DEVELOPMENT OF SALT RESISTANT TRANSGENIC PLANTS BY USING *TANHX1* AND *TASTR* GENES

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DEVELOPMENT OF SALT RESISTANT TRANSGENIC PLANTS BY USING *TaNHX1* AND *TaSTR* GENES

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ABSTRACT

DEVELOPMENT OF SALT RESISTANT TRANSGENIC PLANTS BY USING *TANHX1* AND *TASTR* GENES

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Soil salinity negatively affects agricultural production in Turkey by decreasing the yield and quality. Direct introduction of stress related genes by genetic engineering is one of the most rapid approaches to develop stress tolerant crops. In this study, *TaNHX1* gene was isolated from bread wheat and three different local wheat cultivars were transformed with overexpression vectors containing *TaNHX1* gene by using *Agrobacterium*-mediated and particle bombardment gene transfer techniques. Immature embryo and inflorescence of *Triticum durum* cv. Kızıltan-91 and *Triticum aestivum* cv. Yüreğir-89 and mature embryo of *Triticum durum* cv. Mirzabey-2000 were used as an explant. In this manner, totally 8960 and 5650 explants were used during particle bombardment and *Agrobacterium*-mediated transformation, respectively. Moreover, leaves of *Nicotiana tabacum* cv. Petit Havana were transformed by *TaSTR* gene to develop salt resistant transgenic tobacco plants by using *Agrobacterium*-mediated transformation. Stable expression and inheritance of

the transgenes was confirmed by both genetic and molecular analyses. T_1 progeny showed segregation of the transgenes in a typical Mendelian fashion in most of the plants. Expression of *TaSTRG* in tobacco was evaluated by physiological and biochemical analysis, such as germination test, root length and MDA analysis. In addition to the nuclear transformation, chloroplast transformation of tobacco was performed with *Xyl10B* gene responsible for the synthesis of hyperthermostable xylanase enzyme. Stable integration of transgenes and homoplasmy were confirmed with PCR and Southern blotting.

Keywords: Transgenic wheat, Transgenic tobacco, Salt resistance, *TaSTRG*, *TaNHX1*

ÖZ

TaNHX1 ve *TaSTR* GENLERİ KULLANARAK TUZA DİRENÇLİ BİTKİLERİN GELİŞTİRİLMESİ

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Topraktaki tuzluluk, verim ve kaliteyi azaltmak suretiyle Türkiyedeki zirai üretimi olumsuz yönde etkilemektedir. Olumsuz koşullara dirençli tahılların geliştirilebilmesi için en hızlı yol, bu şartlara dirençliliği sağlayan genlerin genetik mühendisliğiyle direkt olarak bitkilere aktarılmasıdır. Bu çalışmada, ekmeklik buğdaydan *TaNHX1* geni izole edilmiş ve üç farklı lokal buğday çeşidine, bu geni içeren yüksek düzeyli gen ifadesi sağlayan vektörler, *Agrobakter* ve parça bombardımanı tekniğiyle aktarılmıştır. Kızıltan-91(*Triticum durum)* ve Yüreğir-89 (*Triticum aestivum*) çeşitlerinden izole edilen olgunlaşmamış başak taslağı ve olgunlaşmamış embryolar ile Mirzabey-2000 (*Triticum durum*) çeşidinden izole edilen olgunlaşmış embryolar hedef doku olarak kullanılmıştır. Bu bağlamda, toplam 8960 hedef doku parça bombardımanıyla, 5650 hedef doku ise *Agrobakter* ile gen aktarım çalışmalarında kullanılmıştır. Bunlara ilaveten, Petit Havana (*Nicotiana tabacum*) çeşidinin yapraklarına, tuza direçli tütün bitkisi geliştirmek amacıyla

Agrobacterium aracılığıyla *TaSTR* geni aktarılmıştır. Kalıcı gen ifadesi ve transgenlerin kalıtımı genetik ve moleküler analizlerle doğrulanmıştır. İlk kuşaktaki transgenik bitkilerin çoğu Mendel kanunlarına uygun bir dağılım göstermektedir. Tütündeki *TaSTRG* ifadesi, çimlenme testi, kök uzunluğu ve MDA analizi gibi fizyolojik ve biyokimyasal analizlerle değerlendirilmiştir. Çekirdek transformasyonuna ek olarak, tütünde kloroplast transformasyonu, yüksek ısıya dayanıklı xylanase enziminin sentezinden sorumlu *Xyl10B* geni kullanılarak gerçekleştirilmiştir. Transgenlerin kalıcı entegrasyonu ve homoplazmi, PCR ve Southern blotting teknikleriyle doğrulanmıştır.

Anahtar Kelimeler: Transgenik buğday, Transgenik tütün, Tuza dirençlilik, *TaSTRG*, *TaNHX1*

To my soul mate Ayşenur and my source of happiness Ömer Yiğit and Berra

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2,4-D	2,4-dichlorophenoxyacetic acid
accD	β-carboxyl transferase subunit of acetyl-CoA carboxylase
ATPase	Adenosine triphosphatase
BA	Benzylaminopurine
BADH	Betaine aldehyde dehydrogenase
Bar	Bialaphos resistance gene
CAT	Catalase
cDNA	Complementary DNA
CTAB	Cetyl trimethylammonium bromide
GUS	ß-glucuronidase
H_2O_2	Hydrogen peroxide
HptII	Hygromycin phosphotransferase gene
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
MS	Murashige-Skoog basal salt medium
NAA	Naphthalene acetic acid
NaCl	Sodium chloride
NaOCl	Sodium hypochlorite
PEG	Polyethylene glycol
Prrn	Ribosomal RNA operon promoter
rbcL	Ribulose-bisphosphate carboxylase gene
ROS	Reactive Oxygen Species
rps16	Ribosomal protein S16
SOD	Superoxide dismutase
UTR	Untranslated region

CHAPTER 1

INTRODUCTION

Wheat is one of the oldest and most important cereal crops in the world. Wheat also is one of the most widely grown cereals, accounting for over one quarter of the world`s global cereal production (FAO, 2007). It is ubiquitous in the Turkish cuisine and many other cuisines. Wheat is mainly cultivated for its grain, which is ground into flour used to make bread and pasta. A big portion of wheat (95%) is cultivated for bread production. There are three sets of wheat species, differing in ploidy number. *Triticum monococcum* is a primitive diploid species. *Triticum dicoccum*, ancient cultivated species, is a tetraploid wheat. *Triticum aestivum* is the hexaploid wheat species and this is most commonly grown today. There are 30 wheat species produced from artificial and natural hybridization (Goncharov *et al*., 2009). There are two types of wheat in terms of temperature dependence. Winter types require vernelization period, the requirement of a long exposure to low temperatures, to accelerate flowering (Loukoianov *et al*., 2005).

1.1. Environmental Stress Factors for Plants

Environmental stresses are the most limiting factors for agricultural productivity. In addition to the plant specific traits, most of the crop loss is due to the pathogens (biotic stress) and harmful effects of environmental stresses (biotic). Other examples to the biotic stress factors are herbivory and competition. Abiotic stress factors are composed of salinity, drought, high or low temperature, radiation, heavy metals,

deficient or excess of nutrients, pollutants and mechanical stresses. Any of these stresses can negatively affect development of plants and reduce the productivity. If these stress conditions continue for longer period can cause the death of the plant (Levitt, 1980).

