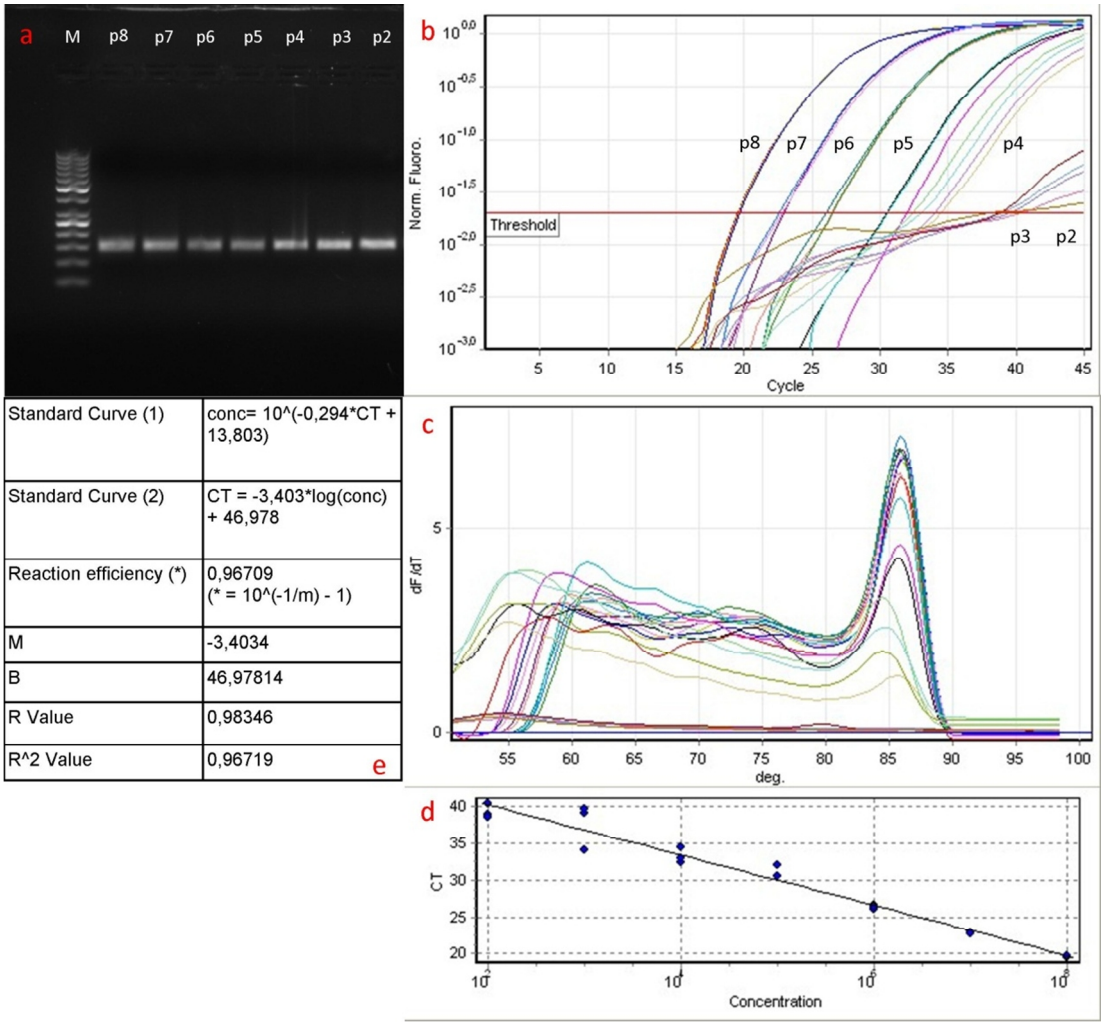


Standard Curve (1)	conc= 10^(-0,275*CT + 10,482)
Standard Curve (2)	CT = -3,639*log(conc) + 38,142
Reaction efficiency (*)	0,88288 (* = 10^(-1/m) - 1)
M	-3,6387
B	38,14241
R Value	0,99919
R^2 Value	0,99839

e

Figure 3.18. Construction of standard curve for *TaNAC69-1*. **a)** PCR amplified fragment of *TaNAC69-1* gene separated on 2% agarose gel. M: 50 bp DNA ladder. **b)** Amplification of different plasmid dilutions. Plasmid dilutions were abbreviated as; p7: 10^7, p6: 10^6, p5: 10^5, p4: 10^4, p3: 10^3 and p2: 10^2 copies. qRT- PCR was performed with three technical replicates for each dilution series. **c)** Melting curve analysis of amplified different plasmid dilutions. Change in fluorescence over time was plotted against temperature. **d)** A representative standard curve generated by different plasmid dilutions. **e)** Quantitation information table which shows reaction efficiency, M, B and R values.

For generation of standard curve of *TtNAM-B2*, 152 bp small gene fragment was also cloned into pENTR™/D-TOPO® cloning vector. After calculation of copy number of plasmid DNA, seven different standards were prepared and run in firstly conventional PCR and real time PCR (Figure 3.19.a). Melting curve analysis of standard curve showed that only one peak belonging to *TtNAM-B2* gene fragment was observed (Figure 3.19.b).



Standard Curve (1)	conc= 10^(-0,294*CT + 13,803)
Standard Curve (2)	CT = -3,403*log(conc) + 46,978
Reaction efficiency (*)	0,96709 (* = 10^(-1/m) - 1)
M	-3,4034
B	46,97814
R Value	0,98346
R^2 Value	0,96719

e

Figure 3.19. Construction of standard curve for *TtNAMB-2*. **a)** PCR amplified fragment of *TtNAMB-2* gene separated on 2% agarose gel. M: 50 bp DNA ladder. **b)** Amplification of different plasmid dilutions. Plasmid dilutions were abbreviated as;

p8: 10^8, p7: 10^7, p6: 10^6, p5: 10^5, p4: 10^4, p3: 10^3 and p2: 10^2 copies. qRT- PCR was performed with three technical replicates for each dilution series. **c)** Melting curve analysis of amplified different plasmid dilutions. Change in fluorescence over time was plotted against temperature. **d)** A representative standard curve generated by different plasmid dilutions. **e)** Quantitation information table which shows reaction efficiency, M, B and R values.

3.2.1.1. Expression Level Changes under Salt Stress

Under the salt stress treatment, although expression profiles of *TaNAC69-1* and *TtNAM-B2* genes resembled each other, which mean that the expression level was peaked at 48 h post treatment (hpt), concentration of transcript levels are different. The concentration of transcript levels of *TaNAC69-1* and *TtNAM-B2* genes were about 12000 copies/µl and 3500 copies/µl, respectively (Figure 3.20). Under the salt and drought stress conditions, relative fold differences were calculated by taking the ratio of control samples to stress treated samples. So, compared to control samples, the transcription level of *TaNAC69-1* began to significantly up regulate at 12 hpt with 20-fold and then peaked at 48 hpt with about 48-fold. However, fold change level of *TtNAM-B2* gene reached to approximately 13.5 fold at 48 hpt (Figure 3.21). Besides tissue specific expression pattern, (Meng *et al*., 2007; Xia *et al*., 2010), expression levels of NAC transcription factors also change when exposed to different biotic and abiotic stresses, which result in playing an important role for growth, development and responses of biotic and abiotic stresses. It is found that *TaNAC69-1* and *TtNAM-B2* genes were strongly induced by salt stress at 48 hpt, which indicates that NAC transcription factors involving in responses to salt stress. Xia *et al*., (2010 a-b) showed that the expression level of novel wheat NAC transcription factor genes, *TaNAC4* and *TaNAC8*, increased transiently at 3 hpt after treating with 200 mM NaCl. The transcripts level of *CarNAC1* from chickpea (*Cicer arietinum* L.) significantly increased under salt treatment (Peng *et al*., 2010). Meng *et al*., (2007) characterized six novel NAC genes (*GhNAC*s) from cotton (*Gossypium hirsutum* L.) and showed their responses to abiotic stresses. The

expression levels for five of the six *GhNAC*s increased following salt treatments. Over-expression of a stress-responsive genes *SNAC1* (Hu *et al*., 2006) and *SNAC2* (Hu *et al*., 2008) in transgenic rice plants significantly improved drought and salt resistance. Our results further support that NAC transcription factor genes play crucial roles in the regulation of salt stress response in various plant species.

NAM gene family plays a central role as transcriptional regulators of multiple processes during leaf senescence, which affects grain nutrient concentrations in developing wheat grain (Uauy *et al*., 2006). However, expression patterns of NAM genes under different abiotic stress conditions at various time series have not been studied yet. Transcription level changes of *TtNAM-B2* gene under the salt stress was firstly examined in this study. Gene expression patterns of *TaNAC69-1* and *TtNAM-B2* genes were similar which suggest that they had a transcriptional activator function and worked together in response to salt stress for wheat plant (Figure 3.20).

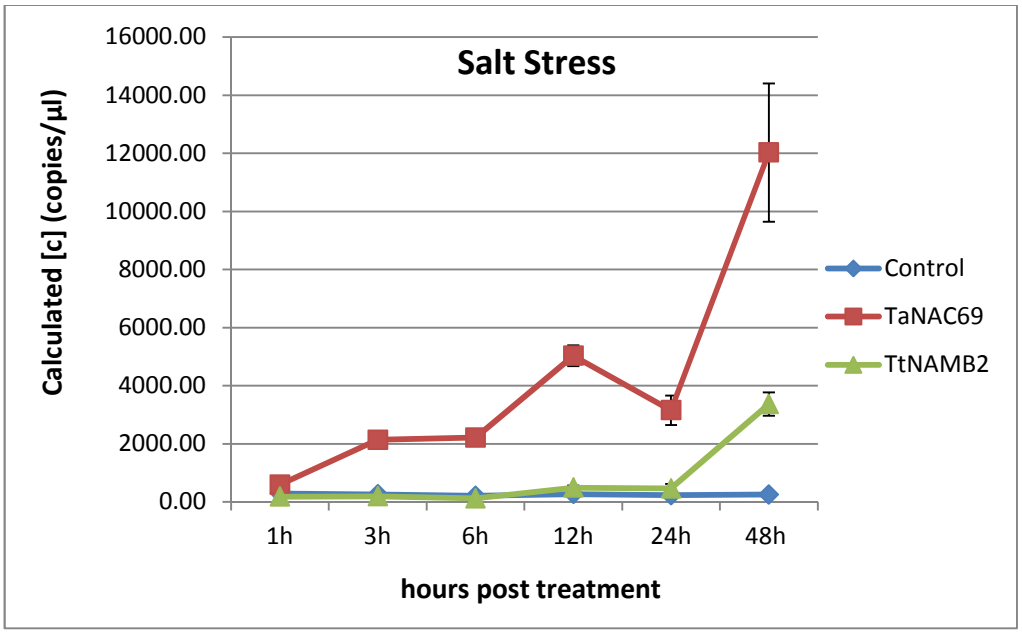


Figure 3.20. Expression pattern of *TaNAC69-1* and *TtNAM-B2* genes on salt stress treated wheat plant at different time periods.

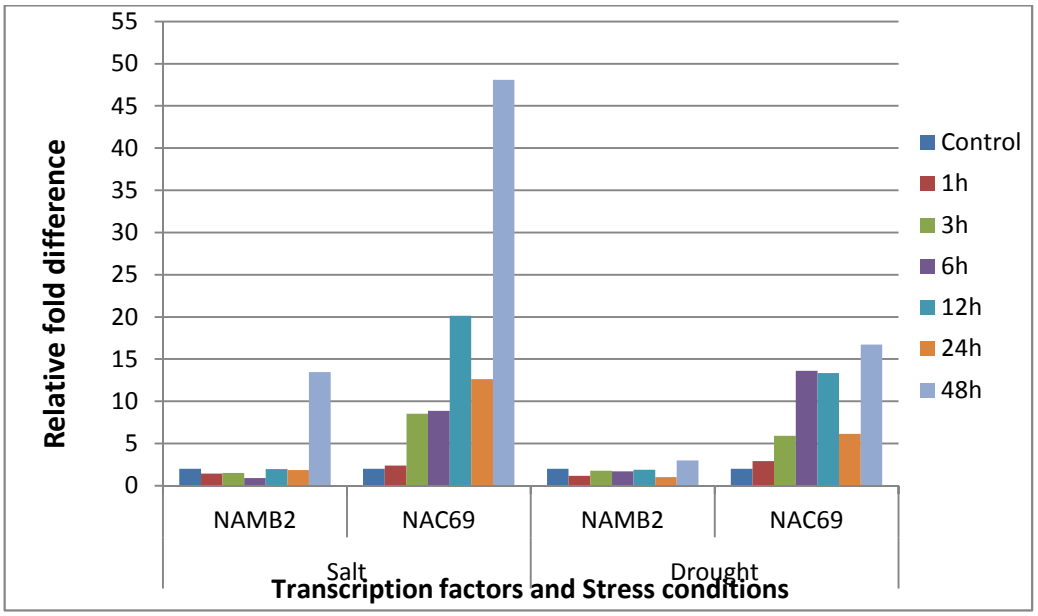


Figure 3.21. Relative fold change differences of *TaNAC69-1* and *TtNAMB-2* genes under the salt and drought stress conditions.

3.2.1.2. Expression Level Changes under Drought Stress

The output from a real-time PCR reaction is in the form of a graph showing the number of PCR cycles against the fluorescence intensity. This is known as an amplification plot. The Ct value (cycle threshold) is defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample which mean that the lower the Ct value for a sample the greater the starting amount of transcript in the sample. Amplification plots of *TaNAC69-1* and *TtNAMB-2* genes under both salt and drought stress conditions were shown in Figure 3.22 and Figure 3.23, respectively. As mentioned earlier, it is important to show the melting curve analysis when working with SYBR Green for amplicon detection. So, melting curve analysis of all the qRT-PCRs in this study were given in Appendix F.

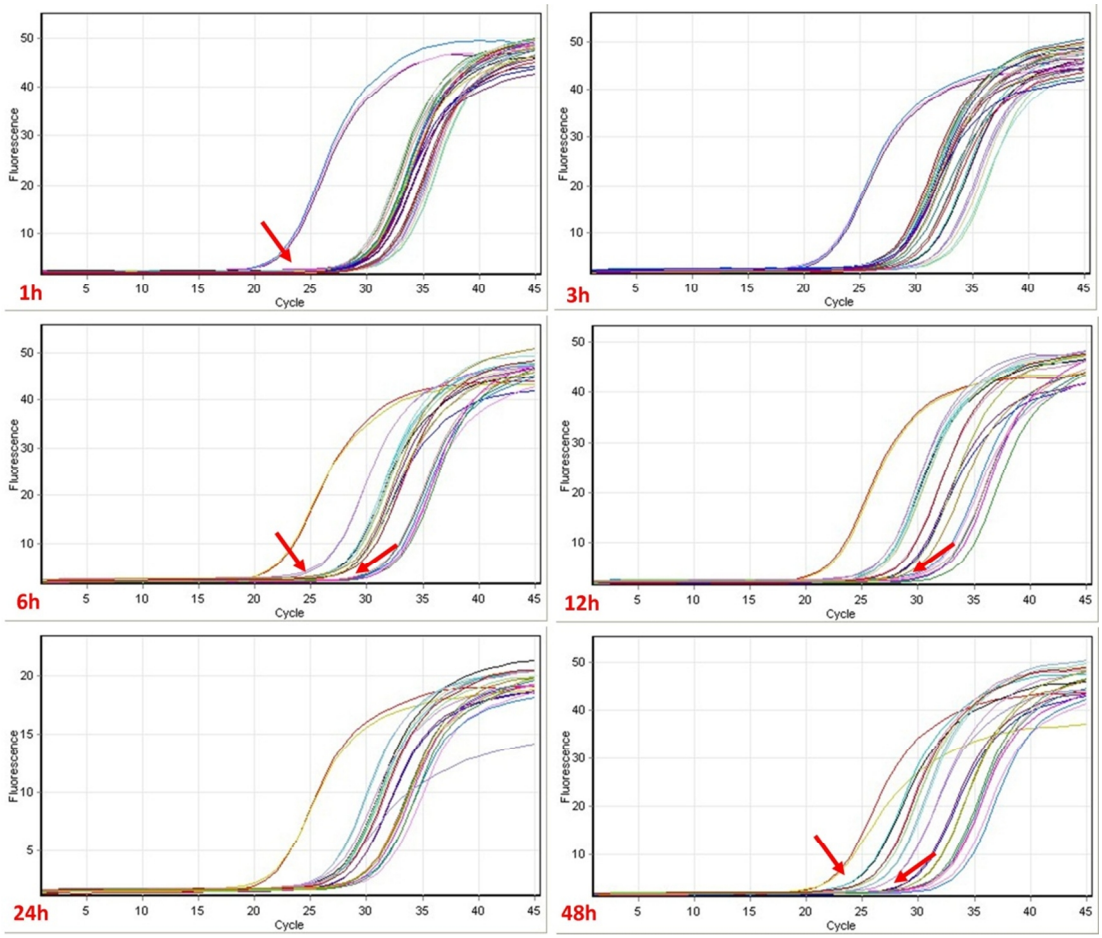


Figure 3.22. Amplification plots of *TaNAC69-1* gene from Kızıltan-91 under both salt and drought stress conditions at different time intervals. Red arrows shows beginning of threshold cycles of salt and drought treated samples.

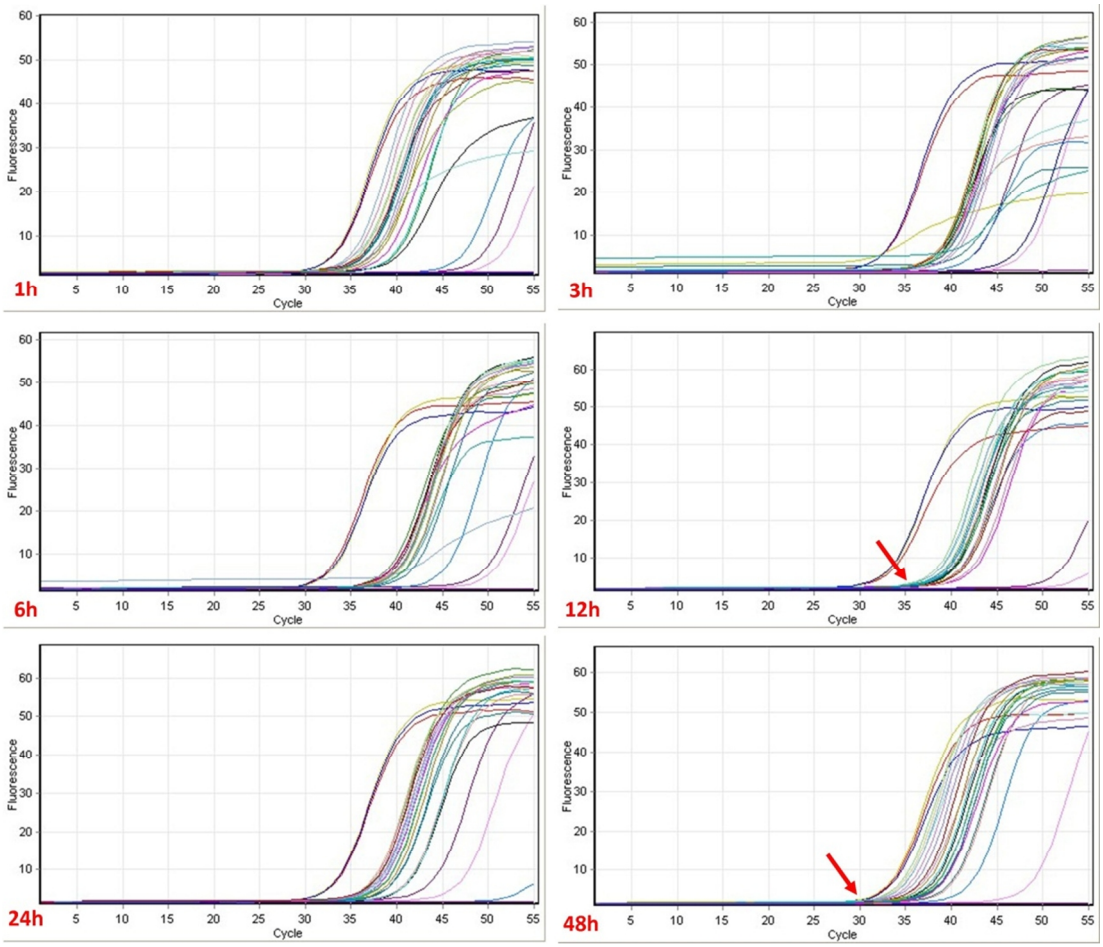


Figure 3.23. Amplification plots of *TtNAMB-2* gene from Kızıltan-91 under both salt and drought stress conditions at different time intervals. Red arrows shows beginning of threshold cycles of salt and drought treated samples.

TaNAC69-1 gene expression level gradually increased from 3 to 12 hpt under the drought stress conditions. Although there was no significant difference between 6, 12 and 48 hpt, maximum expression of *TaNAC69-1* gene was observed at 48 hpt (Figure 3.24). Compared to control samples, the gene expression level of *TaNAC69-1* started to increase from 3 hpt to 12 hpt with 6 and 13 fold-change, respectively. Transcript level of *TaNAC69-1* and *TtNAM-B2* genes peaked at 48 hpt with about 17 and 3 fold changes, respectively (Figure 3.21).

Under the drought stress, expression pattern of *TaNAC8* gene (Xia *et al*., 2010 b) was similar to *TaNAC69-1* gene. In contrast to increase in expression levels of *TaNAC8* and *TaNAC69-1* genes in wheat, drought stress elicitors had no obvious effect on *TaNAC4* expression (Xia *et al*., 2010 a). It has been previously shown that the NAC transcription factors have ability to regulate the drought stress response through both ABA-dependent and ABA-independent pathways (Fujita *et al*., 2004; Tran *et al*., 2004). Peng *et al*., (2010) reported that the expression of *CarNAC1* was induced by dehydration, but not by ABA which indicates that this protein may be associated with drought response in an ABA-independent manner. Other NAC transcription factors, *CarNAC3* and *CarNAC5* from chickpea were also significantly up-regulated by drought stress via the ABA signaling pathway (Peng *et al*., 2009 a; Peng *et al*., 2009 b). Another subgroup member of NAC family genes, *ANAC047* and *GhNAC5*, has been found to be significantly induced by drought stress and ABA (Seki *et al*., 2003; Meng *et al*., 2009). Recently, NAC type transcription factors from various organisms have been widely used to improve drought resistance in transgenic plants. Overexpressing of three different Arabidopsis NAC genes (*ANAC019*, *ANAC055* and *ANAC072*) showed significantly increased drought tolerance (Tran *et al*., 2004). It was shown that *SNAC1* (Stress-Responsive NAC1) was induced by drought specifically in rice guard cells. *SNAC1*-overexpressing transgenic plants showed significantly improved drought resistance under field conditions without phenotypic changes or yield penalty (Hu *et al*., 2006). Gao *et al*., (2009) isolated and characterized a novel *OsNAC52* from rice, which is ABA-dependent NAC-like gene. Over-expression of *OsNAC52* caused expression of downstream genes in transgenic Arabidopsis, resulting in enhanced tolerance to drought stress. Another a rice NAC gene, *ONAC045* was functionally characterized by Zheng *et al*., (2009). Drought, high salt, and low temperature stresses, and ABA induced *ONAC045* gene expression in rice leaves and roots. They reported that transgenic rice plants overexpressing *ONAC045* showed enhanced tolerance to drought and salt treatments. These studies suggest that NAC type transcription factors have a potential for improvement of transgenic plants which resistant to drought and salt stresses.

