TRANSFORMATION OF TOBACCO WITH A NAC TYPE TRANSCRIPTION FACTOR, TANAC69-1 AND CHARACTERIZATION OF TRANSGENIC PLANTS VIA MOLECULAR AND PHYSIOLOGICAL TECHNIQUES

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

AYTEN EROĞLU

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

MARCH 2015

Approval of the Thesis

TRANSFORMATION OF TOBACCO WITH A NAC TYPE TRANSCRIPTION FACTOR, TANAC69-1 AND CHARACTERIZATION OF TRANSGENIC PLANTS VIA MOLECULAR AND PHYSIOLOGICAL TECHNIQUES

submitted by **AYTEN EROĞLU** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy in Biotechnology Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _______________
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Filiz B. Dilek _______________
Head of the Department, **Biotechnology**

Prof. Dr. Meral Yücel _______________
Supervisor, **Biology Dept., METU**

Prof. Dr. Hüseyin Avni Öktem _______________
Co-Supervisor, **Biology Dept., METU (Retired)**

Examining Committee Members

Assoc. Prof. Dr. Sertaç Önde _______________
Biology Dept., METU

Prof. Dr. Meral Yücel _______________
Biology Dept., METU

Prof. Dr. Yasemin Ekmekçi _______________
Biology Dept., Hacettepe University

Prof. Dr. Füsun İnci Eyidoğan _______________
Education Dept., Başkent University

Assist. Prof. Dr. Mehmet Cengiz Baloğlu _______________
Genetics and Bioengineering Dept., Kastamonu Uni.

Date: _______________

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

Name, Last name: Ayten EROĞLU

Signature :

ABSTRACT

TRANSFORMATION OF TOBACCO WITH A NAC TYPE TRANSCRIPTION FACTOR, TANAC69-1 AND CHARACTERIZATION OF TRANSGENIC PLANTS VIA MOLECULAR AND PHYSIOLOGICAL TECHNIQUES

Eroğlu, Ayten

Ph.D., Department of Biotechnology
Supervisor: Prof. Dr. Meral Yücel
Co-Supervisor: Prof. Dr. Hüseyin Avni Öktem

March 2015, 123 pages

Environmental stresses such as drought and salinity greatly affect plant production leading to reduction in yield and quality. Stress tolerant plants can be rapidly produced by transferring stress related genes or transcription factors to crop plants.

In this study, *TaNAC69-1* gene, a NAC type transcription factor, was isolated from *Triticum aestivum* L. cv. Yüreğir-89 and cloned into a Gateway compatible overexpression vector, pEarleyGate 100. Transformation of *Nicotiana tabacum* L. cv. Samsun leaves was carried out via Agrobacterium-mediated transformation method. A total of fourty five T_0 putative transgenic tobacco lines were obtained at the end of independent transformation experiments. Stable expression and integration of *TaNAC69-1* gene into the tobacco genome were evaluated by molecular, biochemical and physiological analysis. Traditional PCR was made by using gene specific and *bar* gene specific primers to screen transgenes. Stable integration of *TaNAC69-1* gene into the genome was also confirmed by Southern blot analysis.

Expression of *TaNAC69-1* gene was examined by qRT-PCR with different time periods under salt and drought stress conditions using *actin* as a reference gene. It was found that drought and salt treatments led to significant up-regulation of *TaNAC69-1* gene expression. Physiological assays such as germination, root and shoot lengths, fresh and dry weights were achieved to compare stress tolerance of wild type and transgenic tobacco lines under salt and osmotic stress conditions. Germination and survival rates of transgenic tobacco seeds were significantly higher than wild type seeds when subjected to NaCl and LiCl treatment. Root and shoot lengths, fresh and dry weights of transgenic plants significantly increased when compared to wild type plants with application of NaCl and LiCl. On the other hand there was no difference between growth parameters of wild type and transgenic lines with mannitol treatment. According to molecular and physiological analysis, overexpression of *TaNAC69-1* gene increased salt and ionic stress tolerance of transgenic tobacco plants. Further experiments should be done with cold and heat stress treatments to better elucidate the function of *TaNAC69-1*gene under abiotic stress conditions.

Keywords: *TaNAC69-1*, Agrobacterium-mediated gene transfer, transgenic tobacco, Southern blot, qRT-PCR, abiotic stress.

ÖZ

TÜTÜNÜN NAC TİPİ BİR TRANSKRİPSİYON FAKTÖRÜ OLAN TANAC69-1 GENİ İLE TRANSFORMASYONU VE TRANSGENİK BİTKİLERİN MOLEKÜLER VE FİZYOLOJİK YÖNTEMLERLE KARAKTERİZE EDİLMESİ

Eroğlu, Ayten

Doktora, Biyoteknoloji Bölümü
Tez Yöneticisi: Prof. Dr. Meral Yücel
Ortak Tez Yöneticisi: Prof. Dr. Hüseyin Avni Öktem

Mart 2015, 123 sayfa

Tuzluluk ve kuraklık stresi bitkilerde verim ve kalite azalmasına yol açarak bitkisel üretimi büyük ölçüde etkilemektedir. Olumsuz koşullara dayanıklı bitkiler strese karşı direnç sağlayan genlerin ya da transkripsiyon faktörlerinin bitkilere aktarılmasıyla hızlı bir şekilde üretilebilmektedir.

Bu çalışmada NAC tipi bir transkripsiyon faktörü olan *TaNAC69-1* geni *Triticum aestivum* L. cv. Yüreğir-89 bitkisinden izole edilmiş ve Gateway uyumlu bir yüksek ifade seviyesi vektörü olan pEarleyGate 100 vektörüne klonlanmıştır. *TaNAC69-1* geni *Agrobacterium*'a dayalı transformasyon yöntemiyle *Nicotiana tabacum* L. cv. Samsun bitkisinin yaprak dokularına aktarılmıştır. Bağımsız yapılan transformasyon denemeleri sonucunda kırk beş transgenik bitki namzeti (T_0) edilmiştir. *TaNAC69-1* geninin kalıcı ifadesi ve genoma entegrasyonu moleküler, biyokimyasal ve fizyolojik testler yardımıyla incelenmiştir. Transgenlerin kalıtımı gen spesifik ve *bar* genine spesifik primerler kullanılarak Polimeraz Zincir Reaksiyonu yöntemiyle

doğrulanmıştır. Southern blot analizi *TaNAC69-1* geninin genoma kalıcı entegrasyonunu kanıtlamak için kullanılmıştır. Genin farklı zaman periyotlarında ifade profilini anlayabilmek için *aktin* geni referans olarak kullanılıp kantitatif gerçek zamanlı PZR yapılmıştır. Kuraklık ve tuz uygulamalarının *TaNAC69-1* geninin ifadesini önemli ölçüde arttırdığı görülmüştür. Yabani tip ve transgenik tütün bitkilerinin kuraklık ve tuz stresi altındaki tepkileri bazı fizyolojik deneyler yardımıyla karşılaştırılmıştır. Transgenik bitkilerin çimlenme ve hayatta kalma oranları NaCl ve LiCl uygulamasında yabani tip bitkilere göre büyük oranda yüksek bulunmuştur. NaCl ve LiCl ile muamele edildiğinde transgenik bitkilerin kök ve gövde uzunlukları ile yaş ve kuru ağırlıkları yabani tip bitkilerle karşılaştırıldığında önemli derecede artmıştır, ancak mannitol uygulamasının bitkilerin büyüme parametreleri üzerine herhangi bir etkisi bulunamamıştır. Moleküler ve fizyolojik analizlere bakarak, *TaNAC69-1* geninin sürekli ifadesinin transgenik tütün bitkilerinin tuz ve iyonik stres toleransını arttırdığı söylenebilir. Abiyotik stress koşulları altında *TaNAC69-1* geninin fonksiyonunu daha iyi anlayabilmek için soğuk ve sıcaklık stresi koşulları altında daha ileri denemeler yapılabilir.

Anahtar Kelimeler: *TaNAC69-1*, *Agrobacterium*'a dayalı gen transferi, transgenik tütün, Southern blot, qRT-PCR, abiyotik stres

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my supervisor Prof. Dr. Meral Yücel for her guidance, patience, encouragements, kindness, valuable advices and insight throughout this research. I wish to express my deepest appreciation to my co-supervisor Prof. Dr. Hüseyin Avni Öktem for his advices, encouragements and criticism throughout the research. I have been always thankfull being a member of their research group. Their support not only influenced my life but also helped me to shape my future career.

I would like to thank my following committee members, Prof. Dr. Füsun Eyidoğan and Assoc. Prof. Dr. Sertaç Önde, for their comments and advices throughout my research. I am also thankfull thesis examining committee members, Prof. Dr. Yasemin Ekmekçi and Assist. Prof. Dr. Mehmet Cengiz Baloğlu, for their critics and valuable suggestions.

I am particularly thankfull Prof. Dr. Michael P. Timko for giving me the opportunity to conduct a part of my research in his laboratory at University of Virginia. I wish to express my appreciation to Tatyana Kotova for continuous help and creating a warm environment during my research.

I would like to express my special thanks to Gülsüm for her endless support and everlasting friendship. She has always listened to me and advised at any subject. And my dear friend Çiçek, I am thankfull to her for sharing my happiness and sorrow and leaving good memories for later times.

I wish to thank Ferhunde Aysin for being always helpful and good humoured, Pelin Canbaz for calming me down and making me feel like a princess, and Derya Gökçay for answering my questions all the time and assistance whenever I needed. I am

thankfull to my friends, Hatice, Dilek, Özlem, Güliz, Aslı, Sema, Erdem, Gamze, Çiğdem, Eser for their love and support.

I would like to thank the Plant Biotechnology and Nanobiotechnology Research Group members. They have created a friendly and relaxed environment in the laboratory and I have always enjoyed doing experiments or just being with them. I am thankfull to Tufan, Tahir, Taner, Musa, Hamdi, Ceyhun, Hamit, Doğa, Dilek, Lütfiye, Selin, Murat, Batuhan, Ceren, Fatma, Emre, Oya and Işkın for their friendship.

I am greatly thankful to my family, especially my mom and my lovely niece Yağmur, with my deepest love and appreciation. I have always felt their support, motivation and endless love at all stages of my life. The life is no worth without them.

I would like to thank The Scientific and Technological Research Council of Turkey for supporting me as a part of National Scholarship Programme for PhD Students. This work was supported by Faculty Development Program (BAP-08-11-DPT-2011K121010).

to my lovely sister, Serpil

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LIST OF ABBREVIATIONS

ABA	Absisic acid
BA	Benzylaminopurine
Bar	Bialaphos resistance gene
cDNA	Complementary DNA
CTAB	Hexadecyl-trimethyl ammonium bromide
DEPC	Diethylpyrocarbonate
DIG	Digoxygenin
DMSO	Dimethyl sulfoxide
LiCl	Lithium chloride
LB	Luria-Bertani Broth
MS	Murashige Skoog medium
NAA	Naphthalene acetic acid
NAC	No Apical Meristem, Arabidopsis Transcription Activation Factor1,2, Cup- Shaped Cotyledon
NaCl	Sodium chloride
NCBI	National Center for Biotechnology Information
PEG	Polyethylene glycol
PPT	Phosphinothricin
qRT PCR	Quantitative Real-Time PCR
ROS	Reactive oxygen species
NaOCl	Sodium hypochlorite
YEB	Yeast extract broth

CHAPTER 1

INTRODUCTION

1.1. Tobacco

Tobacco (*Nicotiana tabacum* L.) is an allotetraploid (2n=48) and herbaceous plant in the family of Solanaceae and commercially grown to produce cigarettes, cigars and other smokable products (Knapp et al., 2004, Ganapathi et al., 2004). *N. tabacum* is known as a hybrid of *N. sylvestris*, *N. tomentosiformis*, and possibly *N. otophora* (Ren & Timko, 2001).

Nicotiana tabacum has been used as a model plant for several years in tissue culture and molecular biology studies because of its easy transformation and producing enough resources in a short time. Murashige and Skoog medium (MS), a nutrient formulation used for tissue culture of a very wide range of plant species, was discovered with the studies of tobacco tissue culture (Murashige & Skoog, 1962). Also *in vitro* tobacco tissue culture studies let to knowledge about growth and differentiation of plants (Ganapathi et al., 2004). First transformation studies were started in tobacco which is a perfect host for *Agrobacterium* producing large galls (Braun, 1958). Gene expression and stability experiments in plants were based on studies started in tobacco (Ganapathi et al., 2004 and the references therein). Researches in tobacco was served as a model to other high value crops closely related to tobacco (Dhingra et al., 2008).

Tobacco is a model plant and several of researches have been conducted in tobacco. *Bacillus thuringiensis* endotoxin was expressed in tobacco giving plants a tolerance to Lepidopteran insects (Barton et al., 1987). *bt2* gene from one of the *Bacillus* strains protected transgenic tobacco plants form damage of tobacco hornworm (Vaeck et al., 1987). Tobacco mosaic virus (TMV) coat protein (CP) was used to

transform tobacco plants. Transgenic plants overexpressing *CP* gene showed retarded disease development and more than 50 % of transgenic plants did not develop disease symptoms (Powell-Abel et al.,1986). A bean *chitinase* gene protected transgenic tobacco plants from disease symptoms of a fungal pathogen, *Rhizoctonia solani* (Broglie et al.,1991). Transgenic tobacco plants engineered for accumulation of betaine aldehyde dehydrogenase withstood increased levels of betaine aldehyde converting it to glycine betaine (Rathinasabapathi et al., 1994). Plants accumulates proline under stress conditions such as salt and drought. [delta]-pyrroline-5-carboxylate synthetase from mothbean was transferred to tobacco plants. Proline production was increased to 10- to 18- fold more than wild type in transgenic lines with a high level of enzyme activity (Kavi Kishor et al., 1995). Constitutive expression of yeast trehalose-6-phosphate synthase gene (*TPS1*) in tobacco protected transgenic plants from effects of drought stress with accumulation of trehalose at high levels in fresh leaves (Romero et al., 1997). Tobacco plants transformed with a class I cytosolic small HSP gene, *TLHS1*, showed cotyledon opening two times more after subjecting high temperature stress (Park & Hong, 2002). Transgenic tobacco plants carrying glycerol-3-phosphate acyltransferase had altered chilling sensitivity with increased tolerance to cold stress (Murata et al., 1992). A chloroplast-localized Cu/Zn superoxide dismutase (SOD) took a part of sustaining photosynthesis in transgenic tobacco plants reducing light-mediated cellular damage caused by reactive oxygen species (Sen Gupta et al., 1993). Svab et al. (1990) reported stable transformation of *Nicotiana tabacum* L. plastid genome. Mitochondrial genome and function (De Paepe et al., 1993, Gutierres et al., 1997), chloroplast biology (Allison et al., 1996) also studied in tobacco plants.

1.2. Abiotic Stresses in Plants

Environmental fluctuations affect plant productivity and are most limiting factors for plant growth and yield (Mahajan & Tuteja, 2005, Schaeffer et al., 2012). Abiotic stress factors such as extreme temperatures leading to freezing or drought, salinity resulting from extensive irrigation, heavy metal pollution, flooding, light intensity or nutrient deficiencies threaten plant life and decrease crop yield (Mittler & Blumwald, 2010). Unlike biotic stress, abiotic sress is a multigenic trait. Genomic, metabolomic, proteomic and physiological networks are activated when plants

respond to abiotic stress and a cascade of molecular events are started to reduce the damage arising from osmotic or oxidative stress (Schaeffer et al., 2012). Figure 1. represents response of plants to abiotic stress (Wang et al., 2003).

1.2.1. Salt Stress

Salt stress mainly caused by sodium chloride are one of the abiotic stress factors which affects plant life negatively (Schaeffer et al., 2012). It is estimated that cultivable lands of earth will be lost up to % 50 by 2050 because of salt-induced damages (Wang et al., 2003). Salt stress leads to osmotic potential disturbance, ion toxicity caused by $Na^{+,}$ Cl^-, and SO_4^-, nutritional disorders and oxidative stress on plant cells (Chinnusamy & Zhu, 2004). Plants use some strategies such as ion exclusion, compartmentalization, osmoprotectan synthesis and increasing protein stability to overcome and reduce salt stress effects (Sugino et al., 1999, Majee et al., 2004).

Soil salts such as nitrates and potassium are necessary constituents of soil as well as essential plant nutrients (Kotuby-Amacher et al., 2000). Irregular crop irrigation combined with poor drainage are the main reason of increase of soil salinity adding extensive amounts of new salts to the soil (Munns et al., 2004). Magnesium (Mg^+), calcium (Ca^+) and sodium (Na^+) ions are found in irrigation water. After evaporation of water , calcium and magnesium precipitate into carbonates and sodium becomes a dominant ion in the soil (Serrano et al., 1999). High concentration of sodium ions in the soil surpass most macronutrients and micronutrient concentrations at least two folds and suppress nutrient-ion activities producing excessive quantity of Na^+/ Ca^+ or Na^+/K^+ (Grattana & Grieveb, 1999). Increase in particularly NaCl and other salts in the soil creates an external osmotic potential preventing plant roots to take water from soil and composing water deficiency just like as drought stress (Bohnert, 2007). If NaCl concentration in the soil exceeds 4dS m^{-1} (decisiemens per meter) ~ 40 mM, soil can be called as saline soil.

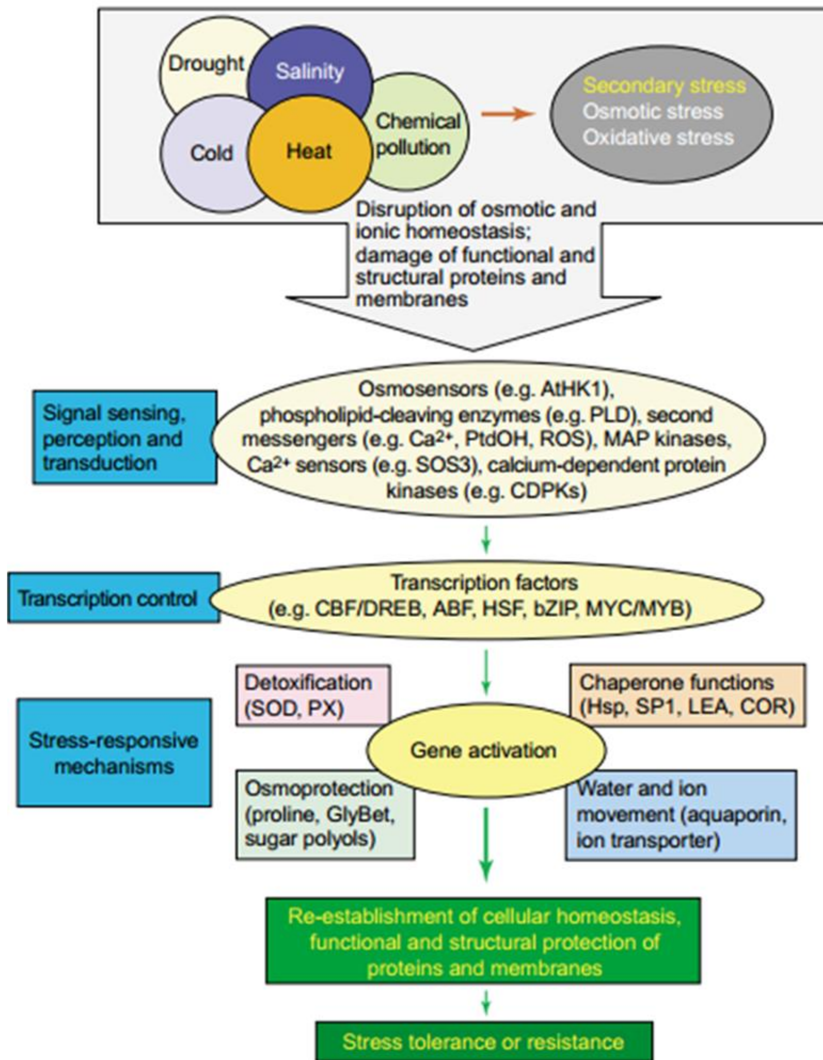

Figure 1. Response of plants to abiotic stress. When receptors sense secondary messengers triggered by abiotic stress, transcription factors related with abiotic stress are activated to stimulate stress response mechanisms to constitute cellular homeostasis (Wang et al., 2003).

Because response of each plant to salinity differ from each other, tolerance of plants to salt stress are different. As well as being differences monocot and dicot plant species, plants in the same group also show different responses to salt stress. Among monocotyledonous species, barley (*Hordeum vulgare*) and tall wheatgrass (*Thinopyrum ponticum*) are more tolerant, rice (*Oryza sativa*) is most sensitive. In dicotyledonous species, common bean (*Phaseolus vulgaris*) is most sensitive, alfalfa

(*Medicago sativa*) and saltbush (*Atriplex* spp.) are very tolerant to salinity (Munns & Tester, 2008, Yadav et al., 2011).

Soil salinity limits crop yield and glycophyte plants, mostly crops, are more vulnerable than halophytes to salt stress. Plants are affected by salt stress in two ways, capacity of roots to extract water from soil are depressed and high amount of salts are toxic to inside the plants inhibiting many physiological and biochemical processes (Hasegawa et al., 2000, Munns, 2002). Munns et al. (1995) proposed a two-phase model to describe growth response of plants to salinity. Timescale is different depending on plant species and level of salinity and it can be days to months. According to this model, at phase 1, osmotic phase, both type of plants reduce growth because of osmotic stress created by saline solution outside the roots, shoot growth, leaf area and cell division reduce, stomatal closure occurs. Root growth does not affected like shoot growth by salt stress. At phase 2, ion specific, ions especially Na^+ are accumulated in the leaf blade being toxic in old leaves. Photosynthetic capacity of old leaves is also decreased preventing carbohydrate supply to new leaves. If plants can not produce young leaves faster than death of old leaves, growth rate of plants reduce. Photosynthetic enzymes, chlorophylls and carotenoids are also damaged by salt stress. In salt sensitive plants, production of reactive oxygen species increase activating antioxidative mechanisms to detoxify these products. Figure 2. shows two-phase growth response of plants to salt stress (Hasegawa et al., 2000, Carillo et al., 2011).

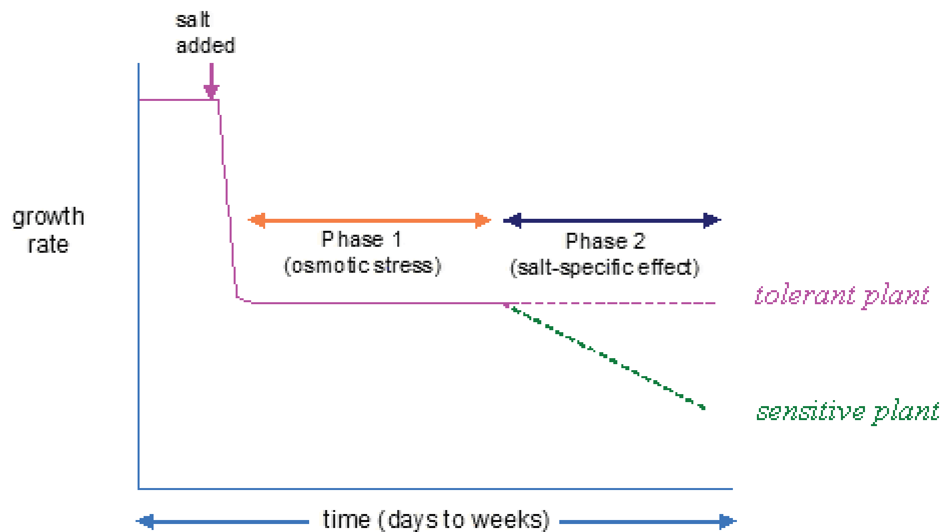

Figure 2. Two-phase growth response of plants to salt stress (Carillo et al., 2011).