In nature, plants may not be totally free from stresses. Only 10% of world`s cultivatable land may be specified as free from stress (Bidhan *et al*., 2011). It is

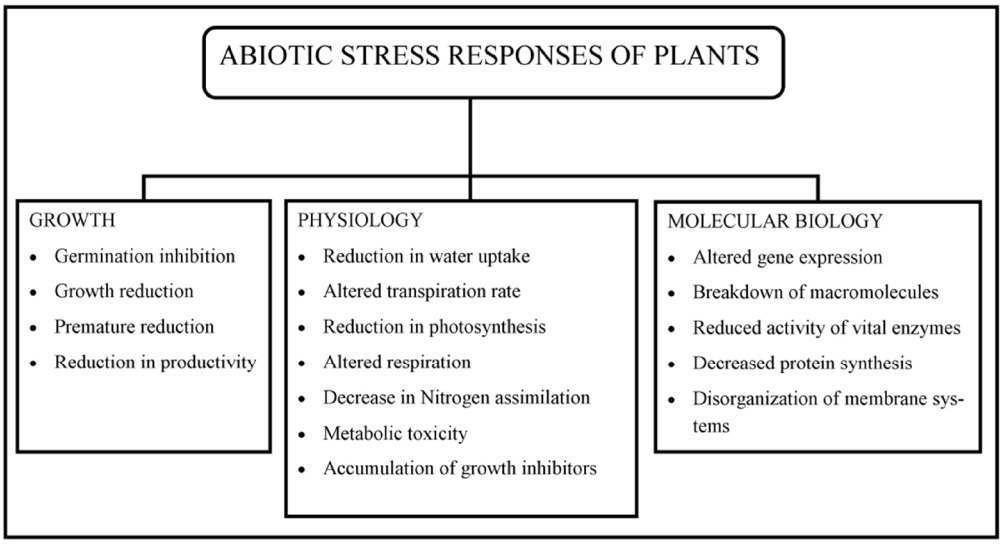

Figure 1. 1. Some of the common plant responces to abiotic stresses (Adapted from Pareek *et al* .2010)

expected that plants can encounter some degree of stress of any factor or factors during their life cycles. Because of their sessile nature, plants must endure these adverse conditions. To combat these stresses, plants develop several effective mechanisms to cope with detrimental effects of stresses, including the production of new molecules and developing molecular mechanisms of stress tolerance (Figure

1.1). However, there are variations between plants in terms of stress tolerance. In order to survive under stress conditions, some plant species can show morphological, physiological, biochemical and molecular differences in response to stress factors (Pareek *et al*., 2010).

Although each stress factor produces own specific effects on plants, a general property of different stress conditions is that they can cause to increase of reactive oxygen species. ROS are recognized as detrimental to the biological systems because they cause to oxidation of lipids, proteins, deoxyribonucleic acid, and carbohydrates. Ultimately, toxic levels of ROS cause a chain reaction of cellular oxidation, resulting in unhealthy situation and lethality. In addition to the oxidative stress related production, ROS are also inevitable outcome of normal physiological processes, such as glycolysis and photosynthesis (Mittler *et al*., 2004). Plant antioxidant systems, both enzymatic and nonenzymatic, have an important function in balancing and preventing oxidative damage (Foyer *et al*., 1994).

1.1.1. Salt Stress

Soil salinity is one of the most important abiotic stress factors reducing agricultural productivity. Saline soil is characterized by high levels of chlorides and sulfates of sodium which may be toxic for plants. Soils are classified as saline when the ECe is 4 dS/m or more, which is equivalent to about 40mM NaCl and generates an osmotic pressure of approximately 0.2 MPa (USDA, 2008). Salinization is the accumulation of soluble salts of sodium, magnesium and calcium in soil so that soil fertility is severely reduce. About 900 million hectares of land all over the world are affected from salinization (FAO, 2007). This amount equal to about 6% of the world`s total land area. Salinization is becoming more and more important in irrigated areas, because salts have been accumulated in upper levels of soil where plant roots located due to the inadequate drainage. Of the current 230 million hectares of irrigated area, 45 million hectares are affected by salinization. This means that 20% of irrigated agricultural lands are salt-affected (FAO, 2007). Natural or Primary salinization

involves accumulation of soluble salts in soils through natural processes due to a high salt content of the parent material or in groundwater. Sodium, potassium, magnesium, calcium, chloride, sulphate, carbonate and bicarbonate ions are accumulated in soil as a result of natural salinization. In addition to the primary salinization, secondary salinization caused by human mediated events such as improper irrigation application is becoming more visible (Munns, 2005). Therefore, the problem of soil salinity is increasing due to the improper irrigation, inappropriate drainage, seawater in coastal areas, and salt accumulation in arid and semi-arid regions by passing years (Ashraf and Wu, 2011).

Based on tolerance to salt stress, plants are generally divided into glycophytes that cannot withstand high salinity and halophytes that can cope with high concentrations of NaCl (Flowers *et al*., 1977). Many groups of plants including wheat are accepted as glycophytic which are sensitive to high concentrations of salt.

1.1.1.1. Effects of salt stress on plants

Sodium is toxic for several organisms, except for halophytic organisms which have specific mechanisms that maintain intracellular sodium concentrations as low. The deleterious effects of high salinity can be seen at the whole plant level, such as suppression of growth, decrease in productivity, increase in root/shoot ratio and even the death of plants (Munns and Termaat, 1986). Generally, the harmful effects of salt on glycophytic plants can be classified as primary and secondary effects. Salt stress reduces water potential, brings about ion imbalance and ion toxicity. These effects are called primary effects of salt stress. Oxidative damage, decreased CO_2 fixation and photosynthesis and inhibition of protein synthesis are emerged as secondary effects of salt stress (Zhu, 2001). Those effects cause to initial growth reduction and decreasing of plant productivity, changes in leaf structure, nitrogen metabolism and carbohydrate fixation. High salt concentration in water may prevent plant growth for two reasons. First, the presence of salt in the soil solution reduces available water amount used by plants, and this brings about reductions in the growth rate. This is

called as the osmotic effect of salinity. Second, if there is high amount of sodium in transpiration stream of plants, there can be damage in cells found in transpiring leaves and this may bring about to decrease in plant growth. This is accepted as the salt-specific effect of high concentration of salt (Greenway and Munns, 1980). Ion specific stresses include decreased cytosolic K^+/Na^+ ratios and higher than normal Na^+ and Cl^- concentrations in the cytosol in which many important biochemical reactions take place. Under normal conditions, plant cells maintain a high cytosolic K^+/Na^+ ratio. Under salinity, Na^+ and Cl^- penetrate the hydration shells of proteins and interfere with the non-covalent interactions between amino acids of proteins. This leads to conformational changes and loss of function of proteins. Ionic toxicity, osmotic stress, and nutritional defects under salinity may lead to metabolic imbalances, which result in oxidative stress.

1.1.1.2. Mechanisms of plant salt stress tolerance

Salt stress tolerances of plants differ extremely; even there are variations within species. When compared to other cereals, rice (*Oryza sativa*) shows the lowest tolerance to high salt concentration. In contrast to rice, barley (*Hordeum vulgare*) shows the highest salt tolerance among cereals (Figure 1.2). There is difference between bread wheat and durum wheat in terms of salt tolerance. Bread wheat (*Triticum aestivum*) is generally defined as mildly tolerant and show high salt tolerance when compared to durum wheat (*Triticum turgidum* ssp. *durum*). Also there is high variation in dicotyledonous species in terms of salt tolerance even higher than monocots (Munns and Tester, 2008).

Main adaptive strategies when plants are exposed to salinity can be grouped as follows (Niu *et al*., 1995; Mansfield, 2002) some of the plants can complete their cycle of development in the most suitable time of the vegetation season. This type of adaptation is called as phenological avoidance. Another adaptation mechanism is salt avoidance with salt exclusion and secretion by the help of specific membrane protein

and special salt glands. In addition to these strategies, most of the excess salt ions can accumulate in vacuoles. Some plants are also developed biochemical tolerance through adaptations of cell organelles and macro-molecular systems to excess of salt. Last adaptive strategy to salt stress is the nutritive tolerance which is related with the capacity for usage of K^+ and Ca^{2+} in normal physiological process in order to alleviate the harmful effects of sodium ions. In the light of this information, mechanisms of salt tolerance can be divided into two main groups: minimization of entry of salt into plant and minimization of the concentration of salt in the cytoplasm (Munns, 2002).