Like salt stress, effect of drought stress on *TtNAMB-2* gene expression in wheat was firstly shown in this study. According to the expression profiles of *TtNAMB-2* gene under drought stress, there was no significant difference between all time periods except for 48 hpt, at which *TtNAMB-2* gene was significantly up-regulated and reached to peak. Together with *TtNAMB-2* gene, *TaNAC69-1* gene responded to drought stress after the 48 hours of PEG treatment which suggest that they act simultaneously against the drought stress (Figure 3.24).

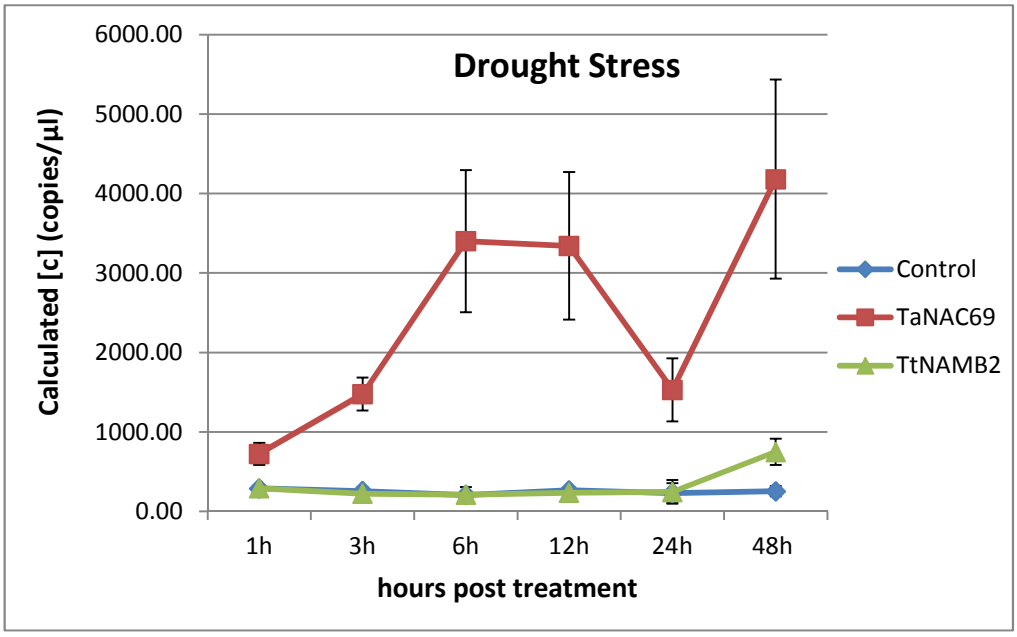


Figure 3.24. Expression pattern of *TaNAC69-1* and *TtNAMB-2* genes from Kızıltan-91 under the drought stress treatment at different time intervals.

The amplification efficiencies of target and reference genes or endogenous plasmid should be determined in real-time PCR. In this study, it was compared by preparing a dilution series of plasmid containing gene of interest. Each dilution series was then amplified in one-step real-time PCR and obtained Ct values were used to construct

standard curves for plasmid. Then, the amplification efficiency (E) for each target was calculated according to the following equation:

$E = 10^{(-1/S)} - 1$ (S = slope of the standard curve)

To compare the amplification efficiencies of each real-time PCR run, the Ct values of plasmid found in each real-time PCR run were subtracted from the Ct values of plasmid used in standard curve. The difference in Ct values was then plotted against the logarithm of the copies/µL plasmid. If the slope of the resulting straight line is lower than 0.1, amplification efficiencies are comparable. So, amplification efficiencies of each real-PCR run throughout this study was calculated. Amplification efficiency comparisons of *TaNAC69-1* and *TtNAMB-2* genes under the salt and drought stress conditions were shown in Figure 3.25 and Figure 3.26, respectively.

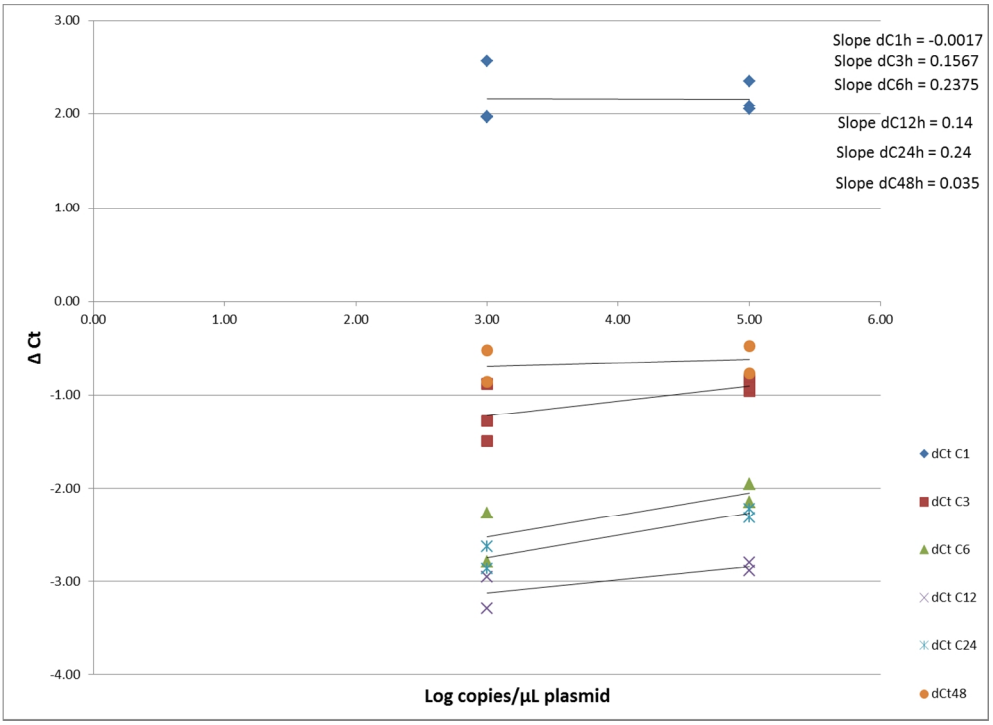


Figure 3.25. Amplification efficiency comparisons of *TaNAC69-1* gene under the salt and drought stress conditions.

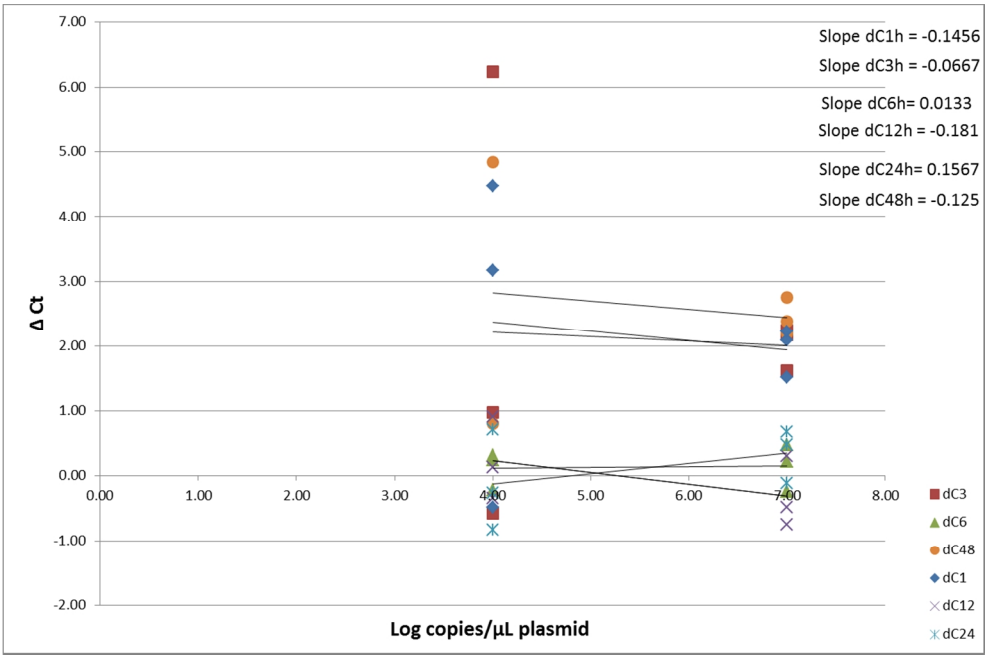


Figure 3.26. Amplification efficiency comparisons of *TtNAMB-2* gene under the salt and drought stress conditions.

3.2.1.3. Expression Level Changes under Cold Stress

The low-temperature treatment (4°C) suddenly raised *TaNAC69-1* gene expression level in wheat cultivar, Kızıltan-91 at 3 hpt and decreased at 6 hpt (Figure 3.27). Then transcript level increased gradually in response to increment time periods. However, no significant fold changes were observed for *TaNAC69-1* gene after the cold stress. Although 4 fold down-regulation was observed during the first 3 hours after cold stress application, *TtNAM-B2* gene expression level was sharply augmented and reached peak level at 12 hpt. The gene expression of *TtNAM-B2* level was kept 3 fold at 24 and 48 hpt (Figure 3.28). Gene expression patterns of *TaNAC4* and *TaNAC8* genes resembled to *TaNAC69-1* gene, which means that they were strongly induced after 3 hours of cold stress treatment and then continued to 12 and 24 hours (Xia *et al*., 2010a; Xia *et al*., 2010b). So, it is suggested that like other

wheat NAC type transcription factors such as *TaNAC4* and *TaNAC8* genes, *TaNAC69-1* gene involves in responses to cold stress. *CarNAC1* and *CarNAC3* share common function in certain processes of physiological metabolism (Peng *et al*., 2010). However, their expression profiles against the cold stress were different each other. Although *CarNAC1* showed significant increase under cold stress application (Peng *et al*., 2010), no obvious effect on the expression of *CarNAC3* (Peng *et al*., 2009a) was observed in chickpea. NAC genes (*GhNAC*s) from cotton (*Gossypium hirsutum* L.) showed different expression patterns under the cold stress. All the *GhNAC*s except for *GhNAC1* gene were strongly induced by low temperature stress. According to protein sequence analysis of *SNAC2* and *SNAC1* genes, they are closely related each other (Ooka *et al*., 2003) and have similarity in response to various abiotic stresses including salt, drought and ABA treatment. However, expression profiles of *SNAC2* and *SNAC1* genes were different under the cold stress application. In spite of the fact that *SNAC1* gene expression was induced by cold, over-expression of *SNAC1* had no significant effect on improving cold tolerance while SNAC2-overexpression caused formation of resistance against low temperature treatments. After the severe cold stress (4-8°C for 5 days) treatment, all wild type plants died whereas about 50% of the transgenic plants were able to survive. It was also observed that cell membrane stability increased in transgenic plants when compared to WT plants (Hu *et al*., 2008). Based on gene expression analysis of *TaNAC69-1* and *TtNAMB-2* genes, they could be participated in plant responses against cold stress.

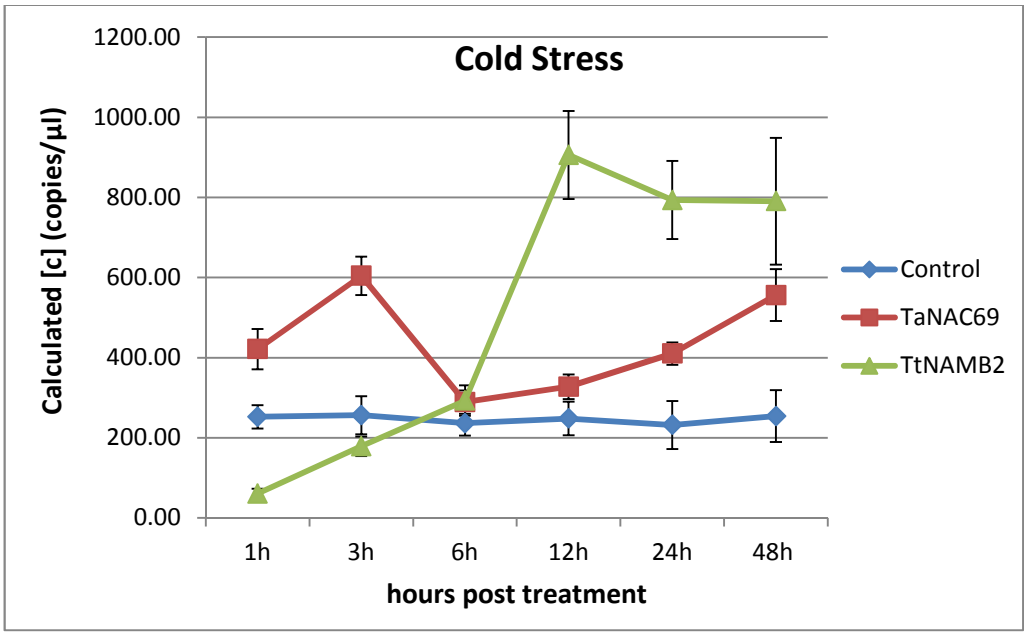


Figure 3.27. Expression pattern of *TaNAC69-1* and *TtNAMB-2* genes from Kızıltan-91 under the cold stress treatment at different time intervals.

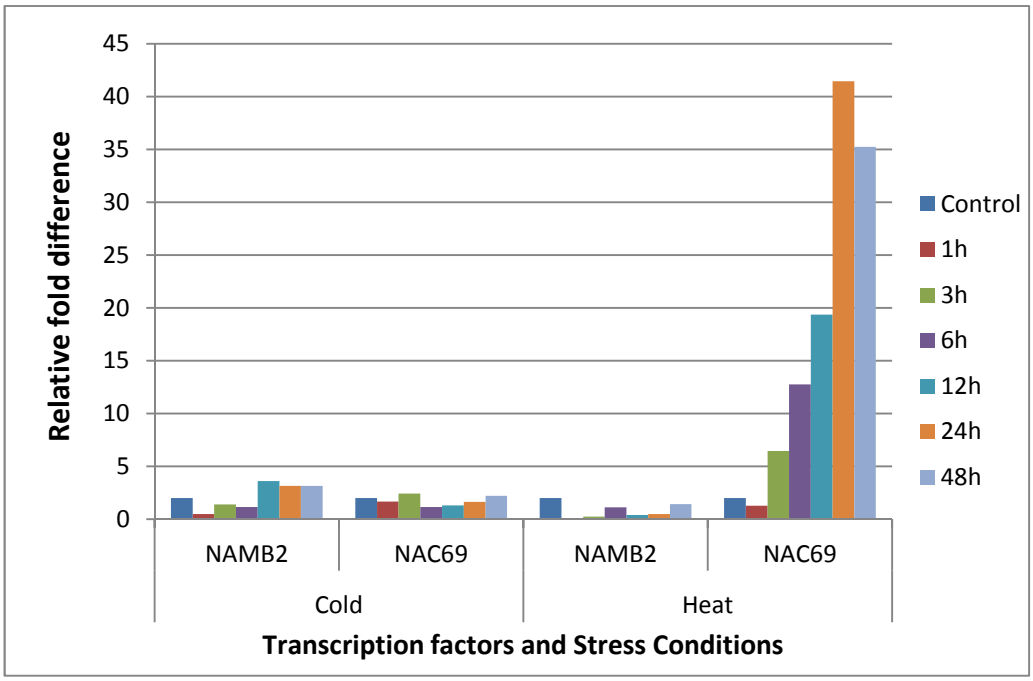


Figure 3.28. Relative fold change differences of *TaNAC69-1* and *TtNAMB-2* genes under the cold and heat stress conditions.

3.2.1.4. Expression Level Changes under Heat Stress

Amplification plots of *TaNAC69-1* and *TtNAMB-2* genes under both cold (4°C) and heat stress (40°C) conditions were shown in Figure 3.29 and Figure 3.30, respectively. The distinction between cold and heat stress amplification plots for *TaNAC69-1* gene was recognized in the Figure 3.29. The amplification curves belonging to heat stress was differentiated after 12 hpt to 48 hpt, which was shown with red arrows. There was a small segregation between cold and heat stress amplification plots for *TtNAMB-2* gene, which was not easily detectable.

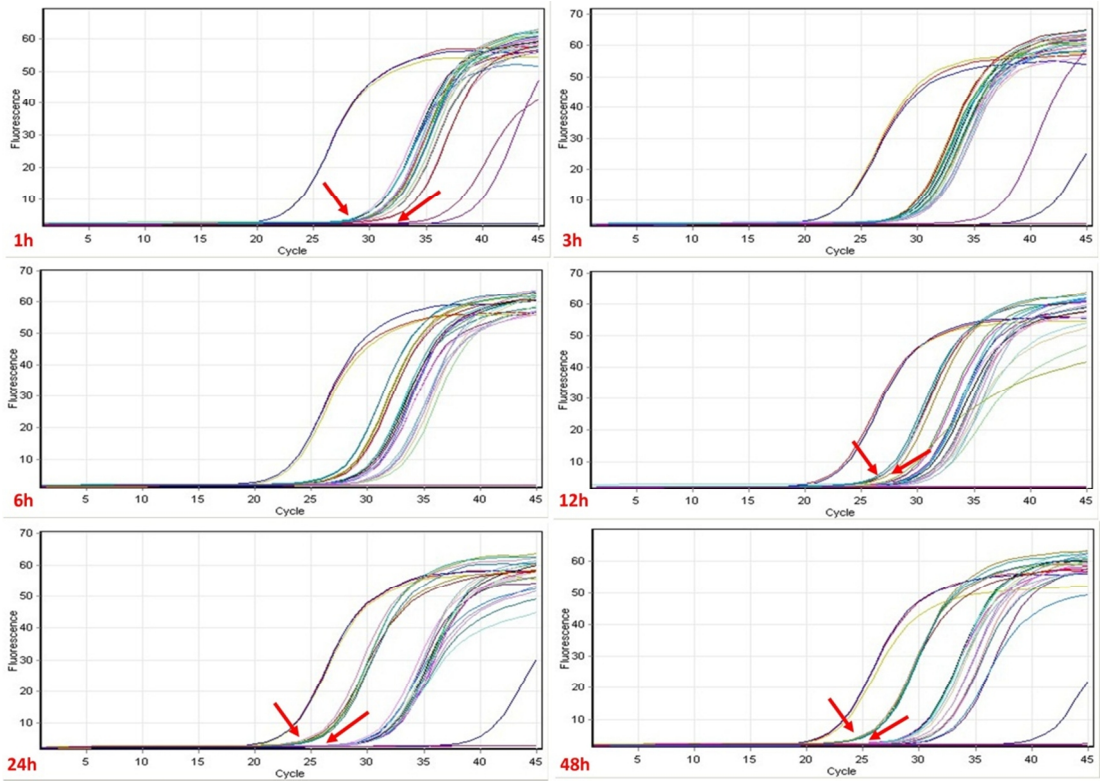


Figure 3.29. Amplification plots of *TaNAC69-1* gene under both cold and heat stress conditions at different time intervals. Red arrows shows beginning of threshold cycles of high temperature treated samples.

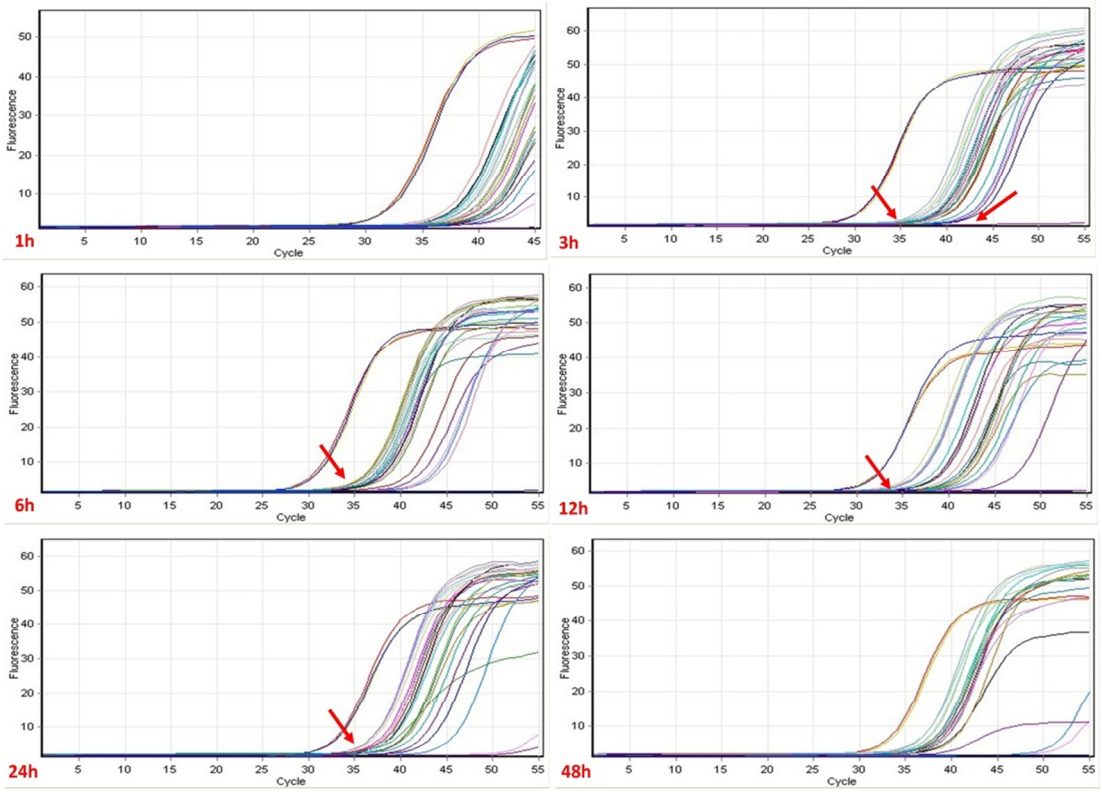


Figure 3.30. Amplification plots of *TtNAMB-2* gene under both cold and heat stress conditions at different time intervals. Red arrows shows beginning of threshold cycles of low and high temperature treated samples.

TaNAC69-1 gene expression level was continuously increased from the initiation of heat stress treatment and peaked at 24 hpt, in which point relative fold difference between control sample and high temperature treated sample was 42 folds. Although there was slight decrease in expression level of *TaNAC69-1* gene at 48 hpt, the relative fold difference was still high with 35 folds. Like salt stress treatment, the concentration of transcript levels of *TaNAC69-1* gene was also high and about 11000 copies/µl. However, no significant changes in expression profiles of *TtNAM-B2* gene was observed under the heat stress condition (Figure 3.31). The expression patterns of chickpea NAC type transcription factors, *CarNAC1* and *CarNAC5*, were

examined under the heat stress (Peng *et al*., 2009b; Peng *et al*., 2010). In contrast to cold stress, heat stress did not affect the expression of *CarNAC1* gene expression. However, significant increase in gene expression level of *CarNAC5* was detected under the high temperature stress.