Plants use three main tolerance mechanism to salt stress namely, osmotic tolerance, Na^+ exclusion and tissue tolerance. Increasing stomatal conductance is related with higher CO_2 assimilation and growth rate and leaf expansion of plants protecting them osmotic effects of salt stress. However cell expansion gives benefit only plants which have adequate soil water such as irrigated lands. Otherwise, in water limited systems, soil water is utilized before the plant reaches reproductive phase. In many plants grown under salt stress conditions, Na^+ reach to toxic level before Cl^- does, because of this most studies focused on Na^+ exclusion. Na^+ is excluded from leaf blades and root tips with the help of specific ion channels and transporters preventing Na^+ accumulation into plant. Compartmentalization of Na^+ and Cl^- ions within the cytoplasm, especially in leaf mesophyll cells and synthesizing and accumulation of osmoprotectans such as amino acids, amines, organic acids, sugars and polyols give plants to tissue tolerance to salt stress (Munns and Tester, 2008, Carillo et al., 2011).

1.2.2. Drought Stress

As water constitutes approximately 90% of plants, water deficit is a major constraint leading to a decrease in crop yield and productivity (Farooq et al., 2009, Hirt & Shinozaki, 2004). Drought stress like salt stress triggers osmotic instability and dehydration in plant cells. One of the consequence of the drought stress is

formation of reactive oxygen species (ROS). Hydroxyl radicals (OH), superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), alkoxy radicals and singlet oxygen are some examples of ROS. They interact with lipids, proteins and deoxyribonucleic acid causing oxidative damage (Munné-Bosch & Penuelas, 2003). As a result of stoma closure, damage of membranes, CO_2 fixation and ATP synthesis enzymes under low water conditions , CO_2 assimilation rate of leaves reduces. (Farooq et al., 2009). Water deficit also affects membrane structure disrupting integrity and selectivity of membrane leading to loss of membrane based enzyme activity. Activity of cytosolic and organelle proteins reduce, beside that proteins can be denatured completely when dehydration occurs. High cellular electrolyte concentration because of protoplasm dehydration may also damage cellular metabolism (Mahajan & Tuteja, 2005). Reduced stem length, fresh and dry weight, impaired germination, leaf senescence, leaf burn and abnormal fruits are some examples of physiological effects of drought stress on plants (Bartels & Sunkar, 2005, Shao et al., 2008, Farooq et al., 2009).

Plants use some mechanisms to avoid and lessen harmful effects of drought stress. Shortened life cycle help plants to reproduce before dry season comes (Araus et al., 2009). Length, density and biomass of roots increase to maintain water uptake from depths. Reducing leaf area, leaf pubescence, waxy bloom on leaves are some adaptations to decrease transpiration under drought stress conditions. Osmoprotectants such as proline, glycinebetaine, polyols and sugar alcohols provide osmotic adjustment. Increased levels of catalase, ascorbate peroxidase, cysteine, superoxide dismutase, glutathione reductase, and ascorbic acid assist to remove reactive oxygen species. Drought stress induce expression of various genes and products of these genes give stress tolerance to plants under drought (Kavar *et al.*, 2007, Farooq *et al.*, 2009). Figure 3. shows physiological, biochemical and molecular responses of plants to water deficit (Shao et al., 2008).

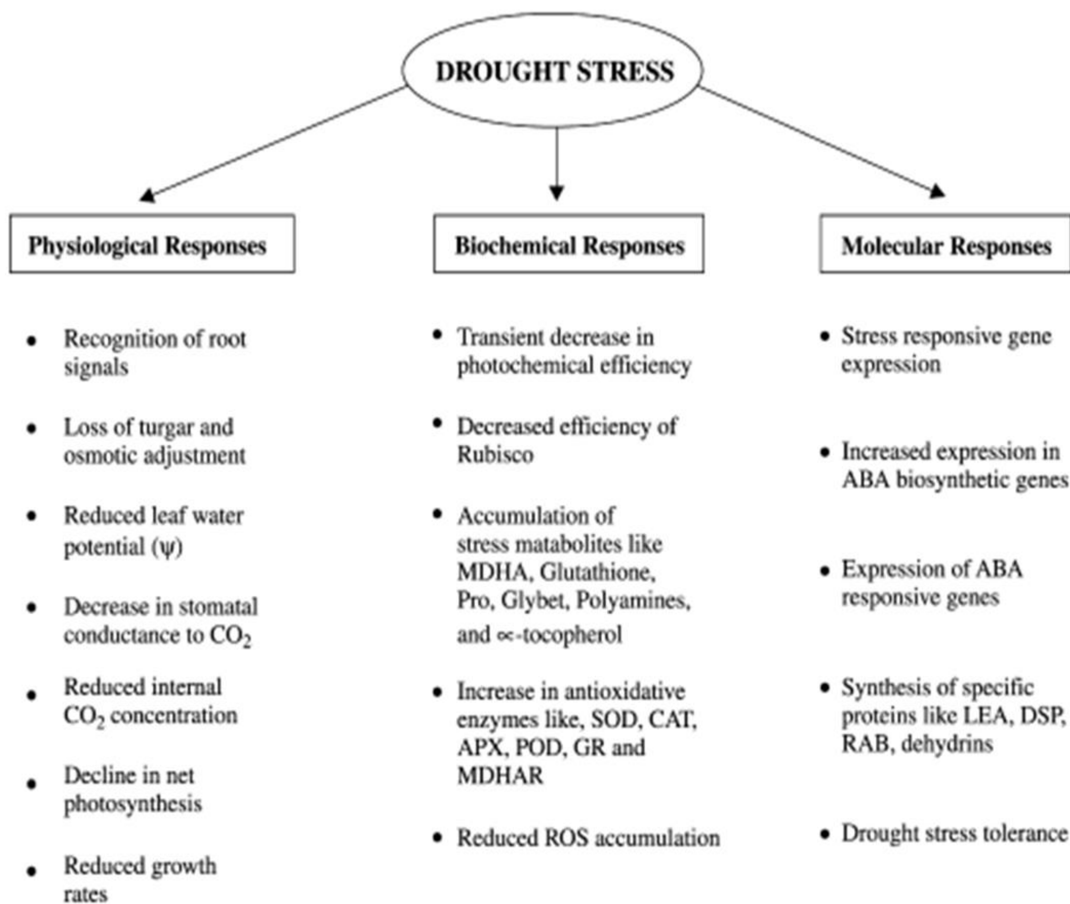

Figure 3. Schematic representation of physiological, biochemical and molecular responses of plants to drought stress (Shao et al., 2008).

1.3. Production of Salt and Dought Stress Tolerant Plants

1.3.1. Tissue Culture and Genetic Engineering

Traditional breeding methods depend on random genetic modifications to cross of desired genes between plant varieties. But crossing and selection of plants are wasting much time and a set of genes, also undesired genes, are transferred between donor and recipient plants, not a single gene. Advences in plant biotechnology, also known as agricultural biotechnology, allow production of new varieties with a desired trait in a short time. To study function of specific genes, researchers introduce foreign genes into plant cells to see expression and interaction of these genes with particular pathways (Topping, 1998, Rommens, 2007).

Transformation and regeneration in tissue culture are the first steps to generate transgenic plants. After stable integration and regeneration of whole plant in culture, selection must be applied to separate transgenic plants from non-transformed ones. Tobacco is a model plant leading discoveries in tissue culture and engineering of plants (Ganapathi et al., 2004). Murashige and Skoog (Murashige & Skoog, 1962) discovered a tissue culture medium with the help of studies in tobacco and this formula is used for a wide range of plant species.

Skoog and colleagues demonstrated importance of auxin and cytokinin balance to differentiate callus into root and shoot (Skoog, 1944, Skoog & Tsui, 1948). Some physical factors such as temperature (Skoog, 1944), photoperiod (Thorpe, 1968), pH, sucrose concentration and cultivars genotype (Rahman et al., 2010) also affect differentiation of callus. Alhough flower primordia (Hicks & Sussex, 1970), petioles (Prabhudesai & Narayanaswamy, 1974), anther (Nitsch, 1969) and pollen (Sunderland & Wicks, 1971) utilized for *in vitro* regeneration, tobacco leaf explants have been most commonly used tissue for generation of new plants (Gupta et al., 1966, Ganapathi et al., 2004).

While expression casettes including suitable promoter, terminator, selectable marker gene and gene of interest can be transferred to tobacco via direct DNA uptake (Paszkowski et al, 1984), electroporation (Shillito et al., 1985), liposome mediated (Deshayes et al., 1985), microinjection (Crossway et al., 1986) and particle bombardment (Klein et al., 1988), Agrobacterium-mediated gene transfer is popular and most widely used method to transfer foreign genes in tobacco plants (Marton et al., 1979, Horsch et al.,1985). Reporter genes are usefull to see if gene of interest is expressed or not in transferred tissues. *Escherichia coli gus* (*uid*A) gene encodes β-glucuronidase enzyme whose product can be easily detected by a histochemical GUS assay (Jefferson et al., 1987) but this assay destroy tissues although *gus* gene is commonly utilized in tobacco transformation studies (Ohta et al., 1990, Hobbs et al., 1990). To overcome this limitation other reporter genes , *gfp* (green fluorescent protein) and luciferase from firefly, are preferred because of their easy activity detection in living cells (Lindsey & Jones, 1989, Haseloff & Amos, 1995, Reisen et al., 2003). Selection genes (marker genes) provide to separate transformed plants with gene of interest from non-transformed ones. *bar* (herbicide resistance), *nptII*

coding neomycin phosphotransferase (kanamycin and neomycin resistance) and *hpt* coding hygromycin phosphotransferase (hygromycin resistance) are selection marker genes generally used for tobacco transformation (Carrer et al., 1993, Iamtham & Day, 2000).

Genetic engineering is a method to gain drought and salt stress tolerance to tobacco plants. Genes functioning in biochemical pathways or signaling process are suitable candidates to produce stress tolerant transgenic plants. Trehalose reduces water potential of plant cells and let them to take water from soil. *TPS1* served in the first step of trehalose production giving tobacco plants to osmotic adjustment and increased tolerance under low water conditions (Romero et al., 1997). When *ABF*, a transcription factor, was overexpressed it gave drought tolerance to tobacco plants decreasing water loss and increasing chlorophyll amount. It also reduced ion leakage and increased POD, SOD and CAT enzymes activity in transgenic tobacco plants (Huang et al., 2010). Mannitol, a sugar alcohol, was produced in transgenic tobacco at elevated levels by overexpression *mt1D* gene from *E. coli*. Transgenic lines with increased mannitol content showed salt tolerance with higher fresh weight in leaves and roots by induction of new leaves and roots (Tarczynski et al., 1992, Tarczynski et al., 1993). *AtNHX1* gene overexpression encoding a vacuolar Na^+/H^+ gave salinity tolerance to transgenic tobacco plants with higher germination rates and increased vacular ATPase activity maintaining the cytosolic H^+ pool (Zhou et al., 2011).

1.3.2. Marker Assisted Selection

Tobacco genetics have been studied using physiological, karyotypical and morphological characters. However because of changing morphological characters with environment and limited number of karyotypical characters, DNA-based molecular markers have been introduced of late years with use in genome mapping and fingerprinting, localization of genes, population genetics and plant breeding (Liu & Zhang, 2008).

Biochemical, morphological and molecular markers are three types of molecular markers used in production of stress tolerant plant species. DNA-based markers are divided into two types as PCR based and non-PCR based. Restriction fragment

length polymorphism (RFLP) is an example of non-PCR based markers. Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP) and microsatellites (Kumar et al., 2009) are some examples of PCR-based markers.

RFLP markers are very useful for breeding and linkage analysis when a linked trait is found in an individual' homozygous or heterozygous state. However RFLP markers requires large amount of DNA, a lot of work, and take much time making them inconvenient to detect agronomic traits (Winter P. & Kahl, 1995, Liu & Zhang, 2008). RAPD is a PCR-based assay utilizing 10 bases long oligonucleotides as primers and separating amplification products on agarose gels. RAPD markers are dominant markers to screen linked traits of interest with their fast, cost and effective production of large number of markers in a short time. RAPD markers can be used gene mapping, population and evolutionary genetics, plant and animal breeding (Williams et al., 1990, Bardakçı, 2001). AFLP is based on digestion of genomic DNA and amplification of selected restriction fragments. AFLP is a sensitive, high reproducible and and quick method to be used in DNA fingerprinting, genomic mapping and cloning studies (Ren & Timko, 2001). Microsatellites are codominant markers found in non-coding regions in the genome. Because of their reproducibility and ease of use, they are very popular to study population genetic studies, fingerprinting and marker assisted selection (Liu & Zhang, 2008).

At the last years, advances in marker technology allowed to identify ancestors of *Nicotiana tabacum*. *N. tabacum* is thought a hybrid of *Nicotiana tomentosiformis, Nicotiana sylvestris*, and possibly *Nicotiana otophora*. AFLP profiles of wild and cultivated tobacco plants showed the presence of polymorphic bands found in *N. tabacum* and other three wild progenitor species (Ren & Timko, 2001). Zhang et al. (2005) examined similarities and relationships between *N. tabacum* L. cultivars using RAPD markers with 63 % polymorphism. Markers can be used also for detection of disease resistance genes. AFLP analysis of 92 *N. tabacum* L. accession identified seven fragments related with resistance to blue mold black root rot (Julio et al., 2006).

1.4. NAC Type Transcription Factors in Plants

Drought and salt stress are major environmental constraints that limit growth and productivity of plants. Because of their sessile nature, plants do not escape from unsuitable environmental conditions and develop some physiological, biochemical and molecular responses to survive under stress conditions (Nakashima et al., 2012). Abiotic stress activates expression of many stress-related genes functioning production of metabolic proteins (functional proteins) or regulating signal transduction (regulatory proteins). Regulatory proteins including transcription factors (TFs) such as NAC proteins, DREBs (dehydration-responsive element-binding proteins) and AREBs (ABA-responsive element-binding proteins) are determined in *Arabidopsis* and rice with respect to their regulatory roles in tolerance of plants to abiotic stress (Yamaguchi-Shinozaki & Shinozaki, 2006, Nakashima et al., 2009).

NAC proteins form one of the largest gene families and involved in diverse processes such as development, defence, biotic and abiotic stress responses of plants (Olsen et al., 2005, Nakashima et al., 2012, Puranik et al., 2012). NAC proteins have a conserved N-terminal NAC domain which is named using first letters of consensus sequences of Petunia NAM (No apical meristem), *Arabidopsis* ATAF (*Arabidopsis* transcription activation factor) and CUC (Cup-shaped cotyledon) and a diverse C terminal transcription regulatory domain (Ernst et al.,2004, Olsen et al., 2005). Figure 4. shows schematic representation of structure of NAC protein (Puranik et al., 2012). 117 NAC genes in *Arabidopsis*, 151 in rice, 79 in grape (*Vitis vinifera*), 163 in poplar, 152 in soybean and 152 in tobacco (*Nicotiana tabacum*) have been identified until so far (Rushton et al, 2012, Hu et al., 2010, Nuruzzaman et al. 2010, Le et al., 2011).

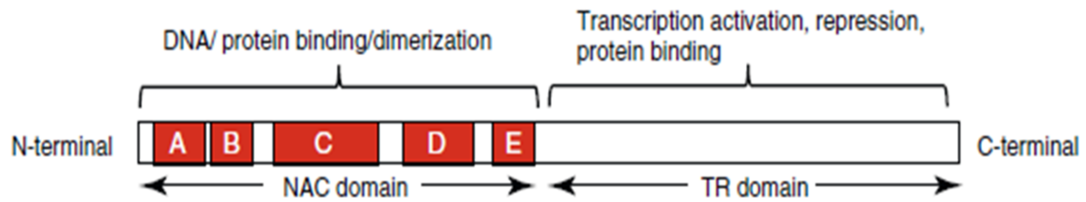

Figure 4. Schematic representation of structure of NAC proteins. Conserved NAC domain has five conserved subdomains (A-E) (Puranik et al., 2012).

NAM gene was the firstly characterized NAC gene from petunia *nam* mutants lacking the SAM (shoot apical meristem) and dying at the seedling stage (Souer et al., 1996). Characterization of *CUC2* gene from *Arabidopsis* came from immediately after *NAM* gene. *cuc2* mutants showed similar SAM lack phenotypes like *nam* mutants (Aida et al., 1997). Cotyledon is fused in *cuc1 cuc2* combined mutants like *nam* mutants and they do not have embryonic shoot apical meristem. If shoots come from mutant calli by regeneration abnormal flower development occurs. NAC-type transcription factors function in abiotic stress tolerance of land plants. *ONAC063*, a NAC transcription factor from rice, inserted in *Arabidopsis*. Expression of *ONAC063* gene was induced not only with high-salinity but also with high-osmotic pressure and high levels of reactive oxygen species. Transgenic *Arabidopsis* seeds expressing *ONAC063* showed higher germination rates under high salinity and osmotic stress. RT-PCR and microarray analyses revealed that expression of some genes such as amylase gene (*AMY1*) increased in transgenic *Arabidopsis* plants expressing *ONAC063* gene (Yokotani et al., 2009).

When plants are exposed to environmental stress, a number of signaling processes starts including activation of transcription factors. *SNAC2*, a stress responsive NAC gene from rice (*Oryza sativa* L. ssp japonica) was isolated and characterized to show stress tolerance using yeast. Expression of *SNAC2* induced with salinity, drought, wounding and ABA treatment. Over-expression of *SNAC2* gene in japonica rice Zhonghua 11 showed that more than half of transgenic plants stayed healthy under severe cold stress with high membrane stability compared with wild type plants. Germination and growth rates of transgenic rice seeds under high salt conditions

were higher than wild type. Transgenic rice plants over-expressing *SNAC2* gene also had tolerance to polyethylene glycol treatment (Hu et al., 2008).

TaNAC69 gene from bread wheat was related to biotic and abiotic stress responses of plants. With a barley drought inducible promoter, *HvDhn4s*, transgenic wheat plants expressing *TaNAC69* gene showed drought induced overexpression especially in leaves and roots. Shoot biomass of transgenic plants under salt stress and water limited conditions was higher. Transgenic plants produced more root biomass and longer roots with PEG-induced dehydration. Expression levels of many stress up-regulated genes increased with constitutive overexpression of *TaNAC69* gene showing tolerance of wheat plants to drought stress (Xue et al., 2011).

NAC type transcription factors take a role also in biotic stress tolerance of plants. Interaction between replicase protein of tobacco mosaic virus (TMV) and *ATAF2* from *Arabidopsis thaliana* was investigated using yeast two-hybrid assay. Expression of *ATAF2* increased when infected with TMV and reduced virus accumulation. Expression levels of marker genes *PR1*, *PR2* and *PDF1.2* which were related to defense raised in *ATAF2* overexpressing transgenic plants (Wang et al., 2009). A tomato *NAC* gene SISRN1 (*Solanum lycopersicum* stress-related *NAC1*) is a protein localized on plasma membrane. When infected with *Botrytis cinerea* or *Pseudomonas syringae* pv. tomato (Pst) DC3000 a 6-8 folds increase in *SISRN1* expression was obtained. Silencing of *SISRN1* resulted in an increase in disease severity showing a positive regulator role in defense response of *SISRN1* gene in tomato (Liu et al., 2014).

1.5. Gateway Cloning Technology

Agrobacterium-mediated gene transfer is a generally preferred method for transformation of plant species. Because binary T-DNA vectors used for Agrobacterium-mediated transformation are big generally, cloning is taking up very much time and labored in some cases. Gateway cloning technology is a quick and reliable alternative compared to other methods to clone genes into large plasmids (Karimi et al., 2002).

Gateway cloning system (Invitrogen, Gaithersburg, MD, USA) utilizes bacteriophage lambda site-specific recombination system to transfer sequences between gateway compatible plasmids.
Gateway cloning technology does not need traditional restriction or ligation reactions and to check orientation of DNA sequences or reading frames. When a DNA sequence enters in an entry vector, it can be recombined easily to other compatible vectors for protein localization, interaction, expression analysis, gene knockdown and functional analysis (Karimi et al., 2002, Earley et al., 2006).

Gateway compatible vectors have recombination sites as *att*BX*att*P or *att*LX*att*R. In Gateway cloning technology, two reactions namely LR and BP recombinations are mediated by LR and BP clonases respectively. BP reaction catalyzed by BP clonase recombines PCR product flanked by *att*B sites and a donor vector flanking with *att*P sites leading to creation of an entry clone flanking with *att*L sites and a byproduct flanking with *att*R sites. Once flanking with *att*L, the insert can be transferred easily to any destination vector containing *att*R recombination sites with LR reaction. Positive (antibiotic resistance) and negative (the cytotoxic *ccdB* gene) selection system can be applied to select resulting construct. *ccdB* is a lethal gene not allowing to grow most of *E. coli* strains. The easiness and speed, also presence of a huge number of destination and entry vectors make Gateway cloning technology very powerful and attractive (Karimi et al., 2002, Earley et al., 2006, Magnani et al., 2006). Figure 5. shows diagram of LR and BP reactions.

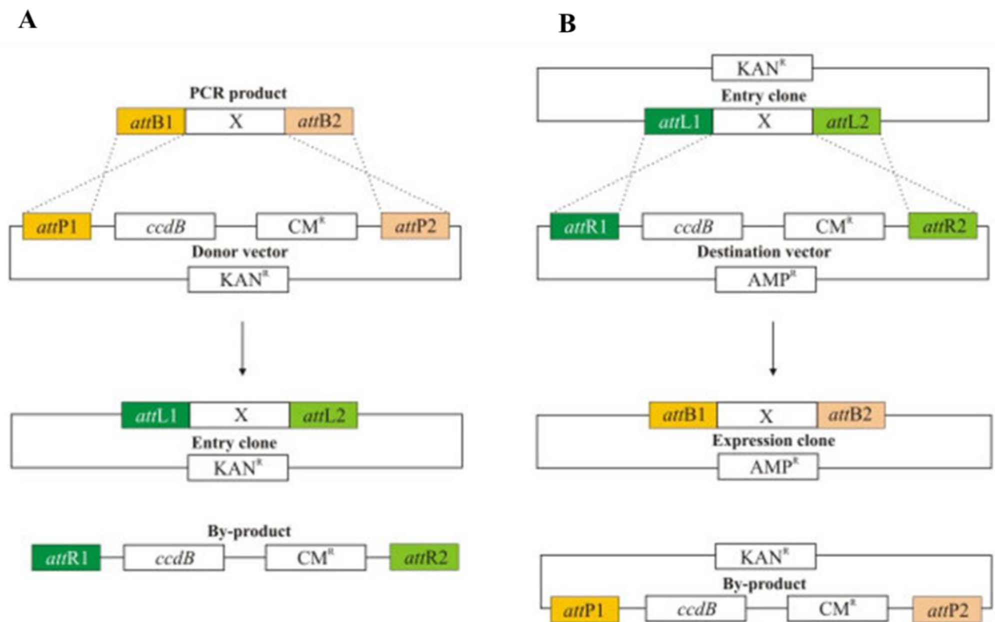

Figure 5. Representative diagram of LR and BP reactions. A) BP reaction of a PCR product flanking witth *attB* sites. B) LR reaction of an entry clone carrying desired DNA fragment with a destination vector (Magnani et al., 2006).

1.6.Quantitative Real-time PCR

The fluorescence-based quantitative real-time reverse transcription PCR (qRT-PCR) is a method which is used for quantification of mRNA levels in molecular medicine, functional genomics, diagnostics, virology, microbiology and biotechnology. qRT-PCR is a powerful tool to detect and compare of RNA levels because of its simplicity, sensitivity and specificity (Bustin, 2002, Bustin et al., 2005). Real-time PCR allows to collect data for amplificaton and detect signals as PCR proceeds reducing the process in a single step (Higuchi et al., 1993).