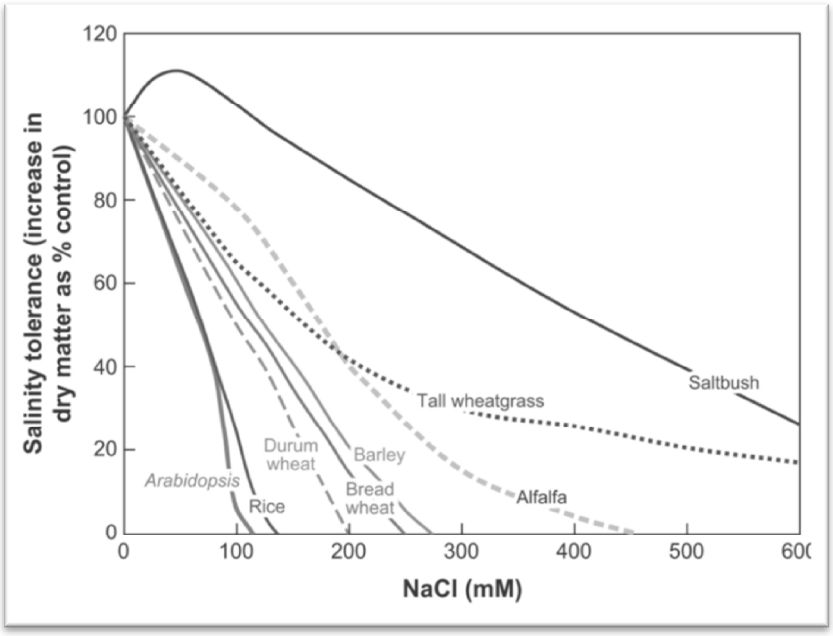

Figure 1. 2. Diversity in the salt tolerance of various species, shown as increases in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl. (Taken from Munns & Tester 2008)

1.1.1.2.1. Sodium sequestration into vacuoles

Since there is big membrane bound vacuoles, organization of plant cells is appropriate for the compartmentation of excess sodium ions. The biggest component of a mature cell is vacuole and can constitute 80% of the total cell volume. Despite the fact that there is generally single large central vacuole in cell, the magnitude and amount of vacuoles can change in different tissues and stages of development (Martinoia, 2000). They have many functions. They play important roles in disruption of cytoplasmic constituents and homeostasis of the plant cells. Additionally, they are involved in the regulation of cytoplasmic ions and pH, the storage of metabolites, control of cell volume and sequestration of toxic ions. They have a role to maintain turgor in vegetative organs by acting in combination with the cell wall (Zouhar and Rojo 2009). Among functions mentioned above, the compartmentation of toxic ions (Na^+, Cl^-) is very important in terms of plant salt tolerance. The sequestration of sodium ions into the vacuoles enables an efficient mechanism to prevent the harmful effects of cytosolic sodium ions. Furthermore, the sequestration of sodium ions (and chloride) into the vacuole enables the plants to use sodium chloride as an osmoticum, maintaining an water potential that impel water into the cells. Blumwald and Poole (1985; 1987) proposed that sequestration of excess sodium in the cytosol can be achieved by Na^+/H^+ antiport protein located on vacuole membrane. So, the transfer of sodium ions into the large membrane bound vacuoles is mediated by a sodium/hydrogen antiporter. The energy for the sequestration of excess ions is provided proton motive force generated by the vacuolar type H^+-ATPase and vacuolar inorganic pyrophosphatase (H^+-PPiase) (Nyrén & Strid, 1991) (Figure 1.3).

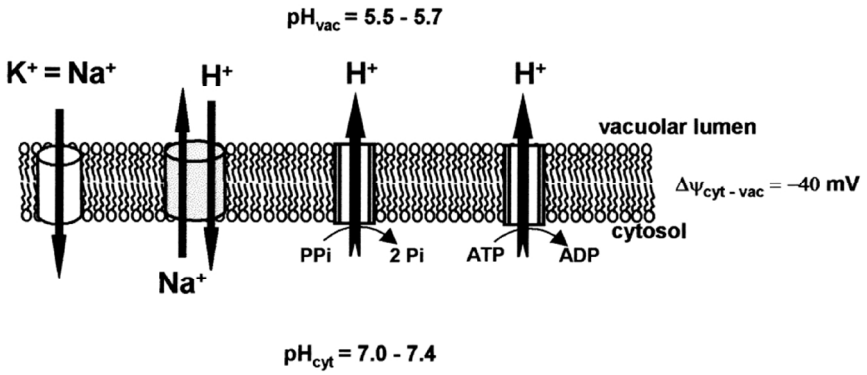

Figure 1. 3. Sodium transport in vacuole membrane

V-H^+-ATPase and V-PPiase hydrolase ATP and pyrophosphate respectively and the energy released is used to generate the proton motive force, which is used by the Na^+/H^+ antiporter to transport the Na^+ into vacuoles against its electrochemical gradient (Rea *et al*., 1992). As a result of sequestration of excess sodium ions into the plant cell vacuole, plant cell can keep cytosolic sodium level in at non-toxic level, so they can alleviate the harmful effects of Na^+ injuries to crucial enzymes in cytosol.

Overexpression of *AtNHX1* gene in *Arabidopsis* showed that transgenic plants were more tolerant to salt stress than the wild type plants by the help of activity of sodium hydrogen antiport protein located on tonoplast (Apse, 1999). This study provided reliable data that sodium hydrogen antiporters have an important function in salt tolerance of plants. In literature, there were many salt tolerant transgenic plants expressing *AtNHX1,* such as canola, tomato, wheat, soybean, tobacco and groundnut (Zhang *et al*., 2001; Xue, 2004; Li *et al*., 2010; Zhou *et al*., 2011; Asif *et al*., 2011).

1.1.1.2.2. Sodium extrusion through plasma membrane

The other adaptive strategy for a plant to cope with high concentration of salt is to expel excess Na^+ out of the cells. The transport of Na^+ out of plasma membrane is an active transport process which is coupled to the downhill transport of H^+ to the cytosol. Na^+ extrusion is carried out Na^+/H^+ antiport by working together H^+-ATPase in higher plants. Na^+/H^+ antiporter is the main element for transporting of Na^+ out of plasma membrane and H^+-ATPase is responsible for the generation of electrochemical H^+ gradient across the plasma membrane (proton motive force). The expelling of sodium ions to the out of cell by the help of sodium hydrogen antiporter located on plasma membrane provides efficient mechanisms to keep intracellular ion homeostasis (Zhu, 2001). However, the energetic cost of this sodium expulsion may be a bottleneck for salt tolerance (Davenport and Tester, 2000).

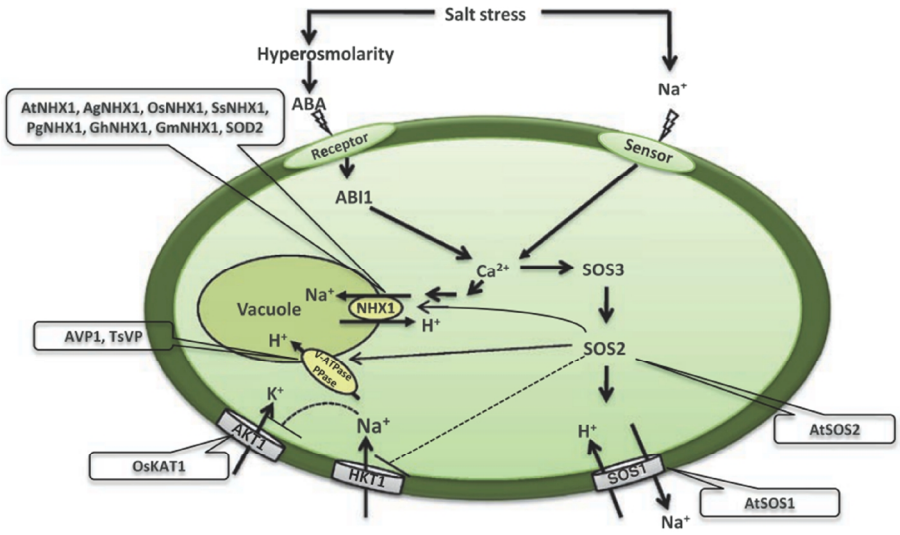

Figure 1. 4. Regulation of cytosolic ion balance in plant cell through Ca^{2+} mediated signal transduction pathways. Taken from Pareek *et al*., (2010)

First response of plant cell to the high concentration of salt is characterized by the transient increase of Ca^{2+} (Knight *et al*., 1997). The mechanism of how Ca^{2+} signal is transduced to regulatory system when plant is affected by salt stress was identified in *Arabidopsis* with salt overly sensitive (SOS) pathway. Most important component of the system are SOS3, SOS2 and SOS1, which form a signaling pathway to transfer the Ca^{2+} signal, so plant can maintain ion homeostasis during salt stress (Zhu, 2003). This signal generated by salt stress is sensed by the membrane associated Ca^{2+}sensor SOS3 and SOS3 act together with protein kinase SOS2 (Halfter *et al*., 2000; Jiping Liu *et al*., 2000). In the existence of calcium ions, the activation of SOS2 was carried out by SOS3. This duplex structure activates SOS1, a integral membrane sodium hydrogen antiport protein which has 7 transmembrane domain, by phosphorylation. This pathway enables plants to restore cellular ionic balance (Figure 1.4).