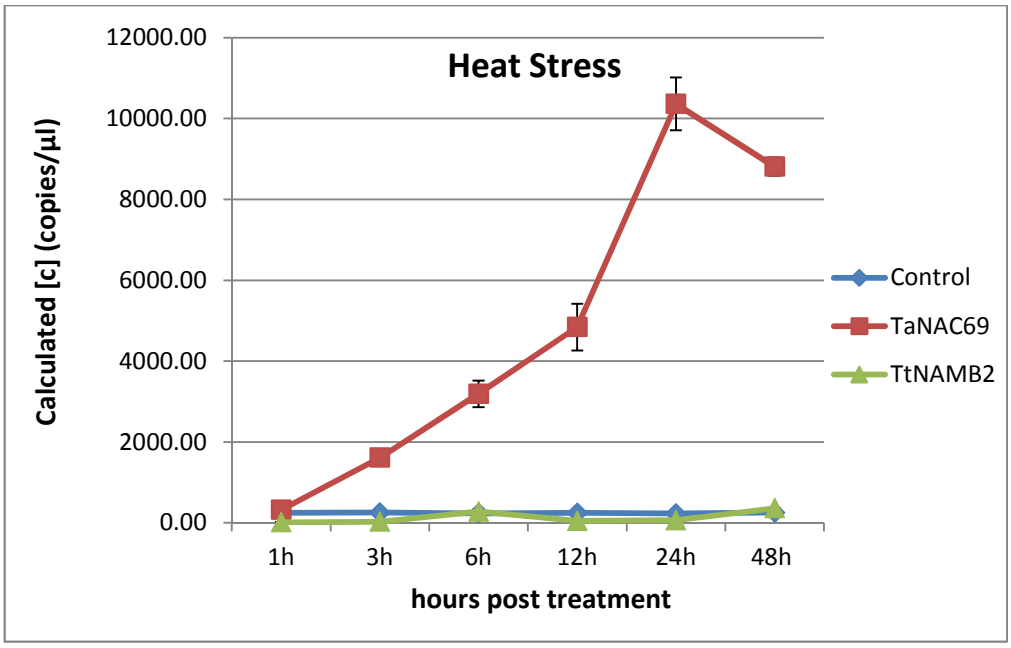


Figure 3.31. Expression pattern of *TaNAC69-1* and *TtNAMB-2* genes from Kızıltan-91 under the heat stress treatment at different time intervals.

Amplification efficiency comparisons of *TaNAC69-1* and *TtNAMB-2* genes under the cold and heat stress conditions were shown in Figure 3.32 and Figure 3.33, respectively.

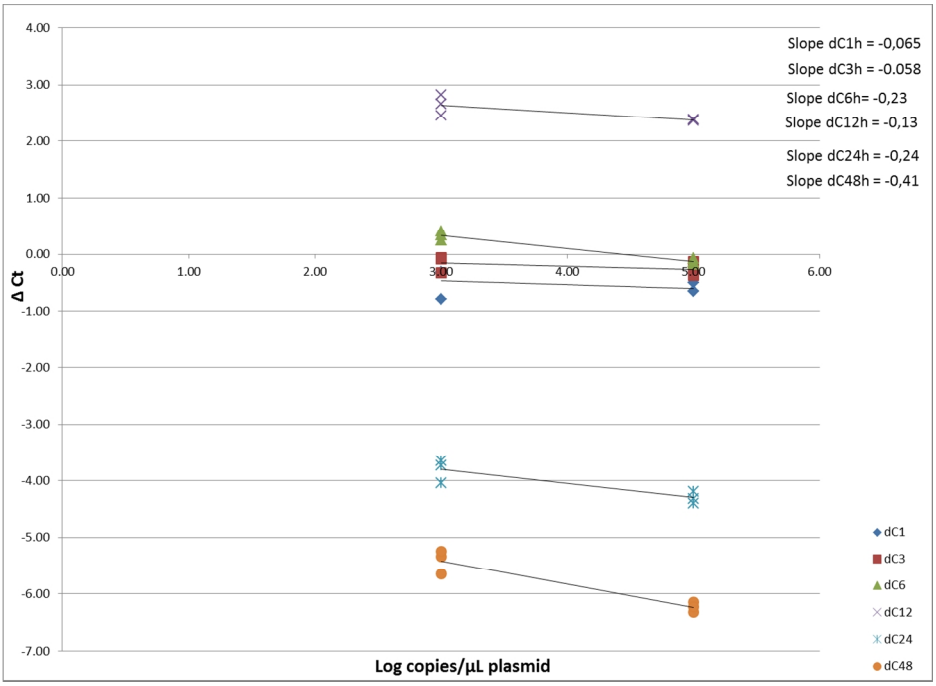


Figure 3.32. Amplification efficiency comparisons of *TaNAC69-1* gene under the cold and heat stress conditions.

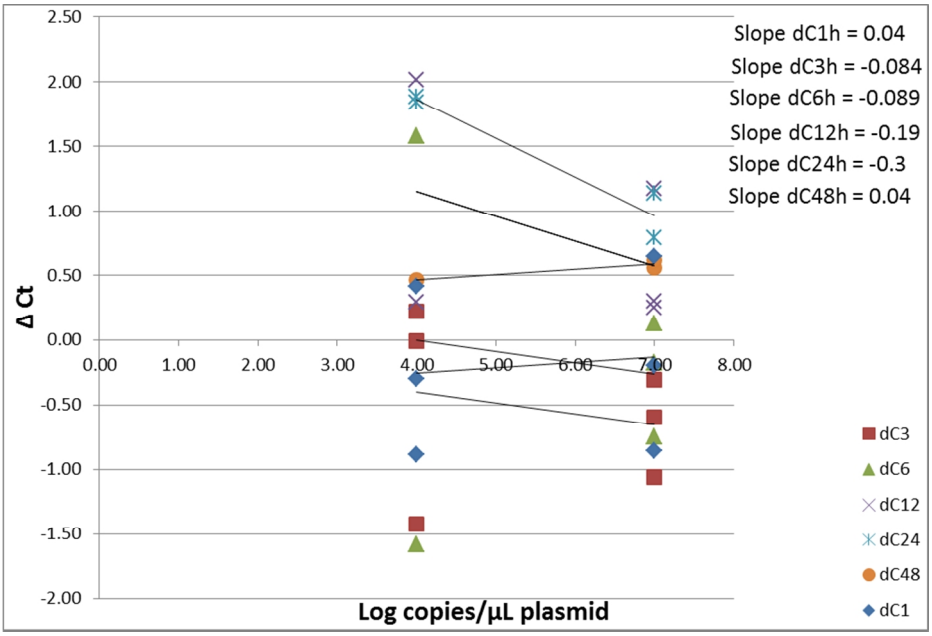


Figure 3.33. Amplification efficiency comparisons of *TtNAMB-2* gene under the salt and drought stress conditions.

3.2.2. Microarray Analysis

Microarray analysis was performed using Affymetrix GeneChip® Wheat Arrays to identify differentially expressed genes under the cold and heat stress conditions. RNA samples were isolated from leaves of wheat cultivar, Kızıltan-91 grown under normal conditions and from leaves subjected to 4 °C for 12 hours and 40 °C for 12 hours. To decrease biological variability and to increase significance of the results statistically, three replicates per condition were used.

The integrity and quality of total RNA samples were checked using Agilent 2100 Bioanalyzer (Figure 3.34) and the concentrations of the samples were determined using Thermo NanoDrop 3300 Fluorospectrometer (Table 3.3). The integrity of RNA plays crucial role for gene expression studies and traditionally has been evaluated using the 28S to 18S rRNA ratio. However, inconsistent results can be obtained using this method. To overcome this issue, The RNA integrity number (RIN) has been designed. The RIN algorithm is used for determination of integrity of RNA samples. This algorithm gives an accurate universal measurements and is applied to electrophoretic RNA measurements. RIN measurements consist of values from 1 to 10 which represent the highest quality RNA. RNA samples have RIN number between 5 and 7 can be used for many types of experiments. The RIN numbers of RNA samples used in microarray study were between 6 and 7 which can be accepted for gene expression studies. All three replicates were analyzed for each condition and their pseudo electrophoresis gel images and electropherograms were shown in (Figure 3.34).

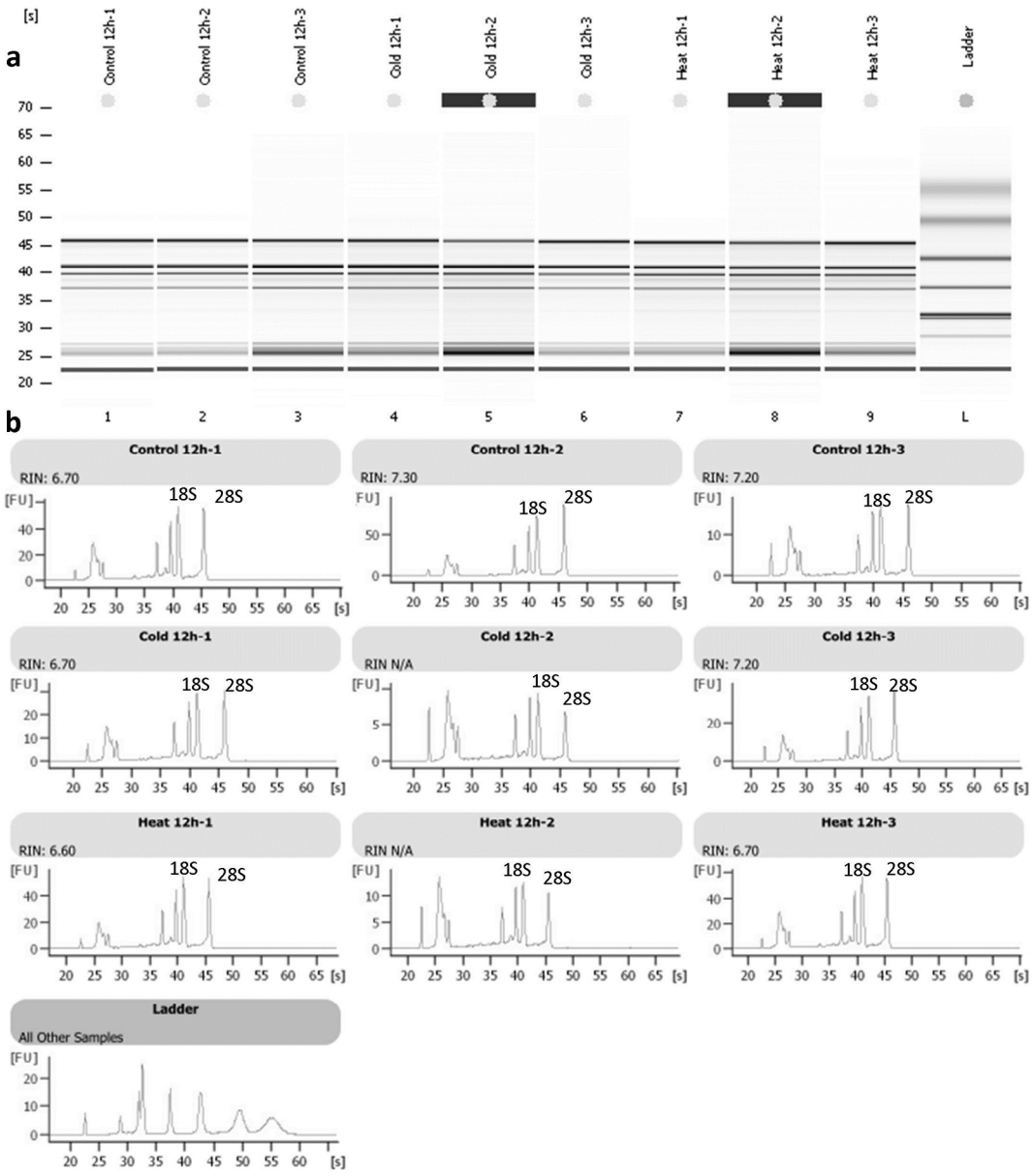


Figure 3.34. Agilent 2100 bioanalyzer **a)** pseudo electrophoresis gel image and **b)** electropherograms of RNA samples used in microarray analysis.

Table 3.3. Concentrations of RNA samples used for microarray analysis.

Sample Names	RNA concentration (µg /µl)
Control 12h-1	1.18
Control 12h-2	1.19
Control 12h-3	2.07
Cold 12h-1	1.74
Cold 12h-2	2.05
Cold 12h-3	1.39
Heat 12h-1	2.37
Heat 12h-2	1.76
Heat 12h-3	1.13

First step for sample preparation of microarray experiment was reverse transcription of RNA samples which were then converted to first-strand cDNA. Using this cDNA as a template, double-stranded DNA (dsDNA) was formed for *in vitro* transcription. Multiple copies of aRNA (called as amplified RNA) were generated from the double-stranded cDNA templates. Then, aRNA samples were labeled with biotin-conjugated nucleotides. After purification step of aRNA samples, each sample was quantified and 15 µg of biotin-labeled aRNA was fragmented using fragmentation buffer. Fragmentation of aRNA target before hybridization onto GeneChip probe arrays has been shown to be critical. Analysis of fragmented aRNA samples were performed using Agilent 2100 Bioanalyzer. Figure 3.35 indicates difference between electropherograms of un-fragmented and fragmented purified control aRNA samples. Un-fragmented aRNA samples have a broader size distribution whereas the fragmented ones have a narrower size distribution. This reduction in the size distribution shows that purified aRNA samples were fragmented appropriately. Although agarose gel is also used for verification of fragmentation, Bioanalyzer gives better results for checking the size distribution and fragmentation of aRNA samples. However, this technique is much more expensive than agarose gel. Therefore, after checking the fragmentation of control aRNA samples with Bioanalyzer, it was decided that the fragmentation procedure works well.

So, the rest of the samples including cold and heat samples, were run on agarose gel and the fragmentation was verified by this way (Figure 3.36). After the fragmentation step, aRNA samples were mixed with hybridization cocktail and loaded together on Affymetrix GeneChip® Wheat Array. Arrays were placed in hybridization oven then sequentially washed and scanned. Data obtained from arrays were analyzed by using GeneChip Operating Software 1.4 and GeneSpring GX 11.

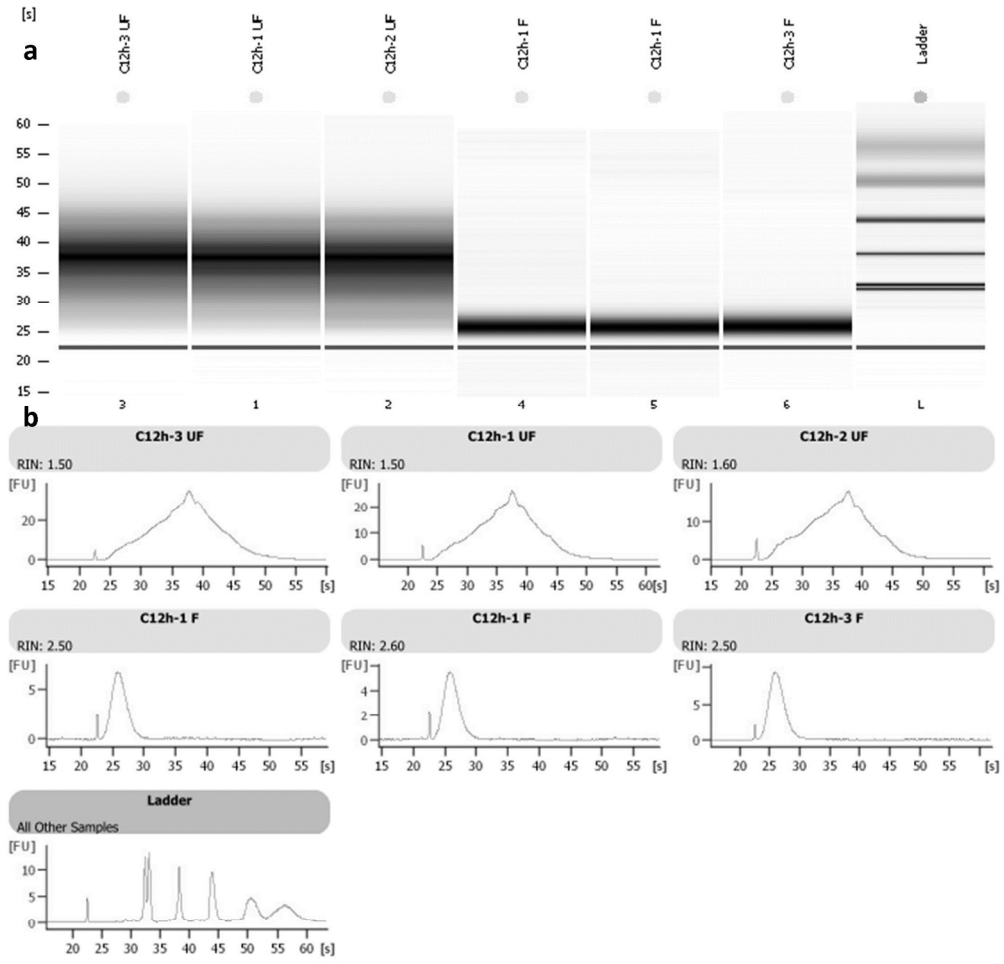


Figure 3.35. Agilent 2100 bioanalyzer **a)** pseudo electrophoresis gel image of un-fragmented and fragmented aRNAs from control samples and **b)** electropherograms on the top shows the un-fragmented (UF) aRNA samples and electropherograms on the bottom shows the fragmented (F) aRNA samples.

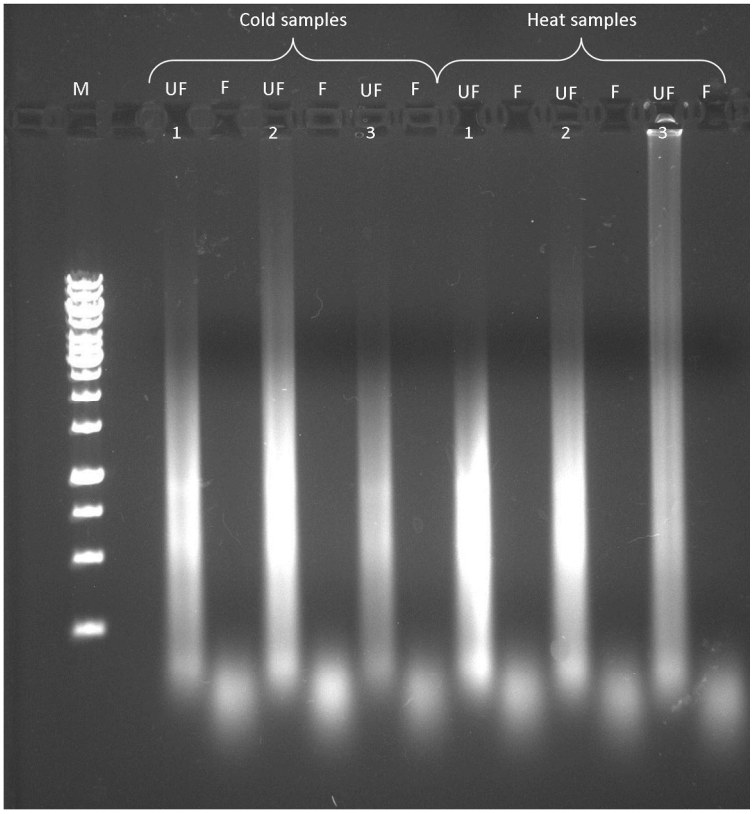


Figure 3.36. Agarose gel electrophoresis of unfragmented (UF) and fragmented (F) aRNAs which belong to three replicates of cold and heat stress treated samples.

3.2.2.1. Differentially Expressed Genes under Cold Stress

Two groups were generated for comparison of microarray data analysis. Cold and heat conditions were separately compared to control condition. RMA algorithm (Robust Multiarray Analysis) was used for microarray raw data normalization. In RMA normalization method, only perfect-match (PM) values are background-corrected, normalized and finally summarized, resulting in a set of expression measures. Significantly expressed probe sets with p-values lower than 0.05 were determined by One way ANOVA. Among significantly expressed probe sets, fold change of at least two was considered as differentially expressed probe sets.

Principal components analysis (PCA) is a statistical technique for determining the key variables in a multidimensional data set that explain the differences in the observations, and can be used to simplify the analysis and visualization of multidimensional data sets (Raychaudhuri *et al*., 2000). It provides the capturing common patterns in data and allows viewing of variation among groups of replicates. Principle Component Analysis (PCA) revealed that three biological replicates within a one group clustered together and separated from other groups (Figure 3.37). The PCA shows that the data dimension was diminished to two principal components and represented as X- and Y-axis with 62.25% and 35.58% of total variance, respectively. Data points of control samples and cold, heat samples were in different areas of the plot, which means that stress factors caused a great variation among the samples. In addition to this, data points of cold and heat stress were found in different areas on the plot. This apparently showed that there was also a big diversity among the stress factors.

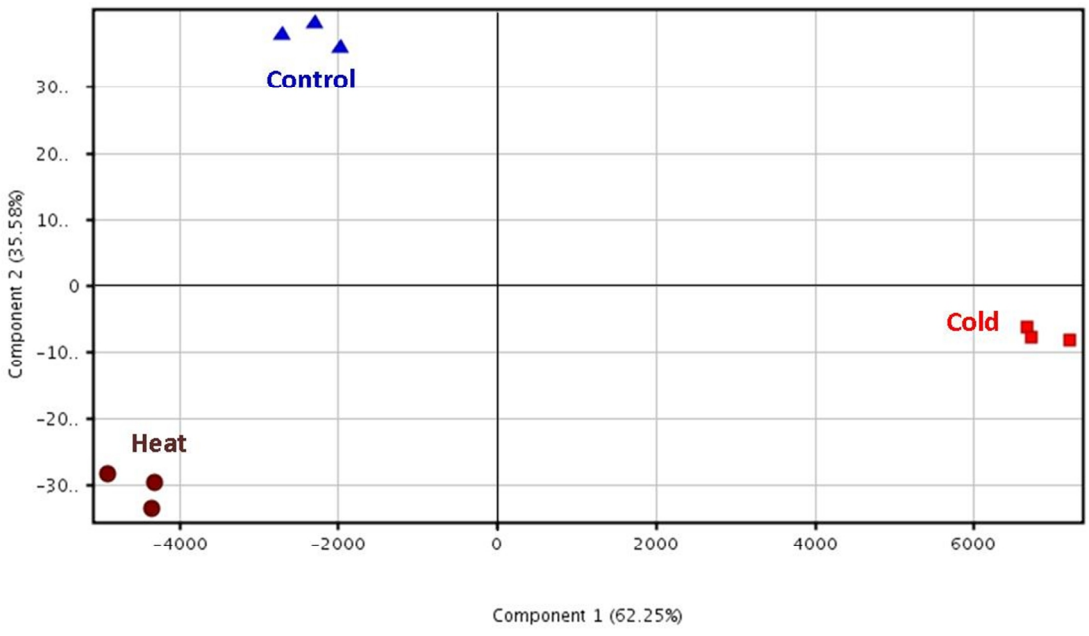


Figure 3.37. Principal Component Analysis of nine wheat Affymetrix arrays.

The Affymetrix GeneChip® Wheat Genome Array contains 61,127 probe sets designed to target 55,052 wheat transcripts from *T. aestivum* (59,356 probe sets and 53,474 transcripts), *T. turgidum* (147 probe sets and 136 transcripts), *T. turgidum ssp. durum* (392 probe sets and 350 transcripts), *T. monococcum* (1,215 probe sets and 1085 transcripts), and *Aegilops tauschii* (five probe sets and four transcripts) (Ergen *et al*., 2009). Out of these more than 60,000 probes, 12,895 (21.5%) were found to be differentially expressed at least p value lower than 0.05 and fold change (FC) higher than 2. The Venn diagram shows the number of overlapping probe sets between cold and heat stress data (Figure 3.38). Alternation in expression level of about 2550 probe sets was common after the cold and heat stress treatments. Approximately 3600 and 5500 probe sets were differentially expressed after the heat and cold stresses, respectively. To demonstrate effect of cold stress on transcriptome profiles of wheat, microarray data sets from control and cold were analyzed. Significantly different probe set lists ($p<0.05$; $FC\geq2$) were generated for control and cold stress data. Under the cold stress condition, the numbers of the up-regulated and down-regulated significantly different probe sets were shown and listed in Figure 3.39. Scatter plots of differentially regulated ($p<0.05$; $FC\geq2$) genes under cold stress condition and their expression values are given in Figure 3.40.