When compared to other methods for gene expression quantification, quantitative real-time PCR have many benefits. Quantitative data can be produced in a confidential way with a dynamic range about 8 orders of magnitude and not need manipulation after amplification. qRT-PCR is more sensitive than RNAse protection assays and blot hybridization approximately 1000 to 100,000 folds. qRT-PCR does not depend much RNA like other methods for expression of gene analysis (Wong &

Medrano, 2005). In qRT-PCR first step is reverse transcription in which conversion of mRNA to cDNA occurs. Amplification of cDNA is second step of qRT-PCR. Third and last step is end-product quantification (Nolan *et al*., 2006). mRNA quantification can be done in two ways in qRT-PCR; one-step or two-step. In one-step qRT-PCR cDNA synthesis and amplification are carried out in a single tube, on the contrary, in two- step reverse transcription is performed firstly in a separate tube, then amplification is made using cDNA synthesized separately. Even though exprerimental variations is supposed to decrease at a minimal level, one-step qRT-protocols are not sensitive like two-step protocols and RNA can degrade easily if it is not treated properly in one-step qRT-PCR (Battaglia et al., 1998).

qRT-PCR phases are classified as linear ground, early exponential, exponential (log linear) and plateau. The first 10-15 cycles are known as linear ground phase in which PCR just begins and emission of fluorescence stays at a level much less than background. In the early exponential phase, the quantity of flouroscence has arrived a threshold level approximately 10 times higher than background level. In ABI Prism (Applied Biosystems) literature, people are called this cycle as C_t (threshold cycle) and as CP (crossing point) in LightCycler® (Roche Applied Science) (Heid et al., 1996, von Ahsen et al., 1999). C_t value shows proportion of target nucleic acid in the original sample and used for calculation of experimental results. During exponential phase (log linear phase), PCR product doubles at every cycle under optimal amplification conditions. At the plateau phase, PCR reaction does not proceed because of finishing all the reaction components and fluorescence intensity is not suitable for data calculation (Bustin, 2000, Wong & Medrano, 2005). Figure 6. shows qRT-PCR amplification phases.

To detect fluorescence in qRT-PCR, DNA binding dyes known as reporter dyes are used. Dyes bind to dsDNA as PCR proceeds and emit fluorescence. As PCR products are accumulated at every cycle, more dye attaches emitting more fluorescence proportional to amplified dsDNA concentration (Wittwer et al., 1997).

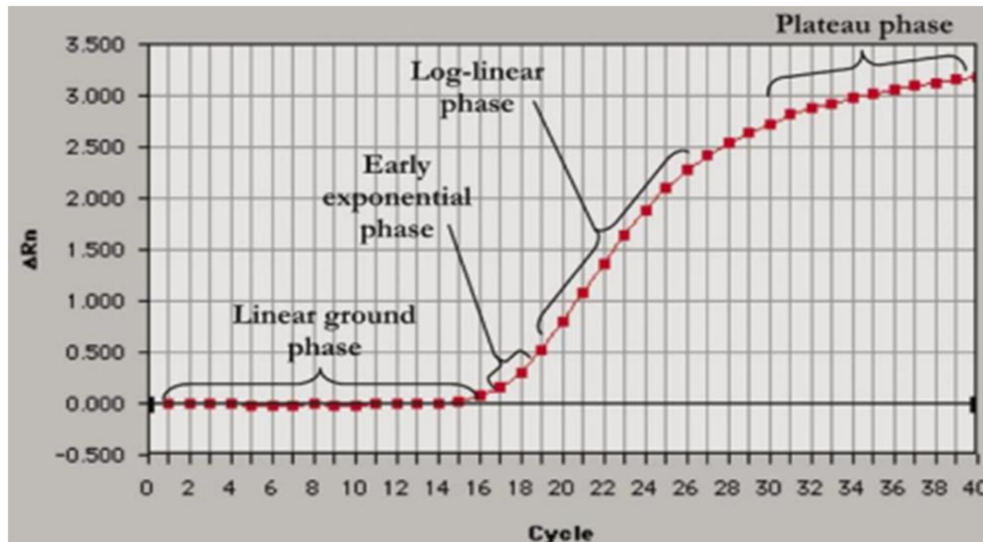

Figure 6. RT-PCR amplification phases (Wong & Medrano, 2005).

1.7. Studies Conducted with Tobacco and TaNAC69-1 Gene in Plant Biotechnology Laboratory

Tobacco is a model plant used in tissue culture and transformation studies. TaNAC69-1, a NAC type transcription factor gene, takes a part of biotic and abiotic stress responses of plants. Some studies conducted with tobacco and TaNAC69-1 gene in Plant Molecular Biology and Biotechnology Laboratory, in METU. Eyidoğan (2001) was transformed tobacco plants with *AtP5CS*, key enzyme in proline pathway, and studied osmotic stress tolerance of plants via proline overproduction. Salt tolerance of *sto* transformed tobacco plants was evaluated by Selçuk (2004). *pflp* gene from sweet pepper known as having antimicrobial characteristic was used to transform tobacco plants. Hypersensitive responses of plants were tested using *Pseudomonas syringae* (Tuncer, 2006). Aysin (2007) transferred Na^+/H^+ antiporter gene (*AtNHX1*) to tobacco and determined salt tolerance of transgenic plants via physiological analysis. Expression profile change of *TaNAC69-1* gene in wheat plants was studied under abiotic stress conditions. Global gene expression profiles was also identified with cold and heat stress treatments (Baloğlu, 2011).

1.8. Aim of the Study

Environmental stresses such as drought and salinity affect plant growth and development negatively. Plants respond to environmental stimuli with changes at biochemical, physiological and molecular level. Understanding the regulation of stress response of plants is an important point to generate stress tolerant plants.

Trancription factors control transcription of other genes involving several different processes. NAC type transcription factors are plant specific transcription factors and involved in plant development, biotic and abiotic stress responses of plants. The aim of this study is to examine the possible roles of *TaNAC69-1* gene to increase tolerance to salt and drought stress in tobacco. To achieve this purpose, firstly cloning of *TaNAC69-1* gene was carried out with the help of Gateway cloning technology. Transformation of *TaNAC69-1* gene from wheat (*Triticum aestivum* L. cv. Yüreğir-89) to tobacco (*Nicotiana tabacum* L. cv. Samsun) was achieved by Agrobacterium-mediated transformation. Southern blot analysis was made to verify integration of the *TaNAC69-1* gene in the tobacco genome. Determination of expression level changes of *TaNAC69-1* gene is one of the powerful tools to develop stress tolerant crop plants. For this purpose expression profile changes of *TaNAC69-1* gene in transgenic tobacco plants were determined using quantitative Real-Time PCR under salt and drought stress conditions. Physiological tests such as germination, root and shoot lenghts, fresh and dry weights were done to see the effects of *TaNAC69-1* gene overexpression on the germination and growth parameters of tobacco. As far as we know, this is the first study analyzing transcript level changes of transgenic tobacco overexpressing *TaNAC69-1* gene.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Materials

A spring wheat variety, *Triticum aestivum* L. cv. Yüreğir-89 was used for *TaNAC69-1* gene isolation and a tobacco cultivar, *Nicotiana tabacum* L. cv. Samsun, was transformed plant in this study. Wheat cultivar seeds were taken from Turkish Ministry of Agriculture and Rural Affairs, Central Research Institute for Field Crops, Ankara. Tobacco seeds were produced in our laboratory.

2.1.2. Bacterial Strains and Plasmids

In this study, *Escherichia coli* DH5α cells as competent cells and a commercial vector, pENTR™/D-TOPO® (Paisely, UK), were used in the cloning studies (Figure 7.). A Gateway compatible plant recombinational cloning vector, pEarleygate 100 , were obtained from Arabidopsis Biological Resource Center (ABRC) (Figure 8.). *TaNAC69-1* gene was transferred to tobacco plants using *Agrobacterium tumefaciens* strain EHA105.

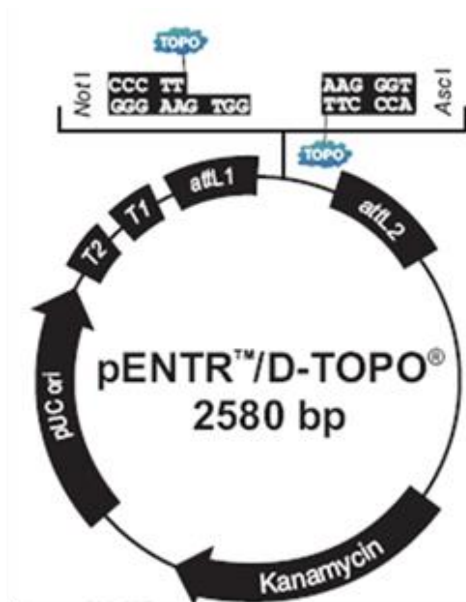

Figure 7. Map of pENTR™/D-TOPO® entry vector

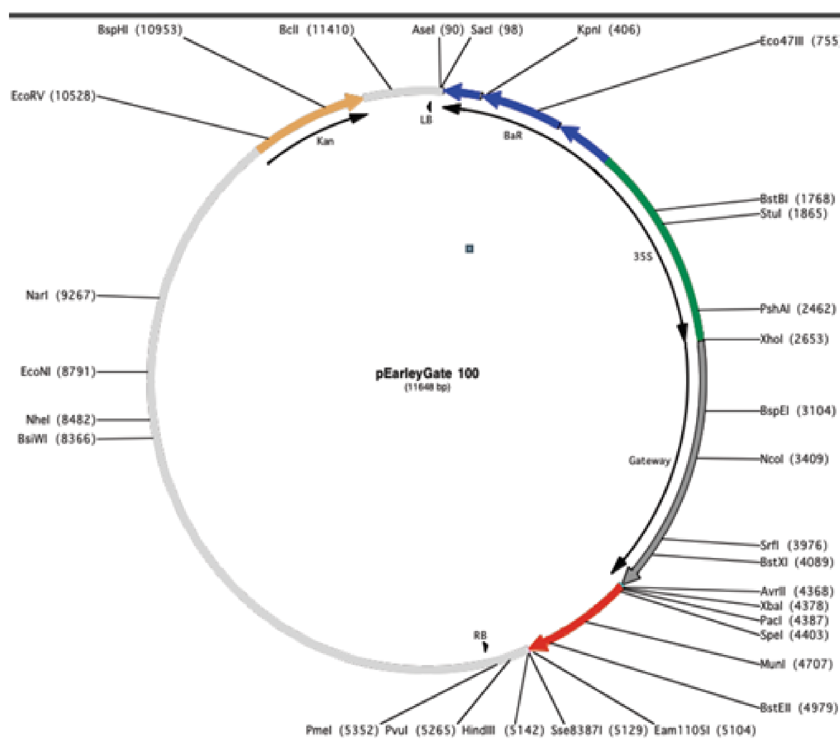

Figure 8. Map of pEarleyGate 100 destination vector.

2.1.3. Bacterial Growth Media

Luria-Bertani (LB) Broth supplemented with suitable antibiotics was used for grow*th of E.coli* strains. To help compotent *E. coli* cells to recover fastly after transformation, SOC medium richer than LB medium was used. *Agrobacterium tumefaciens* strain was grown in yeast extract broth (YEB) medium with appropriate antibiotics. To store bacterial strains, 1.5 % bacterial agar and suitable antibiotics were used and cultures were preserved at 4 °C for several weeks. The bacterial strains including the specific plasmids were also kept in 30 % glycerol at -80 °C for further usage.

2.1.4 Plant Tissue Culture Media

In this study, MS based media (Murashige & Skoog, 1962) were used for plant tissue culture studies. The chemicals were supplied from Duchefa and PhytoTechnology. Table 1. shows the formula and usage of plant tissue culture media.

2.1.5. Chemicals, Kits, and Restriction Endonucleases

Chemicals used to prepare solutions and antibiotics were purchased from Duchefa (Haarlem,The Netherlands), Phyto Technology Laboratories (USA), Sigma (USA), AppliChem (Missouri, USA), Merck (NJ, USA), Prona (PRC), and Deltalar (Ankara, Türkiye). RNA was isolated using Qiagen RNeasy Plant Mini Kit (CA, USA) and Invitrogen Trizol reagent (Paisely, UK). cDNA synthesis was made according to manual of Qiagen QuantiTect Reverse Transcription Kit (CA, USA). Enzymes and other materials used for molecular biology studies were supplied from New England Biolabs (MA, USA), Fermentas (PA, USA) and Amersham Biosciences (NJ, USA).

Table 1. Formula and usage of plant tissue culture media

Media	Composition	Usage
Liquid MS	MS + sucrose (3%)	To dilute *Agrobacterium* culture before transformation
MSA	MS + sucrose (3%) + plant agar (0.8%)+ 1 mg/L BA + 0.1 mg/L NAA	To co-cultivate of *Agrobacterium* treated leaf discs, callus and shoot induction for control leaf strips
MSB	MSA + 10 mg/L PPT + 200 mg/L Timentin	To select transformed leaf strips and regeneration
MSC	MSA or medium MSB according to usage	To induce root formation from shoots, germinate seeds and sub-culture of plants

2.2. Methods

2.2.1. Growth of Plants

The seeds of wheat cultivar, Yüreğir-89, were surface sterilized using 70 % ethanol for 1 minute and 15 % (v/v) sodium hypochlorite for 20 minutes, after that rinsed with distilled water three times . Seeds were sown and germinated in plastic pots . Irrigation of pots were made using ½ strength Hoagland`s solution and pots were kept in a growth chamber with 16 hour light 8 hour dark photoperiod at 25 °C for 14 days. To increase expression level of *TaNAC69-1* gene, plants were treated with 200 mM NaCl for 5 days after the 14th day of growth. After collecting leaves from control and salt stressed plants, they were frozen in liquid nitrogen immediately and stored for further use.

2.2.2. Primer Design to Clone TaNAC69-1 gene

Primers which was specific to *TaNAC69-1* gene were designed according to the manual of pENTR™/D-TOPO® Cloning Kit (Table 2.). Primer specifity was checked using NCBI/Primer-BLAST web tool.

Table 2. *TaNAC69-1* gene specific primers designed for pENTR™/D-TOPO® Gateway cloning vector.

Gene	Primer sequence
TaNAC69-1	5`-CACCATGCCAATGGGCAGCAGC-3` 3`-TCACATGTGCAGCTGCTGGCTG-5`

2.2.3. Total RNA Isolation

Total plant RNA isolation from fresh or frozen wheat and tobacco leaf tissue were made in accordance with the manual of Qiagen RNeasy Plant Mini Kit and using TRIzol reagent (Invitrogen). The RNeasy technology allows efficient purification of total RNA from small amounts of starting material.

RNA quantity and purity was checked in nanodrop spectrophotometer reading absorbances at 260 nm (A260) and 280 nm (A280). The A260/A280 ratio shows RNA purity.Values between 1.8-2.1 shows that RNA is highly purified. Quality of RNA was checked by running gel electrophoresis for 1 hour at 80 V.

2.2.4. Agarose Gel Electrophoresis

Agarose gels were run to check and visualize DNAs, RNAs, plasmid DNAs and PCR products as stated in Maniatis et al., 1989 . Gel concentrations ranging between 0.8-2 % were prepared according to sample size. For this purpose, 1 g agarose was weighed and melted in 1X TAE buffer completely by heating in a microwave oven

for 1 % gel. After cooling the solution around 60 °C, ethidium bromide was added at a final concentration 0.5 µg/mL. The gel was poured into an electrophoresis tray with a suitable comb and let to solidify at room temperature. 1X TAE buffer was used to prepare and run the gel in electrophoresis tank. Samples were mixed with loading buffer (6X). To determine the size of the samples, commercial DNA markers (Fermentas SM0311 or SM0331) was loaded into a separate well. The gel was run under constant voltage (80-100 volts) for 40-50 min. To visualize the gel, UV transilluminator was used and gel was photographed using UVP gel imaging system.

For RNA gels, electrophoresis tank was washed with detergent, rinsed with water, 70 % ethanol was applied to dry. The tank was filled with 3 % hydrogen peroxide, incubated 10 minutes at room temperature and rinsed with DEPC (Diethylpyrocarbonate) -treated sterile water. All solutions were prepared using DEPC-treated sterile water. To remove RNases from glassware, tips and other materials, they were soaked into DEPC- treated water or ethanol for at least two hours, drained and autoclaved.

2.2.5. cDNA Synthesis

RevertAid™ First Strand cDNA Synthesis Kit from Fermentas was used to synthesize cDNAs (complementary DNA) from RNA samples according to manual of the kit. After reading absorbances of RNA samples at 260 nm, 1 µg RNA, oligo dT primer and nuclease-free water up to 12 µl was mixed in an RNase-free tube and incubated at 65 °C for 5 minutes. RiboLock RNase Inhibitor, dNTP Mix, 5X reaction buffer, and RevertAid M-MuLV Reverse Transcriptase were added in the indicated order and the mixture was mixed gently and centrifuged, then incubated at 42 °C for 60 minutes following at 70 °C for 5 minutes to terminate the reaction. The reaction was used immediately for further studies or stored at –20 °C for 1 week or -80 °C for long term.

2.2.6. PCR Amplification

TaNAC69-1 gene was amplified using wheat cDNA samples. Annealing temperature, primer concentrations, amount of cDNA and $MgCl_2$ concentrations

were optimized using Tag DNA Polymerase before starting ampilification. After optimization, a highly thermostable DNA polymerase , *Pfu* DNA Polymerase, was used for amplification of cDNA samples (Table 3., Table 4.).

After amplification, 1 % agarose gel was used to run PCR products. They were visualized under UV light. DNA bands with desired size were cut from the gel, put in an eppendorf tube, and weighed. DNA fragments were isolated from the gel using The Qiagen QIAquick Gel Extraction Kit according to manufacturer's instructions.

Table 3. PCR components used for amplification of *TaNAC69-1* gene

Reagent	**Stock**	**Volume (µl)**
Pfu buffer w/o MgSO4	10X	2.5
dNTP mix	2.5 mM	2
Forward primer	10 µM	1.25
Reverse primer	10 µM	1.25
Pfu DNA polymerase	2.5 units/µl	0.25
DMSO	% 100	1.25
cDNA	50 ng/ µl	3
MgSO4	25	1.5
dH_2O	-	12
Total		25

Table 4. PCR conditions to synthesize *TaNAC69-1* gene

Step		Temperature	Duration	Cycle
Initial Denaturation		95 °C	3 minutes	1
Amplification	Denaturation	94 °C	20 seconds	35
	Annealing	56 °C	20 seconds	
	Extension	72 °C	2 minutes	
Final extension		72 °C	10 minutes	1

2.2.7. Cloning of DNA Fragments into Cloning and Expression Vectors Using Gateway Technology

Traditional cloning of DNA sequences for overexpression or RNAi knock-down experiments into binary plant transformation vectors is laborious and time consuming. To facilitate generation of binary vectors, GATEWAY system for recombinational cloning can be used. Gateway® Technology is a universal cloning technology that provides a highly efficient and rapid route to functional analysis, protein expression, and cloning/subcloning of DNA segments. It allows to transfer DNA segments between different cloning vectors while maintaining orientation and reading frame, effectively replacing the use of restriction endonucleases and ligases. Once your fragment or library has entered the Gateway system, moving fragments to any other Gateway vectors becomes a simple matter.

The recombination reaction requires an entry vector containing a gene of interest flanked by appropriate recombination sites (e.g. attL1 and attL2), a recombination enzyme (Clonase), and a binary destination vector. The binary destination vector contains compatible recombination sites (e.g. attR1 and attR2) integrated downstream of the plant promoter of choice (Himmelbach 2007, Karimi 2002).

2.2.7.1. Cloning of DNA Fragments into Cloning Vector

After purifiying PCR products of *TaNAC69-1* gene, it was cloned into pENTR™/D-TOPO cloning vector according to manual of The pENTR™ Directional TOPO® Cloning Kit. In TOPO cloning system, forward primer contains CCAC overhang and pENTR™/D-TOPO vector contains GTGG overhang. Thus, it is very easy to put PCR product into the vector via directional cloning. Fresh PCR product (0.5:1-2:1 molar ratio to vector), salt solution, sdH_2O, and pENTR™/D-TOPO® cloning vector were put in a PCR tube and mixed gently. The reaction was incubated at room temperature (22-23 ºC) for 30 minutes. This reaction was used to transform chemically competent *E. coli* cells via heat shock method. To check the presence of *TaNAC69-1* gene into recombinant clones, colony PCR was done with gene specific primers. Also sequencing was made using M13 forward and reverse primers. After *TaNAC69-1* was cloned into entry vector, it was transferred into Gateway compatible binary destination vector, pEarleyGate 100, through LR reaction.

2.2.7.2. Cloning of DNA Fragments into Destination Vector

pEarleyGate Gateway-compatible binary destination vector, pEarleyGate 100, was developed by Earley et all (2006). It includes *bar* gene for plant selection, kanamycin for bacterial selection, also attR1 and attR2 sites for LR reaction, 35S promoter and octopine synthase terminater (OCS). The recombination (attL x attR) between entry vector (pENTR™/D-TOPO® including *TaNAC69-1* gene) and destination vector was catalyzed by Gateway® LR Clonase™ II enzyme mix generated an expression clone. Entry clone, binary destination vector and TE buffer were mixed in a PCR tubeö. LR Clonase™ II mix was added for each sample. The reaction was vortexed briefly and incubated 25 °C for 1 hour. After adding Proteinase K solution to terminate the reaction, the reaction mixture was incubated at 37 °C for 10 minutes. Bacterial transformation was made using the reaction mixture. Colony PCR and sequencing were made to analyze the insertion of *TaNAC69-1* gene into expression vector with gene specific primers.

2.2.8. Transformation of Bacterial Cells

2.2.8.1. Preparation of Chemically Competent *E.coli* cells

E.coli DH5α and TOP10 strains were prepared as competent form for transformation studies. LB agar plate was used to streake the cells and they incubated at 37 °C at least 12 hours. Then single colonies of each strain were inoculated in 10 ml liquid LB medium and grown overnight at 37 °C. After initial cultures were ready, subcultures were made in proportion to 1:200 in 200 ml liquid LB medium grown at 37 °C with vigorous agitation (~ 200 rpm) until log phase was reached (OD_{600}=0.6-08). The cultures were incubated on ice for 10 minutes. Starting from this point, everything should be kept on ice or must be prechilled as competent cells are very sensitive to heating. Also media does not contain any antibiotics, it must be worked at sterile conditions and everything should be sterilized before using. Centrifuge was done at 3000 rpm at 4 °C for 15 minutes. Supernatant was discarded and pellet was resuspended in ice cold 80 ml (0.4 volume) TB buffer. Cells were incubated 10 minutes on ice and centrifuged 3000 rpm at 4 °C for 10 minutes. After discarding supernatant, resuspension was made using 8 ml (0.04 volume) prechilled TB buffer and 7 % DMSO was added to prevent cells from bursting. After incubation on ice for 10 minutes, prepared competent cells were distributed as 100 μl aliquots into eppendorf tubes, immediately chilled in liquid nitrogen and stored at - 80 °C for further use.

2.2.8.2. Preparation of Competent *A. tumefaciens* cells

Agrobacterium tumefaciens EHA105 strain was streaked on YEB agar plates and grown at 28 °C for 2 days. A single colony was taken from the plate and transferred to 5 ml YEB liquid medium as starter culture. Bacterial culture was incubated at 28 °C overnight with 200-250 rpm with vigorous shaking. 50 ml batch culture was made from this saturated fresh culture and grown at 28 °C for 1 day with vigorous agitation until OD_{600}=0.5-0.8 was reached. Whole culture was chilled on ice. Bacterial cells were centrifuged to make a pellet at 4000 g for 10 minutes at 4 °C. After discarding supernatant, 10 ml ice cold sdH_2O was added to dissolve the pellet until no clumps remained. The bacterial suspension's final volume was adjusted to 50 ml with ice cold sdH_2O. Second centrifugation and resuspension were made in

the same manner and bacterial yield was collected in 5 ml ice cold 30 % sterile glycerol. 50 µl aliquots were made into eppendorf tubes. They were freezed in liquid nitrogen, and stored at -80 °C.

2.2.8.3. Transformation of Bacteria with Plasmids

Heat shock method was used to transfer cloning and expression vectors carrying *TaNAC69-1* gene to competent *E. coli* cells. Competent *A.tumefaciens* cells were transformed by means of electroporation method.