1.1.1.2.3. Synthesis or accumulation of compatible solutes

Plants accumulate osmolyte under salt stress. Osmolytes generally accepted as organic substance which is used by cell to keep volume of the cell. Sucrose, fructose, proline, glycine betaine, inositols, glycerol and trehalose are the examples of most commonly produced osmolytes under salt stress (Chen and Murata, 2002). Osmolyte are also called as “compatible solutes”, they can keep their activity in cell without affecting the normal cellular process even at high concentraions (Majumder *et al*., 2010). Another suggested function of compatible solutes is eliminating of the effect of reactive oxygen species (ROS), the product of secondary effect of salt stress, by making stabile the membrane and proteins (Diamant *et al*., 2001). In plant cells, osmolytes are accumulated in the cytosol, chloroplast and other cytoplasmic compartments.

1.2. Engineering of Plants Against Salt Stress

Transformation of stress related genes appears to be more applicable and rapid approach when compared to conventional breeding programs to develop stress

tolerant plants. The response of plants to salt stress is highly complex involving simultaneous expression of a number of genes. Plant genetic engineering techniques could be effectively exploited unused potentials to increase the harvestable crop yield. It can be achieved by gene manipulation either through overexpression or silencing of alien/nature genes. Several genes induced in response to salinity have been found from different organisms living in salted environment. Tables 1.1 summarize the studies related with development of salt resistant plants.

Table 1. 1. Examples of salt resistant transgenic plants

Source Species	**Gene Product/Nam e**	**Target Species**	**Reference**
Arthrobacter globiformis	choline oxidase/*codA*	Tomato	(Goel *et al*., 2011)
Atriplex hortensis	Betaine aldehyde dehydrogenase/ AhBADH	Trifoliate orange	(Fu *et al*., 2011) (Liu *et al*., 2011)
Salsola soda	Na^+/H^+ antiporter/SsNHX1	*Alfalfa*	(Li *et al*.,)
Ipomoea batata	Low osmotic stress 5/Los5	Sweetpotato	(Gao *et al*., 2011)
Triticum aestivum L.	MYB gene/TaPIMP1	*Tobacco*	(Liu *et al*., 2011)
Salicornia europaea L.	β-lycopene cyclase gene SeLCY	*Arabidopsis*	(Chen *et al*., 2011)
Hordeum vulgare	Na^+/H^+ antiporter/ HvNHX2	*Arabidopsis*	(Bayat *et al*., 2011)
E. coli	Glycine betaine / betA	Cotton	(Zhang *et al*., 2011)
Tamarix sp	metallothionein	*potato*	(Rahnama *et al*., 2011)
Zoysiagrass	betaine aldehyde dehydrogenase / ZBD1	Ryegrass	(Takahashi *et al*., 2010)
Salicornia europaea	choline monooxygenase /CMO	Tobacco	(Wu *et al*., 2010)
N. tabacum	Osmotin	Tomato	(Goel *et al*., 2010)
E. coli	Glycine betaine / betA	Wheat	(He *et al*., 2010)
O.sativa	Prolin / P5CSF129A	Rice	(Kumar *et al*., 2009)
Spinacia oleracea	Betaine Aldehyde Dehydrogenase (SoBADH)	Cotton	(Luo *et al*., 2008)

Table 1.1. Examples of salt resistant transgenic plants (continued)

H.vulgare	Late embryogenesis abundant (LEA) proteins / HVA1	*Mulberry*	(Lal *et al*., 2008)
A. thaliana	Na^+/H^+ antiporter/AtNHX1	Buckwheat	(Cheng *et al*., 2007)
O.sativa	Dehydroascorbate reductase gene	*Arabidopsis*	(Ushimaru *et al*., 2006)
A. thaliana	Pyrroline-5-carboxylate/*P5CS*	Potato	(Hmidasayari *et al*., 2005)
A. thaliana	Na^+/H^+ antiporter/AtNHX1	*Maize*	(Xiao-yan *et al*., 2004)
Schizosaccharomyces	SOD2	*Arabidopsis*	(Gao *et al*., 2003)
Vigna aconitifolia	Pyrroline-5-carboxylate/	Wheat	(Sawahel and Hassan, 2002)

1.2.1. Osmolyte Synthesizing Genes

Under stress conditions compatible solutes play important role to maintain ion balance and homeostasis. The accumulation of osmolyte in response to osmotic stress can be seen at variety of organism, from bacteria to plants and animals. However, different species produce different osmolyte during osmotic stress. Osmoprotectants can be grouped as betaines, certain amino acids and non-reducing sugars in terms of chemical structure (Takabe *et al*., 2006).

The importance of proline in salt tolerance was shown in several studies. Indica rice expressing *P5CSF129A* produced higher proline accompanied with higher biomass and increased ability to survive under high amount of salt and low level of lipid peroxidation, pointing out that production of high amount of proline may have a function to alleviate the harmful effects of salt stress. Therefore, increased proline level enables plant cell to keep integrity of cell and main physiological processes under high salt concentration (Kumar *et al*., 2009).

One of the most important osmolyte produced by plant under salt stress is glycinebetaine. Glycinebetaine is a very effective osmolyte and accumulation of this type of osmolyte is heavily related with the plants growth in salt stress conditions (Hodes and Hanson, 1993). In high salt stress conditions, glycinebetaine does not involve in cytoplasmic functions. Therefore, the structure and functions of several macromolecules can be effectively stabilized by the help of glycinebetaine. Glycinebetaine protects enzymes and proteins more efficiently when compared to other osmolytes (Waditee *et al*., 2007). Genes involved in glycine betaine synthesis, such as *choline oxidase, betaine aldehyde and betaine aldehyde dehydrogenase genes* have been introduced into several plants by using genetic transformation techniques (Lilius *et al*., 1996; Sakamoto, 2000; Waditee *et al*., 2005; Yang *et al*., 2008). The choline oxidase encoded by codA expressing transgenic tomato plants showed a much higher frequency of seed germination and faster growth of young seedlings under salt stress conditions than non-transformed tomato plants. The salt tolerance of transgenic tomato provided by codA was observed also at the whole plant level (Goel *et al*., 2011).

Zhang *et al*., (2011) investigated the effect of salt stress on *betA* gene transferred cotton. The salt tolerances were evaluated at the seedling stage and during the entire growth period in the field of a T_3 generation. They observed that under salt stress, the amount of ion leakage and levels of MDA in all the transgenic lines were significantly lower than those in the wilt type cotton plants. Also, they stated that net photosynthesis in seedlings was less inhibited by osmotic stress in all the transgenic lines than in the wild type plants.

Liang *et al*., (2009) suggested that overaccumulation of glycine betaine could reduce the accumulation of reactive oxygen species in the transgenic wheat lines transformed with *BADH* gene isolated from *Atriplex hortensis L.* Their results also showed that glycine betaine can maintain or enhance the activities of antioxidant enzymes under the salt stress conditions and this effect of glycine betaine is

advantageous in protecting the cellular and subcellular systems from the cytotoxic effects of ROS.