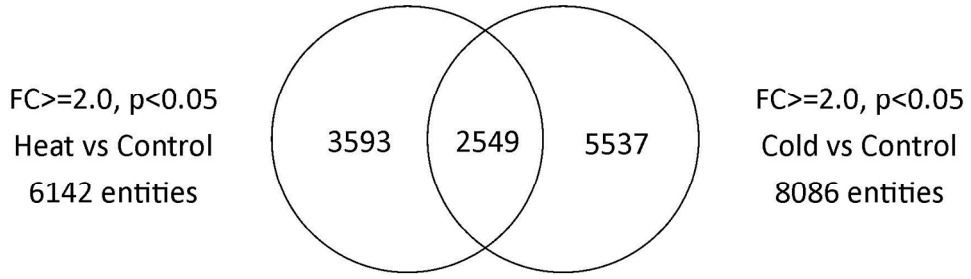
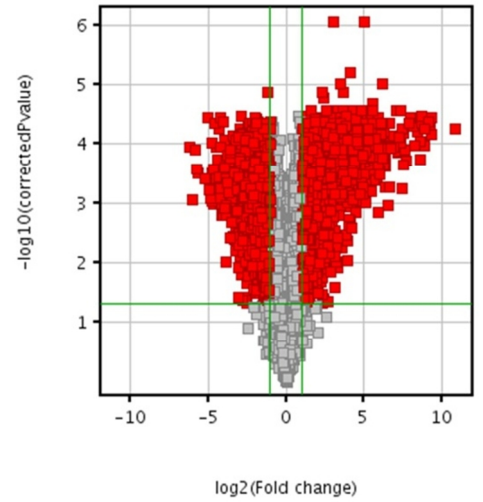


Figure 3.38. Venn diagram showing the overlap of differentially regulated probe sets after the cold and heat stress treatments.



Regulation	Cold Stress (p<0.05; FC≥2)
up-regulated	2784
down-regulated	5302
Total	8086

Figure 3.39. Volcano plot and number of significantly (p<0.05) up and down-regulated probe sets that changed more than 2-fold in cold stress condition compared to control conditions.

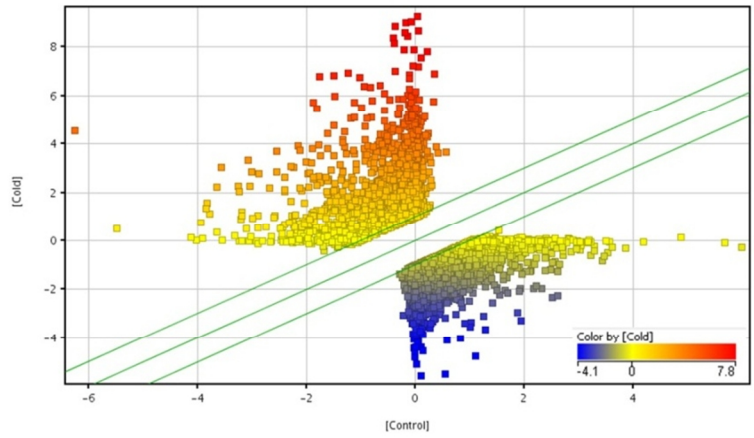


Figure 3.40. Scatter plots of differentially regulated (p<0.05; FC≥2) genes under cold stress condition and their expression values. Diagonal lines indicate twofold difference lines. Points above and below the diagonal lines indicate up- and down-regulated genes, respectively.

Gene Ontology Enrichment Analysis Software Toolkit (GOEAST) was used for determination of annotation and biological processes for significantly different probe set lists ($p<0.05$; $FC\geq2$). The up- and down-regulated probe sets for cold stress treatment were assigned by the web-based database. According to the GO database, under the cold stress treatment up-regulated differentially expressed probes were functionally categorized into 38 groups. These included not only functionally well-defined categories, such as catalytic activity, cell growth, cellular organization, energy, general metabolism, proteolysis, regulation (of biosynthetic process, cellular process, gene expression, macromolecule metabolic process, nitrogen compound metabolic process, nucleic acid metabolic process, transcription, DNA-dependent), response (to abiotic stimulus, chemical stimulus, osmotic stress, stimulus, stress, water) and transcription factor but also transcripts for proteins identified by shared structural domains (calcium binding, DNA/RNA binding domain and membrane binding). Gene Ontology (GO) and biological processes information obtained from the database were shown Figure 3.41.

Differentially down-regulated biological processes under the cold stress treatment were shown in Figure 3.42 and fall into the functionally categorized into 25 groups including biosynthetic process, cellular metabolic process, cellular organization, gene expression, macromolecule metabolic process, protein metabolic process and structural constituent of ribosome. Transcriptome changes under the cold stress treatment show that, the ratio of differentially down-regulated genes was greater than up-regulated ones. This reveals the fact that, when plant exposed to cold stress, it slowed down some major biological processes including synthesis of proteins and macromolecules. On the other hand, expression levels of genes related with response to abiotic and osmotic stress increased to deal with cold stress. Besides this, regulation of some metabolic processes and induction of transcription factors also provide support for wheat plant against the cold stress.

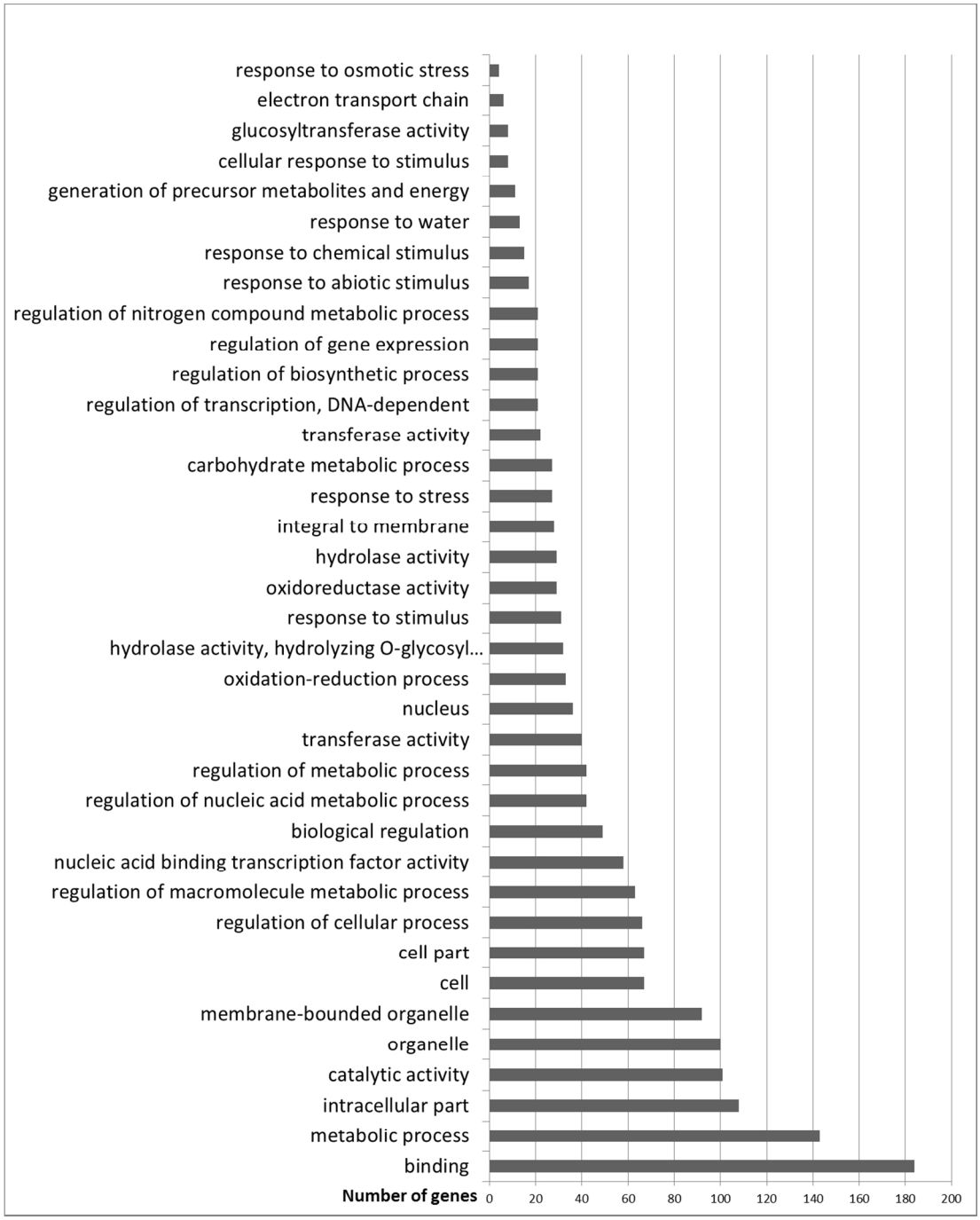


Figure 3.41. Up-regulated biological processes under cold stress treatment compared to control. Significantly different ($p<0.05$; $FC\geq2$) probe sets were used for gene classifications.

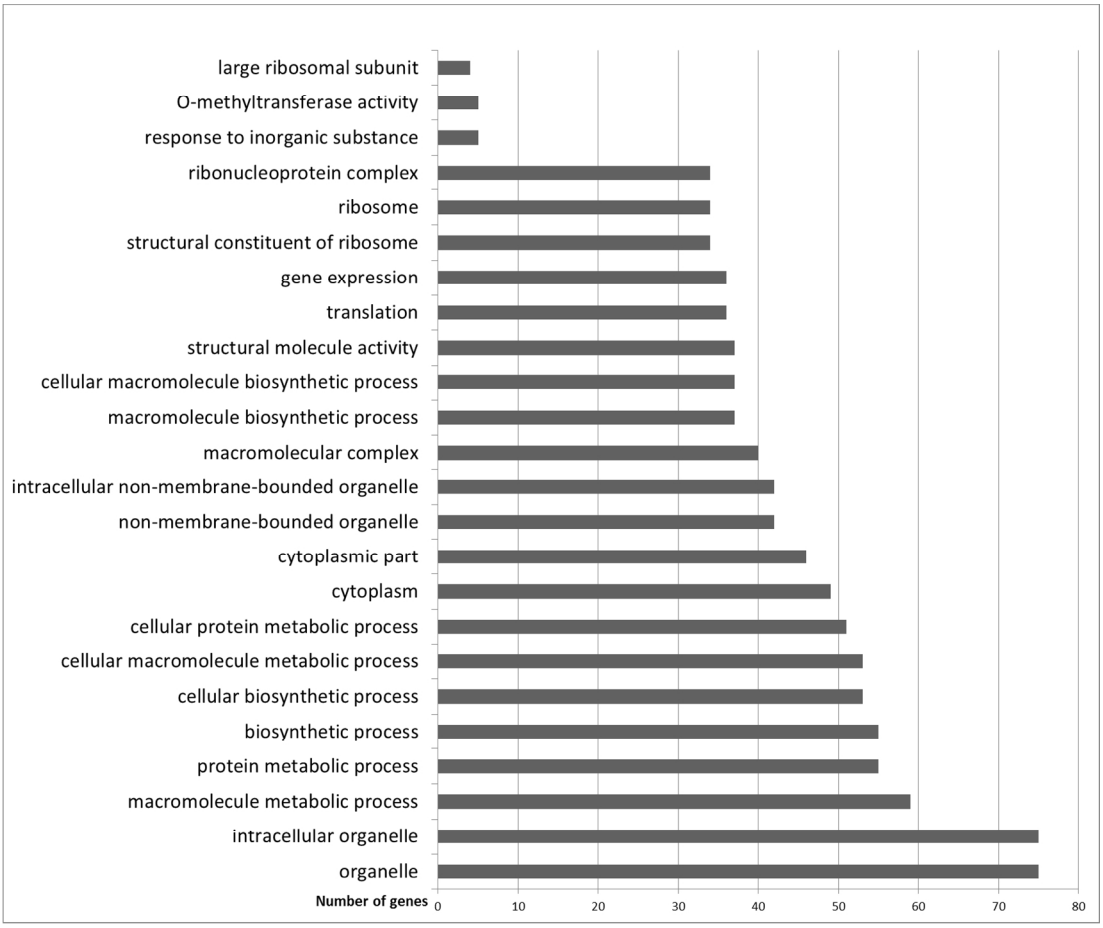


Figure 3.42. Down-regulated biological processes under cold stress treatment compared to control. Significantly different ($p<0.05$; $FC\geq2$) probe sets were used for gene classifications.

To allow for a visual comparison of the control and cold stress treatment, the retrieved expression values of all present genes were imported into MapMan (Sreenivasulu *et al*., 2008; Mangelsen *et al*., 2011). BINs were generated for each pathway depending on the transcript abundancy. High numbers of transcripts were divided to subBINs. Together with BINs and subBINs, 'metabolism overview' and 'regulation overview' maps were generated and shown in Figure 3.43 and Figure 3.44, in which characteristic expression patterns were detected under the cold stress.

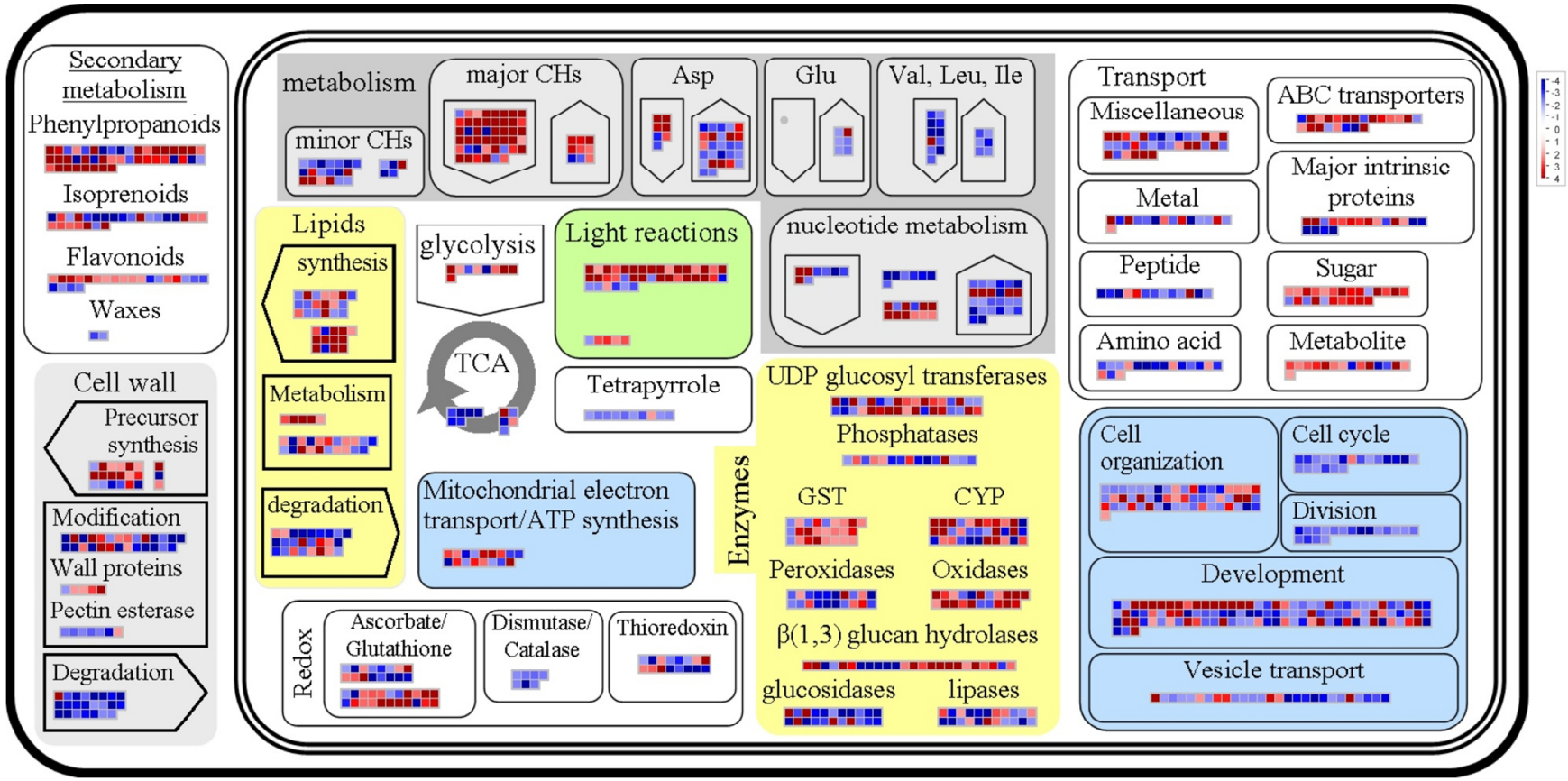


Figure 3.43. MapMan display of metabolism overview for all differentially regulated genes under the cold stress. Up-regulated gene expression in cold-stressed samples versus control samples is shown as an increasingly intense red saturating. Down-regulated gene expression in cold-stressed samples versus control samples is shown as an increasingly intense blue saturating.

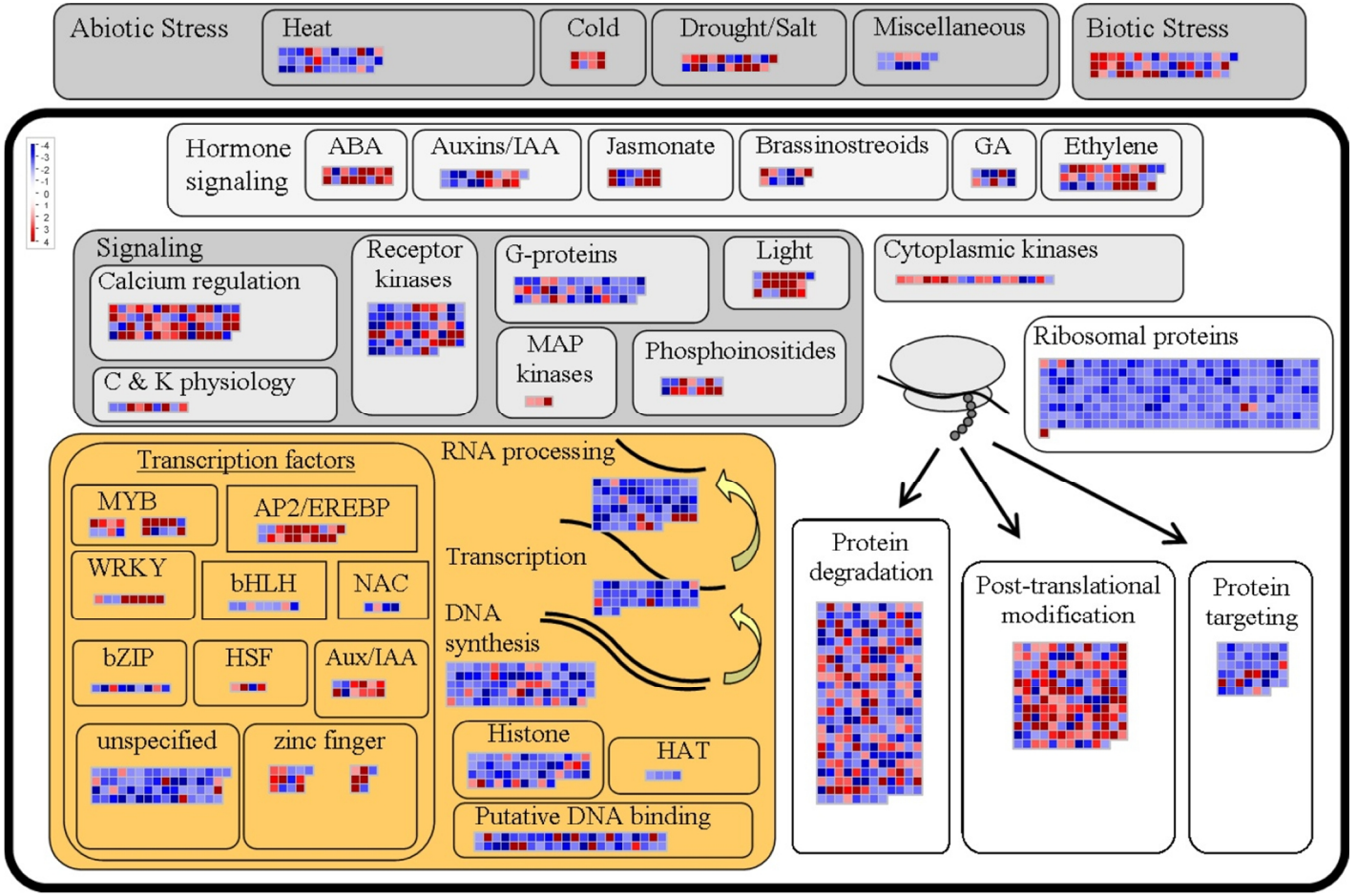


Figure 3.44. MapMan display of regulation overview for all differentially regulated genes under the cold stress. Up-regulated gene expression in cold-stressed samples versus control samples is shown as an increasingly intense red saturating. Down-regulated gene expression in cold-stressed samples versus control samples is shown as an increasingly intense blue saturating.

According to MapMan metabolism and regulation maps, differentially regulated genes ($p<0.05$; $FC\geq2$) were selected to investigate gene expression profile in detail under the cold stress condition.

Freezing tolerance in many plant seriously increases when plants are exposed to a period of low, nonfreezing temperatures which is known as cold acclimation (Zhang *et al*., 2009). In this period, a specific profile of gene expression has been observed which results in influx of calcium into the cytosol is the starting point of these signaling pathways (Monroy & Dhindsa, 1995). Then activation of transducers, expression of inducer of CBF (C-repeat binding factor) expression, induction of CBF genes, and finally expression of downstream COR (cold-regulated) genes are observed (Chinnusamy & Zhu, 2006). Consistent with previous works (Seki *et al*., 2002; Wei *et al*., 2005) many cold stress-responsive genes including COR and LEA homolog genes and different cold acclimation proteins were extremely up-regulated by cold acclimation in our study. Many COR and LEA proteins function as membrane stabilizers and thus enhance cold tolerance in plants (Thomashow, 1999). Wheat cold-acclimation proteins (WSCs) and (WCORs) may function as cold-responsive transcription factors to induce the expression of downstream genes that are related to cold tolerance (Danyluk *et al*., 1996). As a result of induction of these transcription factors, Ice recrystallization (IRI) inhibition genes were also induced after cold acclimation. IRI proteins protect membranes from physical damage by inhibiting ice crystal growth and recrystallization (Atici and Nalbantoglu, 2003). Other stress-responsive genes were also up-regulated during cold acclimation, because cold and other stresses shares many signaling pathways or defense mechanisms (Seki *et al*., 2002; Chinnusamy *et al*., 2006). One of the most important stress-responsive genes is Heat Shock Proteins (HSPs) which was discussed in further section in detail. Table 3.4 show that significantly ($p<0.05$) up-regulated transcripts which involve in abiotic stress responses under the cold stress.

Table 3.4. Significantly up-regulated transcripts which involve in abiotic stress responses under the cold stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control.