2.2.8.3.1. Transformation of *E.coli*

Frozen competent *E. coli* cells was taken from – 80 °C and thawed on ice for a few minutes. Then at least 10 ng plasmid DNA was added to competent cells and this mixture was incubated on ice for 30 minutes. To promote the entrance of plasmid DNA into cells, the mixture was put in a 42 °C water bath for 90 seconds by following incubation on ice for 5 minutes. At the end of this period, 900 µL liquid LB medium was added and cells were incubated at 37 °C incubator for 45 minutes with gentle shaking to recover bacteria. This mixture can be plated directly, but in our case, the cells were centrifuged at at 3000 g for 3 minutes, supernatant was removed as a 900 µL, then the pellet was dissolved in the remaining amount. Pre-warmed LB agar plates with appropriate antibiotics were used to plate transformed bacteria. After incubation at 37 °C overnight when colonies were visible, plates were stored at 4 °C refrigerator for further use.

2.2.8.3.2. Transformation of *Agrobacterium tumefaciens*

Transformation of competent *Agrobacterium tumefaciens* cells was done with electroporation. After thawing frozen competent *A. tumefaciens* EHA105 strain on ice for a few minutes, 1 µg plasmid DNA (maximum 5 µl) was added and mixed gently on ice. The mixture was incubated for 30 minutes on ice and transferred to an electroporation cuvette prechilled before. MicroPulser Electroporation device set 'Agr' mode (Bio-Rad) was used for electroporation. Following pulsing, 1 ml sterile YEB liquid medium was added to cuvette. The solution was mixed gently by

pipetting and taken in an eppendorf tube. Agro cells were grown in a 28 °C incubator for 2 hours with agitation and spread on YEB agar plate with appropriate antibiotic. Plates were incubated at 28 °C for 3-4 days until colonies were grown. Colony PCR was made and positive colonies were sent sequencing (Table 5., 6.).

Table 5. Colony PCR components used to check insertion of *TaNAC69-1* gene in *Agrobacterium* cells.

Component	**Stock**	**Amount**
Taq buffer	10X	2 µl
dNTP mix	2.5 mM	2 µl
$MgCl_2$	25 mM	1.2 µl
Forward gene specific primer	5 µM	1 µl
Reverse gene specific primer	5 µM	1 µl
Nuclease-free water	-	Up to 20 µl
Taq DNA polymerase	5u/ µl	0.3 µl
Total	-	20 µl

Table 6. PCR conditions to analyze insertion of *TaNAC69-1* gene in *Agrobacterium*

Step		**Temperature**	**Duration**	**Cycle**
Initial Denaturation		94 °C	4 minutes	1
Amplification	Denaturation	94 °C	30 seconds	35
	Annealing	64 °C	30 seconds	
	Extension	72 °C	1 minutes	
Final extension		72 °C	5 minutes	1

2.2.9. Plant Transformation Studies

2.2.9.1. Surface Sterilization of Seeds

Surface sterilization of tobacco seeds were done using ethanol and sodium hypochlorite (NaOCl). Tobacco seeds were put in an eppendorf tube and treated with 70 % ethanol for 1 minutes. After seeds were rinsed with sdH_2O for three times, 10 % sodium hypochlorite was added and seeds were incubated in this solution for 30 minutes with gentle agitation. At the end of this period, seeds were rinsed again with sdH_2O for three times. Adequate amount of sterile water was used to keep seeds until the seeds were placed on tissue culture medium.

2.2.9.2. Micropropagation

MSA medium without plant growth regulators was used to germinate wild type tobacco seeds. For transgenic tobacco seeds, 10 mg/L PPT was added to MS medium to select transformed seeds. Germination was occurred into tissue culture room with 16 hours light and 8 hours dark at 23±25 °C approximately one month. At the end of this period, roots of emerging shoots were cut and shoots were transferred to jars with the same medium. Sub-culturing was made every 3 weeks to maintain fresh material for further studies.

2.2.9.3. Determination of Lethal PPT Concentration

Prior to start germination studies, effective concentration of plant selection agent (PPT) was determined. DL-Phosphinothricin (PPT), ammonium salt of glufosinate, is a herbicide used to select genetically engineered plants in tissue culture medium. In literature, there were some studies used PPT selection for tobacco transformation. Acccording to them, generally 5-10 mg/L PPT was enough to select transformed plants. In this study, 0, 5 and 10 mg/L PPT were applied to wild type tobacco plantles in tissue culture to see the effective dose of selection agent. While wild type plantles gave healthy roots on control media, plantles were died and not give any roots on selective media at the end of 2 weeks.

2.2.9.4. Preperation of Tissue Culture Media

MS salt basal medium (Duchefa) was used for regeneration, transformation and selection studies of tobacco plants. Medium was prepared according to manufacturer's instructions and pH was adjusted to 5.6-5.8 using 1 N KOH. Medium was autoclaved at 121 °C for 20 minutes. Filter sterilized plant hormones (NAA and BA) were added appropriate amounts according to purpose. Composition and purpose of the tissue culture media were given in Table 1.

2.2.9.5. Leaf Disc Transformation of *Nicotiana tabacum*

The transformation studies of tobacco leaves was done according to a method of Öktem et al. (1994). Edges, midribs and petioles of young leaves from 4-6 weeks old tobacco plants were cut into 0,5-1 cm^2 pieces. Leaf discs were incubated for 15 minutes with liquid MS including 1:10 times diluted *A. tumefaciens* cells which were grown overnight (OD600 = 0.6-0.8) at 28 °C in YEB medium. Liquid MS medium was used to treat control leaf discs.

At the end of this period, the leaf discs were removed from media and blotted on sterile filter paper to remove the excess *Agrobacterium* solution. Leaf discs were transferred on MS media including 1mg/ml BA and 0,1mg/ml NAA, then cocaltivated at dark for 3 days. After the co-cultivation period ended, leaf discs were transferred into liquid MS medium containing 300 mg/ml timentin and washed for 3 hours with gentle shaking at 120 rpm. The leaf discs were dried on sterile filter paper to remove excess medium and transferred on to MS media including 1mg/ml BA, 0,1mg/ml NAA, 200 mg/ml timentin to prevent bacterial growth and 10 mg/ml PPT for plant selection. Control leaf discs were put on MS medium with no antibiotic and selection agent. The leaf discs were kept at 25 °C with 16 hours light period. Plates were refreshed at every 10 days.

After 3-4 days from the transformation, when calli gave healthy shoots, regenerated explants were removed from callus with cutting from attachment points and transferred to baby jars including MS medium with 200 mg/ml timentin and 10 mg/ml PPT for transformed tobacco shoots and MSA medium for wild type tobacco

shoots. When roots become strong enough, regenerated plants were transferred to pots and put into growth chamber with 16 hours light 8 hours dark period at 25 °C. After regenerated plants adapted to soil, they transferred to greenhouse to induce flower development. Flowers were covered with clear plastic bags to prevent cross-pollination. When capsules dried completely, seeds of wild type and transgenic tobacco plants were collected.

2.2.10. Molecular Analysis of Transgenic Plants

2.2.10.1. Plant Genomic DNA Isolation

In order to confirm to presence of *TaNAC69-1* gene in T_0 putative transgenic plants, PCR analyses were performed with *TaNAC69-1* gene specific primers. For this purpose, genomic DNA was isolated with CTAB (Hexadecyl-trimethyl ammonium bromide) DNA extraction method based on Saghai-Maroof et al (1984). Fresh or frozen 2-3 plant leaves (1.5-2 grams) were ground to a fine powder using liquid nitrogen in prechilled mortar and pestle. CTAB buffer was pre-heated in 65 °C waterbath and added 1 ml for each sample. Samples were ground a little bit and transferred to sterile 2 ml eppendorf tubes. The tubes were incubated at 65 °C in a waterbath at least 45 minutes and inverted 3-4 times during this period. After incubation, the tubes were centrifuged at 3000 rpm for 10 minutes at 4 °C. Supernatant was taken to a new sterile 1.5 ml eppendorf tube and 0,8 volume phenol:chloroform:isoamylalcohol (25:24:1) was added from lower phase. The tubes were mixed by vortexing and centrifuged at the same manner. The upper phase was transferred to a new 1.5 ml eppendorf tube following addition of 0,8 volume chloroform: isoamylalcohol (24:1). Centrifuge was done at 3000 rpm for 10 minutes at 4 °C. The supernatant was mixed with 1 volume cold isopropanol in a new 1.5 ml eppendorf tube and inverted for precipitation of DNA. The tubes were kept at -20 °C at least 2 hours, but overnight incubation was preferred at most of the times. At the end of the incubation period, the tubes were spinned at 3000 rpm for 5 minutes at 4 °C to collect DNA as pellet. Supernatant was removed and pelleted DNA was washed with 1 ml cold 70 % ethanol. A final centrifuge was done at 3000 rpm for 5 minutes at 4 °C, then the pellet was dried in a hood and dissolved completely in sterile H_2O. OD_{260}/OD_{280} was determined via nanodrop spectrophotometer to check the purity of the samples. 1% agarose gel was run to verify the integrity of the

samples. PCR analyses were carried out using 500 ng DNA. 10 lines were used for PCR analyses. It was expected to obtain a PCR product approximately 1068 bp. The DNA was stored at –20 °C for further use.

2.2.10.2. Total RNA Isolation

TRIzol® reagent was used to isolate total RNA. Previously water was treated with 1 % v/v DEPC (diethylpyrocarbonate) to inactivate RNA degrading enzymes (RNases). Then all other materials used for RNA isolation were put into DEPC-treated water under hood at least for two hours to evaporate DEPC and autoclaved to remove traces of it. 0.2 g fresh or pre-frozen leaf tissue was disrupted with a pre-cooled mortar and pestle using liquid nitrogen. The fine powder was transferred to a pre-chilled centrifuge tube. Tubes were stored in liquid nitrogen until all samples were grinded. 1 mL Invitrogen TRIzol® reagent was added and vortexed for 20 minutes at room temperature using a block shaker. Tubes were centrifuged at 21000 g for 8 minutes in a pre-cooled centrifuge at 4 °C. 900 ul of supernatant was transferred to a new tube. 200 ul chloroform was added to each tube, mixed by vortex and incubated for 3-4 minutes at room temperature. The tubes were centrifuged at a speed of 21000 g for 20 minutes at 4 °C. 450 ul of upper phase were taken. 200 ul chloroform was added, tubes were shaked vigorously and incubated at room temperature for 10 minutes. Then centrifuge was done at 21000 g for 10 minutes at 4 °C to collect RNA as pellet. Supernatant was poured and 1 ml % 75 cold ethanol was added to each tube to wash pellet. Tubes were vortexed briefly and incubated at room temperature for 3 minutes. After that, tubes were centrifuged at 21000 g for 5 minutes at 4 °C. Supernatant was taken off and tubes were centrifuged for 30 seconds to collect the pellet at the bottom of the tubes. Visible liquid were removed and tubes were air dried in a hood for 10 minutes. 30-40 ul DEPC-treated or RNase free water was added. If pellets were not dissolved easily, tubes were incubated at 65 °C for 10 minutes. Nanodrop spectrophotometer was used to check RNA quantity and purity. RNA gel was run for 1 hour at 80 V to analyze the quality of RNA. The prepared RNAs were stored at -80 °C for further use.

2.2.10.3. Southern Blot Analysis

Southern blot analysis was done using genomic DNA of wild type and T_0 transgenic tobacco plants. Before loading DNA samples on agarose gel, they were digested with *Hind*III (Fermentas) enzyme which had a single cut region in the T-DNA. 100 units *Hind*III enzyme was used to cut 20 µg genomic DNA. Each reaction was incubated at 37 °C overnight, mixed with 6X loading dye and loaded on ethidium bromide free 0.8 % agarose gel. As DNA size marker, λ/*Hind*III was loaded into the first well. After finishing sample loading, the gel was run with 0.5 X TBE at 20 V overnight. To stain the gel, 0.5 µg/ml ethidium bromide solution was prepared and the gel was soaked in this solution for 15 minutes at dark with gentle agitation. The gel was washed and destained with dH_2O, at least 15 minutes with agitation and photographed .

To denature the DNA, the gel was washed twice with dH_2O in a plastic box. After draining water, 0.25 N HCl solution was put in the box and the gel was treated with this solution for 15 min with gentle agitation at room temperature. Following removal of HCl solution, the gel was washed twice using dH_2O to remove residual of HCl. The gel was agitated with denaturation solution for 15 minutes, then rinsed with dH_2O and neutralization solution was added. DNA and gel matrix were neutralized with this solution for 30 min and the gel was washed with dH_2O.

To transfer DNA to naylon membrane, a platform was prepared in a plastic tray and a bridge was built with a Whatman 3MM paper. The paper was wetted using 10X SSC solution and bubbles formed between platform and paper was removed with a plastic rod. Two other piece of Whatman 3MM paper as the same size with the gel was put in the same way. The gel was positioned upside down and was wetted with 10X SSC. Positively charged naylon membrane was soaked in 10X SSC and placed on the top of the gel. Two sheets 3MM Whatman paper was put on the membrane and air bubbles were removed using a plastic road. Approximately 8-10 cm high blotting paper stack was laid down on top of the 3 MM papers. 10X SSC buffer was and put in the tray with half filling and the tray was wrapped with cling film to prevent evaporation of buffer. A 500 g weight was placed on the paper stack. DNA

was transferred overnight and the blot was dismantled at the following day. Then the DNA was immobilized on the membrane by UV irradiation.

DNA samples were marked with DIG labeling system. For this purpose, PCR reaction was prepared to label *bar* gene fragment using DIG DNA labeling mixture unlike control reaction prepared with dNTP. The PCR components, primers and conditions for amplification of *bar* gene was given in Table 7., 8. and 9. PCR products were run on 1.5 % agarose gel. DIG labeled PCR product was heavier than control PCR product, then it run a shorter distance on the agarose gel. After conformation, 17 µl DIG labeled PCR product was mixed with 10 ml hybridization buffer and this mixture was stored in – 20 °C for further use.

Table 7. PCR components used for synthesis of *bar* gene probe.

Component	**Stock**	**Amount**
Taq buffer	10X	2 µl
DIG DNA labelling mix	10X	2 µl
Forward *bar* gene primer	10 µM	1 µl
Reverse *bar* gene primer	10 µM	1 µl
Template DNA (plasmid)	25 ng/µl	1 µl
Taq DNA polymerase	5 u/ µl	0.5 µl
dH_2O	-	12.5 µl
Total	-	20 µl

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Table 8. Primer sequences for *bar* gene.

Gene	Direction	Primer sequence
bar	Forward	ACCGGCAGGCTGAAGTCCAGCTG
	Reverse	TCGCCGAGGTGGACGGCGAGGTC

Table 9. PCR conditions for synthesis of *bar* gene.

Step		Temperature	Duration	Cycle
Initial Denaturation		94 °C	4 minutes	1
Amplification	Denaturation	94 °C	30 seconds	35
	Annealing	58 °C	30 seconds	
	Extension	72 °C	1 minute	
Final extension		72 °C	5 minutes	1

For hybridization, a mesh was put under the membrane and the membrane was wetted with 2X SSC. The membrane was placed glass hybridization tube and 10 ml DIG hybridization buffer was added. After incubation at 42 °C for 2 hours at 20 V in hybridization oven, denatured probe solution was put in the tube with overnight incubation at 42 °C. To denature DIG labeled probe, the probe was boiled for 5 minutes and chilled on ice. At the end of hybridization, the membrane was taken from glass tube and and washing was started firstly with low stringency buffer. The membrane was placed in a plastic box and incubated with low stringency buffer for 5 minutes with gentle agitation. After repeating this step one more time using fresh buffer, the solution was poured and the membrane was washed twice with prewarmed high stringency buffer under agitation for 15 minutes. Maleic acid buffer with 0.3 tween 20 was used to rinse out the membrane for 5 minutes at room temperature. After transferring the membrane in a plastic bag, it was incubated with 10 ml 1% blocking solution (Roche) for 30 minutes at room temperature on a shaker.

After that the membrane was treated with anti-Digoxigenin-AP solution for another 30 minutes rinsed with maleic acid buffer twice for 15 minutes in a plastic box. After washing finished, the membrane was tranfferred in a plastic bag and CDP-Star solution (Biolabs) was put in the bag with incubation for 15 minutes at dark because of light sensitiveness of CDP-Star solution. To remove CDP-Star solution from the bag, one end was cut and removal of excess solution was carried out completely. To image the signal on the membrane, X-ray film or CCD imaging system were used.

2.2.10.4. Two-Step qRT-PCR, Measurement Expression Level Changes of *TaNAC69-1* Gene

qRT-PCR enables to quantify a targeted DNA molecule simultaneously. Detection of amplified DNA molecule is made as reaction proceeds in real time. Reverse transcription and PCR are done in separate tubes in qRT-PCR. SYBR Green-based detection method was used for quantification of *TaNAC69-1* gene. A comparison between expression of the target and the control gene (generally a housekeeping gene) was made with relative quantification method. *Actin* was used as control gene for real-time qPCR.

2.2.10.4.1. Growth of Plants and Stress Applications

Surface sterilized wild type and transgenic tobacco seeds were germinated on duchefa boxes with MS media. For transgenic tobacco seeds, 10 mg/L PPT was added to tissue culture medium. Seeds were put in a growth chamber at 23 ± 2 °C for 1 month with 16 hours light and 8 hours dark period. Stress treatments of plantles were started on the 30th day of growth. For salt and drought stresses, 250 mM NaCl and 10 % polyethylene glycol (PEG-6000) solutions were prepared adding them in ½ strength Hoagland’s Solution. For control applications, ½ strength Hoagland’s Solution was used. Control (non-treated) and stress (treated) plants were transferred into this solutions and kept in plant growth chamber. Leaf tissues were used for gene expression studies and sample collection was made at 0th, 12th, 24th and 48th hour of stress application. Experiments were done using three biological replicates and leaf samples were frozen in liquid nitrogen for further use.

2.2.10.4.2. Total RNA Isolation and Characterization

Plant total RNA was isolated using QIAGEN RNeasy Plant Mini Kit. 100 mg plant leaf was used as starting material. The quantity of RNA was checked with NanoDrop 3300 Fluorospectrometer (Thermo Scientific). The A260/A280 ratio between 1.8-2.1 shows highly purified RNA. To determine the quality of RNA samples, RNase-free agarose gel was run at 80 V for 45 minutes. Sharpness of 18 S and 28 S bands showed the integrity of RNA samples.

2.2.10.4.3. cDNA Synthesis

cDNA synthesis was made according to QIAGEN QuantiTect Reverse Transcription Kit's manual. To eliminate genomic DNA, 500 ng RNA was mixed with gDNA Wipeout buffer and RNase-free water. This mixture was incubated at 42 °C for 2 minutes, then immediately placed on ice. Then master mix was prepared using Reverse Transcriptase, RT Buffer and RT Primer mix. Template RNA was added to master mix and total reaction was incubated at 42 °C for 20 minutes, then at 95 °C for 3 minutes to inactivate Reverse Transcriptase. Reverse-transcription reaction was directly used for qRT-PCR or stored at – 20 °C for further use.

2.2.10.4.4. qRT-PCR

TaNAC69-1 gene expression level changes was measured under salt and drought stress. Changes in mRNA expression levels of *TaNAC69-1* gene was measured by relative quantification also known as comparative quantification. Relative quantification determines the ratio of amount of target with regard to amount of control (a housekeeping gene). Any transcript with a known sequence can be a reference for relative quantification method. *Actin* was used as endogenous reference gene for qRT-PCR measurement.

A dilution series of pool cDNA was prepared to compare amplification efficiency of *TaNAC69-1* gene and *actin* (endogenous reference gene). Quantitative RT-PCR was carried out using 50 ng, 5 ng, 0.5 ng, and 0.05 ng pool cDNA with primer pairs of *TaNAC69-1* and *actin* gene. qRT-PCR reactions were performed with three technical

replicates of each dilution using QuantiTect SYBR Green PCR Kit. PCR components, conditions and primer sequences were given in Table 10., 11. and 12. respectively. Three no template control containing all qRT-PCR components without template was used to check any DNA contamination because of pipetting errors or in the master mix. Standart curves for *TaNAC69-1* and *actin* gene were constructed using the C_T values obtained from amplification of dilution series in qRT-PCR.

After comparing amplification rate of *TaNAC69-1* and *actin*, quantitative real-time PCR was performed using cDNAs of wild type and transgenic tobacco lines. Five biological and three technical replicates were used for qRT-PCR reactions with 10 ul QuantiTect Master Mix, 1 µL cDNA, 0.5 µM primers and total volume was completed to 20 µL. After obtaining C_T values of samples coming from control and stressed plants, amount of TaNAC69-1 gene were determined comparing standart curve and C_T values. Normalized amount of target gene was calculated by dividing the amount of target by the amount of reference.

Table 10. PCR components used for amplification of *TaNAC69-1* and *actin* gene in qRT-PCR

Reagent	**Volume/reaction**
Forward primer	1 µl
Reverse primer	1 µl
QuantiTect master mix	10 µl
cDNA	Variable (0.05-50 ng)
dH_2O	Up to 20 µl

Table 11. PCR conditions for dilution series of pool cDNA of *TaNAC69-1* and *actin* gene for qRT-PCR

	PCR conditions			
Gene name	**Step/Segment**	**Temperature**	**Duration**	**Cycle**
TaNAC69-1	Pre-incubation	95 °C	15 min	1
	Quantification	94 °C 54 °C 72 °C	15 sec 30 sec 20 sec	40
	Melt curve	65-95 °C	5 sec/1 °C	1
actin	Pre-incubation	95 °C	15 min	1
	Quantification	94 °C 54 °C 72 °C	15 sec 30 sec 20 sec	40
	Melt curve	65-95 °C	5 sec/1 °C	1

Table 12. Primer sequences of *TaNAC69-1* and actin gene used in qRT-PCR.

Gene name	**Direction**	**Primer sequence**	**Tm (°C)**	**Amplicon size (bp)**
TaNAC69-1	Forward	5'-CCGAAAACCCACTCATCTAC-3'	53.0	168
	Reverse	5'-TCTTCCTCTTCATGCCGTTA-3'	53.4	
actin	Forward	5'-TTCTTCGTTTGGACCTTGCT-3'	54.6	150
	Reverse	5'-AGTGTCAAGCTCCTGCTCGT-3'	54.7	

2.2.11. Physiological Characterization of Transgenic Plants

Three transgenic tobacco T_2 lines (NT2, NT6, NT33) were chosen for stress experiments. Firstly to determine limiting concentrations of NaCl, LiCl and mannitol for salt, osmotic and ionic stress experiments, wild type plants were germinated on MSA media including 0, 50, 100, 150, 200, 250, 300 mM NaCl; 0, 100, 200, 300, 400 mM mannitol; and 0, 50, 100 mM LiCl. After determining concentrations which inhibit plant growth, germination test and further physiological analysis were carried out using 250 mM NaCl, 100 mM LiCl and 300 mM mannitol.

2.2.11.1. Germination test

250 mM NaCl, 300 mM mannitol and 100 mM LiCl were used to test germination and survival ability of seeds of wild type and transgenic tobacco lines under salt, osmotic and ionic stress. After surface sterilization of wild type and each transgenic tobacco lines with 70 % ethanol for 1 minute then 10 % hypochlorite for 20 minutes, seeds were sowed on MSA media including 250 mM NaCl, 300 mM mannitol and 100 mM LiCl. MSA medium was used as control. Approximately 50 seeds were put in a plate, then plates were incubated in tissue culture room for 15 days with 16 hours light, 8 hours dark period at 25 °C and pictures were taken.

2.2.11.2. Root and Shoot Length Measurements

Wild type and transgenic tobacco plantles grown on MSA medium were used for NaCl, LiCl and mannitol treatment. 15 days old plantles with the similar growth were taken from media with roots and transferred to MSA medium including 250 mM NaCl, 100 mM LiCl and 300 mM mannitol. 10 plantles were used from each line for each treatment. Plates were put in a tissue culture room with 16 hours light and 8 hours dark period for 1 month at 25 °C. At the end of this period , root and shoots were seperated from each other, and shoot and root lenghts were determined.