One of the best-characterized osmoprotectants is trehalose, which is a non-reducing disaccharide of glucose and plays an important role for stabilization of biological structure under osmotic stress as an osmoprotectant (Crowe *et al*., 1992). Trehalose is biosynthesized by trehalose-6-phosphate synthase (*TPS*) and trehalose-6-phosphate phosphatase (TPP) in plants. However, the role of trehalose in plants is not yet fully elucidated. Under osmotic stress, trehalose was shown to accumulate at high levels in resurrection plants and was therefore proposed to function in stress protection (Wingler, 2002). The protection roles of trehalose in plants under salt stress were reported by some researcher in recent years. Jiang *et al*., (2010) have cloned and characterized trehalose-6-phosphate synthase gene in maize. They reported that semi-quantitative RT-PCR and real-time quantitative PCR indicated that the expression of this gene is up-regulated in response to both salt and cold stress.

Transgenic tobacco plants expressing *TPS1* and *TPS2* genes from *Saccharomyces cerevisiae* were generated by Karim *et al*., (2007). They used both constitutive and inducible promoter during cloning studies. They observed that co-expression of *ScTPS1* and *ScTPS2* provide high tolerance to drought and salt stress without any growth abnormalities.

Plants generally accumulate compatible solutes to keep cell turgor. Mannitol is one of the most frequently encountered osmolyte in plants under osmotic stress. Mannitol is a mono-saccharide consists of six hydroxyl groups. The bacterial *mtlD* gene encoding mannitol-phosphate de-hydrogenase, which enables the conversion of fructose to mannitol, can be transferred to plants by genetic engineering to induce the synthesis of mannitol in plant cells. *Populus tomentosa* plants expressing a bacterial *mtlD* gene can accumulate more mannitol and they showed improved salt tolerance under 75mM salt stress (Hu *et al*., 2005). The *mtlD* gene has also been

transferred to tomato where it enhanced the mannitol accumulation that contributes to the enhanced tolerance to NaCl stress under high salinity conditions (Rahnama *et al*., 2011).

1.2.2. Oxidative Stress Related Genes

Salt stress brings about oxidative damage, ion toxicity and disruption of cellular homeostasis through the production of reactive oxygen species (ROS). ROS induces various complex biochemical, molecular, cellular, and physiological changes in plants including damage of DNA, proteins and membrane lipids (Munns and Tester, 2008). Therefore, many studies on the antioxidant capacity of the cell have been carried on scavenging of ROS under abiotic stresses. In order to scavenge reactive oxygen species, plants have developed a wide range of enzymatic and non-enzymatic mechanisms. Among the enzymatic systems, superoxide dismutase (SOD, EC 1.15.1.1) is one of the crucial enzymes in the plant's defense mechanism that converts superoxide anion radicals to hydrogen peroxide (H_2O_2), provide protection against the detrimental effects of ROS (Fridovich, 1975). Plant cells have three major isoforms of SOD depending on the metal cofactors Cu/Zn-SOD in the cytosol and chloroplasts, Mn-SOD in the mitochondria and Fe-SOD in plastids (Bowler *et al*., 1994).

Gill *et al*., (2010) were evaluated the response of *Arabidopsis thaliana* expressed cytosolic Cu/Zn-SOD (*PaSOD*) isolated from *Potentilla atrosanguinea.* They suggested that overexpression of a cytosolic *PaSOD* in *Arabidopsis* can increase the tolerance to salt stress in terms of germination rate and seedling growth. Also, they proposed that SOD mediated lignification of the conducting tissue might be yet another mechanism of tolerance to abiotic stress in plants and this parameter may be used as a quick and simple screen to filter stress tolerant species.

Another study was conducted by Kavitha *et al*., (2010) with transgenic tobacco plants expressing monodehydroascorbate reductase (MDAR) from halophyte

Avicennia marina. According to their results tobacco lines expressing *Am-MDAR* showed a significant higher monodehydroascorbate reductase activity compared to control plants. They found that the level of ascorbic acid detected in the transgenic plants was substantially higher compared to control plants while the level of monodehydroascorbate was found to be lesser. Nagamiya *et al*., (2007) and Prodhan *et al*., (2009) were able to produce salt tolerant japonica and indica rice by over expressing the catalase gene (*katE*) isolated from *Escherichia coli*. Since, catalase is one of the major antioxidant enzyme which break down hydrogen peroxide by producing H_2O and O_2, so it provides a surviving mechanism to the salt effected plants.

1.2.3. Signal Transduction Genes

Plants grown under natural conditions suffer various environmental stresses such as drought, high salinity and extreme temperatures (Yamaguchi-Shinozaki and Shinozaki, 2006). According to previous studies, osmotic stress is a major cause resulting in these abiotic stresses and the regulation of signaling transduction plays an important role in tolerance to osmotic stress in plants (Zhu, 2002; Bartels and Sunkar, 2005; Nakashima *et al*., 2009). Protein kinases involved in regulating signaling transduction by phosphorylation/dephosphorylation were considered to mediate acclimation to environment changes in eukaryotic organisms (Boudsocq and Laurière, 2005). A large superfamily, which consists of seven types of serine–threonine protein kinases, such as sucrose non-fermenting 1-related protein kinases (*SnRKs*), has been identified in the past few years (Hrabak *et al*., 2003).

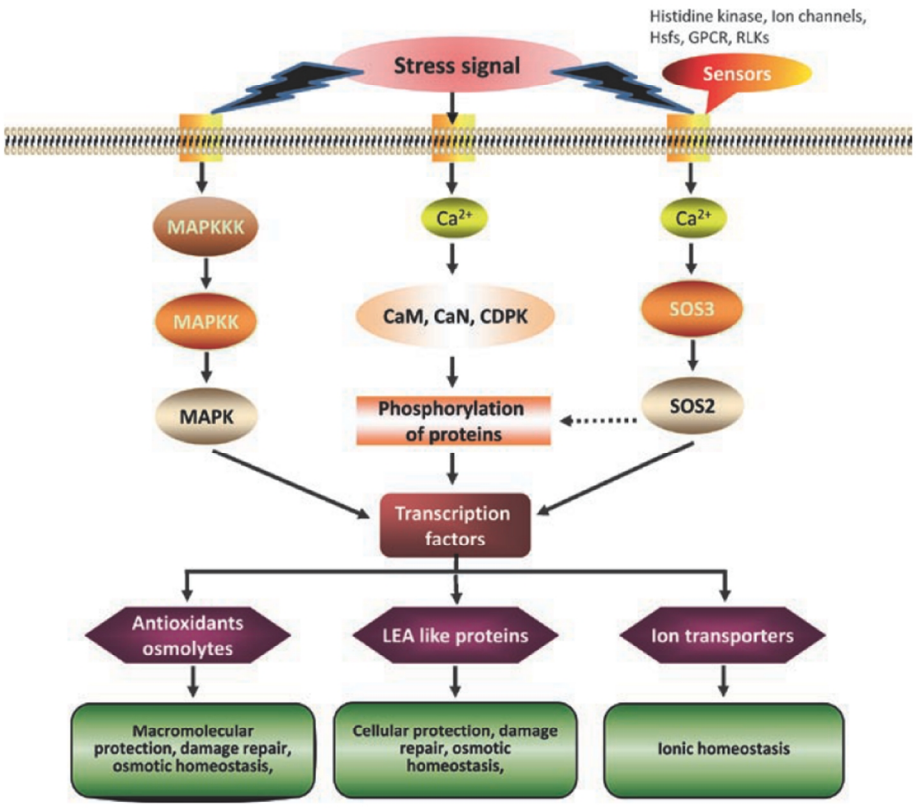

Figure 1. 5. Signal transduction pathways involved in abiotic stress responses (Pareek *et al*., 2010)

Transcription factor genes play important roles in stress survival by serving as master regulators of a set of downstream stress-responsive genes. Transcription factors regulate downstream gene expression via binding to specific elements in promoter of these genes, consequently, enhance stress tolerance in plants. A novel chrysanthemum NAC transcription factor gene was isolated and characterized by Liu *et al*., (2011). They transferred gene into tobacco to evaluate the function of transcription factor. The*35S:DgNAC1* expressing transgenic tobacco of the enhanced tolerance of high salt stress without the severe growth retardation and dwarfing was observed in their study.