Probe Set ID	**Putative Annotation**	**FC**
ABIOTIC STESS/COLD		
Ta.21768.1.S1_at	Ice recrystallization inhibition protein 1 precursor	610.7
Ta.123.1.S1_x_at	Cold acclimation protein WCOR80	323.0
Ta.13183.1.S1_x_at	Cold regulated protein	43.4
TaAffx.9441.1.S1_at	Cold-responsive LEA/RAB-related COR protein	36.6
Ta.13183.1.S1_s_at	Cold regulated protein	32.9
TaAffx.54307.1.S1_x_at	Cold-responsive LEA/RAB-related COR protein	24.6
Ta.351.1.S1_at	Cold acclimation induced protein 2-1	21.5
Ta.18487.2.S1_at	Cold-responsive protein	18.3
Ta.2826.1.S1_at	Cold-responsive protein	17.0
Ta.18574.1.A1_x_at	Ice recrystallization inhibition protein 1 precursor	15.7
TaAffx.132296.1.A1_x_at	Cold-responsive protein	13.0
Ta.613.1.S1_at	Cold-responsive LEA/RAB-related COR protein	12.5
Ta.122.1.S1_x_at	Cold acclimation protein WCOR410b	12.3
Ta.2541.1.S1_x_at	Cold acclimation protein WCOR615	10.5
TaAffx.3462.1.S1_at	Cold acclimation induced protein 2-1	10.0
Ta.1991.1.S1_at	Cold acclimation protein WCOR410	6.7
Probe Set ID	**Putative Annotation**	**FC**
ABIOTIC STESS/HEAT		
TaAffx.70677.1.S1_at	Heat shock protein DnaJ	45.4
Ta.16248.1.S1_at	Heat shock protein 16.9B	39.3
Ta.22973.1.S1_x_at	Small heat shock protein HSP17.8	3.3
Ta.10259.1.S1_at	Heat shock protein (hsp70A)	2.1

Transcription factors play crucial roles for response to environmental stimuli. Through a signaling cascade, they bind to specific regulatory sites upstream of constituent genes in a regulatory network by direct physical interaction or in combination with other proteins (Xia *et al*., 2010). Some of the significantly ($p<0.05$) up-regulated transcription factors under the cold stress belong to ARF, AP2/EREBP, bZIP, C_2H_2-type zinc finger, DRE, NAC, MYB and WRKY families.

The NAC type transcription factors constitute one of the largest families of plant-specific transcription factors and are known to possess diverse roles in plant development and in the recognition of environmental stimuli (Olsen *et al*., 2005). Cold induced the expression of NAC domain containing protein represented by Ta.5367.2.S1_x_at, Ta.28539.1.A1_x_at and Ta.9497.1.S1_at. According to Table 3.5, their fold changes differ between 7 and 23, which mean that NAC type transcription factors were induced in response to cold stress.

DRE-binding factors 2 (DREB2) specifically binds to DRE/CRT sequence found in the promoter region of many stress inducible genes. In Arabidopsis, expression of DREB2 genes is induced by dehydration and high salinity. Four up-regulated probe sets assigned for DRE-binding factors were identified as a result of cold stress. DREB2 induces the expression of stress-inducible gene, late embryogenesis-abundant (LEA) protein. LEA proteins are involved in osmotic stress response and induced by salt, drought, cold and ABA. LEA protein was highly up-regulated after the cold stress treatment. It can be concluded that, LEA/dehydrin genes may have a protective function under cold stress.

Table 3.5. Significantly up-regulated transcripts related with transcription factors under the cold stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control.

Probe Set ID	Putative Annotation	FC
TRANSCRIPTION FACTORS		
Ta.25026.1.S1_at	Late embryogenesis-abundant (LEA) protein	278.7
TaAffx.1024.1.A1_at	CRT/DRE binding factor 15	166.0
Ta.24728.1.S1_at	Actin-binding protein WCOR719 (Wcor719)	136.3
Ta.30495.1.A1_s_at	T51830 transcription factor DREB1A	102.5
Ta.30336.1.S1_x_at	Wdhn13 mRNA for LEA D-11 dehydrin	77.3
Ta.25843.1.A1_at	T51830 transcription factor DREB1A	71.9
TaAffx.98930.1.A1_at	CRT/DRE binding factor 12	67.4
TaAffx.9231.2.S1_s_at	CBFIVd-B22	38.7
TaAffx.122374.1.A1_at	CRT/DRE binding factor 2	30.1
Ta.2882.1.S1_at	EF-hand Ca^{2+}-binding protein CCD1	29.1
Ta.5367.2.S1_x_at	NAC transcription factor	23.0
Ta.28539.1.A1_x_at	NAC transcription factor	22.2
TaAffx.109327.1.S1_at	CBFIVd-A22	18.9
Ta.25920.1.A1_at	MYB-related protein	18.4
TaAffx.9231.2.S1_x_at	CBFIVd-B22	13.3
TaAffx.130052.2.S1_at	C_2H_2 zinc finger protein	13.0
Ta.103.1.S1_at	Zinc-finger protein WZF1	12.6
Ta.10822.2.S1_a_at	$C_3H_2C_3$ RING-finger protein	11.2
Ta.29449.1.S1_s_at	Zinc finger protein 1	10.3
Ta.10822.1.S1_at	$C_3H_2C_3$ RING-finger protein	10.2
Ta.28791.1.A1_at	Dehydration response element binding protein	10.2
Ta.961.2.S1_a_at	WRKY-type DNA binding protein	8.6
Ta.9497.1.S1_at	NAC transcription factor	7.4
Ta.6227.1.S1_at	GTP-binding RAB2A	4.4
Ta.28852.1.S1_at	Auxin response factor (ETT1-alpha)	4.0
Ta.1347.1.S1_at	SAR1GTP-binding secretory factor	4.0
Ta.9366.1.S1_x_at	Zinc finger protein OBP4 - like	3.6
Ta.24806.1.S1_x_at	bZIP transcription factor-like protein	3.4
Ta.2781.1.S1_at	CRTDRE-binding factor (CBF1)	3.3
Ta.30470.1.A1_at	AP2 domain transcription factor-like protein	2.7

Low temperature is the one of the most important limiting factors that causes decrease in photosynthetic activity. In our study, the highest effected genes from the cold stress (between 500 and 1800 fold changes) belong to early light-inducible proteins (ELIPs) families which directly involves in photosynthesis (Table 3.6). ELIP are nuclear-encoded light-inducible proteins which are detected within the thylakoid membrane system (Kolanus *et al*.,1987). Simultaneous with ELIP protein accumulation, D1 protein in the photosystem II (PSII) reaction center decreases in abundance and there is a decrease in PSII activity (Bruno and Wetzel, 2004). RNA hybridization and microarray analysis which are consistent with our results, reveals that transcript level of ELIP increases in response to a variety of stress-related signals coming from salt, heat, abscisic acid, cold, desiccation, aluminum, high CO_2, and senescence (Adamska, 2001; Binyamin *et al*., 2001; Harari-Steinberg *et al*., 2001; Montane *et al*., 1997; Provart *et al*., 2003).

Table 3.6. Significantly up-regulated transcripts which involve in photosynthesis under the cold stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control.

Probe Set ID	**Putative Annotation**	**FC**
PHOTOSYNTHESIS		
Ta.9600.1.S1_x_at	Low molecular mass ELIP	1787
Ta.25398.1.S1_at	Low molecular mass ELIP	602.2
Ta.18362.1.A1_at	ELIP- HV58	586.1
Ta.23419.3.S1_x_at	High molecular mass ELIP	442.3
Ta.26973.1.S1_at	Low molecular mass ELIP	55.9
TaAffx.114127.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	15.7
TaAffx.128757.1.S1_at	Photosystem II protein D1	10.4
Ta.3795.1.S1_x_at	Chlorophyll ab-binding protein WCAB	5.0

Following the low temperature treatment of wheat seedlings, some enzymes related with metabolic pathways were seriously influenced. Among the enzymes families, carbohydrate metabolism enzymes were significantly up-regulated. Actually, in stress conditions, plants support synthesis of low and high molecular weight carbohydrates. So, plants avoid a deficiency in carbohydrates and running out of cell energy. In this study, sucrose and starch synthesis were up-regulated whereas some lipid metabolism enzymes were down-regulated after the cold stress treatment. Table 3.7 shows differentially regulated transcripts related with major metabolic enzymes.

Table 3.7. Significantly up- and down-regulated transcripts related with major metabolic enzymes under the cold stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation and positive values show up-regulation.

Probe Set ID	Putative Annotation	FC
MAJOR METABOLIC ENZYMES		
Ta.2789.1.S1_at	Sucrose:fructan 6-fructosyltransferase	91.5
Ta.2789.1.S1_a_at	Sucrose:fructan 6-fructosyltransferase	80.2
Ta.2788.1.A1_at	Sucrose 1-fructosytransferase	77.7
Ta.20429.1.S1_at	Phenylalanine ammonia-lyase	73.2
Ta.14281.1.S1_at	Defensin	61.6
Ta.25274.1.A1_x_at	Glycosyl hydrolases	44.0
Ta.23272.1.S1_at	UDP-glucoronosyl protein	24.2
Ta.2780.1.S1_at	RNA for NADPH-thioredoxin reductase	19.3
Ta.28700.1.S1_at	Chalcone synthase	18.2
Ta.14145.1.S1_at	Glycosyltransferase	16.9
Ta.8269.1.S1_at	Cyclin dependent protein kinase	16.4
Ta.30543.1.S1_at	UDP-D-glucose epimerase 2	13.3
Ta.144.1.S1_s_at	wpk4 protein kinase	11.7
Ta.7151.1.S1_x_at	Beta-fructofuranosidase	10.9
Ta.7104.1.S1_at	Phosphoethanolamine N-methyltransferase	10.7
Ta.7151.1.S1_at	Beta-fructofuranosidase	9.0
Ta.1258.2.S1_x_at	S-adenosyl-L-homocysteine hydrolase (SH6.2)	8.4
Ta.144.1.S1_at	wpk4 protein kinase	7.4

Table 3.7. (continued)

Probe Set ID	Putative Annotation	FC
METABOLIC ENZYMES		
Ta.14421.1.S1_at	putative malate oxidoreductase	6.5
Ta.5480.2.S1_at	Uricase	6.3
Ta.6204.1.S1_at	Aminoalcoholphosphotransferase	6.2
Ta.5480.1.S1_x_at	Uricase	6.0
Ta.3760.2.A1_a_at	Beta 1,3-glucanase (Glc1)	5.8
Ta.24918.1.S1_at	Serinethreonine-protein kinase	5.5
Ta.1258.1.S1_a_at	S-adenosyl-L-homocysteine hydrolase (SH6.2)	5.2
Ta.5480.3.S1_at	Uricase	5.0
Ta.137.1.S1_at	Beta-amylase /DEF	4.7
Ta.1842.1.S1_a_at	Phosphoethanolamine methyltransferase	4.4
Ta.22333.1.S1_at	Fatty acyl coA reductase	-26.7
Ta.27751.5.S1_at	Beta-D-glucan exohydrolase isoenzyme	-13.5
Ta.211.1.S1_at	O-methyltransferase	-11.4
Ta.23090.1.A1_at	Chitinase 2	-10.6
Ta.191.1.S1_at	Thiol protease	-5.4
Ta.2783.1.S1_at	Chitinase 2	-4.8
Ta.9296.1.S1_at	Obtusifoliol 14-alpha-demethylase	-3.3
Ta.151.1.S1_at	Dihydrodipicolinate synthase (DHDPS)	-2.7
Ta.23170.1.A1_s_at	Dihydrodipicolinate synthase 2	-2.6
Ta.16227.1.S1_at	Glycoprotein 3-alpha-L-fucosyltransferase	-2.5
Ta.1055.1.S1_at	Catalase isozyme	-2.4
Ta.114.1.S1_at	Acetyl-coenzyme A carboxylase	-2.4
Ta.23763.1.S1_at	Lipoxygenase	-2.3
Ta.3722.1.S1_at	Histidyl-tRNA synthetase	-2.2
Ta.591.1.S1_at	Pyrroline-5-carboxylate reductase	-2.1
Ta.232.1.S1_at	Phytochelatin synthase (PCS1)	-2.0
Ta.162.1.S1_at	L-isoaspartyl methyltransferase	-2.0

There were also some transcription factors such as MADS-box transcription factor and No Apical Meristem protein (NAM) which were repressed after the cold stress treatment. The MADS-box transcription factor-encoding genes are expressed mainly during plant reproductive development, where they play important roles in

controlling floral organ initiation and identity (Duan *et al*., 2006). NAM genes are members of the NAC gene family which play a central role in senescence, including nutrient remobilization to the developing grain (Jamar *et al*., 2010). According to microarray analysis, down-regulation of these transcription factors means that plant reproduction and senescence were delayed under the low temperature condition.

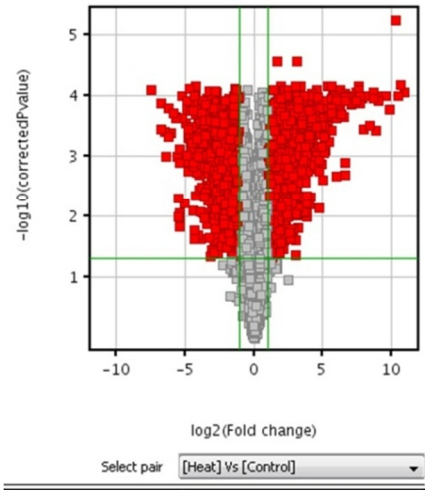
Table 3.8. Significantly down-regulated transcripts related with transcription factors under the cold stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation.

Probe Set ID	**Putative Annotation**	**FC**
TRANSCRIPTION FACTORS		
Ta.17523.2.S1_at	MADS-box transcription factor	-12.2
Ta.16423.1.S1_at	No Apical Meristem protein (NAM)	-10.4
Ta.13724.1.S1_at	T51273 promoter-binding factor	-4.0
Ta.27790.1.S1_s_at	Histone H1 WH1B.1	-3.5
Ta.7602.1.S1_at	Nucleosome assembly protein I	-2.5
Ta.7312.1.S1_at	Cyclophilin-like protein (Cyp-70)	-2.2
Ta.24961.1.S1_x_at	BBC1-like protein	-2.1
Ta.13447.1.S1_at	Cyclophilin-like protein (Cyp-70)	-2.0

3.2.2.2. Differentially Expressed Genes under Heat Stress

Microarray data sets from control and heat were analyzed to examine effect of heat stress on transcriptome profiles of wheat plant. Significantly up- or down-regulated probe set lists ($p<0.05$; $FC\geq2$) were determined for control and heat stress data. According to lists obtained from volcano plot analysis, approximately 2600 and 3500 probe sets were up-regulated and down-regulated, respectively (Figure 3.45). Scatter plots of differentially regulated ($p<0.05$; $FC\geq2$) genes under heat stress condition and their expression values are given in Figure 3.46.

GO analysis was also performed to differentially expressed heat stress samples to identify their annotation and biological processes. The up- and down-regulated probes were analyzed separately in GOEAST annotation web-based tool. Under the heat stress, up-regulated probes formed a 40 distinct groups which include genes related with cellular organization, cellular respiration, electron transport chain, energy, peroxisome, response (to abiotic stimulus, chemical stimulus, stress, water), binding activity (of cation, copper ion, metal ion, zinc ion) and stress related enzymes (aminotransferase, catalase, glutathione transferase, oxidoreductase) (Figure 3.47). Transcriptome analysis under the heat stress indicates that the number of down-regulated genes was higher than up-regulated ones. According to GO result of down-regulated heat stress samples, 54 different functional groups were identified, which contain genes associated with cellular organization, cellular process (biosynthetic process, lipid metabolic process, macromolecule biosynthetic process, metabolic process, protein complex assembly), cytoplasm (cytoplasmic part, cytoskeletal part), fatty acid metabolism, organelle (intracellular non-membrane-bounded organelle, intracellular organelle, large ribosomal subunit), lipid metabolism, macromolecule metabolism, microtubule (microtubule cytoskeleton, microtubule-based movement, microtubule-based process), protein metabolism (complex assembly, complex biogenesis, complex subunit organization, polymerization), sexual reproduction, and transcripts for binding.



Regulation	Heat Stress (p<0.05; FC≥2)
up-regulated	2623
down-regulated	3519
Total	6142

Figure 3.45. Volcano plot and number of significantly ($p<0.05$) up and down-regulated probe sets that changed more than 2-fold in heat stress condition compared to control conditions.

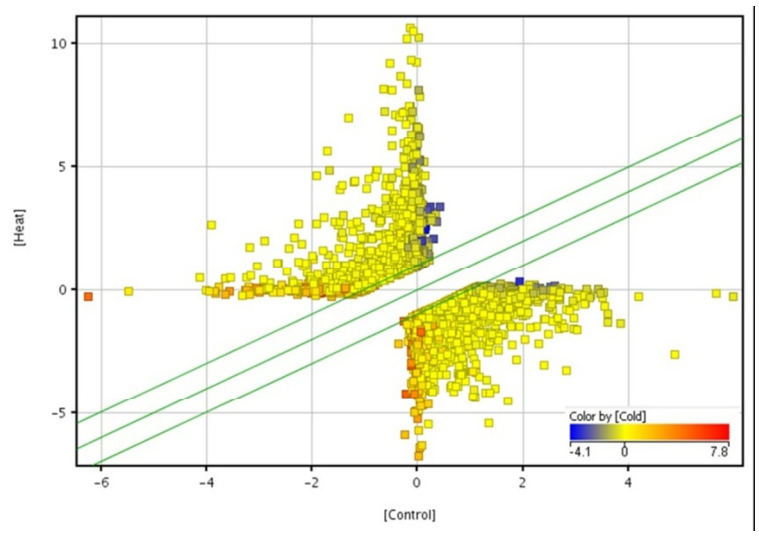


Figure 3.46. Scatter plots of differentially regulated ($p<0.05$; $FC\geq2$) genes under heat stress condition and their expression values. Diagonal lines indicate twofold difference lines. Points above and below the diagonal lines indicate up- and down-regulated genes, respectively.

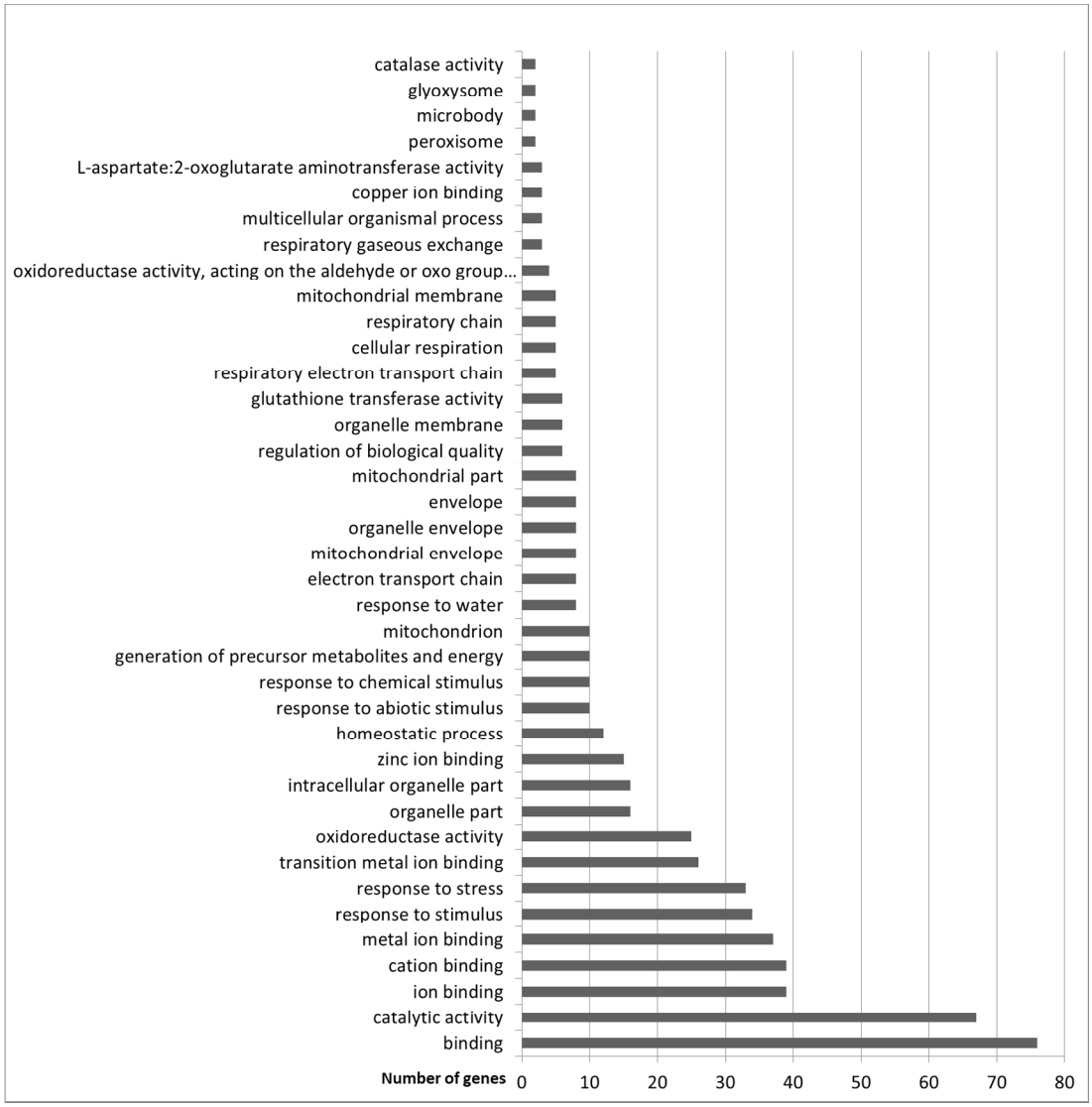


Figure 3.47. Up-regulated biological processes under heat stress treatment compared to control. Significantly different ($p<0.05$; $FC\geq2$) probe sets were used for gene classifications.