2.2.11.3. Root and Shoot Fresh Weight Measurements

Wild type and transgenic tobacco plantles grown on MSA medium were used for NaCl, LiCl and mannitol treatment . After 15 days of growth period, 10 plantles from each line with similar growth were transferred to 250 mM NaCl, 100 mM LiCl and 300 mM mannitol including medium. Plantles were grown for 1 month in a tissue culture room with 16 hours light, 8 hours dark period at 25 °C. After 1 month, plants were removed from the medium, roots were separated from shoots. Shoot and root fresh weights were determined.

2.2.11.4. Root and Shoot Dry Weight Measurements

15 days old wild type and transgenic tobacco plantles grown on MSA medium were used for NaCl, LiCl and mannitol treatment. 10 plantles with similar growth stage from each line were taken from the petri plates and transferred to 250 mM NaCl, 100 LiCl and 300 mM mannitol including MSA medium. Plantles were incubated in a tissue culture room for 1 month with 16 hours light and 8 hours dark period at 25 °C. At the end of this period, plants were removed from the medium, and root and shoots separated from each other. They were put in a 60 ^{0}C oven for 2 days and dry weights of the samples were measured.

CHAPTER 3

RESULTS AND DISCUSSION

NAC type transcription factors are involved in plant development and abiotic stress tolerance. *TaNAC69-1* gene, a NAC type transcription factor, are isolated from *T. aestivum* L. cv. Yüreğir- 89 and cloned into a dicot expression vector. To check the insertion of gene into tobacco genome, southern blot was performed. Quantitative Real-time PCR was made to see the effects of salt and drought stress on expression of *TaNAC69-1* gene. Physiological experiments were performed using NaCl, mannitol and LiCl to determine effects of abiotic stress at physiological level.

3.1. Cloning of *TaNAC69-1* Gene

Triticum aestivum L. cv. Yüreğir-89 plants was used for total RNA isolation of *TaNAC69-1* gene. To increase expression level of *TaNAC69-1* gene, salt stress was applied to *T. aestivum* L. cv. Yüreğir-89 plants at growing stage. Total RNA was isolated using TRIzol reagent. Quality and quantity of RNA samples were checked by nanodrop spectrophotometer and running on agarose gel electrophoresis (Figure 9.).

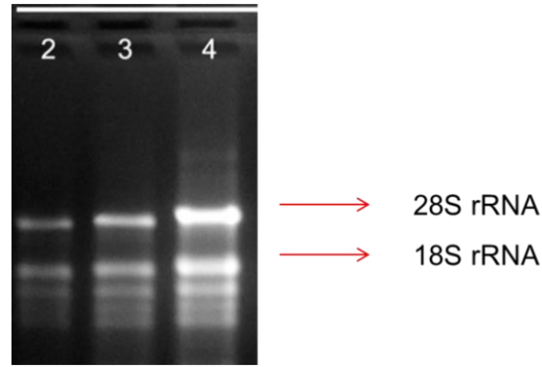

Figure 9. Gel electrophoresis results of total RNA isolated from *T. aestivum* L. cv. Yüreğir-89. RNA samples were loaded on RNase-free 1 % agarose gel and run at 80 V for 45 minutes.

T. aestivum L. cv. Yüreğir-89 cDNA samples were used for amplification of *TaNAC69-1* gene. cDNA synthesis was made according to manual of RevertAid™ First Strand cDNA Synthesis Kit. *TaNAC69-1* gene was amplified using gene specific primers and run on 1 % agarose gel at 100 V for 1 hour. Amplified PCR product of *TaNAC69-1* gene was extracted from the gel and sent sequencing with gene specific primers. Figure 10. shows PCR result of *TaNAC69-1* gene amplified by gene specific primers.

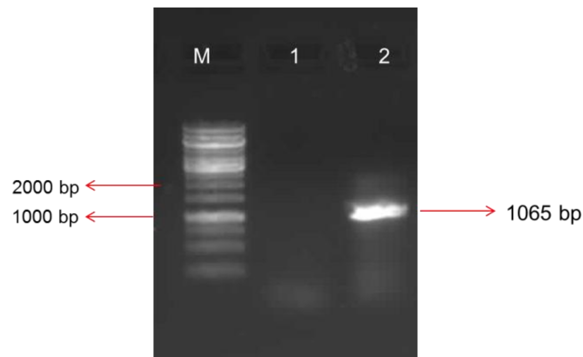

Figure 10. Gel electrophoresis result of PCR amplified *TaNAC69-1* gene. M: GeneRuler 1 kb DNA ladder (SMO311), lane 1: negative control, lane 2: PCR amplified product of *TaNAC69-1* gene. Amplicon length was about 1065 bp.

Following sequencing, blunt-end PCR product was cloned directionally into pENTR™/D-TOPO cloning vector. This reaction requires no post PCR procedures, no enzyme digestion or no ligase. After that, chemically competent *E. coli* cells were transformed with the vector carrying *TaNAC69-1* gene via heat shock method. *E. coli* cells were spread on a selective LB plate and incubated at 37 °C. Selected colonies were grown in LB liquid culture , plasmid isolation was carried out and isolated plasmids were sent sequencing to verify insertion of the gene in the plasmid.

attB-flanked entry clone of *TaNAC69-1* gene was transferred into Gateway-compatible binary destination vector, pEarleyGate 100, via LR reaction with site specific recombination. Competent *E. coli* transformation was made by means of heat shock. After growing *E. coli* cells on selective plates, colony PCR was made with selected colonies using gene specific and *bar* gene primers. Plasmid isolation was done from positive colonies and *A. tumefaciens* EHA105 strain was transformed with destination vector via electroporation. Verification of transformation of Agro cells with *TaNAC69-1* gene was done by colony PCR and sequencing with gene specific primers. Figure 11. shows gel electrophoresis result of colony PCR made by using *TaNAC69-1* gene specific primers. All of the colonies gave positive result after colony PCR.

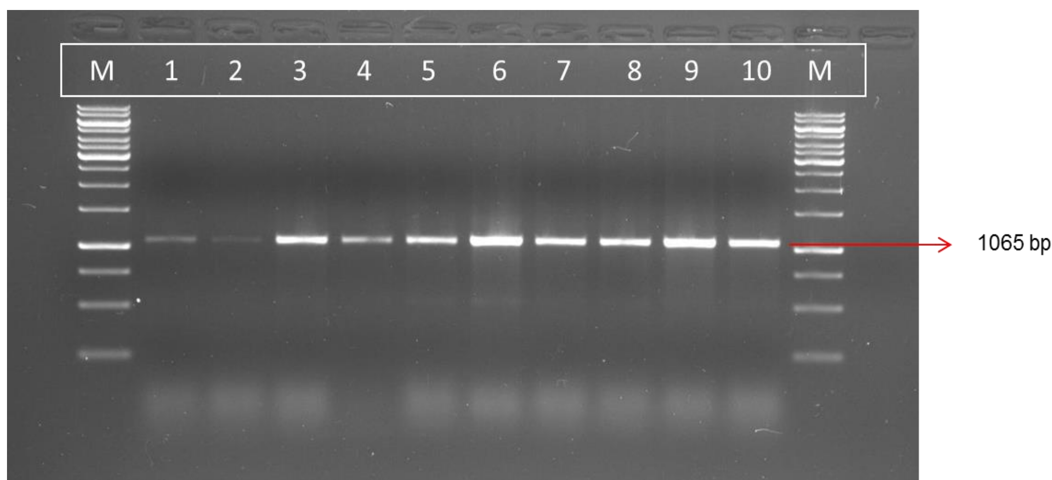

Figure 11. Gel electrophoresis result of PCR amplification for colony PCR of *Agrobacterium* EHA 105 strain carrying *TaNAC69-1* gene. Amplicon length was about 1065 bp. M: GeneRuler 1 kb DNA ladder (SMO311), lane 1-10: Amplified PCR product of *TaNAC69-1* gene.

3.2. Determination of Lethal PPT Concentration

L-Phosphinothricin (PPT) is a herbicide which is an inhibitor of glutamine synthase (GS) enzyme activity, thus toxic levels of ammonia accumulate into the cells. PPT is generally used in tissue culture studies to select resistant transgenic plants which show resistance to this herbicide via *bar* gene insertion. *bar* gene encodes an enzyme, phosphinothricin acetyl transferase (PAT), which acetylates the amino group of glufosinate and leads to inactivation of PPT.

There were some studies used PPT selection for tobacco transformation. Acccording to them, generally 5-10 mg/L PPT was enough to select transformed plants (De Block et al., 1987, Lutz et al., 2001, Kang et al., 2005). In this study, 0, 5 and 10 mg/L PPT were applied to wild type tobacco plantles in tissue culture to see the effective dose of selection agent. It was observed that there was no root and new leaf formation in the media including 5 and 10 mg/L PPT. At the end of 3 weeks, plantles were died completely in selection media. Therefore, PPT with 10 mg/L concentration was chosen to select transformed tobacco plants for further studies.

Figure 12. shows response of plantles grown on control and selection agent including media.

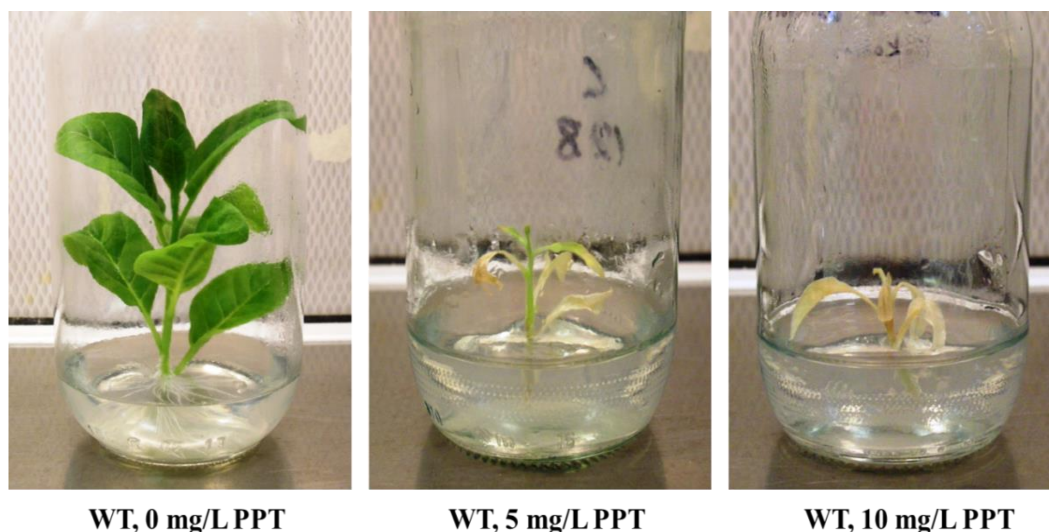

Figure 12. Determination of effective PPT concentration for transformation studies using subcultured wild type tobacco plantles. Picture was taken after 15 days of PPT treatment. WT:wild type tobacco.

After transformation transgenic plants were used to test whether PPT concentration was adequate or not. For this purpose wild type and transgenic tobacco plant seeds, leaf parts and shoots were plated on MS media including 0, 5 and 10 mg/L PPT. Wild type and transgenic seeds germinated, plant leaves were formed calluses, and shoots gave roots on control MS media. On the other hand on 5 and 10 mg/L PPT including media wild type plant seeds were germinated but died completely some time later, leaf discs turned yellow, and shoots did not give any roots. On the same media transgenic plants formed calluses from leaf discs, seeds germinated and gave healthy roots. Therefore, PPT with 10 mg/L concentration was chosen to select transformed tobacco plants for further studies. Figure 13., 14. and 15. showed callus formation, seed germination and root development of wild type and transgenic tobacco lines on control and PPT including MS media.

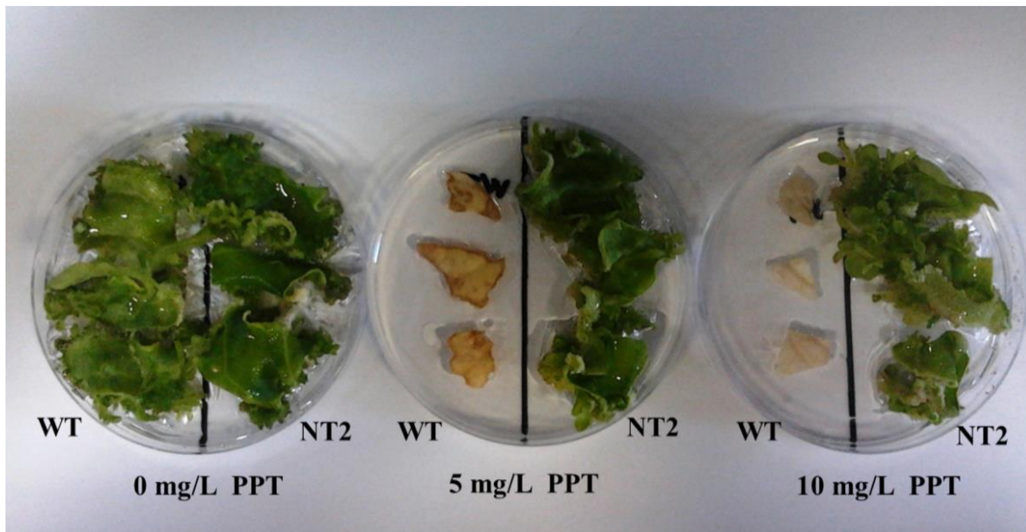

Figure 13. Callus formation from leaf discs of wild type and transgenic tobacco lines on control, 5 and 10 mg/L PPT including MS media. Picture was taken after 15 days of PPT treatment. WT: wild type tobacco, NT2: T0 putative transgenic tobacco line 2.

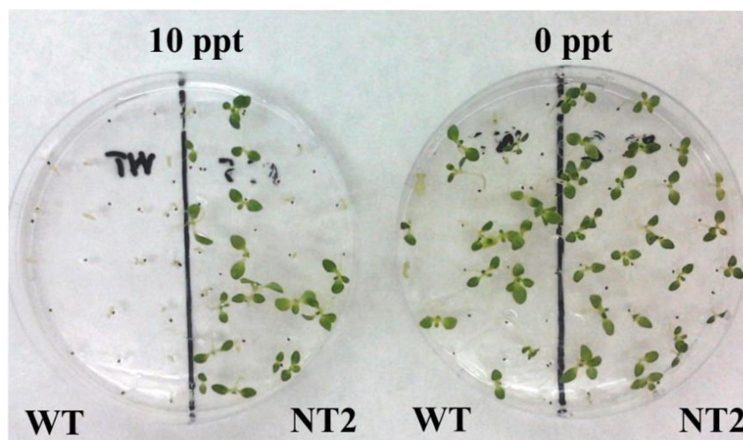

Figure 14. Germination of wild type and T0 transgenic tobacco seeds on control and 10 mg/L PPT including media. 10 mg/L PPT including MS medium on the left, control MS medium on the right, WT: wild type tobacco, NT2: T0 transgenic tobacco line 2. Picture was taken after 10 days following seeding.

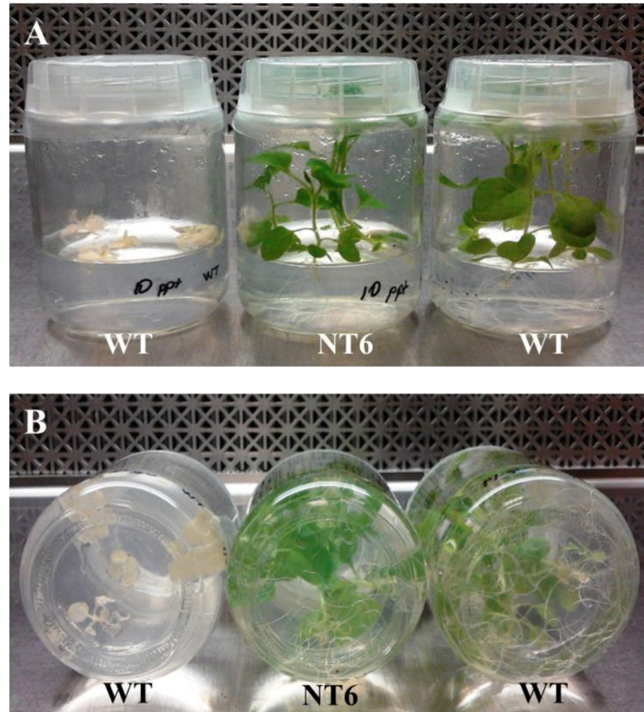

Figure 15. Growth and root development of wild type and transgenic tobacco plantles on control and selective MS media. Selective media including 10 mg/L PPT on the left and middle, control media on the right. WT: wild type, NT6: T0 transgenic tobacco line 6. Picture was taken after 2 weeks.

3.3. Transformation of Tobacco Plants

Agrobacterium mediated transformation protocol (Öktem et al., 1994) was used to transform tobacco plants with *A. tumefaciens* EHA105 strain carrying *TaNAC69-1* gene. Tobacco plants grown under same conditions and with same age were used for transformation studies. After incubation of leaf discs with *Agrobacterium* solution and liquid MS for untreated ones, all explants were put in nonselective media for 3 days. At the end of co-cultivation period, leaf discs were washed with liquid MS media containing 300 mg/L timentin to remove bacteria.

Selective media were used for leaf discs treated with *Agrobacterium* cells and non-transformed (negative control) ones, and non-selective media for positive control leaf discs. After 2-3 weeks callus formation was started to be seen from transformed and

positive control leaf discs. On the other hand, negative control leaf discs were turned yellow and no callus formation was observed.

Despite *bar* gene prevented transformed leaf discs from toxic effects of PPT, growth was retarded in transformed cells, and positive controls produced callus and shoots faster than transformed ones. Also leaf discs treated with *Agrobacterium* were survived on 10 mg/L PPT containing medium more than 70 %. But untreated leaf discs were completely died on selective medium at this period.

After 4-5 weeks following transformation, calli were started to give healthy shoots. When shoots grew enough to remove from calli, they were cut from attachment points and transferred to root induction medium including 200 mg/L timentin and 10 mg/L PPT for transgenic shoots. Control shoots were placed on MS medium without antibiotic, plant hormones and selection agent. Root formation was started from transgenic and control shoots in two weeks and it showed that transformation was successful because transgenic shoots gave healthy roots on selective media. Transgenic shoots were transferred to soil after growing roots strong enough.

Transformation studies were repeated for at least 3 times. A number of 45 transgenic tobacco lines were reached at the end of several transformation experiments. Figure 16. shows representative pictures of callus formation, shoot formation from callus and root development of a tobacco plantlet in MS media.

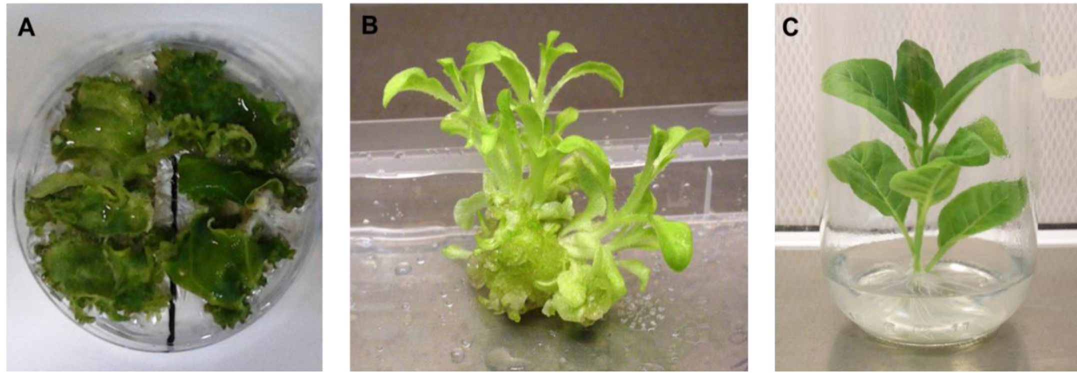

Figure 16. Representative pictures of A: callus formation, B: shoot formation from callus, C: root development of tobacco plantlet in MS media.

3.4. Development in Soil

Development of regenerated plantles in soil and production of fertile seeds to check integrity of and stability of gene in the plant genome are important parts of transformation studies. Because plantles were sensitive and grown at controlled conditions in the jars, they covered with plastic bags to prevent evaporation after transferring the soil and put in a growth chamber at 25 °C with 70 % humidity to adapt plantles to soil. Totally 27 plantles were transferred to soil for further growth and development. Approximately 1 month later, plants were taken to greenhouse and started flowering within 2.5-3 months. Control plants grew up and produced seeds faster than trangenic ones. Seed collection was made about 5 months later. Figure 17. showed pictures of tobacco plants growing and flowering in the greenhouse.

Figure 17. Putative T0 transgenic tobacco plants growing in the greenhouse conditions. A:growth of 2 independent lines of T0 transgenic tobacco plants. Picture shows 1 month old plants after transferring the soil. B & C: Flowering of T0 transgenic tobacco plants. Pictures show 3 months old plants after transferring the soil.

3.5. Analysis of Transgenic Plants

3.5.1. Germination Test

To determine whether or not *TaNAC69-1* gene was transferred to T_0 progeny successfully, germination test was done. For this purpose, 9 T_0 transgenic tobacco lines were randomly selected and their seeds were germinated on MS media including 10 mg/L PPT. Approximately 120 seeds were sown on the media and number of germinated seeds and survived plantles were counted after 1 week and 2 weeks, respectively. Table 13. shows number of germinated and survived seeds of wild type and T_0 transgenic tobacco seeds on 10 mg/L PPT including media.

Table 13. Germination and survival rates of wild type and selected T_0 transgenic tobacco seeds grown on MS media including 10 mg/L PPT. WT: wild type, NT2-NT42: selected T_0 transgenic tobacco lines.

Name of line	# of seeds	% germination	% survival
WT	119	55,63	0
NT2	123	97.82	72.56
NT5	117	93.65	76.43
NT6	116	98.47	82.98
NT13	120	95.34	73.67
NT17	122	97.49	89.48
NT24	123	89.75	69.32
NT33	118	91.63	75.46
NT35	115	87.56	64.59
NT42	119	88.74	71.63

Figure 18. represents the appearances of 2 weeks old wild type and selected T_0 lines of transgenic tobacco plantles on selective media after seed sowing. Wild type seeds were used as control and they germinated on selective media but died completely in two weeks. On the other hand all transgenic tobacco lines germinated and grew easily on selective media. Among them, NT33 line grew faster than wild type and other transgenic tobacco lines.

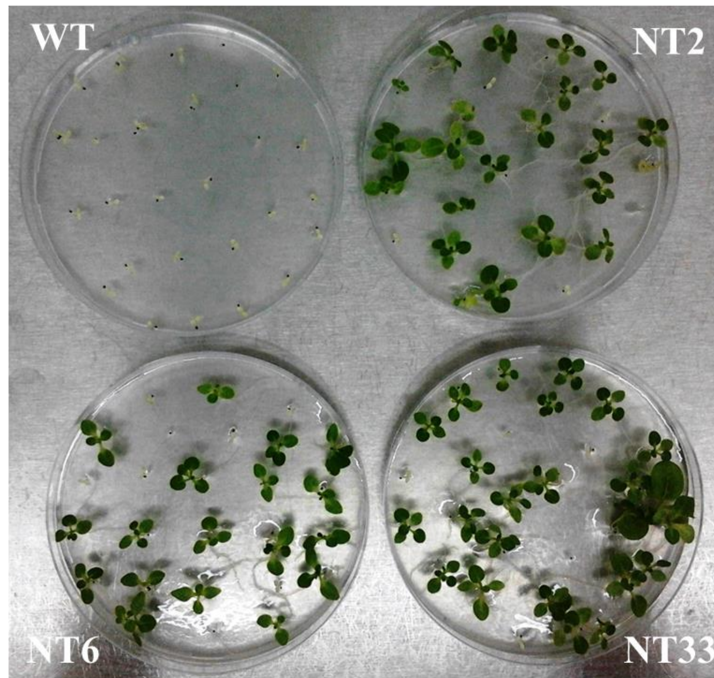

Figure 18. Germination and growth of wild type and selected T0 transgenic tobacco seeds on 10 mg/L PPT including media. Pictures were taken after 2 weeks following seed sowing. WT: wild type tobacco, NT2: transgenic tobacco line 2, NT5: transgenic tobacco line 5, NT33: transgenic tobacco line 33.