Another example for salt tolerance enhanced with transcription factor was proposed by Zhang *et al*.,(2010). They cloned sucrose non-fermenting1-related protein kinase 2 (*SnRK2*) from wheat and functions of this gene were characterized under different stresses, including salt stress. They reported that overexpression of *TaSnRK2.8* resulted in enhanced tolerance to drought, salt and cold stresses. There were good evidences such as longer primary roots and various physiological characteristics, including higher relative water content, strengthened cell membrane stability, significantly lower osmotic potential, more chlorophyll content, and enhanced PSII activity to confirm the function of *TaSnRK2.8* in *Arabidopsis.*

1.2.4. Antiporter genes

Plants can use three strategies to keep cytosolic sodium concentration under the control: sodium exclusion, compartmentation, and secretion. Sodium transport out of the cell was carried out by plasma membrane-bound Na^+/H^+ antiporter proteins. The sequestration of sodium is achieved by vacuolar Na^+/H^+ antiporter proteins that move potentially harmful ions from cytosol into large, internally acidic, tonoplast-bound vacuoles. Antiports use the proton motive force generated by vacuolar H^+-translocating enzymes, H^+-adenosine triphosphatase (ATPase) and H^+-inorganic pyrophosphatase (PP_iase), to couple downhill movement of H^+ with uphill movement of Na^+ (Rea *et al*., 1992).

Transgenic *Arabidopsis* plants expressing *HvNHX2* from barley had normal growth in the presence of 200 mM NaCl while wild type plants showed leaf necrosis according to Bayat *et al*., (2011). In addition to this finding also they reported that when transgenic *Arabidopsis* seeds were grown on MS medium containing NaCl, transgenic plants overexpressing *HvNHX2* accumulated more Na^+ in the shoots, and had longer roots in the early seedling stage. Wang *et al*., (2011) isolated V-ATPase subunit B gene (vacuolar H^+-ATPases) from salt-tolerant wheat mutant RH8706-49 and transferred to *Arabidopsis thaliana* cv. Colombia. B-subunit of $V–H^+$-ATPase is a regulatory subunit and also involved in the formation of catalytic sites. They used

sequence information of homologous V–H^+-ATPase subunit B in rice and *Arabidopsis* to determine wheat *TaVB* out of an NCBI wheat EST library. They observed that seeds belong to transgenic *Arabidopsis* transformed with *TaVB* gene had a higher germination rate and better growth responses under salinity stress (180mM NaCl).

A new Na^+/H^+ antiporter gene was identified and cloned by Yu *et al*., (2007) from wheat by using RACE and called as *TaNHX2*. The function of *TaNHX2* was analyzed by complementation in the yeast mutant Δnhx1. They proposed that the yeast cell with overexpressed *TaNHX2* showed higher salt, osmotic and freezing tolerance. These results provide evidence that *TaNHX2* may play an important role in salt and osmotic stresses. The first member of the vacuolar H^+-PPase pump (*TVP1*) gene was cloned by (Brini *et al*., 2005) from wheat cDNA library. Along with *TVP1* gene also *TaNHX1* gene was cloned. They reported that expression analysis of salt-stressed wheat plants showed substantial up-regulation of *TaNHX1* transcript levels as compared to control plants, while transcript accumulation for *TVP1* was not greatly affected by exposure of plants to salt stress.

Gao *et al*., (2010) reported a novel aquaporin gene *TaNIP* (*Triticum asetivum* L. nodulin 26-like intrinsic protein) involving in salt tolerance mechanism in plants. They characterized and cloned *TaNIP* gene through the gene chip expression analysis of a salt-tolerant wheat mutant RH8706-49 under salt stress. According to localization analysis, *TaNIP* proteins tagged with green fluorescent protein were detected in cell plasma membrane. They observed that *TaNIP*-overexpressing *Arabidopsis* plants have higher K^+, Ca^{2+} and proline concentration and lower Na^+ concentration than control plants. The overexpression of *TaNIP* in transgenic *Arabidopsis* also up-regulated the expression of a number of stress-associated genes. As a result of their study they suggested that *TaNIP* plays an important role in salt tolerance in *Arabidopsis*.

1.3. Genetic engineering of crop plants

Genetic engineering is one of the most important tools for developing new transgenic plants with desirable traits. It is possible to transfer genes into plants from unrelated organisms by the development of plant genetic engineering. Traits can be manipulated by inserting a gene or genes originating from any organism. The addition of new traits can be accomplished with conventional breeding and plant biotechnology or plant genetic engineering.

The main purpose of the biotechnology is to manipulate the genome of important plants, typically by adding a few genes at a time. Traits can be manipulated by inserting DNA originating from any organism with that trait of interest into the target plant. Thus far in crop biotechnology, much work has been accomplished in conferring traits to plants such as the ability to survive herbicide treatment, insect resistance, disease resistance, and stress tolerance. However, there is growing interest in producing drugs and industrial proteins in plants as well as enhancing the nutrition of plant products (Newell, 2000). Conventional breeding techniques utilize processes of crossing, back crossing and selection. However, there is crossing barriers derived from incompatibility and species differences. Advances in plant genetic engineering have resulted in introduction of desired foreign gene(s) to overcome problems of sexual incompatibility and species barriers between organisms. By the help of this technology, new traits such as: the potential to resist biotic and abiotic stresses, enhancement in nutritional quality of the product, herbicide, pesticides resistance and increased productivity. Additionally, there is growing interest in producing drugs and industrial proteins in transgenic plants. Recent genetic engineering technology allows not only transfer of a single gene, but also a couple of genes in a much more precise, controllable, and predictable way than is achievable with conventional breeding. Plant biotechnology has played a significant role in modern agriculture in the past two decades. Since the first genetically modified (GM) crop was commercialized in 1996, global hectarage of

biotech crops has continued to grow, reaching 134 million hectares in 2009 (James, 2010).

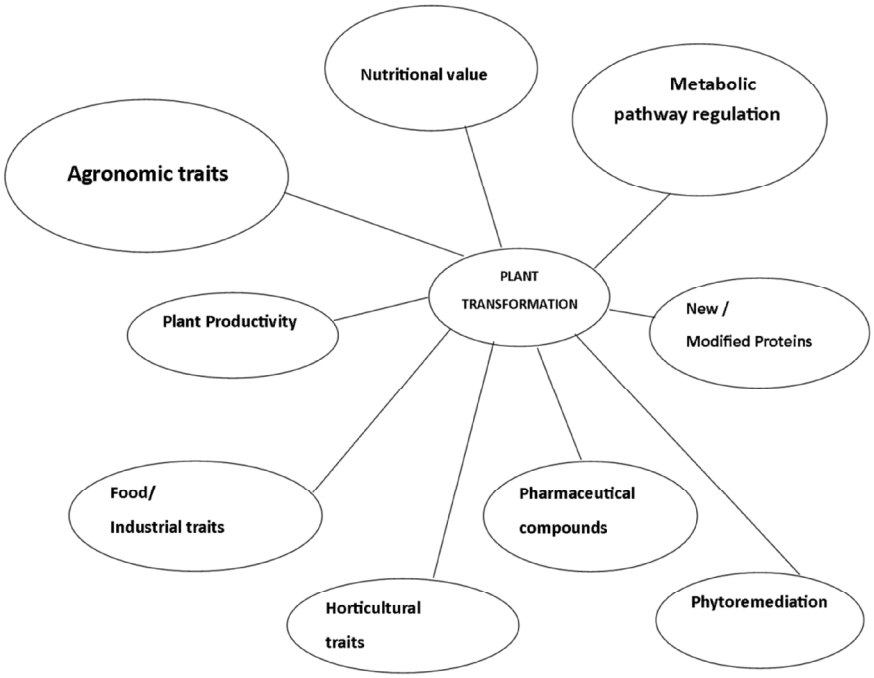

Figure 1. 6. Application of plant transformation (Adapted from Newell, 2000)

In typical plant cell, DNA is located in three subcellular compartments: such as nucleus, plastids and mitochondria. Transferring of a gene or genes into plant genetic compartment can be defined as plant transformation. Methods used for gene transfer into plant cell can be divided into two major categories: indirect and direct DNA deliveries. Exogenous DNA molecule can be transferred by viruses or bacteria, e.g., *Agrobacterium tumefaciens* and *Agrobacterium rhizogenesis (Tzfira and Citovsky, 2006)*, therefore this type of transformation is called indirect DNA transformation. Particle bombardment or biolistic are good example for direct DNA introduction to the plant cells. *Agrobacterium*-mediated transformation, PEG-mediated

transformation and particle bombardment methods are most commonly used for nuclear transformation. However, PEG-mediated transformation and particle bombardment are only used for routine chloroplast transformation studies. Also there are alternative transformation methods used in plant transformation, including electroporation, microinjection, silicon carbide (Rakoczy-Trojanowska, 2002).