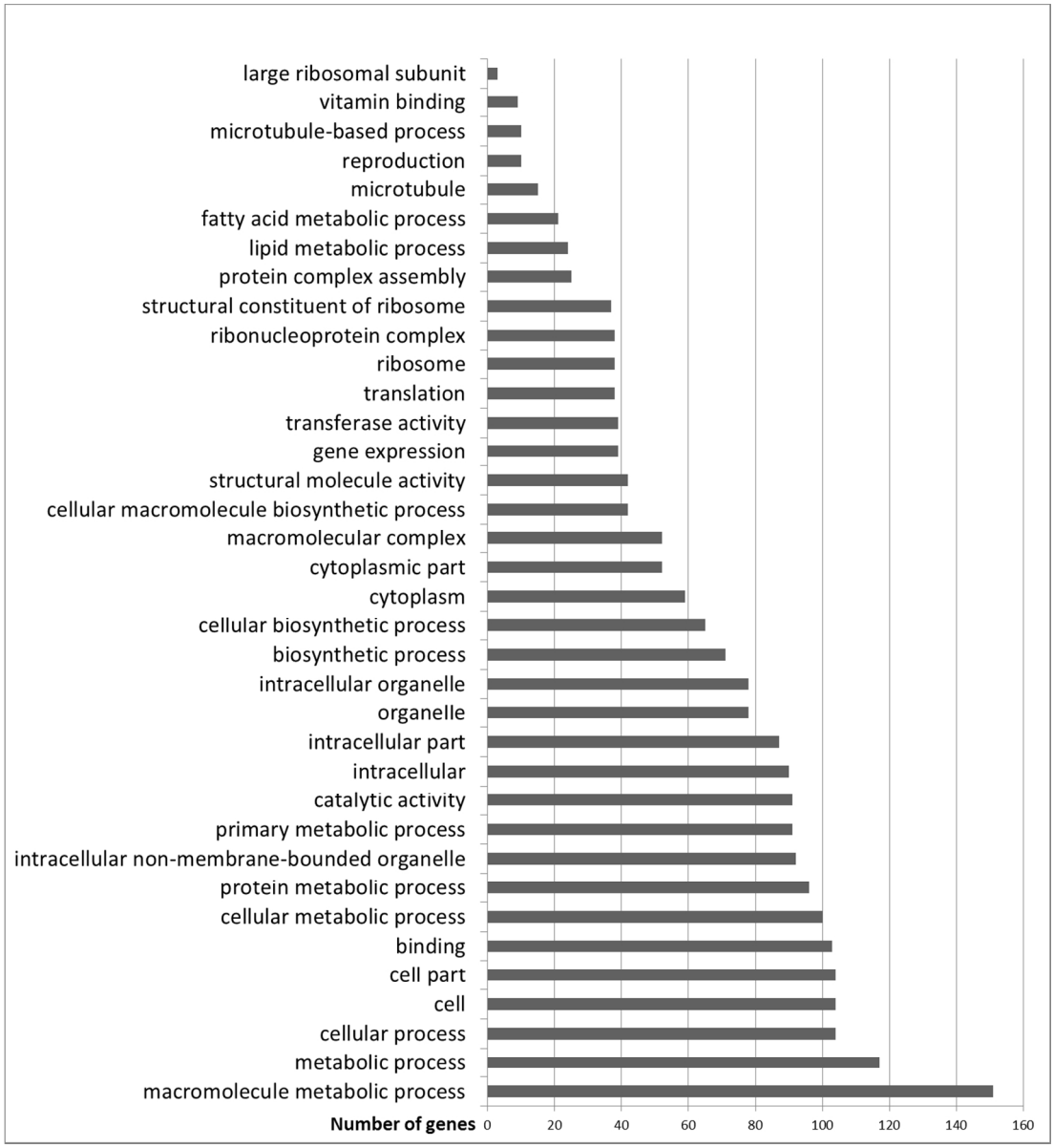


Figure 3.48. Down-regulated biological processes under heat stress treatment compared to control. Significantly different ($p<0.05$; $FC\geq2$) probe sets were used for gene classifications.

Transcriptome characterization of differentially regulated heat-stressed probe sets were displayed in the MapMan metabolism overview and regulation overview maps, which are shown in Figure 3.49 and Figure 3.50, respectively.

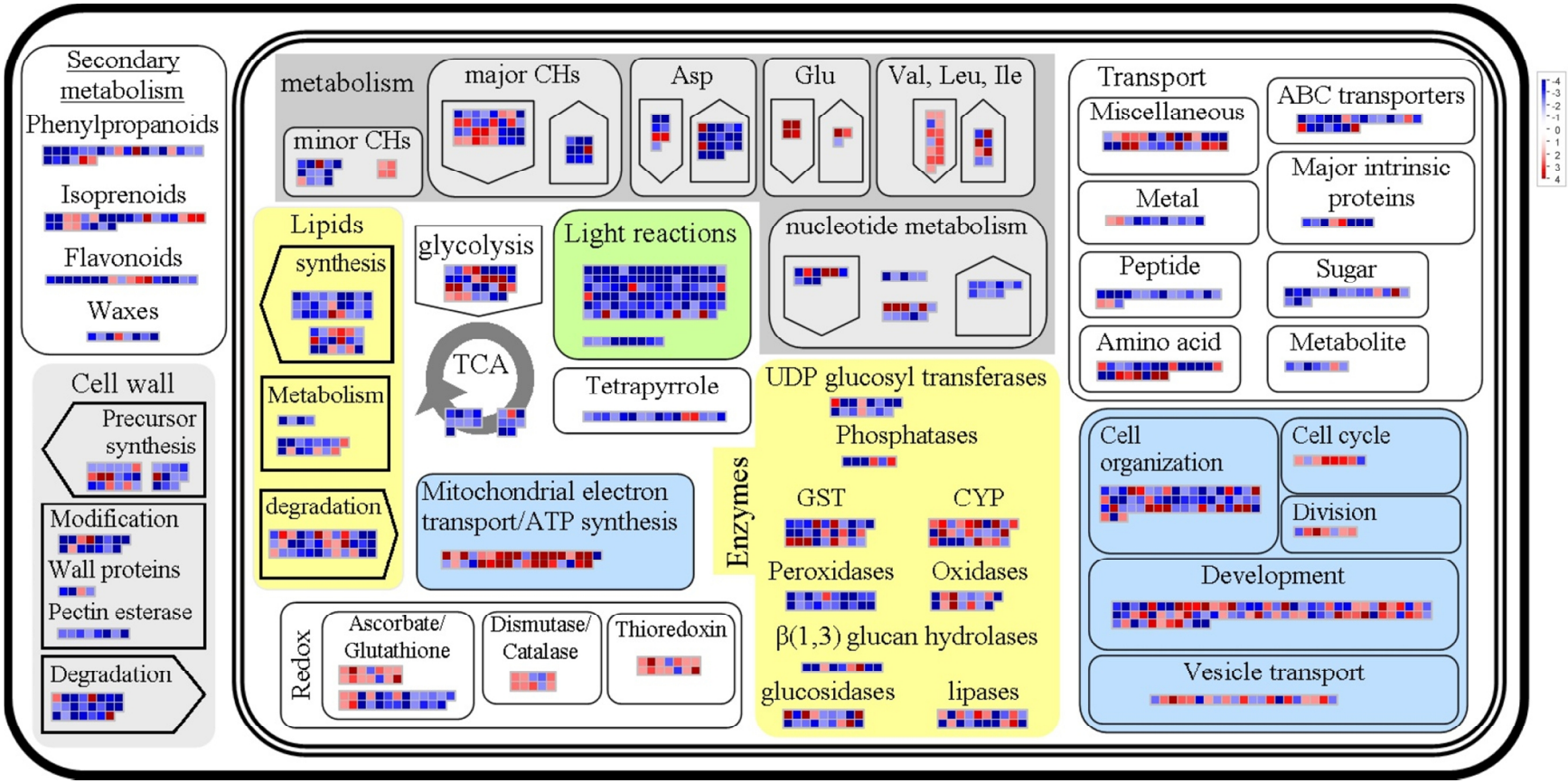


Figure 3.49. MapMan metabolism overview map showing differences in transcript level between control and heat stress. Up-regulated gene expression in heat-stressed samples versus control samples is shown as an increasingly intense red saturating. Down-regulated gene expression in heat-stressed samples versus control samples is shown as an increasingly intense blue saturating.

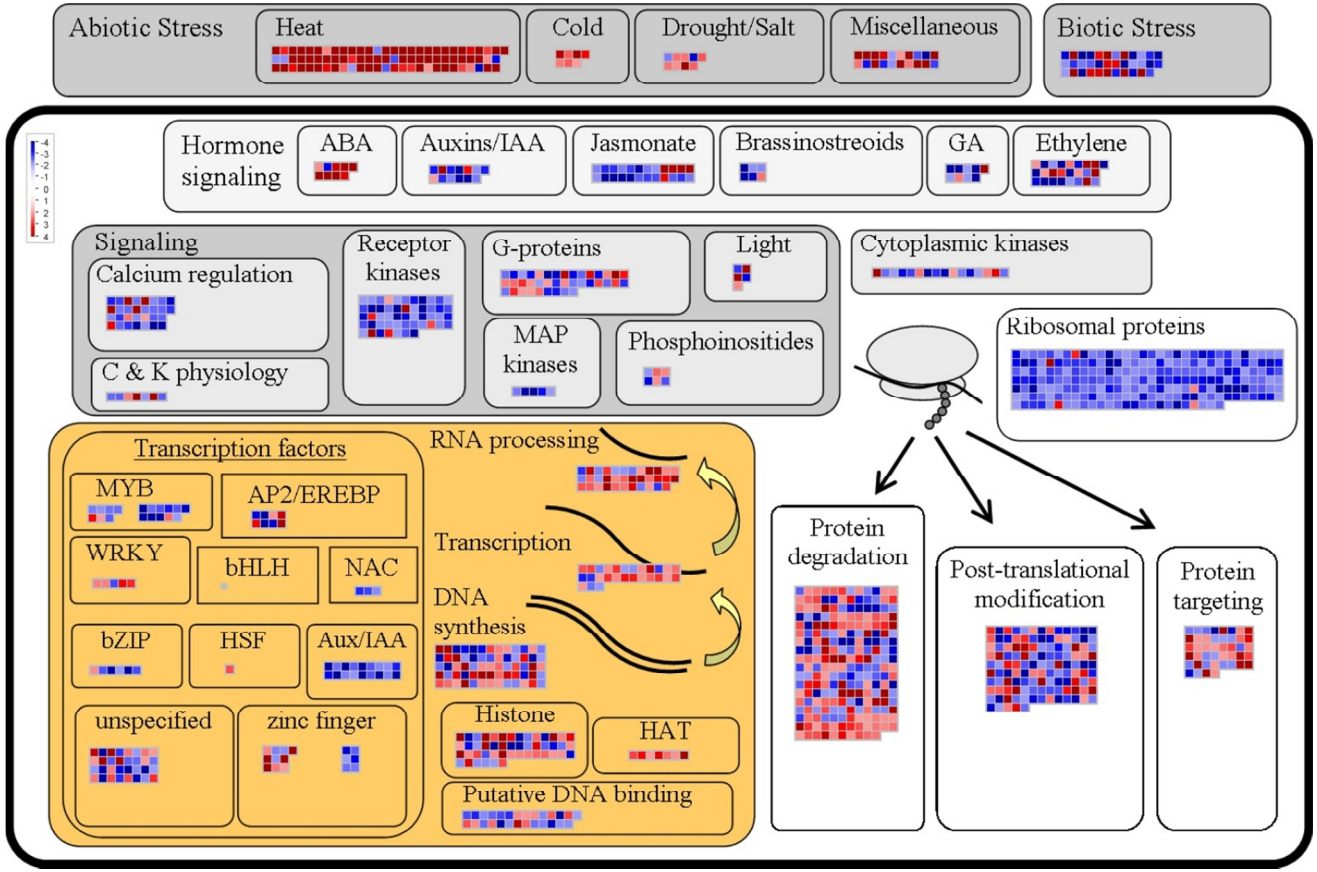


Figure 3.50. MapMan regulation overview map showing differences in transcript level between control and heat stress. Up-regulated gene expression in heat-stressed samples versus control samples is shown as an increasingly intense red saturating. Down-regulated gene expression in heat-stressed samples versus control samples is shown as an increasingly

Heat shock proteins (Hsps) protect the plant against severe effects of heat stress. The best-characterized response of plants to high temperature stress is the production of Hsps. Within seconds of reaching a critical temperature 5–10°C above the optimum for growth, transcription of HSPs is induced (Chen *et al*., 1990). Depending on their molecular weight, HSPs are categorized into six structurally distinct classes: Hsp100, Hsp90, Hsp70, Hsp60 (or chaperonins), □ 17-to 30 kDa small Hsps (sHsps) and ubiquitin (8.5 kDA) (Sun & MacRae, 2005). Hsps are characterized as structurally unstable proteins and are involved in the refolding of denatured proteins and preventing their aggregation and also membrane protection (Renaut *et al*., 2006). According to microarray analysis, about 40 different heat shock protein transcripts were identified. Although samples were collected 12 hours later after the heat stress application, high number of Hsps were induced, which clearly indicates that the first response of plant to high temperature was induction of high number of Hsps. In addition to this, their expression levels were also enormous. The transcript level of small Hsps including HSP26, HSP17.4 and HSP22 were very high which means that compared the control samples their expression levels ranged from 1200 to 1800 fold changes. Besides sHsps, big ones such as Hsp100, Hsp80, Hsp70 and Hsp60 were also significantly up-regulated following heat stress treatment.

Table 3.9. Significantly up-regulated transcripts of Heat Shock Proteins which involve in heat stress responses. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control.

Probe Set ID	**Putative Annotation**	**FC**
ABIOTIC STESS/HEAT (HEAT SHOCK PROTEINS)		
Ta.202.1.S1_at	Heat shock protein HSP26-m allele	1813.3
TaAffx.73024.1.S1_at	Heat shock protein 17.4 kDa class I	1555.7
Ta.8665.1.S1_at	Heat shock protein 22.0 kDa class IV	1400.4
Ta.201.1.S1_at	Heat shock protein HSP26-i allele,	1198.1

Table 3.9. (continued)

Probe Set ID	Putative Annotation	FC
ABIOTIC STESS/HEAT (HEAT SHOCK PROTEINS)		
Ta.28630.1.S1_at	Heat shock protein 16.9 kDa class I	861.3
Ta.9140.2.S1_at	Heat shock protein 82	718.5
Ta.24332.1.S1_at	Low molecular weight heat shock protein	630.2
Ta.2747.1.S1_at	Heat shock protein HSP26-g allele	455.0
TaAffx.100415.1.A1_at	Heat shock protein 17.6kDa	395.8
Ta.8714.1.S1_at	Heat shock protein 22.0 kDa class IV	391.2
Ta.6123.1.A1_s_at	Heat shock protein 22.0 kDa class IV	279.7
Ta.28631.1.S1_at	Heat shock protein 16.9 (hsp16.9-2LC2)	228.9
TaAffx.18332.1.S1_at	DnaK family protein	202.8
Ta.28083.1.S1_at	Heat shock protein 17.3.	191.0
Ta.22973.1.S1_x_at	Small heat shock protein HSP17.8	163.9
Ta.261.1.S1_at	Heat shock protein 101 (HSP101c)	135.0
Ta.12042.1.S1_at	Heat shock protein HSP20	105.6
Ta.6123.1.A1_at	Heat shock protein 70kD	75.0
Ta.27661.1.S1_at	Heat shock protein 16.9 (hsp16.9-10LC1)	74.7
Ta.132.1.S1_a_at	Heat shock protein 16.9 (hsp16.9-1LC1)	74.4
Ta.639.1.S1_at	Peptidylprolyl isomerase	51.9
Ta.23663.1.S1_s_at	Heat shock protein 16.9 (hsp16.9-3LC1)	50.6
Ta.24230.1.S1_at	Histone H2A.1	48.2
Ta.203.1.S1_at	Small heat shock protein Hsp23.5	46.5
Ta.204.1.S1_at	Small heat shock protein Hsp23.6	35.8
Ta.9115.3.S1_x_at	Chaperone protein clpB 1	29.3
TaAffx.37294.1.S1_at	Heat shock protein DnaJ	26.9
Ta.256.1.S1_at	Heat shock protein 101 (Hsp101b)	23.8
Ta.30802.1.A1_at	Heat shock protein HSP70	23.2
Ta.9115.1.S1_at	Chaperone protein clpB 1	22.5
TaAffx.65575.1.A1_at	Heat shock factor RHSF2	21.1
Ta.30802.1.A1_x_at	Heat shock protein 70	19.9
Ta.6123.2.S1_at	Heat shock protein 70kD	18.2
Ta.6964.1.S1_at	Chaperonin CPN60-1, mitochondrial	17.8
Ta.28758.1.S1_at	Heat shock protein 101	16.8
Ta.24515.1.S1_at	Heat shock protein 70 kDa	11.5
Ta.21335.2.A1_x_at	Heat shock protein 70 kDa	11.4
Ta.522.1.S1_at	Heat shock protein 70 (HSP70)	3.6
Ta.217.1.S1_x_at	Heat shock protein 80	3.3
Ta.28630.1.A1_at	Heat shock protein 16.9 (hsp16.9-13LC1)	2.8

As mentioned earlier, transcription factors initiates signaling cascades resulting in response to several abiotic stresses in plants. Following the heat stress treatment, various transcription factors were induced, like cold stress treatment. Multiprotein bridging factor 1 (MBF1) showed the highest expression change compared to control samples. Multiprotein bridging factor 1 (MBF1) is a transcriptional co-activator which mediates transcriptional activation by bridging between an activator, bZIP factor, and a TATA-box binding protein (TBP). Its expression is specifically elevated in response to pathogen infection, salinity, drought, heat, hydrogen peroxide, and application of abscisic acid or salicylic acid (Tsuda *et al*., 2004). Microarray analysis reveals that both co-activator and activator molecules were significantly up-regulated. In addition to response of Hsps, transcription factors also played a central role against the high temperature stress.

Table 3.10. Significantly up-regulated transcripts related with transcription factors under the heat stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation.

Probe Set ID	**Putative Annotation**	**FC**
TRANSCRIPTION FACTORS		
Ta.12225.2.S1_at	Multiprotein bridging factor 1C	81.4
TaAffx.70031.1.A1_at	ZPR1 zinc-finger domain	31.6
Ta.14269.1.A1_at	Heavy metal transport/detoxification family	29
Ta.14467.2.S1_at	bZIP transcription factor	15.3
Ta.1274.2.S1_at	Calcyclin-binding protein	15.3
Ta.23797.1.S1_x_at	LEA1 protein (LEA1)	12.6
TaAffx.28200.1.S1_at	Zinc finger family protein	10.0
Ta.5367.2.S1_x_at	NAC domain protein	3.8
Ta.5367.1.S1_s_at	NAC domain protein	3.1
Ta.961.2.S1_a_at	WRKY-type DNA binding protein	3.0
Ta.24806.1.S1_x_at	bZIP transcription factor	2.0

Cytochromes P450 (P450s) constitute the largest family of enzymatic proteins in higher plants. P450s are mono-oxygenases meaning that they insert one oxygen atom into inert hydrophobic molecules to make them more reactive and hydrosoluble. In addition to their physiological functions in the biosynthesis of hormones, lipids and secondary metabolites, P450s help plants to deal with harmful exogenous chemicals such as pesticides and industrial pollutants (Werck-Reichhart *et al*., 2000). Besides metabolic function of P450s, Arabidopsis cytochrome P450 genes were induced after the various biotic and abiotic stress conditions (Narusaka *et al*., 2004). According to microarray results, 10 different probe sets related with cytochromes were detected. All of them were meaningfully up-regulated under the heat stress. Increase in transcription level of P450s in plant tissue may explain induction of hormone synthesis. In connection with this, JA and GA biosynthesis were also induced response to heat stress.

Table 3.11. Significantly up-regulated transcripts related with cytochromes under the heat stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation.

Probe Set ID	**Putative Annotation**	**FC**
CYTOCHROME		
Ta.28701.1.A1_at	Cytochrome c oxidase subunit	94.6
Ta.21438.1.A1_at	Cytochrome P450	92.8
Ta.9600.1.S1_x_at	Low molecular mass early light	63.3
TaAffx.114431.1.S1_s_at	Cytochrome c oxidase subunit 3	51.5
TaAffx.113847.1.S1_at	Cytochrome oxidase subunit II	49.7
TaAffx.15011.1.S1_at	Cytochrome c maturation subunit Fc	28.5
TaAffx.116369.1.S1_x_at	Cytochrome P450	22.1
TaAffx.113847.2.S1_at	Cytochrome-c oxidase	17.7
Ta.28645.1.S1_at	Cytochrome oxidase subunit I	13.7
TaAffx.116369.1.S1_s_at	Cytochrome P450	11.9
Ta.26997.1.S1_at	Chlorophyll A-B binding family protein	11.2

Following the high temperature stress application of wheat seedling, genes encoding enzymes related with carbohydrate, fatty acid/lipid, nitrogen, amino acid metabolism and photosynthesis were directly affected (Table 3.12 and Table 3.13). Main degradation enzymes in these pathways were repressed after the heat stress treatment which results in deceleration of metabolism. So, plant may protect itself against high temperature through this way. For example, expression of beta-glucosidase and beta-amylases involved in starch degradation was down-regulated in heat stressed samples. Down-regulation of these enzymes may cause reduction in free sugar composition.

Table 3.12. Significantly up- and down-regulated transcripts related with major metabolic enzymes under the heat stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation and positive values show up-regulation.

Probe Set ID	**Putative Annotation**	**FC**
MAJOR METABOLIC ENZYMES/CARBOHYDRATE METABOLSIM		
Ta.12118.1.S1_a_at	Beta-glucosidase	-76.1
TaAffx.38476.1.S1_at	UDP-Glycosyltransferase	-52.6
Ta.3094.2.S1_at	GDP-mannose 3,5-epimerase 1	-15.7
Ta.5177.1.A1_at	Glucose-6-phosphate dehydrogenase 1	-12.2
Ta.24427.1.S1_at	Glucanendo-1,3-beta-glucosidase isoenzyme	-12.1
Ta.4601.2.S1_at	Beta-glucosidase	-11.0
Ta.1166.2.S1_a_at	Fructose-1,6-bisphosphatase,	-10.7
Ta.3.1.S1_at	Beta-amylase	-6.8
Ta.511.1.S1_at	Glyceraldehyde-3-phosphate dehydrogenase	-6.5
Ta.439.1.S1_at	Fructose-1,6-bisphosphatase	-3.3
Ta.14421.1.S1_at	Malate oxidoreductase	-3.2
Ta.28068.1.S1_at	Glyoxalase I	-3.1
Ta.22899.1.S1_at	UDP-glucuronyltransferase-like protein	-2.1
Ta.304.2.S1_a_at	Fructose 1-,6- biphosphate aldolase (ald gene)	4.0
Ta.2788.1.A1_at	Sucrose:sucrose 1-fructosytransferase	3.0

Table 3.12. (continued)

Probe Set ID	Putative Annotation	FC
MAJOR METABOLIC ENZYMES/FATTY ACID METABOLSIM		
Ta.22333.1.S1_at	Fatty acyl coA reductase	-178.5
Ta.1967.2.A1_x_at	Lipoxygenase	-108.5
Ta.1967.1.S1_x_at	Lipoxygenase	-73.6
Ta.7830.1.S1_at	Lipoxygenase	-25.8
Ta.9528.1.A1_at	Fatty acyl coA reductase	-18.8
Ta.4873.1.S1_at	Plastid omega-3 fatty acid desaturase	-16.9
Ta.12415.1.A1_at	Fatty acyl coA reductase	-14.6
Ta.23763.1.S1_at	Lipoxygenase	-14.5
Ta.1456.1.A1_at	Fatty acyl coA reductase	-14.4
Ta.701.1.S1_at	Omega-3 fatty acid desaturase	-6.5
Ta.187.1.S1_at	Cytosolic acetyl-CoA carboxylase	-4.5
Ta.13443.1.S1_at	Fatty acyl coA reductase	-2.9
Ta.114.1.S1_at	Acetyl-coenzyme A carboxylase	-2.3
Ta.24254.3.S1_x_at	Omega-3 fatty acid desaturase	-2.0
MAJOR METABOLIC ENZYMES/AMINO ACID METABOLSIM		
Ta.3504.1.A1_at	Asparaginase	-48.7
Ta.581.2.S1_a_at	glycine decarboxylase subunit	-5.1
Ta.20429.1.S1_at	Phenylalanine ammonia-lyase	-3.0
Ta.24508.1.S1_x_at	Glutamine synthetase	-2.2
Ta.7091.1.S1_at	Pyrroline-5-carboxylate synthetase	8.6
Ta.9466.1.S1_a_at	Aspartate aminotransferase	3.2
MAJOR METABOLIC ENZYMES/NITROGEN METABOLSIM		
Ta.5633.1.S1_at	Nitrate reductase	-10.7
Ta.5633.1.S1_x_at	Nitrate reductase	-10.6
Ta.24768.1.A1_x_at	Ferredoxin--nitrite reductase	-8.4
MAJOR METABOLIC ENZYMES/STRESS RELATED		
TaAffx.110629.1.S1_at	Glutathione S-transferase	-10.0
Ta.6534.1.S1_s_at	Trehalose-6-phosphate synthase	-4.5
Ta.3418.1.S1_x_at	Glutathione transferase F5	-3.1
Ta.6534.1.S1_at	Trehalose-6-phosphate synthase	-3.0
Ta.28354.4.S1_at	Glutathione transferase	-2.6
MAJOR METABOLIC ENZYMES/PROTEIN KINASE		
Ta.25531.2.A1_x_at	Serine/threonine kinase-like protein	-29.9
Ta.25531.2.A1_at	Serine/threonine kinase-like protein	-23.4

Table 3.13. Significantly up- and down-regulated transcripts related with photosynthesis/light reactions under the heat stress. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts in cold stress compared to control. Negative values show down-regulation and positive values show up-regulation.