3.5.2. Molecular Analysis

3.5.2.1. PCR Analysis with *TaNAC69-1* and *bar* Gene Specific Primers

Polymerase chain reaction (PCR) is a technique used in molecular biology studies to amplify a piece of DNA generating millions of copies of it. PCR analysis was made to be sure about integration of *TaNAC69-1* gene into T_0 transgenic tobacco lines. After genomic DNA isolation was carried out with CTAB DNA extraction method, PCR analysis was made using *TaNAC69-1* and *bar* gene specific primers. Negative control was prepared with PCR sample including all elements of PCR but any DNA. Wild type control was also used to verify that wild type plants were not carry the gene of interest. As positive control, pure plasmid carrying *TaNAC69-1* gene was used. PCR reactions were made 3 times to achieve reliable results. PCR results were shown in Figure 19. and 20. All of the transgenic lines gave positive results in PCR with *TaNAC69-1* and *bar* gene specific primers. On the other hand, wild type sample and negative control including no DNA template did not give any PCR bands. This

results showed that *TaNAC69-1* gene was successfully transfrerred to into the genome of T_0 transgenic tobacco lines.

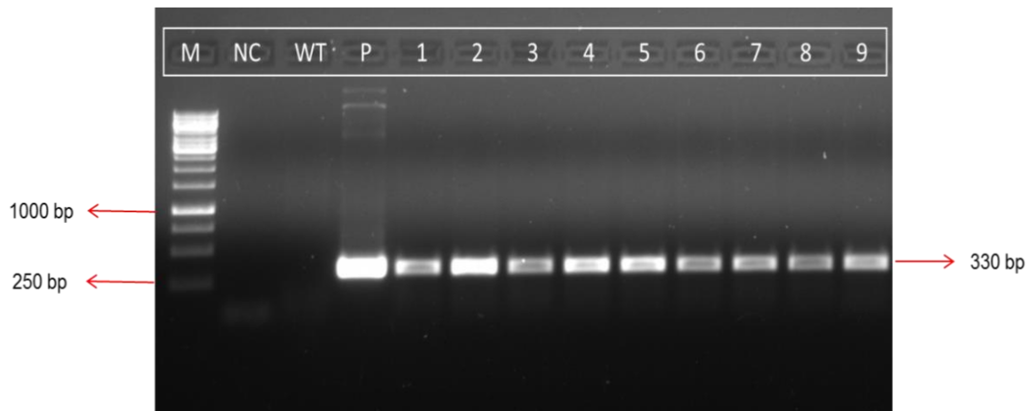

Figure 19. PCR amplification result of putative T_0 transgenic and wild type tobacco plants with *bar* gene specific primers. M: GeneRuler 1 kb DNA ladder (SMO311), NC: negative control, WT: wild type tobacco plant, 1-9: Amplified PCR product of *bar* gene. Amplicon length was about 330 bp.

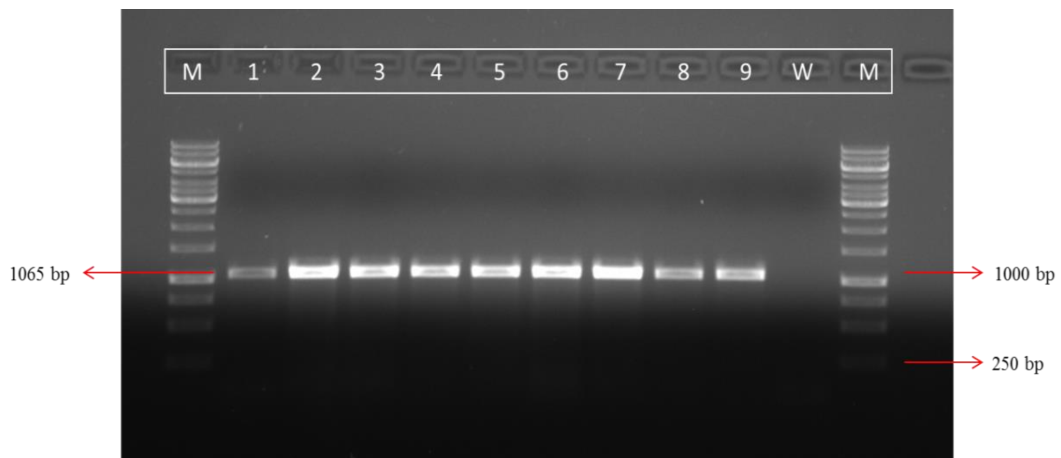

Figure 20. PCR amplification result of putative T_0 transgenic and wild type tobacco plants with *TaNAC69-1* gene specific primers. M: GeneRuler 1 kb DNA ladder (SMO311), 1-9: Amplified PCR product of *TaNAC69-1* gene, W: wild type tobacco plant, Amplicon length was about 1065 bp.

3.5.2.2. Southern Blot Analysis

Southern blot is a technique which is used to detect a specific fragment of DNA into DNA samples. It uses transfer of DNA samples to a membrane and detection of them via probe hybridization. For this purpose, isolation of DNA samples from wild type and T_0 transgenic tobacco lines was made by CTAB DNA extraction method. Purity of DNA samples was checked in a nanodrop spectrophotometer looking their OD_{260}/OD_{280} values. % 1 agarose gel was run to analyze integrity of the samples. Concentrations of DNA samples were shown in Table 14. Figure 21. showed the genomic DNAs separated on 1% agarose gel.

Table 14. DNA concentrations of wild type and selected transgenic tobacco lines used for Southern blot analysis.

Line	**OD_{260}/OD_{280}**	**OD_{260}/OD_{230}**	**Conc (ng/µl)**
WT	2.11	2.08	1804.2
NT2	2.06	2.12	2268.1
NT5	2.06	2.09	3453.4
NT6	2.07	2.16	3312.7
NT13	2.05	2.05	3139.2
NT17	2.08	2.07	2968.4
NT24	2.06	2.05	2563.6
NT33	2.08	2.01	3234.9
NT35	2.03	2.10	3149.0
NT42	2.07	2.10	1628.6

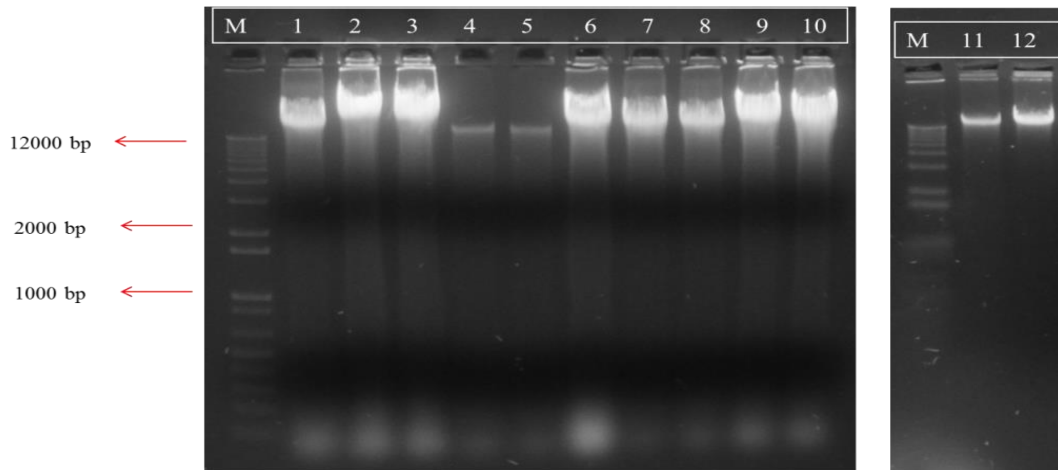

Figure 21. Genomic DNA of wild type and selected T0 transgenic tobacco lines. M: Invitrogen 1 kb Plus DNA ladder, 1:NT2, 2:TN5, 3:NT6, 4:NT8, 5:NT11, 6:NT13, 7:NT17, 8:NT24, 9:NT33, 10:NT35, 11:NT42, 12:WT.

DIG labeling system was used to mark DNA samples. DIG-labeled *bar* probe and and not-labeled control PCR products were run on agarose gel to be sure labelling was done successfully. Size of DIG-labeled probes were bigger than control PCR products, thus they run less distance on agarose gel (Figure 22.). Comparing labeled and control products we would say labelling was succesfull.

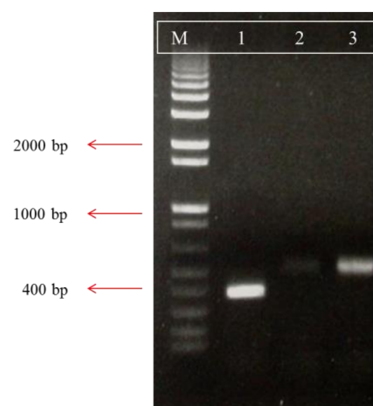

Figure 22. Agarose gel result of DIG-labeled *bar* and non-labeled control PCR products . M: Invitrogen 1 kb Plus DNA ladder, 1:non-labeled *bar* PCR product, 2&3:DIG-labeled *bar* probes.

Before Southern Blot, 20 µg genomic DNA from wild type and transgenic tobacco samples digested using *Hind*III enzyme. Digested samples were run on 0.8% agarose gel. Figure 23. shows separation of digests of wild type and *TaNAC69-1* lines on agarose gel. After gel electrophoresis, DNA samples were transferred to naylon membrane and hybridized with *bar* gene probes. Figure 24. showed the insertion of single, double and multiple copies of insertion of bar probes in *TaNAC69-1* transgenic tobacco lines.

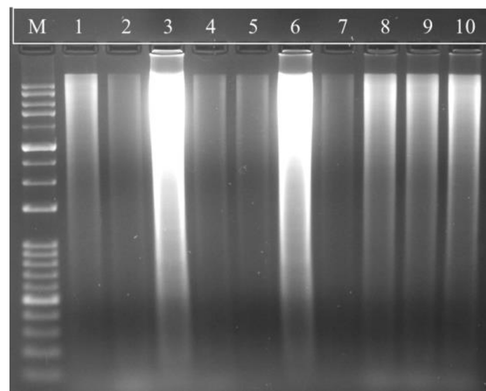

Figure 23. *Hind*III digested genomic DNA of wild type and T0 transgenic tobacco lines separated on 0.8 % agarose gel. M: λ *Hind*III DNA ladder, 1:WT, 2:NT2, 3:NT5, 4:NT6, 5:NT13, 6:NT17, 7:NT24, 8:NT33, 9:NT35, 10:NT42.

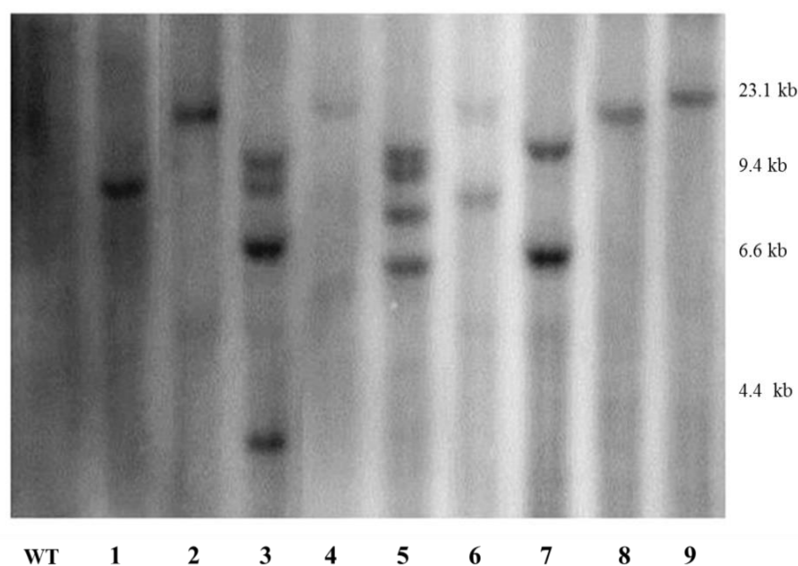

Figure 24. Southern blot of *bar* in the genome of T_0 transgenic tobacco lines. WT: wild type tobacco, 1:NT2, 2:NT5, 3:NT6, 4:NT13, 5:NT17, 6:NT24, 7:NT33, 8:NT35, 9:NT42 T_0 transgenic tobacco lines.

3.5.2.3. qRT-PCR Analysis for Expression Profile of *TANAC69-1* gene

To figure out the expression profile of *TaNAC69-1* gene under salt and drought stress conditions, RNA samples collected from leaves of wild type and 5 different transgenic tobacco lines at different time periods including 0th, 12th, 24th and 48th hours was used to perform qRT-PCR analysis. Data analysis was performed according to relative quantification method.

Primer design is an important point for easy amplification and fluorescence signal detection of PCR product. So primer pairs with a ~ 50 % GC content, ~ 20 bp length and a product size of 158 and 168 bp were designed for *TaNAC69-1* and reference gene, *actin*, respectively. To be used in RT-PCR analysis, primers must produce single specific amplicons in PCR experiments. Thus primers for *TaNAC69-1* and *actin* gene was firstly amplified in conventional PCR and products run on 2.5 %

agarose gel to see if there were any unspecific products or primer dimers. Figure 25. and 26. show PCR amplification result of *TaNAC69-1* and *bar* gene primers.

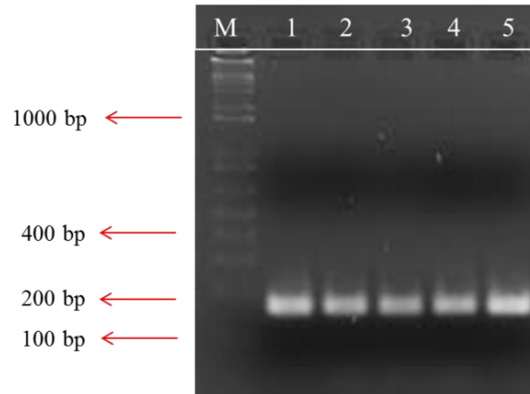

Figure 25. PCR amplification result of cDNA samples with *TaNAC69-1* gene specific primers. M: Invitrogen 1 kb Plus DNA ladder (SMO311). 1-5: Amplified PCR product of *TaNAC69-1* gene. Amplicon length was about 168 bp.

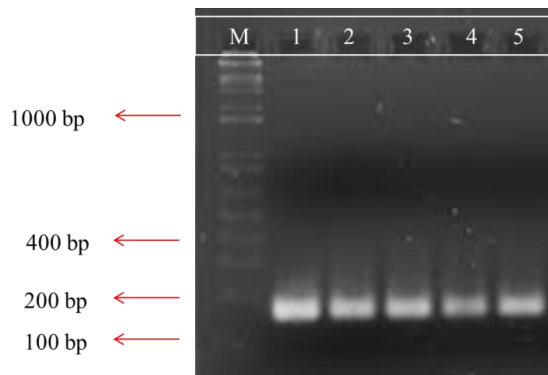

Figure 26. PCR amplification result of cDNA samples with *actin* gene specific primers. M: Invitrogen 1 kb Plus DNA ladder (SMO311), 1-5: Amplified PCR product of *actin* gene. Amplicon length was about 158 bp.

After checking primer pairs, a dilution series with cDNA samples were prepared and qRT-PCR was done using *actin* and *TaNAC69-1* gene specific primers. Amplification plots and standart curves were generated for each dilution series and each gene. Figure 27. and 28. shows a representative standart curve and amplification plot for actin gene.

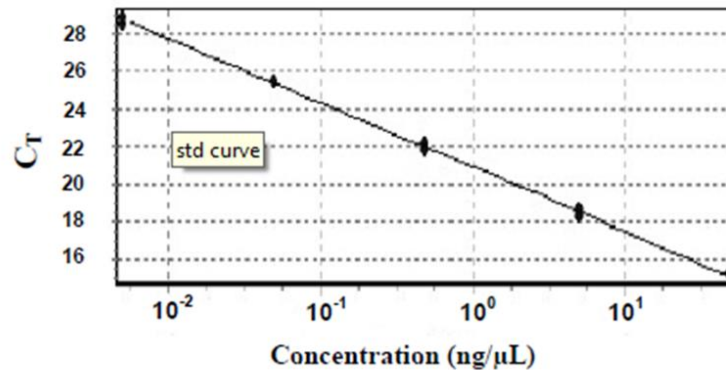

Figure 27. A representative standart curve generated using different dilutions of cDNA amplified with *actin* gene primers.

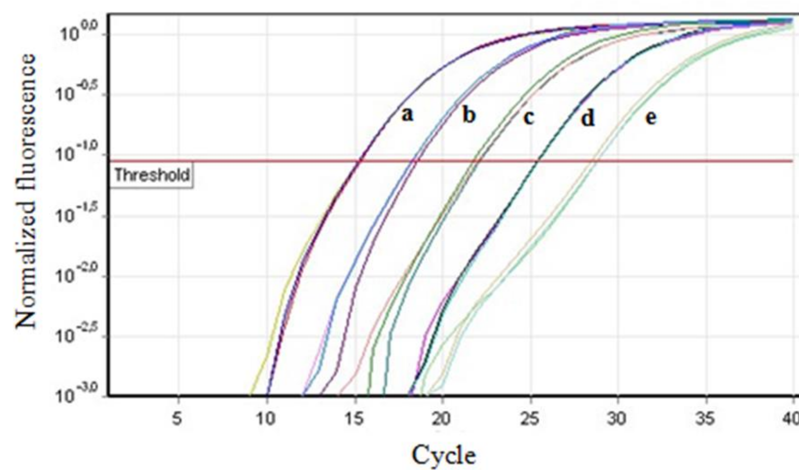

Figure 28. Amplification plot of different dilutions of cDNA generated by using *actin* gene primers. Dilutions of cDNA was a: 50 ng/μL, b: 5 ng/μL, c: 0.5 ng/μL, d: 0.05 ng/μL, e: 0.005 ng/μL.

SYBR Green dye binds to double stranded DNA products amplified by gene specific primers but it can bind also primer dimers or contaminating genomic DNA and give fluorescence. Because of this, a melting curve should be run after RT-PCR. Obtaining one narrow peak for each PCR product shows that one pure product is amplified. Melt curve analysis of *TaNAC69-1* and *actin* gene showed similar melt temperatures with one narrow peak. Figure 29. shows a representative melting curve for *actin* gene.

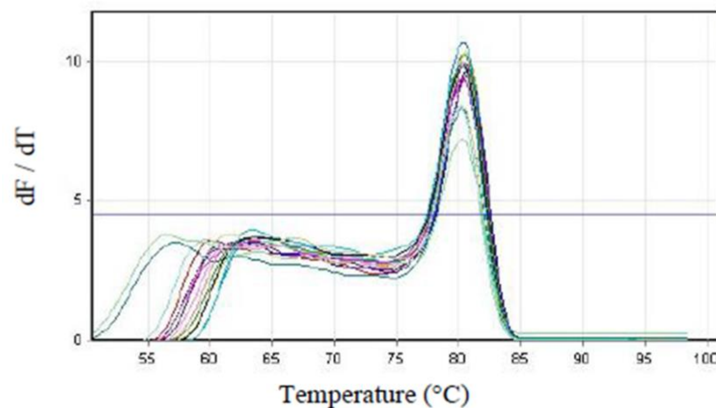

Figure 29. A representative melting curve for *actin* gene amplified with different dilutions of cDNA. Change in fluorescence over time was plotted against temperature.

The fold change difference in TaNAC69-1 gene relative to reference gene, actin, was calculated by Δ(ΔCT) method. Figure 30. and 31. show relative fold change difference of *TaNAC69-1* gene with different time periods. Five transgenic tobacco lines including NT2, NT5, NT6, NT17 and NT33 were used for expression analysis. Compared to control samples, expression level of *TaNAC69-1* gene in all transgenic tobacco lines was started to increase at 12 h about 10 to 24 fold and reached to maximum level at 48 h about 22 to 57 fold under salt stress conditions. Fold change showed a similar pattern under drought stress conditions but a lower fold change difference compared to salt stress. Expression level of *TaNAC69-1* gene was increased to a maximum level about 13 to 27 fold in drought stress conditions.

NAC transcription factors are involved in stress responses of plants and expression levels of them change with biotic and abiotic stress. They also have different expression patterns in different tissues (Fang et al., 2008). A rice NAC gene, *ONAC045* had higher expression in young roots than other parts of plant. When plants exposed to 200 mM NaCl *ONAC045* gene expression increased to maximum level in roots at 24 h (Zheng et al., 2009). Tran et al. (2004) found that NAC transcription factor family proteins namely *ANAC019, ANAC055,* and *ANAC072* bound to *Arabidopsis thaliana erd1* (early responsive to dehydration stress 1) gene promoter region. Expression of these three genes were induced by drought and high salinity. *TaNAC69* genes (*TaNAC69-1, TaNAC69-2,TaNAC69-3*) from bread wheat showed up-regulation in the roots of wheat plants under salt stress. Expression levels of *TaNAC69-1* and *TaNAC69-3* genes were raised to fourfold and ninefold with 250 mM NaCl in the roots (Xue et al., 2011). Baloğlu *et al* (2012) showed that *TaNAC69-1* gene expression upregulated to ninefold at 3 h and reached to a maximum level at 48 h with 250 mM NaCl treatment in durum wheat similar to our results in tobacco plants. A chrysanthemum NAC transcription factor, *DgNAC1*, had an elevated expression in chrysanthemum plants and upregulation in transgenic tobacco under NaCl treatment (Liu et al., 2011). *GmNAC11* and *GmNAC20* genes from *Glycine max* were related to stress response and development of soybean plants. Overexpression of *GmNAC11* and *GmNAC20* genes in transgenic Arabidopsis and and soybean transgenic hairy roots provided salt tolerance (Hao et al., 2011).

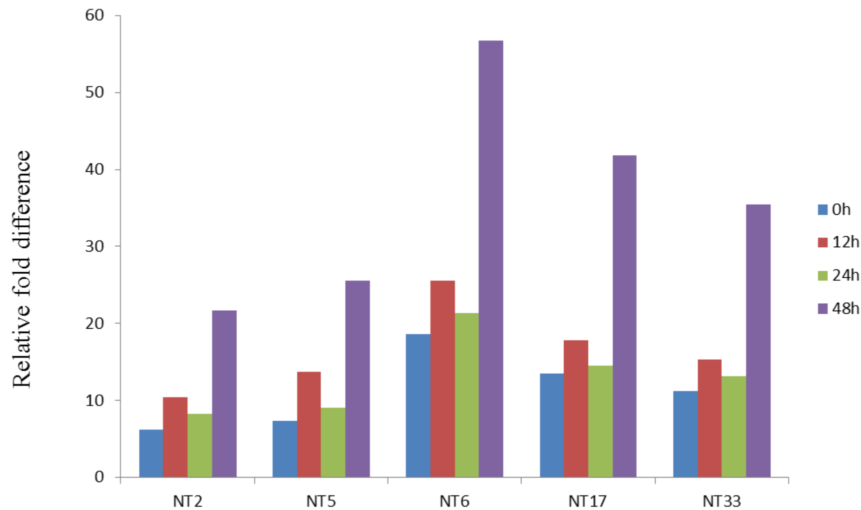

Figure 30. Relative fold change difference of *TaNAC69-1* gene under salt stress conditions with different time periods. NT2-NT33:T_0 transgenic tobacco lines.

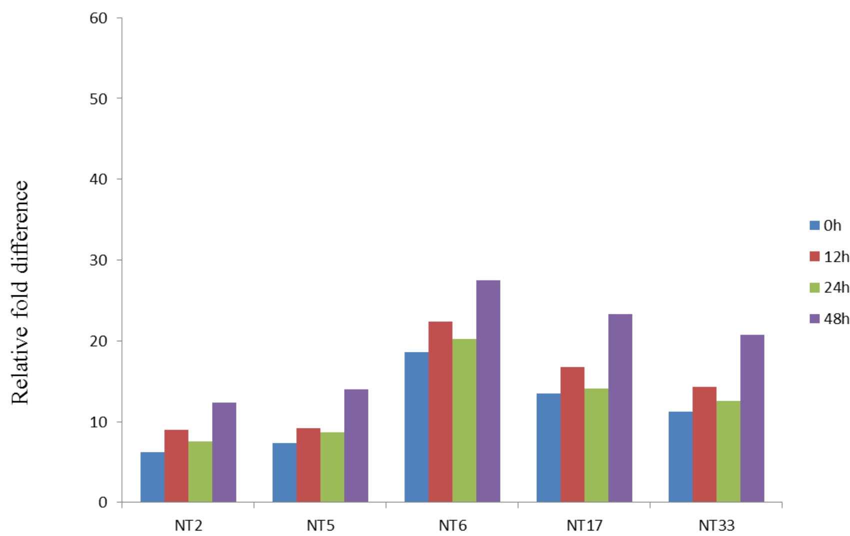

Figure 31. Relative fold change difference of *TaNAC69-1* gene under drought stress conditions with different time periods. NT2-NT33: T0 transgenic tobacco lines.