Although *Agrobacterium*-mediated and particle bombardment methods are commonly used for plant transformation, transgenic plants produced by these techniques subjected to biosafety concern because of insertion of vector backbone and undesired gene particles (Barampuram and Zhang, 2011). This unwanted integration cause to multiple copies of transgene, making the control of transgenic plants more complicated. In recent years, there has been important development in generation of vector backbone and selectable marker free transgenic plants. There are several methods developed for the production of marker free transgenic plants. Co-transformation followed by segregation, site-specific recombination-mediated marker deletion system (Cre-LoxP, FLP/FRT, R/RS), transposon-mediated elimination method and intrachromosomal recombination systems were used to generate marker free transgenic plants (Darbani *et al*., 2007).

1.3.1. Tissue Culture and Regeneration

Successful plant transformation of plants including cereals largely relies on the capacity of transformed tissues to proliferate on selective medium and following regeneration of plants from transformed cells. Regeneration capacity in tissue culture differs from one species to another even within the species. Plant tissue culture can be defined as cultivation of isolated plant cells, tissues and organs under sterile conditions to regenerate whole plants. Tissue culture is comprehensive term to describe all types of plant cultures, such as callus, cell, protoplast, anther, meristem, embryo and organ cultures (George *et al.*, 2008). It heavily depends on the concept of cell totipotency which is ability of single cells to divide, to produce all the differentiated cells characteristic of organs, and to regenerate into a whole plant

(Razdan, 2003).The different techniques of culturing plant tissues may offer certain advantages over traditional methods of propagation. Tissue cultures represent the major experimental systems used for plant genetic engineering, as well as for studying the regulation of growth and organized development through examination of structural, physiological, biochemical and molecular bases underlying developmental processes.

There are several factors affecting in vitro culture of wheat including growth conditions of the donor plant, and composition of the nutrient medium. In most species including cereals, plant regeneration depends on genotype (Maddock *et al*., 1983; Özgen *et al*., 1998) the type of explant (Ozias-Akins and Vasil, 1982; Maddock *et al*., 1983; Redway *et al*., 1990) and media composition (Mathias and Simpson, 1986; Fennell *et al*., 1996). Application of some methods is, in many cases, restricted to only a particular set of genotypes, types of explants, culture media.

1.3.1.1. Studies on Wheat Regeneration Systems

A highly efficient and reproducible in vitro regeneration system is an absolute prerequisite for producing transgenic plants. Particularly in the cereals routine application of molecular improvement independent of the chosen method of transformation is still impeded by the lack of readily available highly efficient and long-term regenerable cell and tissue culture systems (Sharma *et al*., 2004). Plant regeneration in tissue cultures can follow two different pathways. These are organogenesis involving the development of axillary buds following inhibition of apical dominance and somatic embryogenesis (Vasil, 1987).

The formation of callus from an explant roughly contains 3 stages: induction, cell division, and differentiation. During induction phase, the metabolism is prepared for cell division. At the actively cell division phase, the cells of the explant are reverted

to meristematic or dedifferentiated state. Third phase is the appearance of cellular differentiation and expression of certain metabolic (Dodds and Roberts, 1985).

1.3.1.2. Explants used in wheat transformation

The explants used in wheat transformation include immature embryos (Khanna and Daggard, 2003; Hu *et al*., 2003; Cheng *et al*., 2003; Cao *et al*., 2011a; Stephenson *et al*., 2011), inflorescence (He and Lazzeri, 2001;Amoah, 2001;Kavas *et al*., 2008), mature embryos (Delporte *et al*., 2001; Patnaik and Khurana, 2003; Wang *et al*., 2009; Ding *et al*., 2009; Battal, 2010), and apical meristem (Supartana *et al*., 2006), etc. But, the most widely used explant is the immature embryo because of its superior regeneration potential than the other tissues.

1.3.1.2.1. Immature embryos

In cereal crops, immature embryos are accepted the most responsive explant in tissue culture because of their ability to form embryogenic callus and subsequently large number of plants. For the first time, complete plantlets were obtained from immature embryos of wheat by Shimada (1978). Later, Yamada and Shimada, (1979) optimized various factors, such as age and size of the embryos and auxin concentration for callus induction and found that embryos isolated 14 days after anthesis induced high intensity of green spots formation. Gonzalez *et al*.,(2001) were evaluated influence of genotype and culture medium on callus formation and plant regeneration from immature embryos of 12 different durum wheat cultivars. They have reported that the regeneration of plantlets was higher from compact than from soft calli, with a strong dependence on genotype and type of induction medium utilized.

Pellegrineschi *et al*., (2004) have obtained optimal callus induction and plant regeneration in bread and durum wheat by manipulating NaCl concentration in the induction medium. Callus yield and regeneration frequencies were higher in durum

wheat embryos that were incubated in media containing 2 mg/L 2,4-D and 2 mg/L NaCl. Stephenson *et al*., (2011) identified and cloned two NF-YB members from *Triticum aestivum* (*TaNF-YB3* & *7).* They prepared constructs carrying these two genes for particle bombardment by immature embryo derived calli. They observed that transgenic wheat lines constitutively overexpressing *TaNF-YB3* had a significant increase in the leaf chlorophyll content, photosynthesis rate and early growth rate.

Cao *et al*., (2011) generated transgenic wheat plants expressing the powdery mildew resistance gene *Pm21* by bombarding immature embryos. As a consequent of this study, they evaluated the function of *Stpk-V,* a putative serine/threonine protein kinase gene, by gene mapping, stable transformation, transient expression assay, and gene silencing studies. They found that a susceptible wheat variety Yangmai158 transformed with *Stpk-V* showed high and broad-spectrum powdery mildew resistance.

1.3.1.2.2. Mature embryos

Heyser *et al*., (1985) have investigated long-term, high frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of *Triticum aestivum* using mature and immature embryos. They found that the relative amounts of embryogenic and non-embryogenic callus in mature and immature embryos could be significantly altered by different 2,4-D concentrations in the medium. Zale *et al*.,(2004) compared the behavior of a diverse set of wheat genotypes in their tissue culture response using mature embryos. They found significant differences in plant regeneration, culture efficiency, and regeneration capacity when mature embryos of 47 wheat cultivars, breeding lines, and the common wheat progenitors were compared. Also they declared that there was no significant correlation between total callus induction and regeneration capacity or culture efficiency indicating that these variables were not related.

Wang *et al*.,(2009) reported the successful wheat transformation by using mature embryo derived calli and intact mature embryo with *Agrobacterium*-mediated transformation. They observed that based on the transient GUS assays and the regeneration of transgenic T_0 plants, the mature embryo halves prepared from freshly imbibed seeds rather than the calli derived from scraped embryos are generally more suitable recipient tissues for *Agrobacterium* mediated T-DNA transfer in wheat.

1.3.1.2.3. Immature inflorescences

Immature inflorescences are an alternative explant for plant regeneration studies. Advantages of using inflorescence tissue versus scutella are that explants are harvested from younger plants reducing greenhouse or growth chamber requirements. In 1982, Ozias-Akins and Vasil reported tissue cultures of *Triticum aestivum* initiated from young inflorescences and immature embryos possessed the potential for regeneration of whole plants. They declared that both a friable and a compact type of callus were produced on MS medium supplemented with 2 mg/L 2,4-D. The friable callus contained meristematic centers in which the peripheral cells halted dividing, elongated, and could be easily separated. They stated that embryogenic callus from inflorescence tissue and from immature embryo have morphological similarities.