Probe Set ID	Putative Annotation	FC
PHOTOSYNTHESIS/LIGHT REACTIONS		
Ta.22984.2.S1_x_at	Chlorophyll a/b-binding protein WCAB	-106.0
Ta.3249.3.A1_at	Chlorophyll a/b-binding protein WCAB	-82.7
Ta.28496.1.A1_x_at	Chlorophyll a/b-binding protein WCAB	-37.6
Ta.30702.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	-29.8
Ta.28496.1.A1_at	Chlorophyll a/b-binding protein, putative	-27.1
Ta.1139.1.S1_x_at	Chlorophyll a/b-binding (CAB) protein	-17.3
Ta.27751.6.S1_at	Chlorophyll a/b-binding protein type II	-16.9
TaAffx.53098.1.S1_x_at	Chlorophyll a/b-binding protein	-15.3
Ta.3795.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	-4.4
Ta.2402.3.S1_x_at	Chlorophyll a/b-binding protein	-4.3
Ta.2402.1.S1_a_at	Chlorophyll a/b-binding protein	-4.3
Ta.22468.1.S1_at	ATP synthase protein MI25	128.5
Ta.28812.1.S1_at	NADH dehydrogenase subunit 2	54.2
TaAffx.87200.1.S1_at	NADH-ubiquinone oxidoreductase chain 6	49.5
TaAffx.70660.1.S1_at	NADH-ubiquinone oxidoreductase chain 6	49.4
TaAffx.20.1.S1_at	NADH-ubiquinone oxidoreductase chain 4	5.7

After the cold and heat stress treatments of the wheat seedlings, change in expression level of about 2550 common probe sets was also examined closely. They were analyzed and annotated in different biological processes. They were mainly related with cold and heat stresses responsive genes, transcription factors, antioxidant enzymes and main degradation enzymes in carbohydrate, fatty acid/lipid, nitrogen, amino acid metabolism and photosynthesis. Genes associated with some protective proteins such as cold regulated proteins and heat shock proteins were significantly up-regulated after both stress treatments (Table 3.14).

Table 3.14. Significantly up- and down-regulated common transcripts related with cold and heat stresses responsive genes. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
Ta.123.1.S1_x_at	**Cold acclimation protein WCOR80**	**323.0**	2.6
Ta.245.1.S1_at	Cold-responsive protein WCOR14	66.7	-13.0
Ta.13183.1.S1_x_at	Cold regulated protein	43.4	5.8
Ta.2148.1.S1_x_at	Cold-responsive protein	25.8	-3.9
Ta.759.1.S1_at	Cold acclimation protein WCOR413	21.9	3.8
Ta.351.1.S1_at	Cold acclimation induced protein	21.5	8.2
Ta.18487.2.S1_at	Cold-responsive protein	18.3	-3.9
Ta.28917.2.S1_a_at	Cold acclimation protein WCOR518	13.2	-2.7
TaAffx.132296.1.A1_	Cold-responsive protein	13.0	-3.2
Ta.122.1.S1_x_at	Cold acclimation protein WCOR410b	12.3	4.1
TaAffx.3462.1.S1_at	Cold acclimation induced protein	10.0	6.3
Ta.18487.1.S1_x_at	Cold-responsive protein	9.3	-18.7
TaAffx.107864.1.S1_	Cold acclimation induced protein	8.6	2.9
Ta.1138.1.S1_at	Cold-responsive LEA/RAB-related	8.3	-3.1
Ta.28917.1.S1_at	Cold acclimation protein WCOR518	7.8	-7.0
Ta.7479.1.S1_a_at	Cold-regulated protein	3.4	-18.0
Ta.7479.3.S1_x_at	Cold-regulated protein	3.2	-11.4
TaAffx.5352.1.S1_at	Cold acclimation WCOR413	2.5	2.3
Ta.19248.1.S1_at	Cold acclimation protein WCOR413	2.4	2.6
Ta.6123.1.A1_s_at	**70kDa heat shock protein**	-2.3	**279.7**
TaAffx.18332.1.S1_at	70kDa heat shock protein	-2.1	202.8
Ta.22973.1.S1_x_at	Small heat shock protein HSP17.8	3.3	163.9
Ta.27661.1.S1_at	17.4 kDa class I heat shock protein	-2.3	74.7
Ta.6964.1.S1_at	Chaperonin CPN60	-2.2	17.8
Ta.28758.1.S1_at	Heat shock protein 101	-2.8	16.8
Ta.2934.2.S1_a_at	Heat shock protein Hsp70	-3.4	5.2
Ta.10203.1.S1_at	Heat shock protein DnaJ	-2.1	4.4
Ta.9424.2.S1_at	Hsp70 binding protein	2.4	3.7
Ta.6380.1.A1_at	Ubiquinone biosynthesis protein	66.6	3.1
Ta.9548.1.S1_a_at	Ubiquitin-conjugating enzyme	4.8	2.6
Ta.11133.1.S1_at	LMW heat shock protein	5.3	2.6
Ta.24065.1.A1_x_at	LMW heat shock protein	5.0	2.2

Under the cold stress treatment, all transcripts related with cold acclimation, responsive and regulated proteins were induced significantly, whereas most of them were down-regulated under the heat stress conditions. This clearly indicates that the pattern of gene expression during cold stress was very dissimilar to the heat shock response (Table 3.14). Although common probe sets related with heat shock proteins were up-regulated after the heat stress condition, no significant change was observed after the cold stress treatment for heat shock proteins. It can be concluded that transcript levels of heat shock proteins increased as a result of sudden temperature jump to protect proteins from high temperature effects.

Abscisic acid (ABA) is a plant hormone which regulates transpiration, stress responses including drought, cold, and heat stresses and germination of seeds. The identification of ABA-associated genes from wheat is significant for gaining better insights about the cold and heat stress-response mechanisms. ABA-related genes were significantly induced under both stress treatments (Table 3.15). So, because of interactions with other phytohormones and transcription factors, ABA plays a crucial role in response to plant abiotic stresses.

Table 3.15. Significantly up- and down-regulated common transcripts related with ABA-associated genes. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
Ta.27945.1.S1_x_at	ABA-responsive protein	63.5	43.8
Ta.16038.1.S1_at	Abscisic acid-induced-like protein	51.0	7.4
TaAffx.128488.2.S1_s_at	Abscisic acid-induced-like protein	35.4	3.9
Ta.13907.2.S1_a_at	Abscisic acid-induced-like protein	34.9	2.0

Transcription factors differentially regulated under the both stress conditions were listed in Table 3.16. They were bZIP transcription factors, C_2H_2 zinc finger proteins, EF hand family proteins, Late embryogenesis abundant proteins, MYB family transcription factors, NAC domain-containing protein and WRKY-type transcription factors.

The basic leucine zipper motif (bZIP) regulates plant developmental and physiological processes and abiotic and biotic stresses responses (Jakoby *et al*., 2002; Correa *et al*., 2008). The quick response of wheat plants to cold stress resulted in induction of bZIP transcription factors. However, their responses were different under the heat stress condition.

In plants, like plant hormone ABA, calcium (Ca^{2+}) is also an important messenger which mediates the action of many hormonal and environmental signals, including biotic and abiotic stresses (Day *et al*., 2002). EF-hand-containing proteins are Ca^{2+}-binding proteins in plants. Up-regulation of these proteins after the cold stress treatment showed that EF-hand-containing proteins had a central role to deal with cold stress.

MYB transcription factors are one of the most abundant transcription factor families in plants and have a certain role in defense mechanisms, stress responses and in hormone signaling. With the other type pf transcription factors including NAC type transcription factors and DRE-binding factors 2 (DREB2), MYB transcription factors were also significantly induced after the low temperature application. On the other hand, their regulations reduced after the high temperature application. So, it can be concluded that heat shock proteins were main protective agents against high temperature shifts rather than transcription factors. However, wheat plants mainly response to low temperature stress condition with transcription factors.

Table 3.16. Significantly up- and down-regulated common transcripts related with transcription factors. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
TaAffx.18447.3.S1_s_at	**bZIP transcription factor**	**37.1**	**-7.5**
Ta.30801.1.S1_s_at	bZIP transcription factor	3.9	-2.8
TaAffx.130052.2.S1_at	**C_2H_2 zinc finger protein**	**13.0**	**2.6**
TaAffx.137523.1.A1_at	C_2H_2 zinc finger protein	6.5	3.3
TaAffx.128894.1.S1_at	C_2H_2 zinc finger protein	6.5	-2.6
TaAffx.110394.1.S1_at	Ca^{2+}/H^{+}exchanging protein	-4.4	-2.6
Ta.320.2.S1_a_at	Calcium-binding protein	16.3	-2.4
Ta.5511.1.S1_at	**EF hand family protein**	**67.3**	**4.4**
Ta.2882.1.S1_at	EF-hand Ca^{2+} binding protein	29.1	2.4
Ta.2882.1.S1_s_at	EF-hand Ca^{2+} binding protein	18.5	2.0
TaAffx.108538.1.S1_at	Homeobox-leucine zipper protein	8.2	2.1
Ta.1814.1.S1_at	**Late embryogenesis abundant**	**34.5**	**-2.6**
Ta.9718.1.S1_at	Late embryogenesis abundant	34.2	-15.5
Ta.449.1.S1_at	Late embryogenesis abundant	6.4	4.4
Ta.13811.1.S1_at	**MYB family transcription factor**	**25.2**	**-4.2**
Ta.25920.1.A1_at	MYB related protein	18.4	-2.4
Ta.7524.2.A1_a_at	MYB family transcription factor	16.1	-4.2
Ta.7524.1.A1_at	MYB family transcription factor	10.7	-6.6
Ta.27337.1.S1_at	MYB29 protein	9.1	-4.7
Ta.11849.1.S1_at	MYB family transcription factor	8.5	-2.4
Ta.27013.1.S1_at	MYB family transcription factor	7.3	-6.0
Ta.26049.1.S1_a_at	MYB family transcription factor	5.3	3.3
TaAffx.57480.1.S1_at	MYB family transcription factor	-4.7	-2.7
Ta.5367.2.S1_x_at	**NAC domain-containing protein**	**23.0**	**3.8**
Ta.28539.1.A1_x_at	NAC domain-containing protein 67	22.2	3.5
Ta.5367.1.S1_s_at	NAC domain-containing protein 67	7.9	3.1
Ta.16423.1.S1_at	NAC domain protein NAC1	-10.4	-5.6
Ta.961.2.S1_a_at	**WRKY DNA binding domain**	**8.6**	**3.0**
Ta.4678.2.S1_at	WRKY4 transcription factor	8.8	3.2
Ta.5600.1.S1_a_at	**Zinc finger protein**	**11.8**	**-2.0**
Ta.12400.1.A1_at	Zinc-finger protein	6.9	2.8
Ta.17381.1.S1_at	Zinc-finger protein	-19.2	-10.7

Some protective enzymes were also involved in response to cold and heat stresses. Glutathione S-transferase (GST) is an antioxidant enzyme which detoxifies herbicide and xenobiotic compounds in plants by covalently connecting glutathione to a hydrophobic substrate, resulting in formation of less reactive and more polar gluatathione s-conjugate. Wheat microarray analysis showed that they had no significant influences on cold and heat stresses. Although cytochrome P450 is a detoxification enzyme for pesticides and industrial pollutants, especially under the heat stress condition genes encoding for cytochrome P450 were significantly up-regulated (Table 3.17).

Table 3.17. Significantly up- and down-regulated common transcripts related with antioxidant enzymes. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
TaAffx.110629.1.S1_at	**Glutathione S-transferase**	**-2.4**	**-10.0**
TaAffx.110629.1.S1 _at	Glutathione S-transferase	-2.3	-9.3
Ta.8545.1.S1_at	Glutathione S-transferase	5.5	-8.0
Ta.303.2.S1_x_at	Glutathione S-transferase	2.3	-6.8
Ta.303.3.S1_x_at	Glutathione S-transferase	2.2	-6.6
Ta.3418.1.S1_x_at	Glutathione S-transferase	-2.5	-3.1
Ta.3418.2.S1_x_at	Glutathione S-transferase	-2.3	-3.1
Ta.28354.4.S1_at	Glutathione S-transferase	3.8	-2.6
TaAffx.108452.1.S1_at	Glutathione S-transferase	4.7	2.0
Ta.27096.1.S1_at	Glutathione-S-transferase	3.3	2.6
Ta.1457.1.S1_x_at	Glutathione S-transferase	2.4	3.8
Ta.1457.1.S1_s_at	Glutathione S-transferase	3.3	5.5
Ta.24106.1.S1_x_at	**Peroxidase 2**	**-4.5**	**-8.3**
Ta.29531.2.S1_x_at	Peroxidase	-3.6	-2.7

Table 3.17. (continued)

Probe Set	Putative Annotation	FC	
		Cold	Heat
Ta.21438.1.A1_at	**Cytochrome P450**	**2.4**	**92.8**
TaAffx.116369.1.S1_at	Cytochrome P450	2.3	22.1
Ta.25469.1.S1_at	Cytochrome P450	15.2	4.4
Ta.13841.1.S1_at	Cytochrome P450	9.4	2.8
TaAffx.1119.1.A1_at	Cytochrome P450	16.1	-2.1
TaAffx.12575.1.A1_at	Cytochrome P450	3.0	-2.3
Ta.9806.1.S1_x_at	Cytochrome P450	4.4	-2.5
Ta.303.2.S1_at	Cytochrome P450	2.1	-6.5

After treatment of wheat seedling with low and high temperature stresses, genes associated with main degradation enzymes in different metabolic pathways were also examined. Genes encoding for amylase enzymes were significantly induced under both stress applications. This enzyme breaks down starch into monosaccharide sugar molecules which are used for production of soluble sugars as an osmolytes in response to osmotic stress. The cold stress causes the initiation of freeze-induced dehydration. Besides cold regulation proteins and transcription factors, one of the mechanisms to deal with cold stress was up-regulation of amylase genes which provides synthesis of osmolytes for resistance to cold stress condition (Table 3.18).

According to microarray results, dehydrin proteins belonging to group 2 LEA proteins (Saavedra *et al*., 2006), were also significantly up-regulated after both stress treatments (Table 3.18). High level production of dehydrin proteins is associated with low temperatures and drought stress. They may act as a membrane stabilizer during freeze induced dehydration and may protect membranes from damage (Puhakainen *et al*., 2004).

Phenylalanine ammonia lyase (PAL) is a key enzyme which responsible for synthesis of precursor of various phenylpropanoids including lignins, coumarins and

flavonoids (Kostenyuk *et al*., 2002). Microarray analysis revealed that increase in PAL gene expression level was closely associated with the low temperature application. So, it can be deduced that another protection mechanism against the cold stress is production of phenylpropanoids.

Lipoxygenases (LOXs) are dioxygenases generally found in plants. They add molecular oxygen to polyunsaturated fatty acids to produce an unsaturated fatty acid. Linolenic and linoleic acids are the most commonly used substrates for LOX in plant cells (Porta and Rocha-Sosa, 2002). According to microarray data, especially under the heat stress, gene expression level of lipoxygenases was significantly reduced (Table 3.18). This may result in accumulation of unsaturated fatty after both stress treatments. So, it can be concluded that the proportion of unsaturated fatty acids tends to increase in the plasma membrane during low and high temperature stresses.

The 12-oxo-phytodienoic acid reductase (OPRs) is an enzyme which is a part of the octadecanoid pathway in which conversion of linolenic acid to the plant hormone jasmonic acid occurs (Zhang *et al*., 2005). Expression profile of OPRs indicates that under both stress conditions, genes associated with this enzyme were significantly induced (Table 3.18). Together with giberellic acid, biosynthesis of jasmonic acid was also induced in response to cold and heat stresses.

Table 3.18. Significantly up- and down-regulated common transcripts related with main degradation enzymes in different pathways. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
Ta.25274.1.A1_x_at	Acid beta-fructofuranosidase	44.0	-2.1
Ta.10389.1.S1_a_at	Aldose reductase	20.7	-2.1
Ta.10281.1.S1_a_at	Alpha-amylase	3.1	2.1
Ta.16135.1.A1_at	**Beta-amylase**	**18.8**	2.6
TaAffx.68872.1.S1_at	Beta-amylase	17.9	3.4
Ta.4494.1.S1_x_at	Beta-amylase	8.5	2.2
Ta.4035.3.S1_at	Beta-glucosidase	45.9	9.6
Ta.12118.1.S1_a_at	Beta-glucosidase	-76.1	-76.1
TaAffx.131747.1.S1_at	**Dehydrin**	**75.7**	2.1
TaAffx.46097.2.S1_at	Dehydrin	15.5	8.0
TaAffx.45258.1.A1_at	Dehydrin	10.0	2.7
Ta.2704.1.S1_at	Dehydrin	9.5	13.6
Ta.22333.1.S1_at	**Fatty acyl coA reductase**	-26.7	**-178.5**
Ta.12457.1.S1_x_at	Fatty acid hydroxylase	-20.1	-2.2
TaAffx.98909.1.A1_at	NADP-dependent oxidoreductase	30.9	-5.5
TaAffx.98909.1.A1_at	NADP-dependent oxidoreductase	25.5	-2.5
Ta.14421.1.S1_at	NADP-dependent malic enzyme	6.5	-3.2
TaAffx.92008.1.A1_at	**Phenylalanine ammonia-lyase**	**196.7**	-2.4
Ta.7934.2.S1_x_at	Phenylalanine ammonia-lyase	164.3	2.3
Ta.20429.1.S1_at	Phenylalanine ammonia-lyase	73.2	-3.0
Ta.1967.2.A1_x_at	**Lipoxygenase**	-2.5	**-108.5**
Ta.1967.1.S1_x_at	Lipoxygenase	-3.3	-73.6
TaAffx.104812.1.S1_at	Lipoxygenase	-2.9	-15.5
Ta.23763.1.S1_at	Lipoxygenase	-2.3	-14.5
Ta.5408.1.S1_at	Serine/threonine protein kinase	38.8	4.8
Ta.11120.1.S1_a_at	Serine/threonine protein kinase	38.2	-4.1
Ta.2788.1.A1_at	Sucrose 1-fructosytransferase	77.7	3.0
Ta.2657.1.S1_s_at	UDP-glucose 6-dehydrogenase,	18.5	-3.9
Ta.2657.1.S1_x_at	UDP-glucose dehydrogenase	15.0	-2.9
Ta.1207.1.S1_at	**12-oxophytodienoic acid reductase**	**155.8**	3.9
Ta.1207.1.S1_s_at	12-oxophytodienoate reductase	76.6	6.1
Ta.1207.1.S1_x_at	12-oxophytodienoic acid reductase	52.6	4.2

Low and high temperature stress treatments influenced some genes related with photosynthesis. Among them, genes encoding for chlorophyll a-b binding protein and ELIPs were mostly affected from these stress conditions. High temperature causes the reduction of photosynthesis. Also microarray results support reduction in photosynthesis due to heat stress application. All probe sets associated with chlorophyll a-b binding protein were down-regulated under the heat stress condition whereas they were induced after the low temperature treatment (Table 3.19).

Table 3.19. Significantly up- and down-regulated common transcripts related with photosynthesis/light reaction. Probe set ID, putative annotation of each transcript and fold change (FC) are listed. Values represent fold changes of transcripts after cold and heat stresses compared to normal growth. Negative values show down-regulation and positive values show up-regulation.

Probe Set	Putative Annotation	FC	
		Cold	Heat
Ta.22984.2.S1_x_at	**Chlorophyll a/b-binding protein**	**4.1**	**-106.0**
Ta.22984.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	3.9	-87.2
Ta.28496.1.A1_x_at	Chlorophyll a-b binding protein 2	8.0	-37.6
Ta.30702.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	7.5	-29.8
Ta.29587.3.A1_at	Chlorophyll a-b binding protein	2.5	-27.4
Ta.22101.1.A1_at	Chlorophyll a-b binding protein	4.7	-7.0
Ta.3249.2.S1_x_at	Chlorophyll a/b-binding protein WCAB	3.0	-6.5
Ta.4346.1.A1_x_at	Chlorophyll a-b binding protein,	4.5	-6.2
Ta.3795.1.S1_x_at	Chlorophyll a/b-binding protein WCAB	5.0	-4.4
TaAffx.128506.1._at	Chlorophyll a/b-binding protein WCAB	2.3	-3.6
Ta.30494.1.A1_at	Chlorophyll a/b-binding protein WCAB	4.7	-2.2
TaAffx.114127.1._at	Chlorophyll a/b-binding protein WCAB	15.7	-2.1
TaAffx.128795.28._at	Cytochrome b/f complex subunit 5	4.8	-3.2
Ta.9600.1.S1_x_at	**Low molecular mass ELIP**	**1787.6**	**63.3**
Ta.26997.1.S1_at	**High molecular mass ELIP**	**59.4**	**11.2**
TaAffx.128566.1._at	**Photosystem II protein W protein**	**29.4**	**-2.9**
TaAffx.128757.3._at	Photosystem II thylakoid membrane	10.8	-2.8
TaAffx.128757.1._at	Photosystem Q(B) protein	10.4	-2.3

CHAPTER 4

CONCLUSIONS

Plant growth, development and productivity are adversely affected from environmental stresses, resulting in reduction of average yields for most of economically important crop plants. Any conventional, biotechnological or 'omics' technologies have therefore concentrated on increasing the grain yield, quality characteristics and minimizing crop loss coming from biotic and abiotic stress conditions.

This study was mainly focused on examination of expression profiling of NAC-type transcription factors under the severe environmental stresses. *TaNAC69-1* gene provides salt, drought and cold tolerance for plants. On the other hand, *TtNAMB-2* gene greatly influences grain protein content, Fe, and Zn concentrations in wheat plant. To develop stress resistant transgenic plants, NAC-type transcription factor genes, *TaNAC69-1* and *TtNAMB-2* were firstly isolated from *T.aestivum* and *T.turgidum*, respectively. Then they were cloned into different monocot and dicot expression vectors to be used for further wheat and tobacco genetic transformation. The time periods expression profiles of *TaNAC69-1* and *TtNAMB-2* genes under drought, salt, cold and heat stresses were determined using quantitative Real-time PCR. It was found that, the *TaNAC69-1* gene was significantly up-regulated by salt, drought, cold and heat stresses, which indicate that *TaNAC69-1* gene, may participate in responses to these environmental stimuli. The present results suggest that there is significant cross-talk in the expression of *TaNAC69-1* gene under abiotic stress conditions. So, it is assumed that the *TaNAC69-1* transcription factor might be connecting element involved in cross-talk between stress signaling pathways.

Although *TtNAMB-2* gene is related with enhancement of grain protein content, expression patterns of *TtNAMB-2* gene under drought, salt, cold and heat stresses were also inspected to indicate a role in plant response to abiotic stresses. Except for heat stress, expression of *TtNAMB-2* gene significantly induced under drought, salt and cold stress conditions. Besides its effect of grain protein content, *TtNAMB-2* gene can be considered as transcriptional activator involved in wheat response to abiotic stresses.