NAC transcription factors also play role in drought stress tolerance of plant varieties. *OsNAC6* gene from rice is a transcriptional activator and overexpression of *OsNAC6* caused growth retardation and reduced yield but an increased tolerance to dehydration and salinity in transgenic rice plants (Nakashima et al., 2007). *ATAF-1*, *Arabidopsis* NAC family gene, regulated to drought stress tolerance of plants by

overexpression but also led to dwarf phenotype and sterility (Wu et al., 2009). Sugarcane *SsNAC23* gene, homologous to *Arabidopsis ATAF1* and rice *OsNAC6*, had an increased expression under water stress conditions (Nogueira et al., 2005). Tran et al. (2009) identified 31 unigenes coding GmNAC proteins in soybean. Nine of them showed enhanced expression with dehydration stress having different levels of induction in both root and shoot. Isolation and characterization of a chickpea NAC gene *CarNAC3* was made by construction of cDNA libraries of PEG-treated and untreated seedling leaves. Expression of *CarNAC3* induced by drought stress but a differential expression in different tissues (Peng et al., 2009). Arabidopsis *RD26* gene encoding a NAC protein was upregulated under dehydraton with ABA-dependent signaling pathway (Fujita et al., 2004). *OsNAC52* gene from *Oryza sativa* was overexpressed in transgenic plants depending ABA signaling pathway. Transgenic *Arabidopsis* plants overexpressing *OsNAC52* gene represented enhanced tolerance to drought via activation of expression in downstream genes (Gao et al., 2010). Mao et al. (2012) characterized a wheat *TaNAC2* gene in *Arabidopsis* plants. Increased tolerance to drought, salt and freezing in *Arabidopsis* was obtained with overexpression of *TaNAC2* gene predicting a possible role to utilize this gene in transgenic breeding studies. We can conclude taking into account these studies and our findings NAC transcription factors increase tolerance of plants against salt and drought stress and they can be used to generate stress tolerant plants.

3.5.3. Physiological Analysis of Wild Type and Transgenic Tobacco Plants

To see the responses of wild type and transgenic tobacco lines to salt, osmotic and ionic stresses, firstly germination test was done . After germination test, 15 day old wild type and transgenic tobacco plantles were subjected to NaCl, LiCl and mannitol. Following exposure to stress, shoot and root lengths, shoot and root fresh weights, shoot and root dry weights of plantles were determined. 15 plantles from wild type and each transgenic tobacco lines were used for each independent experiment. Three T_2 transgenic tobacco lines were chosen for stress experiments according to germination, Southern blot and qRT-PCR analysis. Each experiment was repeated for at least three times for reliable results.

3.5.3.1. Germination Test

To figure out the effect of overexpression of TaNAC69-1 gene on germination under salt, ionic and osmotic stress, seeds from wild type tobacco and each transgenic tobacco lines were surface sterilized with 70 % ethanol for 1 minute then 10 % hypochlorite for 20 minutes and were put on MS media including 0, 50, 100, 150, 200, 250 and 300 mM NaCl, 0, 50 and 100 mM LiCl, and 0, 100, 200, 300 and 400 mM mannitol. They were let them to germinate in tissue culture room with 16 hours light, 8 hours dark period at 25 °C for 30 days. After that, 250 mM NaCl, 100 mM LiCl and 300 mM mannitol were chosen for germination test because they were minimum concentrations that limit plant germination and growth.

Germination and survival rates of wild type and transgenic tobacco seeds were determined using 250 mM NaCl, 100 mM LiCl and 300 mM mannitol. For control, only MS medium was used. Seeds were let them to germinate in tissue culture room with 16 hours light, 8 hours dark period at 25 °C for 30 days. Figure 32., 33. and 34. show pictures of wild type and transgenic tobacco lines germinated and grown on NaCl, LiCl and mannitol including MS media. Figure 35. and 36. show germination and survival rates of wild type and transgenic tobacco seeds on control, NaCl, LiCl and mannitol including MS media.

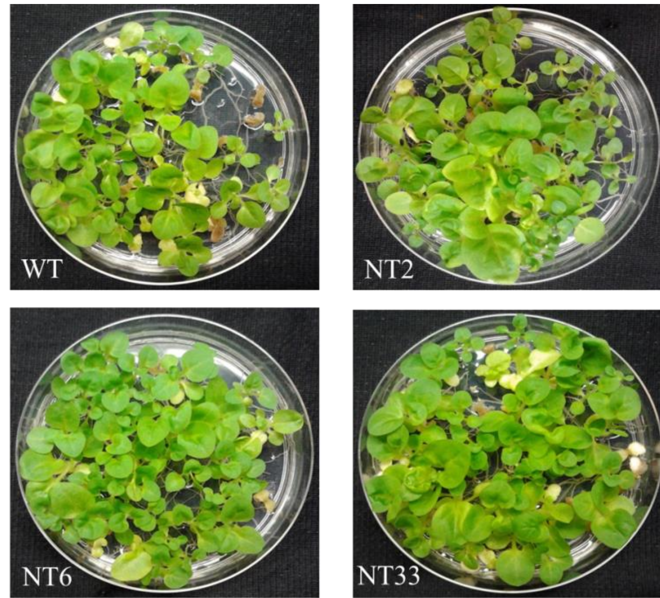

Figure 32. . Germination and growth of wild type and T_2 transgenic tobacco seeds on 250 mM NaCl including media. WT: wild type, NT2-NT33: transgenic tobacco lines. Picture was taken after 1 month.

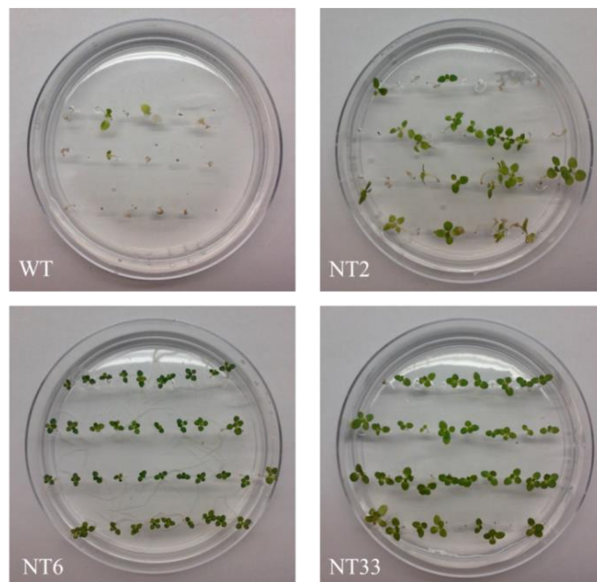

Figure 33. Germination and growth of wild type and T_2 transgenic tobacco seeds on 100 mM LiCl including media. WT: wild type, NT2-NT33: transgenic tobacco lines. Picture was taken after 1 month

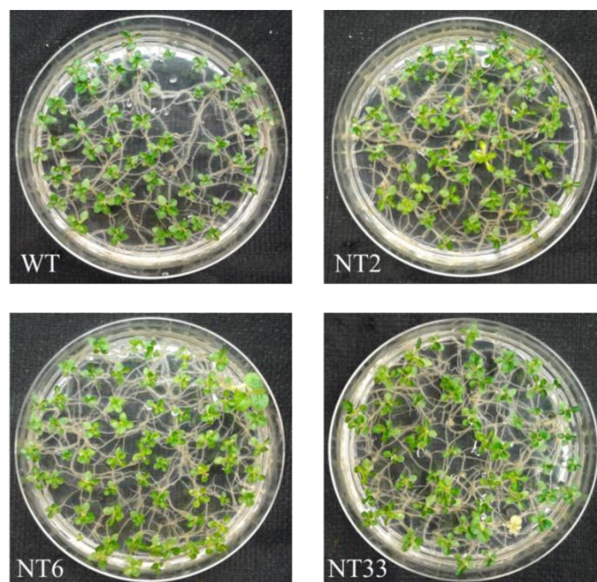

Figure 34. Germination and growth of wild type and T_2 transgenic tobacco seeds on 300 mM mannitol including media. WT: wild type, NT2-NT33: transgenic tobacco lines. Picture was taken after 1 month.

There were no differences between germination and survival rates of wild type and transgenic tobacco seeds germinated on control and 300 mM mannitol including media. On 250 mM NaCl including media, only germination rate of NT6 transgenic tobacco line was higher than wild type and other transgenic tobacco lines. On the other hand transgenic tobacco lines survived better on the same media. Germination and survival rates of transgenic tobacco seeds on 100 mM LiCl including media were significantly higher than wild type tobacco seeds. These results suggest that *TaNAC69-1* gene may take a role of salt and ionic stress responses of transgenic plants but does not give osmotic tolerance. Borsani et al. (2001) investigated the function of a salicylate hydroxylase (*NahG*) gene in *Arabidopsis*. Wild type and transgenic *Arabidopsis* seeds germinated under 100 mM NaCl and 270 mM mannitol treatment. Shoots of wild type plants showed extensive chlorosis, on the other hand transgenic plants stayed green and produced healthy leaves under same conditions. Tobacco plants were transformed with a cell wall associated peroxidase (*TPX2*) which is an enzyme responsible for altering cell wal architecture. Transgenic plants overexpressing *TPX2* had higher germination rates under excess salt or osmotic stress conditions holding more water for germination (Amaya et al., 1999).

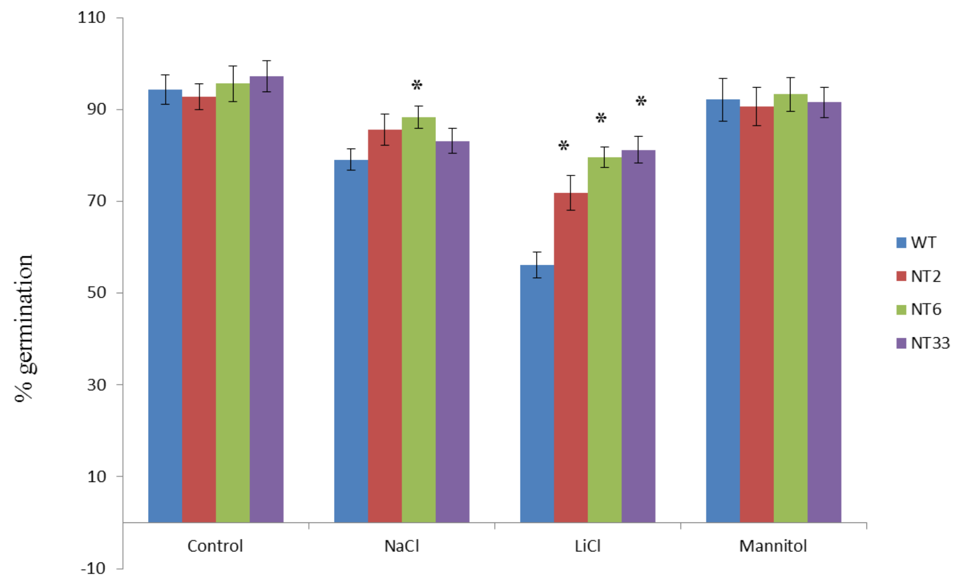

Figure 35. Germination rates of wild type and transgenic tobacco lines germinated on control, 250 mM NaCl, 100 mM LiCl and 300 mM mannitol including MS media. WT:wild type tobacco, NT2- NT33:transgenic tobacco lines

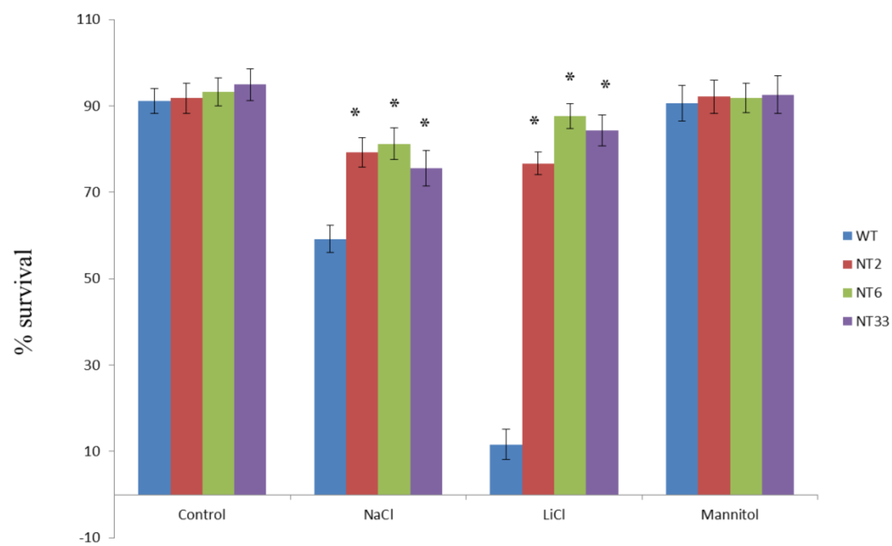

Figure 36. Survival rates of wild type and transgenic tobacco lines germinated on control, 250 mM NaCl, 100 mM LiCl and 300 mM mannitol including MS media. WT:wild type tobacco, NT2- NT33:transgenic tobacco lines.

3.5.3.2. Growth Characteristics of Wild Type and Transgenic Tobacco Plants under Stress Conditions

Three T_2 transgenic tobacco lines were chosen for stress experiments according to germination, Southern blot and qRT-PCR analysis. After germination of wild type seeds on MS media including 0, 50, 100, 150, 200, 250 and 300 mM NaCl, 0, 50 and 100 mM LiCl, 0, 100, 200, 300 and 400 mM mannitol, 250 mM NaCl, 100 mM LiCl and 300 mM mannitol were chosen for stress experiments. To see effects of salt, ionic and osmotic stresses on wild type and transgenic tobacco lines, 15 day old tobacco plantles were subjected to NaCl, LiCl and mannitol. Following exposure to stress, shoot and root lengths, shoot and root fresh weights, shoot and root dry weights of plantles were determined.

High salt concentrations affect negatively plant growth and development disrupting water homeostasis and ion distribution and leading to damage at molecular level, growth retardation and at the end death. Because of this sustaining growth is an important factor under salt stress conditions (Zhu, 2001). At the beginning salt stress create osmotic effect similar to water stress effect with no genotypic differences among plant species. After that, salt starts to accumulate at toxic levels depending on plant genotype (Lauchli & Grattan, 2007). Salinity stress also causes nutritional disorders. Plants give different responses to salinity depending on genotype, plant species, plant age, tissue, composition and ionic strength of solution (Läuchli & Epstein, 1990). Salt stress leads to cell dehydration and shrinkage, reduction in cell elongation, division, leaf and root appearance and size increasing with time. Visual injuries can be also seen under excessive salt uptake (Munns, 2002). In our study growth parameters of wild type and transgenic tobacco plants were watched to analyze salt tolerance of them.

Salt stress creates both osmotic and ionic stress on plants. A sodium analog, lithium, is used to constitute ionic stress environment under saline conditions to understand whether negative effects of salt stress on plants come from osmotic or ionic effect of salinity (Shah *et al*, 2002, Nakayama *et al*, 2005, Tester & Davenport , 2003). Lithium is very toxic to plant cells at one-tenth when compared to concentration of NaCl, but they use similar transport pathways leading to same inhibiton on plant

growth (Plett *et all*, 2010). Li^+ toxicity causes spotted chlorosis on plant leaves, reduced leaf area and plant height, necrotic lesions on the edge of leaves and at the further stage through the blade (Cortina & Culiáñez-Macià, 2005).

To create osmotic stress effect salts or organic solutes have been added to culture medium. Mannitol is a sugar alcohol and have a role to control osmotic potential in stress experiments inducing water deficiency (Zang & Komatsu, 2007). Mannitol does not inhibit cell processes with accumulation high concentrations due to becoming a compatible solute (Tholakalabavi *et all*, 1994). Mannitol has some adverse effects on plants like growth reduction, a decrease in leaf number, reduction in leaf surface area, water and K^+ contents (Slama *et all*, 2007). In this study, growth parameters of wild type and transgenic tobacco lines were evaluated with mannitol treatment.

3.5.3.2.1. Root and Shoot Length Measurements

15 days old 10 plantles from wild type and T_2 transgenic tobacco plantles grown on control MS media were transferred to control, 250 mM NaCl, 150 mM LiCl and 300 mM mannitol including MS media. Plantles were grown in a tissue culture room with 16 hours light, 8 hours dark period at 25 °C for 1 month. Only plantles with roots were used to determine growth parameters. Roots and shoots were separated and lengths of them were measured. Figure 37. and 38. shows root and shoot lengths of wild type and transgenic tobacco plantles.

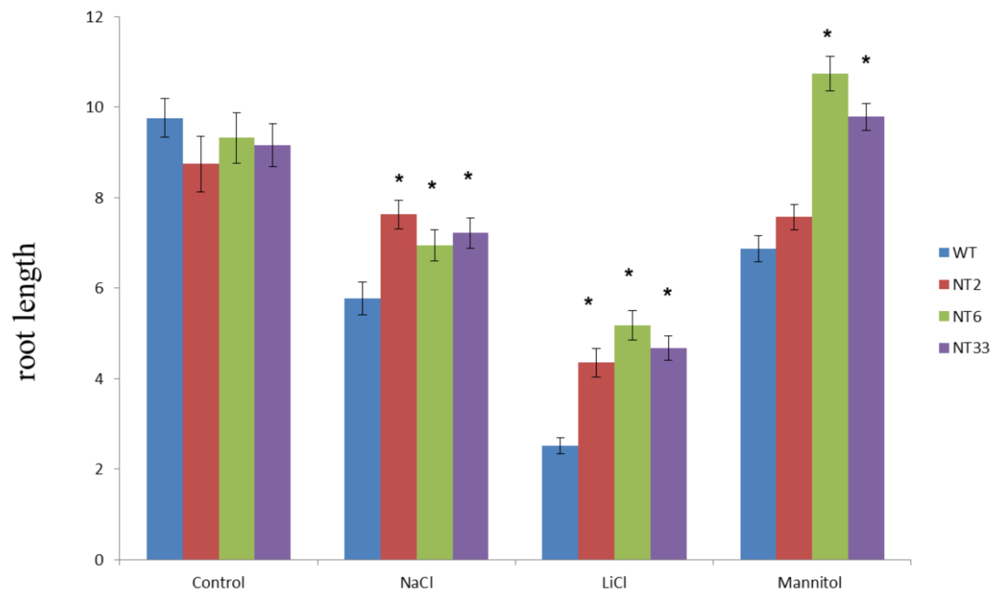

Figure 37. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on root lengths of wild type and T2 transgenic tobacco plants. WT:wild type, NT2-NT33:T2 transgenic tobacco lines.

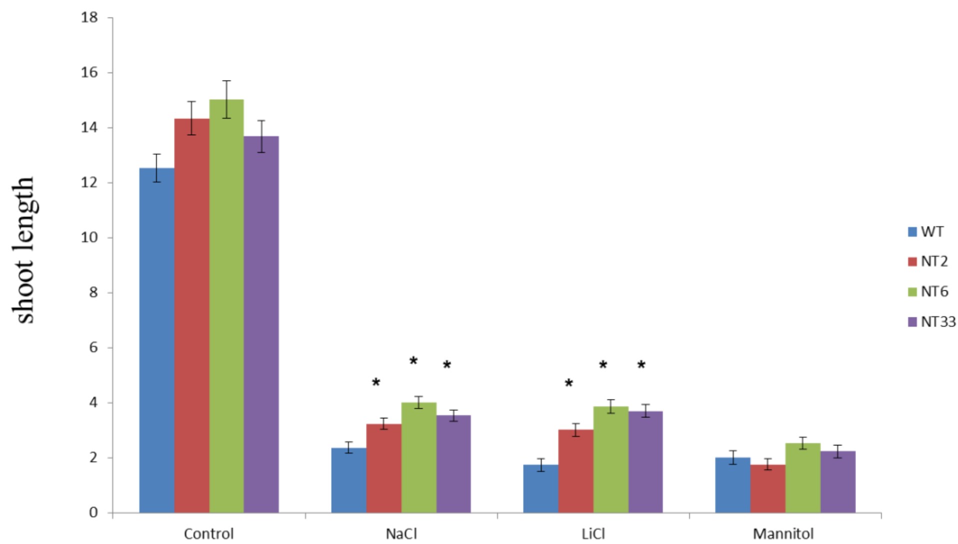

Figure 38. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on shoot lengths of wild type and T_2 transgenic tobacco plants. WT:wild type, NT2-NT33:T2 transgenic tobacco lines.

There was no significant difference between root lengths of wild type and transgenic tobacco lines growing on control media. Root lengths of transgenic tobacco lines treated with 250 mM NaCl and 100 mM LiCl were significantly higher than wild type tobacco plantles. On mannitol including media, NT6 and NT33 transgenic tobacco lines had longer roots than wild type and NT2 transgenic tobacco line. Shoots lengths of all transgenic tobacco lines grown on NaCl and LiCl including media were higher than wild type plantlets significantly. On the other hand, no significant difference were observed between shoot lengths of wild type and transgenic tobacco lines.

An increase in physiological parameteters of transgenic tobacco lines can be a result of taking a part of *TaNAC69-1* gene in salt stress tolerance. *AlSAP* from a halophyte grass*, Aeluropus littoralis,* was overexpressed in tobacco. Transgenic plants having tolerance to drought and salt stress. On the other hand wild type tobacco plants had four- to five-fold reduction in plant biomass with 100 mM LiCl treatment (Ben Saad et al., 2010). Rice leaf growth was examined under NaCl, KCl and mannitol treatment. It was shown that addition of this chemicals into the rooting medium stopped cell elongation into one minute (Yeo et al., 1991). In another study with rice, two weeks old seedlings were treated with different concentrations of mannitol up to 400 mM for 48 hours. An inhibition of cell elongation, withering at the leaf blade and reduction in height of seedlings were observed depending on increasing concentrations of mannitol (Zang & Komatsu, 2007). Xue et al. (2011) found that shoot biomass of transgenic wheat plants overexpressing *TaNAC69* gene was higher than wild type plants under mild salt stress and low water conditions.

3.5.3.2.2. Root and Shoot Fresh Weight Measurements

After germinating and growing for 15 days, 10 plantles from wild type and each T_2 transgenic tobacco line were taken from control media and transferred to control, 250 mM NaCl, 100 mM LiCl and 300 mM mannitol including MS media. Plantles were put in a tissue culture room for further growth and development for 1 month with 16 hours light, 8 hours dark period at 25 °C. Plantles with roots were taken from the media at the end of this period fresh weigths of roots and shoots were measured.

Figure 39. and 40. shows shoot and root fresh weights of wild type and transgenic tobacco lines, respectively.