In this study, it was also aimed to explore effect of cold and heat stress treatments on global gene expression profiles of wheat plant. Analysis of transcriptome profile shows that different expression patterns were obtained after the cold and heat stress applications, which directly affect transcriptional network associated with defense, metabolism and development. Among them, LEA, WCOR, WCAB, Transcription Factors (ARF, AP2/EREBP, bZIP, C_2H_2-type zinc finger, DRE, NAC, MYB and WRKY families), 1-pyrroline-5-carboxylate synthetase, glutathione-S-transferase, ferittin, heat shock proteins, and cytochrome P450s were differentially regulated in response to cold and heat stress conditions. Many cold stress-responsive genes including COR and LEA homolog genes and different cold acclimation proteins (WSCs and WCORs) were extremely up-regulated following cold acclimation period. WSCs and WCORs triggered the expression of downstream genes such as recrystallization (IRI) inhibition genes. Approximately 40 different heat shock protein transcripts were induced following heat stress period. Besides small heat shock proteins, expression level of other heat shock proteins significantly increased. The fold changes in their expression levels ranged from 850 to 20, which indicate that heat shock proteins played a central role in response to heat stress treatment in wheat plant. In addition to these protective proteins, transcription factors also played a central role to deal with severe effects of low and high temperature conditions. Up- and down regulation of these transcription factors may activate or repress different downstream target genes that may contribute to the stress response.

About 2550 common probe sets were differentially regulated under both cold and heat stress conditions in wheat seedlings. Their annotation results indicate that they

were chiefly associated with cold and heat stresses responsive and protective genes, transcription factors, antioxidant enzymes and main degradation enzymes in carbohydrate, fatty acid/lipid, nitrogen, amino acid metabolism and photosynthesis. As a protective agent in wheat seedlings, gene expression levels of cold acclimation proteins and heat shock proteins significantly increased after low and high temperature treatments, respectively. Abscisic acid (ABA) has interactions with other phytohormones and transcription factors and plays central role in response to plant abiotic stresses.

The next goal of this research will investigate the link between NAC-type transcription factors and their downstream target genes using all the microarray data produced in this study. Understanding function of these candidate genes will provide development of agriculturally important crop plants, resistant to environmental stresses like salt, drought and cold. Also identification of molecular markers for marker assistant selection will be another further aim of this research. Using microarray data, genes which show similar expression patterns under the cold and heat stress conditions will be selected as a molecular marker candidate. Their DNA sequences and expression changes will be compared with data from different abiotic stress conditions. So, molecular markers associated with abiotic stress tolerant will be identified. Development of such a valuable molecular markers will allow production of agriculturally important crop plants with abiotic stress tolerance.

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APPENDIX A

COMPOSITIONS OF BACTERIAL CULTURE MEDIA

Luria-Bertani Broth (1 L)

Yeast extract	5 g
Tryptone	10 g
NaCl	10 g
Bacterial agar	15 g

The pH of the medium is adjusted to 7.4 and autoclaved at 121°C for 20 minutes.

S.O.C. Medium

Tryptone	2%
Yeast extract	0.5%
NaCl	10 mM
KCl	2.5 mM
MgCl2	10 mM
MgSO4	10 mM
Glucose	20 mM

The pH of the medium is adjusted to 7.0 and autoclaved at 121°C for 20 minutes. Add the sterile glucose immediately before use.

YEB Medium (1 L)

Nutrient broth	13.5 g
Yeast extract	1 g
Sucrose	5 g
$MgSO_4.7(H_2O)$	0.493 g

The pH of the medium is adjusted to 7.2 and autoclaved at 121°C for 20 minutes.

APPENDIX B

HOAGLAND'S SOLUTION

Composition	Stock solution	mL Stock solution /1L
2M KNO_3	202g/L	2.5
2M $Ca(NO_3)_2x4H_2O$	236g/0.5L	2.5
FeEDTA	15g/L	1.5
2M $MgSO_4x7H_2O$	493g/L	1
1M NH_4NO_3	80g/L	1
1M KH_2PO_4	136g/L	0.5

Minors:

H_3BO_3	2.86g/L
$MnCl_2x4H_2O$	1.81g/L
$ZnSO_4x7H_2O$	0.22g/L
$CuSO_4$	0.051g/L
$Na_2MoO_4x2H_2O$	0.09g/L

Make up stock solutions and store in separate bottles with appropriate label. Add each component to 800mL deionized water then fill to 1L.

APPENDIX C

TAGUCHI ARRAYS

Concentration levels for components used in a Taguchi array. Components and different levels are shown as numbers and letters, respectively.

Reaction	[1]	[2]	[3]	[4]
1	A	A	A	A
2	A	B	B	B
3	A	C	C	C
4	B	A	B	C
5	B	B	C	A
6	B	C	A	B
7	C	A	C	B
8	C	B	A	C
9	C	C	B	A

APPENDIX D

BUFFERS FOR PREPARATION OF COMPETANT E.coli CELLS

Transformation buffer I (200 mL)

Compound	Amount	Final molarity/conc.
Potassium acetate	0.588 g	30 mM
Rubidium chloride	2.42 g	100 mM
Calcium chloride	0.294 g	10 mM
Glycerol	30 ml	87% v/v

Transformation buffer II (100 mL)

Compound	Amount	Final molarity/conc.
MOPS	0.21 g	10 mM
Calcium chloride	1.1 g	75 mM
Rubidium chloride	0.121 g	10 mM
Glycerol	15 ml	87% v/v

The pH of the medium is adjusted to 6.5 and performed filter sterilization.

APPENDIX E

TaNAC69-1 and *TtNAM-B2* GENES mRNA SEQUENCES

TaNAC69-1 mRNA SEQUENCE

```
LOCUS       AY625682               1423 bp    mRNA    linear   PLN 03-MAR-
2005
DEFINITION  Triticum aestivum NAC domain transcription factor (NAC69-1)
mRNA,
            complete cds.
ACCESSION   AY625682
VERSION     AY625682.1  GI:51702423
KEYWORDS    .
SOURCE      Triticum aestivum (bread wheat)
ORGANISM    Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 1423)
AUTHORS     Xue,G.P.
TITLE       A CELD-fusion method for rapid determination of the DNA-binding
            sequence specificity of novel plant DNA-binding proteins
JOURNAL     Plant J. 41 (4), 638-649 (2005)
PUBMED      15686526
REFERENCE   2  (bases 1 to 1423)
AUTHORS     Xue,G.P.
TITLE       NAC domain transcription factor from wheat
JOURNAL     Unpublished
REFERENCE   3  (bases 1 to 1423)
AUTHORS     Xue,G.P.
TITLE       Direct Submission
JOURNAL     Submitted (17-MAY-2004) Plant Industry, CSIRO, 306 Carmody Rd.,
St.Lucia, Brisbane, Qld 4067, Australia

FEATURES             Location/Qualifiers
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                     /gene="NAC69-1"
                     /note="TaNAC69"
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ORIGIN

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TtNAM-B2 mRNA SEQUENCE

```
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2006
DEFINITION  Triticum turgidum subsp. durum NAC transcription factor NAM-B2
            (NAM-B2) gene, complete cds.
ACCESSION   DQ869676
VERSION     DQ869676.1  GI:115336266
KEYWORDS    .
SOURCE      Triticum turgidum subsp. durum (durum wheat)
ORGANISM    Triticum turgidum subsp. durum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta;
Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 1498)
AUTHORS     Uauy,C., Distelfeld,A., Fahima,T., Blechl,A. and Dubcovsky,J.
TITLE       A NAC Gene regulating senescence improves grain protein, zinc,
and iron content in wheat
JOURNAL     Science 314 (5803), 1298-1301 (2006)
PUBMED      17124321
REFERENCE   2  (bases 1 to 1498)
AUTHORS     Uauy,C., Distelfeld,A., Fahima,T., Blechl,A. and Dubcovsky,J.
TITLE       Direct Submission
JOURNAL     Submitted (25-JUL-2006) Department of Plant Sciences,
University                              of California at Davis, One
Shields Ave, Davis, CA 95616, USA
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ARASPAPTKQFLAPSSSTPFNWLDASTVGILPQARNFPGFNRSRNVGNMSLSSTADMA

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ORIGIN

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APPENDIX F

MELT CURVE ANALYSIS OF ALL THE qRT-PCR

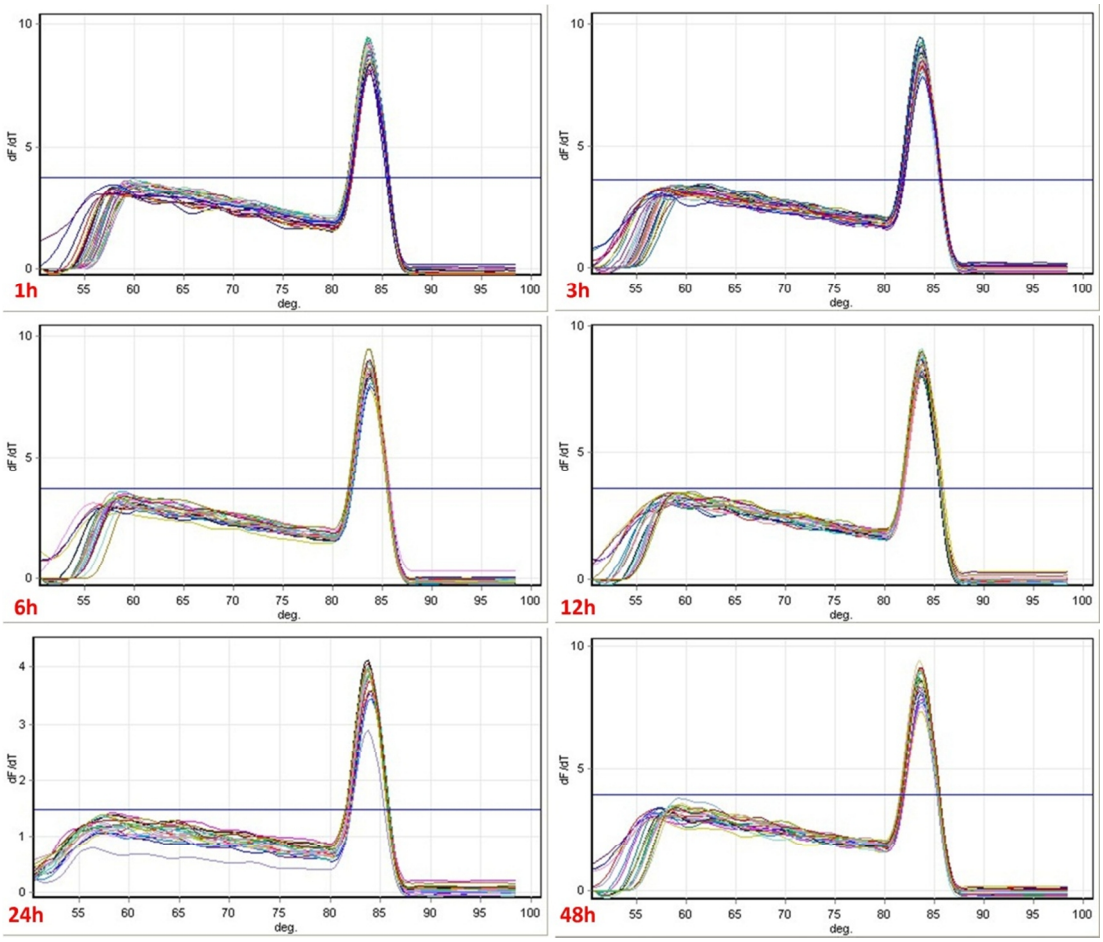

Figure F.1. Melting curves of *TaNAC69-1* gene under the both salt and drought stress conditions.

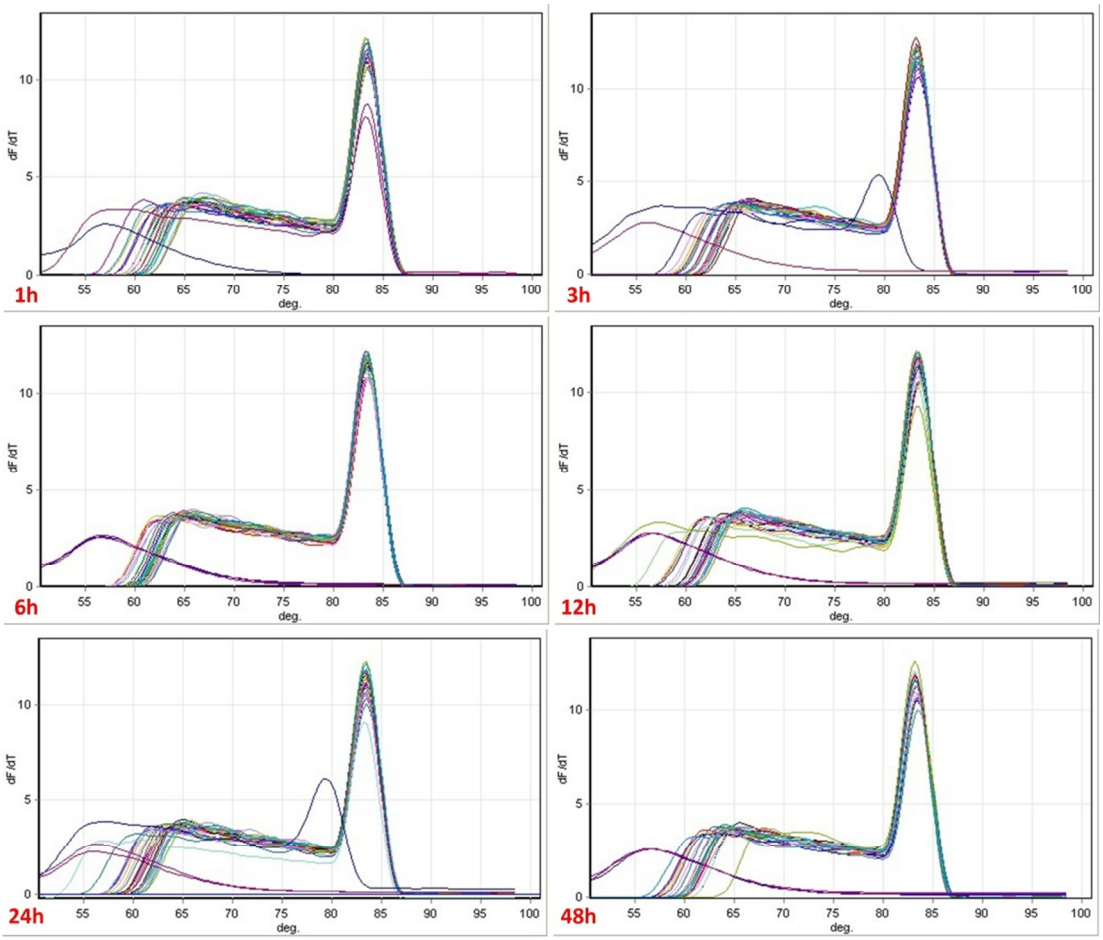

Figure F.2. Melting curves of *TaNAC69-1* gene under the both cold and heat stress conditions.

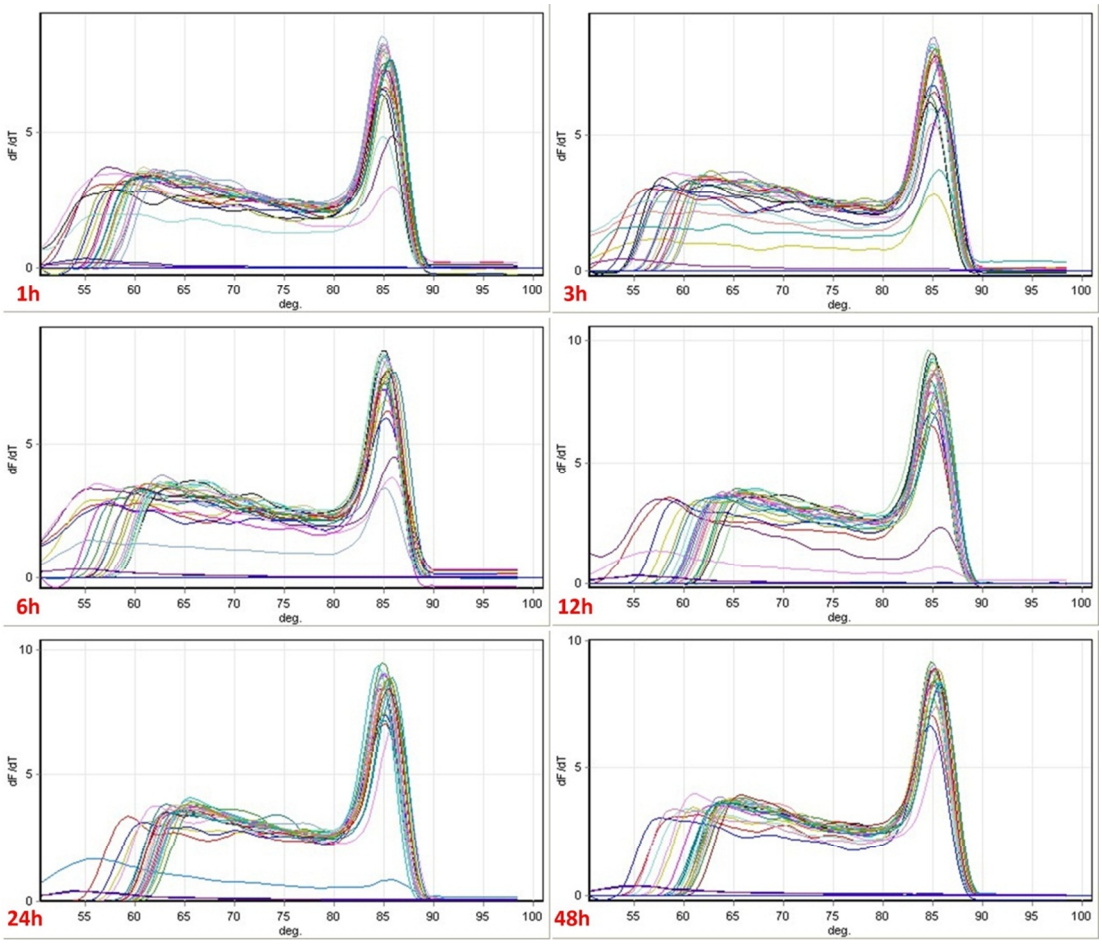

Figure F.3. Melting curves of *TtNAMB-2* gene under the both salt and drought stress conditions.

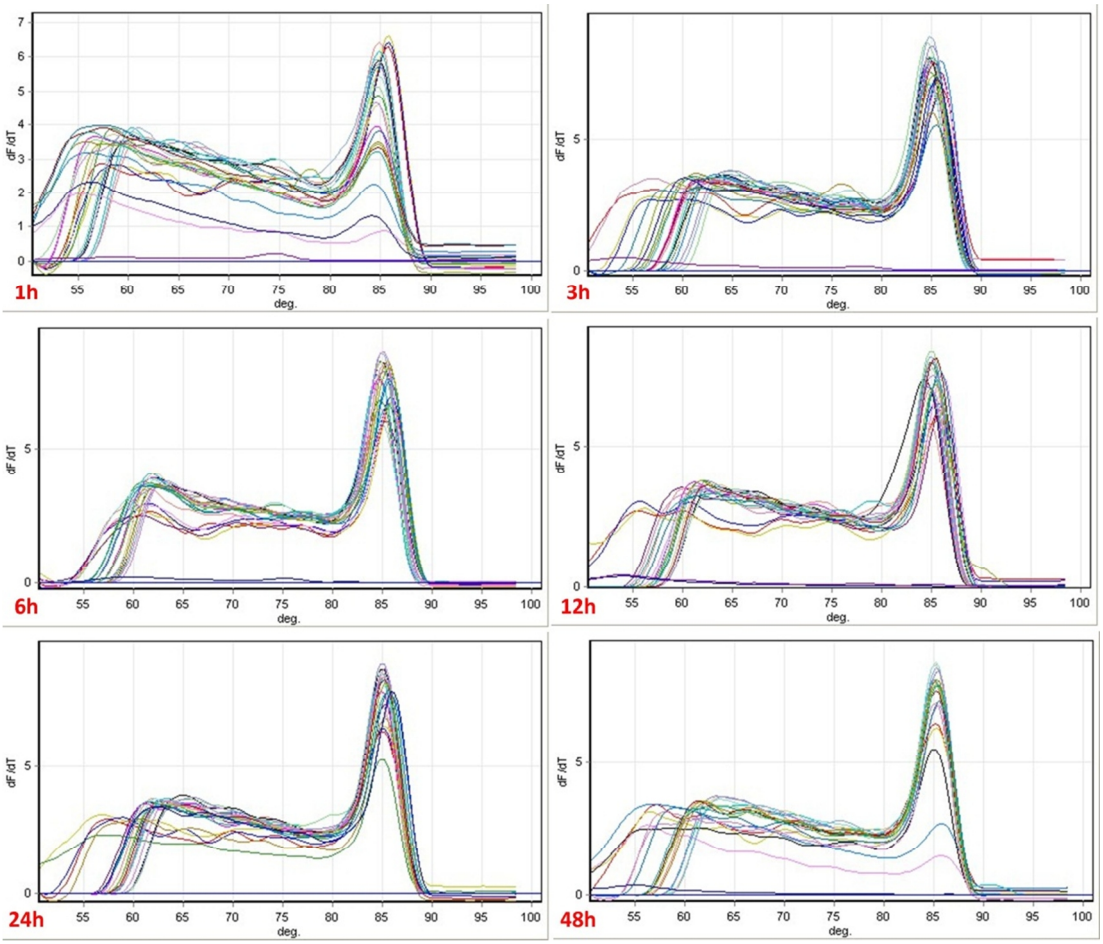

Figure F.4. Melting curves of *TtNAMB-2* gene under the both cold and heat stress conditions.

CURRICULUM VITAE

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EDUCATION

Degree	Institution	Year of Graduation
MS	Middle East Technical Uni. Biology Department	2005
BS	Ankara Uni. Biology Department	2002

WORK EXPERIENCE

Year	Place	Enrollment
2002-present	METU Biology Department	Research assistant

PUBLICATIONS

Baloglu MC, Yücel M, Öktem HA. (2007) Assessment of Conditions Influencing Agrobacterium mediated Transient Expression of *uidA* Gene in Leaf disks of Sugarbeet (*Beta vulgaris*). Cell, Genetics and Molecular Biology (formely Journal of Genetics and Molecular Biology) Vol. 18, No. 3, 169-178.

Baloglu MC, Negre-Zakharov F, Öktem HA, Yücel M. (2011) Molecular Cloning, Characterization and Expression Analysis of a Gene Encoding a Ran Binding Protein (RanBP) in *Cucumis melo* L., Turkish Journal of Biology, 35:387-397.

Gurel S, **Baloglu MC**, Gurel E, Öktem HA Yücel M. (2011) A two-stage pretreatment of seedlings improves adventitious shoot regeneration in sugar beet (*Beta vulgaris* L.). Plant Cell Tiss Organ Cult 106:261–268.

BOOK

Bayrac AT, **Baloglu MC**, Kalemtas G, Kavas M. (2007) Genetiği Değiştirilmiş Organizmalar, MetuPress.

PROJECTS

TÜBİTAK TOVAG 108O786 Nolu TÜBİTAK Projesi: Nac Tipi Transkripsiyon Faktörleri Kullanılarak Abiyotik Stres Dirençli Transgenik Buğday Çeşitlerinin Geliştirilmesi ve Elde Edilen Bitkilerde Abiyotik Stres Koşullarında Gen İfade Profillerinin Mikroarray Yöntemiyle İncelenmesi, 2009-2011.