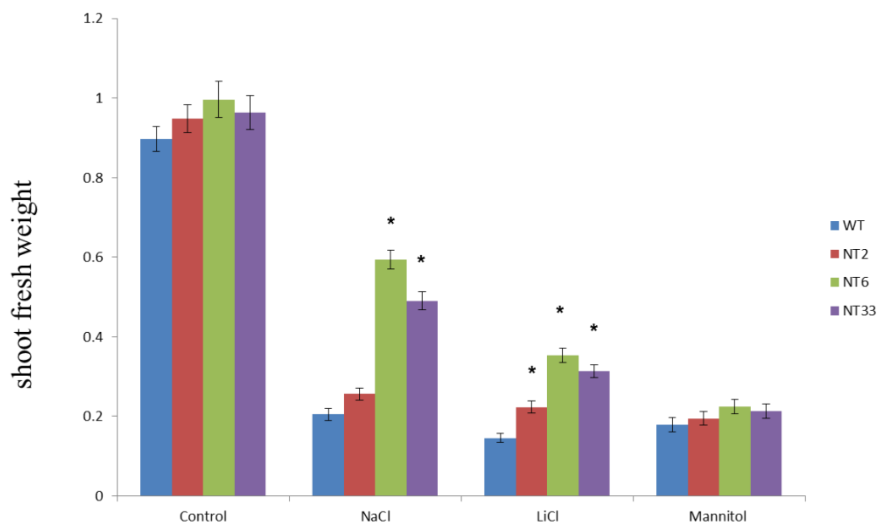

Figure 39. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on shoot fresh weights of wild type and T_2 transgenic tobacco plants. WT:wild type, NT2-NT33:T_2 transgenic tobacco lines.

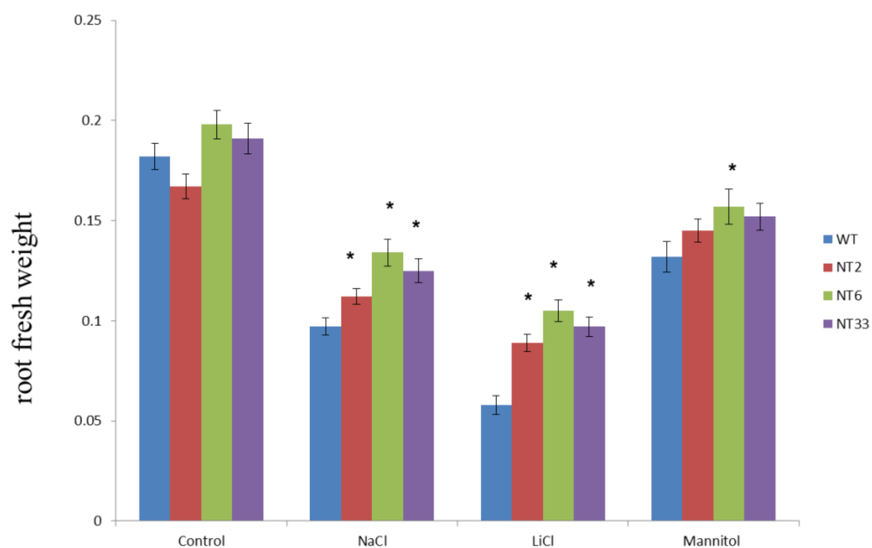

Figure 40. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on root fresh weights of wild type and T_2 transgenic tobacco plants. WT:wild type, NT2-NT33:T_2 transgenic tobacco lines.

Transgenic tobacco lines were produced significantly more shoot and root biomas with LiCl treatment. Shoot fresh weights of NT6 and NT33 lines, root fresh weights of all transgenic tobacco lines were higher than wild type tobacco under NaCl stress conditions. On mannitol including media, only NT6 transgenic tobacco line produced more root biomass. No significant difference was observed in terms of root and shoot fresh weights of wild type and transgenic tobacco lines grown control and mannitol including MS media.

Wild type tobacco plants showed greater reduction in shoot and root fresh weight when compared to transgenic lines under salt and ionic stress conditions. Growth reduction can be a result of water deficiency or ion toxicity because of excessive intake of especially Na^+ and Cl^- ions. Nutritional imbalance can be another factor with withered uptake, transport and distiribution of minerals like K^+ and Ca^{+2} (Lutts et al., 1996). Reduction in shoot and root fresh and dry weights of wild type tobacco plants when compared to transgenic lines with better response to LiCl treatment may be because of toxic effects of LiCl on plant growth. Wild type tomato plants showed leaf chlorosis, necrotic lessions at the leaf margin spreading through curly leaf blade with LiCl treatment (Cortina & Culiáñez-Macià, 2005). Tobacco seeds overexpressing *NtC7* gene were germinated on NaCl, LiCl and mannitol including media. Seed germination and further growth were inhibited by NaCl and LiCl suggesting tolerance of transgenic tobacco plants only osmotic stress (Tamura *et all*, 2003). In our study, there were no significant differences between root and shoot fresh weights of wild type and transgenic tobacco lines treated with 300 mM mannitol compared to non-treated plants. It could be attributed to mainly water deficiency created by osmotic effect of mannitol. Effect of mannitol on growth of cell suspension cultures of *Populus deltoids* var. Occidentalis (poplar) was examined by Tholakalabavi *et all* (1994). Mannitol suppressed the growth of cell cultures leading to osmotic stress

3.5.3.2.3. Shoot and Root Dry Weight Measurements

15 days old 10 independent wild type and T_2 transgenic tobacco plantles with similar age grown on control MS media were chosen for 250 mM NaCl, 100 mM LiCl and 300 mM mannitol treatment. After growing in a tissue culture room for 1 month

with 16 hours light, 8 hours dark period at 25 °C, plantles with roots were removed from MS media, their root and shoot fresh weights were measured. Root and shoot dry weights of plantles were recorded after drying in an oven at 60 ^{0}C for two days. Roots were very small to measure their dry weights, so root samples for each line were gathered together, weighed and divided by number of plants. Figure 41. and 42. shows shoot and root dry weights of wild type and transgenic tobacco lines, respectively.

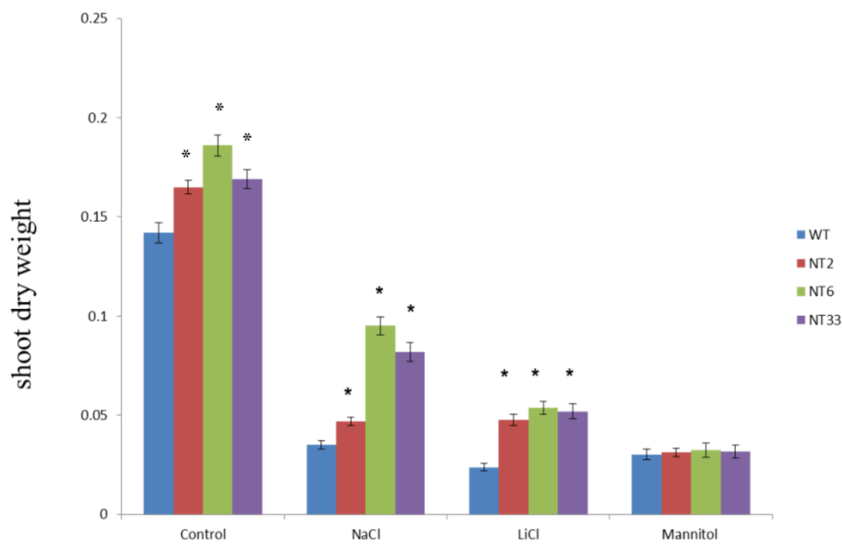

Figure 41. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on shoot dry weights of wild type and T_2 transgenic tobacco plants. WT:wild type, NT2-NT33:T_2 transgenic tobacco lines.

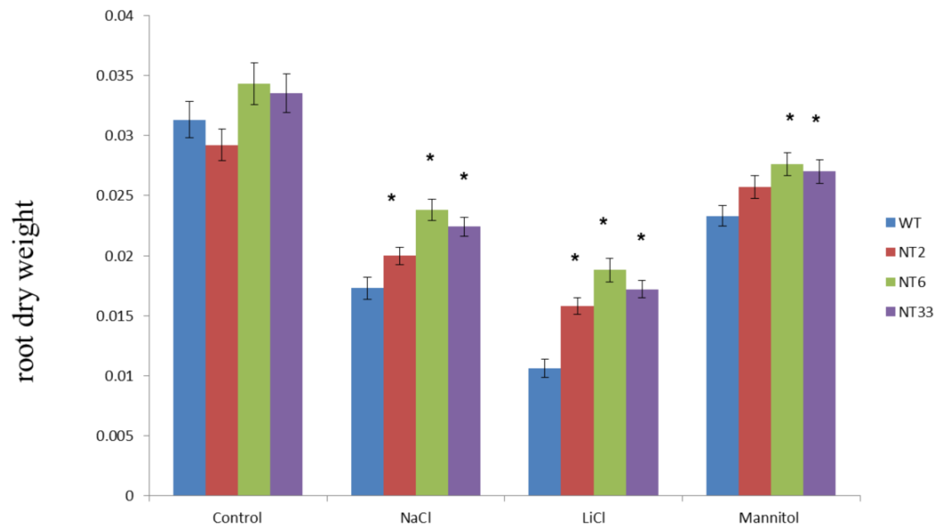

Figure 42. Effect of 250 mM NaCl, 100 mM LiCl and 300 mM mannitol on root dry weights of wild type and T_2 transgenic tobacco plants. WT:wild type, NT2-NT33:T_2 transgenic tobacco lines.

Shoot dry weights of T_2 transgenic tobacco lines with control, NaCl and LiCl treatments were significantly higher than wild type plantles. Shoot dry biomass of wild type and transgenic tobacco lines were not differ from each other under mannitol treatment. All transgenic tobacco lines produced more root biomass under NaCl and LiCl treatment. NT6 and NT33 transgenic tobacco lines had significantly higher root dry weights when compared to wild type. No significant difference were found between root dry weights of wild type and transgenic tobacco lines under control conditions.

Better responses of transgenic tobacco lines to NaCl treatment can show a possible role of TaNAC69-1 gene creating tolerance to salt stress. Ghaulam *et al* (2002) investigated salt stress tolerance of 5 sugar beet cultivars. Plants were exposed to 0, 50, 100 and 200 mM NaCl for 30 days. At the end of this period reduction of leaf size, root and shoot fresh and dry weights, relative water content and K^+ concentrarions, but increase in Na^+ and Cl^- ion concentrations were observed depending on increasing concentrations of salt. Tomato plants had reduced leaf area, stomatal density and conduction, transpiration, CO_2 assimilation, plant dry weight and height under saline growth conditions (Romero-Aranda et al., 2001). Dry

weights of transgenic tobacco lines were significantly higher than wild type tobacco under LiCl treatment. *AlSAP* from a halophyte grass, *Aeluropus littoralis,* was overexpressed in tobacco. Transgenic plants having tolerance to drought and salt stress. On the other hand wild type tobacco plants had four- to five-fold reduction in plant biomass with 100 mM LiCl treatment (Ben Saad et al., 2010). In our study, no significant difference was observed between shoot dry weights of wild type and transgenic tobacco lines treated with 300 mM mannitol. It could be attributed to mainly water deficiency created by osmotic effect of mannitol. Effect of mannitol on growth of cell suspension cultures of *Populus deltoids* var. Occidentalis (poplar) was examined by Tholakalabavi et al. (1994). Mannitol suppressed the growth of cell cultures leading to osmotic stress. To study effects of osmotic stress on a halophyte plant, Sesuvium portulacastrum, PEG and mannitol treatments were used for 12 days. Reduction of leaf growth, leaf water content and leaf biomass were the negative effects of osmotica on plant growth (Slama et al., 2007). It can be concluded that being no significant difference between growth parameters of wild type and transgenic tobacco lines shows that TaNAC69-1 gene does not give tolerance to transgenic plants under osmotic stress conditions.

CHAPTER 4

CONCLUSION

Environmental stresses such as drought, salinity and extreme temperatures affect growth and productivity of plants leading to reduction in crop yield for most of the agronomically important plants. Biotechnological or conventional methods have been used to minimize crop loss and increase quality of plants. Transfer of stress related genes or transcription factors to plants is one of the ways to produce resistant plants to biotic or abiotic stress factors.

In this study, we focused on producing salt and osmotic stress resistant transgenic tobacco plants by using a NAC type transcription factor, *TaNAC69-1* gene. For this purpose, *TaNAC69-1* gene was firstly isolated from *Triticum aestivum* L. cv. Yüreğir- 89. It was cloned into a Gateway compatible expression vector, pEarleyGate 100, with a 35S promoter via Gateway cloning technology. Tobacco leaf discs were transformed by using *Agrobacterium tumefaciens* EHA105 strain. PPT selection was applied with a 10 mg/L concentration at regeneration and rooting stage to select transformed plants. At the end of four independent transformation studies, 45 putative transgenic tobacco plants was obtained. PCR analysis was made to screen transgenes using gene specific and selection gene specific primers. Nine of these lines were transferred to soil to achieve T_0 generation. Germination and survival rates of wild type and transgenic tobacco plants were determined under selection of PPT. Although wild type seeds were germinated on selective media, wild type plantles died at the end of two weeks. On the other hand, transgenic plantles had a survival rate at least 65 % at the same media. Integration of *TaNAC69-1* gene into the genome was evaluated by Southern blot analysis. Germination test, PCR and Southern blot analysis showed that *TaNAC69-1* gene was transferred to tobacco plants and integrated into the genome successfully.

Expression profile change of *TaNAC69-1* gene at different time periods was detected by qRT-PCR under drought and salt stress conditions using *actin* as a reference gene. *TaNAC69-1* gene expression started to increase at 12 hours after 250 mM NaCl application and reached a maximum level at 48 hours. A similar pattern was obtained with PEG (6000) application but a lower expression level change. These results may be shown participation of *TaNAC69-1* gene for salt and drought stress responses of plants. In the present studies, expression of *TaNAC69-1* gene is related to up-regulation of other stress related genes under abiotic stress. So, it is supposed that *TaNAC69-1* gene may provide connection between signal sensing and responsive mechanisms of plants to stress.

Expression of *TaNAC69-1* gene was also figured out by physiological assays such as germination, root and shoot lengths, root and shoot fresh and dry weights of tobacco plants under salt, osmotic and ionic stress conditions. There was no significant differences between germination rates of wild type and transgenic tobacco lines under control and 300 mM mannitol except 100 mM LiCl on which all transgenic lines germinated with very high rates unlike wild type. Survival rates of plantles under control and mannitol including media showed a similar pattern like germination, but on NaCl and LiCl including media transgenic tobacco plantles survived significantly better than wild type plantles. Root and shoot lengths of only NT2 and NT33 transgenic lines were significantly higher than wild type growing on NaCl including media. NT6 and NT33 transgenic lines had more shoot fresh and dry weights on the same media, root fresh and dry weights of all transgenic lines were higher when compared to wild type. On the LiCl including media, physiological parameters of all transgenic lines greater than wild type plants. Despite there was no significant difference between shoot lengths of wild type and transgenic lines on mannitol including media, transgenic lines produced more root lengths. But there was no significant difference between wild type and transgenic lines in terms of shoot and root fresh and dry weights on mannitol including media. So, we can conclude that *TaNAC69-1* gene increase salt and ionic stress tolerance of transgenic plants but doe not give osmotic resistance.

Following research in this study will be investigation of expression level changes of *TaNAC69-1* gene under heat and cold stress using qRT-PCR. Because NAC type transcription factors have different expression in different tissues, expression of the gene in both shoot and root tissues will be examined with salt, drought, heat and cold stresses. Microarray analysis may be carried out for understanding function of *TaNAC69-1* and related genes under stress conditions. Detailed analysis of these genes and link between *TaNAC69-1* gene can contribute to be developed crop plants more tolerant to environmental stresses especially salt and ionic stress. Investigation of stress related NAC type transcription factors of tobacco plants should be a further aim of this study .

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APPENDIX A

BACTERIAL CULTURE MEDIA

LB medium (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

$MgCl_2$ 10 mM

$MgSO_4$ 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

$MgSO_4 \cdot 7(H_2O)$ 0.493 g

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

APPENDIX B

BUFFERS TO PREPARE CHEMICALLY COMPETENT E. COLI CELLS

Transformation Buffer I, 200 ml

Compound	Amount	Final molarity
Potassium acetate	0.588 g	30 mM
Rubidium chloride	2.42 g	100 mM
Calcium chloride	0.294 g	10 mM
Manganese chloride	2.0 g	50 mM
Glycerol	30 ml	15 % v/v

Adjust pH 5.8 and filter sterilize.

Transformation Buffer II, 100 ml

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

Adjust pH 6.5 and filter sterilize.

APPENDIX C

HOAGLAND'S MEDIUM

After addition of proper amounts of solutions as given in Table A., adjust the pH of the medium to 5.8 by using NaOH or HCl. Sucrose may be added as 10 g/L if the culture is axenic. Autoclave the medium. (FeEDTA addition to the medium should be performed after autoclaving.)

Table A.

COMPOSITION	STOCK SOLUTION	USE (ml/L)
$MgSO_4.7H_2O$	24.6 g/100ml	1.0 ml
$Ca(NO_3)_{2.}4H_2O$	23.6 g/100ml	2.3 ml
KH_2PO_4	13.6 g/100ml	0.5 ml
KNO_3	10.1 g/100ml	2.5 ml
Micronutrients	See table B	0.5 ml
FeEDTA	See table C	20.0 ml

Table B. Preparation of micronutrient stock solutions

MICRONUTRIENT	STOCK SOLUTION
H_3BO_3	2.86 g/L
$MnCl_{2.}4H_2O$	1.82 g/L
$ZnSO_{4.}7H_2O$	0.22g/L
$Na_2MoO_{4.}2H_2O$	0.09 g/L
$CuSO_{4.}5H_2O$	0.09 g/L

Table C. Preparation of FeEDTA Stock Solution

ADDITION	STOCK SOLUTION
$FeCl_{3.}6H_2O$	0.121 g / 250 ml
EDTA	0.375 g / 250 ml

Dissolve both $FeCl_{3.}6H_2O$ and EDTA in dH_2O and then complete the volume to 250 ml.

APPENDIX D

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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/cultivar="Babax"
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CDS 140..1204
/gene="NAC69-1"

/note="TaNAC69"
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/protein_id="AAU08785.1"
/db_xref="GI:51702424"

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RRGS
RRGSSMRLDDWVLCRIHKKCGNLPNFSSSDQEQEHEQESSTVEDSQNNHTV
SSPKSEAFDGDGDDHLQLQQFRPMAIAKSCSLTDLLNTVDYAALSHLLLDGA
GASSSDAGADYQLPPENPLIYSQPPWQQTLHYNNNNGYVNNETIDVPQLPEA
RVDDYGM NGDKYNGMKRKRSSGSLYCSQLQL
PADQYSGMLIHPFLSQQLHM"

ORIGIN
1 gcagcatttt tatgcagtag ccatcttctc ctcctcctcc ccccgccttc cagctagcca
61 cctagctcac tatcacatca tccagcagcc cacaccaact catattgatt ccgttcccac
121 ctgctcgcca tcgccagcca tgccaatggg cagcagcgcc gccatgcccg ccctccctcc
181 cggcttccgg ttccacccca ccgacgagga gctcatcgtc cactacctcc gcaggcaggc
241 cgcgtccatg cccagccccg tgcccatcat cgccgaggtc aacatctaca agtgcaaccc
301 atgggacctc cccggcaaag ctttgttcgg ggagaatgag tggtacttct tcagcccccg
361 ggatcgcaag taccccaacg gcgcgcgccc gaaccgcgcc gccgggtccg gctactggaa
421 ggccaccggc accgacaagg ccatcctgtc cacgccggcc aacgagagca tcggggtcaa
481 gaaggcgctc gtgttctaca ggggcaagcc gcccaagggc gtcaagaccg actggatcat
541 gcacgagtac cgcctcaccg cagccgacaa ccggaccacc aagcgcagag gatcctccat
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1381 aattacgggg gtttattgtg aaataaatta atccagtatt atc

APPENDIX E

CTAB EXTRACTION BUFFER

2X CTAB

2% (w/v) CTAB

1.42 M NaCl

20 mM EDTA (pH:8)

100 mM TrisHCl (pH:8)

0.2% (w/v) PVP 40

5 mM Ascorbic Acid

0.02% 2-mercaptoethanol

β-mercaptoethanol must be added to medium just before use.

APPENDIX F

ELECTROPHORESIS BUFFERS

10X TBE Buffer (1L)

Tris Base	108 g
Boric acid	55 g
0.5 M EDTA (ph:8.0)	40 ml

50X TAE Buffer (1L)

Tris Base	242 g
Glacial acetic acid	57.1 ml
0.5 M EDTA (ph:8.0)	100 ml

APPENDIX G

SOLUTIONS USED IN SOUTHERN BLOT ANALYSIS

Denaturation Solution

1.5 M NaCl

0.5 M NaOH

Neutralization Solution

1.5 M NaCl

0.5 M Tris Base

20X SSC Stock (1L)

Component	**Amount**	**Stock concentration**
NaCl	175.3 g	3M
Na_3 citrate.$2H_2O$	88.2 g	300 mM

Adjust pH 7 and autoclave.

DIG Hybridisation Buffer

%50 Formamide (v/v)

5X SSC

1X Blocking Reagent (Roche)

0.02% SDS

0.1% N-Lauroylsarcosine

5 % dH2O

Low Stringency Buffer (500 ml)

2X SSC

0.1 % SDS

High Stringency Buffer (500 ml)

0.1X SSC

0.1 SDS

Maleic acid Buffer

0.1 M Maleic acid

0.15 M NaCl

Adjust pH 7.5 and autoclave.

Washing Buffer (500 ml)

Component	**Amount**
Maleic acid buffer	498.5 ml
0.3 % Tween 20 (v/v)	1.5 ml

APPENDIX H

PLANT TISSUE CULTURE MEDIA COMPOSITION

	M5519
Component	**mg/L**
Ammonium Nitrate	1650
Boric Acid	6.2
Calcium Chloride anhydrous	332.2
Cobalt Chloride.6H2O	0.025
Cupric Sulfate.5 H2O	0.025
Na2EDTA	37.26
Ferrous Sulfate.7H2O	27.8
Magnesium Sulfate	180.7
Magnesium Sulfate.H2O	16.9
Molybdic Acid.2H2O	0.25
Potassium Iodide	0.83
Potassium Nitrate	1900
Potassium Phosphate monobasic	170
Zinc Sulfate.7H2O	8.6
Organics	
Agar	-
Glycine	2
Myoinositol	100
Nicotinic Acid	0.5
Pyrodoxine.HCl	0.5
Sucrose	-
Thiamine.HCl	0.1
Grams of Powder (1 liter)	4.4

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Eroğlu, Ayten

Nationality: Turkish (TC)

Date and Place of Birth: 15 February 1979, Kütahya, Türkiye

Marital Status: Single

email: ayteneroglu@gmail.com

EDUCATION

Degree	Institution	Year of Graduation
MS	Dumlupınar Uni. Biology Dept.	2006
BS	Ege Uni. Biology Dept.	2004

WORK EXPERIENCE

Year	Place	Enrollment
2006-present	METU Biology Department	Research assistant
2012-October 2013-September	UVA-Timko Laboratory	Visiting scholar

PUBLICATIONS

Baloğlu M. C., Battal A., Aydın G., **Eroglu A**., Öz M.T., Kavas M., Öktem H. A., Yücel M. (2013). Vector construction strategies for transformation of wheat plant. Journal of Animal and Plant Sciences, 23(3), 906-912.

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Eroğlu A., Baloğlu M.C., Öz M.T., Öktem H.A., and Yücel M., Agrobacterium mediated transformation of tobacco with a NAC-type transcription factor, *TaNAC69-1*, 2011, Plant Transformation Technologies II and Plant Gene Discovery Technologies, Vienna, AUSTRIA (Poster bildiri)

Baloğlu MC, Kavas M, Öz MT, Battal A, **Eroğlu A**, Kayıhan C, Öktem HA, Yücel M Cloning of wheat NAC-type transcription factors and Agrobacterium mediated transformation of wheat mature and immature embryos. Plant Transformation 198 Technologies II, International Conference, Book of Abstracts pp 71, Vienna, Austria, February 19-22, 2011 (Poster bildiri)

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1, and analysis of transgenic plants under salt and drought stress, XXI National Biology Congrees, 2012, İzmir, TURKEY (Poster bildiri)

PROJECTS

DPÜ-BAP 2006-3

Emet Borik Asit Fabrikası atıklarının arpa ve buğday bitkilerinde çimlenme ve vejetatif büyüme üzerine etkileri, 2006-2008.