Amoah *et al*., (2001) have investigated factors influencing *Agrobacterium*-mediated transient expression of gus gene in wheat inflorescence tissue. During transformation studies, they used *Agrobacterium tumefaciens* strain AGL harboring the binary vector pAL156. They studied the effects various factors on delivery and transient expression of gus gene including the duration of precultured, vacuum infiltration, the effect of sonication treatments and *Agrobacterium* cell density. They obtained optimal T-DNA delivery from inflorescence tissues precultured for 21 days and sonicated.

1.3.2. Particle bombardment

Particle bombardment is one of the direct gene transfer methods used for generation of transgenic plants. This method was developed in 1980s to manipulate plants showing recalcitrance for *Agrobacterium*-mediated transformation. Afterwards, the technique has been widely used to produce transgenic plants in a wide range of plant species (Breitler *et al*., 2002). The first particle bombardment device was developed by Sanford (1988) and his coworkers. The first successful application of particle bombardment in wheat transformation was performed with Lonsdale *et al*., (1990) by using mature embryos. The technique involves coating microcarriers with the gene of interest and then accelerating them at high velocities, to penetrate into the plant cell. Inert metal particles, such as tungsten and gold, are used as microcarriers. There are many factors affecting the success of transformation with particle bombardment technology. These factors include the type of vector with a small size and high copy number, additionally the quantity and quality of the delivered DNA. The deepness of penetration is affected by size and types of accelerated microcarrier. Therefore, selection of microcarriers is very important to reach desired success. There are commercially available gold particles ranging from 0.6 to 3.0 μm in diameter. Helium pressures, vacuum level, the size of the particles and the position of target tissues are very important parameters affecting the success of transformation. So, they have to be optimized before to start any bombardment studies (Taylor and Fauquet, 2002). Pretreatment of the target tissues has a significant effect on the frequency of recoverable transgenic plants. Embryogenic and meristematic tissues are the most commonly employed target tissues for the production of genetically transformed plants. Another advantage of microprojectile bombardment is possibility of transferring large DNA fragments up to 150 kb (Phan *et al*., 2007).

In addition to the many advantages of this technology, there are a few drawbacks, such as the integration of multiple copies of transgene, in addition to superfluous DNA sequences that are associated with the plasmid vector. Integration of several

copies of transgenes and superfluous DNA can cause to silencing of the gene of interest in the transformed plant (Lowe *et al*., 2009). There are several examples of biolistic transformation in wheat. According to recently published study, *RsAFP2* gene was introduced into wheat by using particle bombardment (Li *et al*., 2011). The defensin *RsAFP2*, a small cyteine-rich antifungal protein from radish (*Raphanus sativus*), was shown to inhibit growth of agronomically important fungal pathogens, such as *F. graminearum* and *R. cerealis.* They reported that transgenic wheat plants expressing *RsAFP2* gene showed increased resistance to these fungi.

Liu *et al*., (2011) reported a study related with production of marker free transgenic wheat and transgenic wheat plants with increased flour quality. In this context, they prepared minimal gene expression cassette with *HMW-GS 1Bx14* gene and transferred to immature embryo of local wheat cultivar by using particle bombardment. They obtained relatively low transformation rate as 0.28% when compared to other wheat transformation studies. They proposed that this low transformation rate might be due to absence of selection pressure.

Another wheat transformation study carried out with particle bombardment was reported by Sestili *et al*., (2010). They generated plant transformation vector carrying the RNAi construct of SBEIIa. SBEs are transglycosylase enzymes that catalyze the formation of α-1,6 linkages within the polymer by cleaving an internal alpha-1,4 linkage. In monocots, three starch branching isoforms are present: SBEI, SBEIIa and SBEIIb. They observed that silencing of SBEIIa in durum wheat causes obvious alterations in granule morphology and starch composition, leading to high amylose wheat.

1.3.3. *Agrobacterium*-mediated transformation

In this method, biological agents namely *Agrobacterium tumefaciens* and *Agrobacterium rhizogenesis* were used to transfer gene of interest to the target cells or tissues. *Agrobacterium tumefaciens* is a gram negative soil bacterium that has

unique ability to form crown gall at the wound sites of many dicotyledonous plants. Large Ti (tumor inducing) plasmids provide the tumor-inducing capability of virulent *Agrobacterium* cells. Similarly, Ri (root-inducing) megaplasmids are found in virulent strains of *A. rhizogenes*, the causative agent of "hairy root" disease. Both Ti- and Ri-plamids contain a form of "T-DNA" (transferred DNA). During the infection process a segment of the Ti (tumor-inducing) plasmid, called T(transferred)-DNA, is transferred from *Agrobacterium* to the plant cell nucleus in which it is integrated into the chromosomal DNA and expressed. The important requirements for *Agrobacterium*-mediated transformation firstly include the production of some active compounds like acetosyringone by the explants in order to induce the *vir* genes present on the Ti plasmid and then the induced agrobacteria must have access to competent plant cells that are capable of regenerating adventitious shoots or somatic embryos at a reasonable frequency. While it was originally thought that *Agrobacterium* was only capable of infecting dicotyledonous plants, it has recently become clear that some supervirulent strains of *Agrobacterium* are also capable of infecting wounded cells of monocots such as barley and wheat under laboratory conditions. In monocotyledone transformation, the induction of *vir* genes (virA, virB, virC, virD, virE, virF, virG, and virH) which are necessary for gene transfer has been achieved by wounding and also by using chemical inducers like acetosyringone. In addition to the availability of supervirulent strain, development of superbinary vector carrying a 15.2 kb KpnI fragment from the virulence region of pTiBo542 made it possible to transform monocotyledone plants (Komari, 1990). *Agrobacterium*-mediated transformation offers many advantages in plant transformation (Hansen & Wright, 1999, Jones, 2005, Shibata & Liu, 2000):

1. The ability of transfer relatively long T-DNA segment
2. The higher frequency of stable transformation with many single copy insertions
3. The high co-expression of introduced genes
4. The easy manipulation of transgene
5. The lower experimental cost

In order to obtain successful results from *Agrobacterium*-mediated transformation several factors must be considered, including the plant genotypes, sources of explants, *Agrobacterium* strains, medium salt strength and pH, duration and temperature of *Agrobacterium* explant interactions (inoculation and cocultivation), and use of T-DNA-inducing compounds (Cheng *et al*., 2004; Wu *et al*., 2003). Variety of explants was used for successful wheat transformation by using *Agrobacterium tumefaciens* including immature embryo (Wu *et al*., 2008), mature embryo (Ding *et al*., 2009), immature inflorescence (Amoah, 2001) and shoot tips (He *et al*., 2010).

He *et al*., (2010) reported that generation of drought resistant transgenic wheat plants by introducing of *betA* gene encoding choline dehydrogenase from *Escherichia coli* into shoot tips with *Agrobacterium*-mediated transformation. They demonstrated that drought stress resulted in increasing soluble sugars and free proline in all lines of wheat, while the transgenic lines accumulated considerably more soluble sugars and proline than the wild type after 7 days of drought treatment. The transgenic lines exhibited enhanced resistance to drought stress and produced more biomass under drought conditions than the wild type. Moreover, the transgenic lines had earlier seed germination and more developed roots under normal conditions.

Zale *et al*., (2009) developed floral dip transformation method to obtain transgenic wheat plants. In that experiment, transformation efficiency was estimated at 0.44%. Three well-characterized transformants have been isolated in the germplasm line Crocus, and Southern analyses has shown the transgenes to be integrated in the wheat genome and are stable for three–six generations. The transgenes showed the expected segregation ratios for a nuclear insertion event, and this was verified by transmission through pollination. Transgene expression was observed in the T_5 and T_6 generations.

1.3.4. Chloroplast Transformation in Higher Plants

In plant transformation studies, the gene of interest generally introduced into the nucleus; however, also plastids are other subcellular compartments which are good candidate for the integration of transgenes. The chloroplast is the place of photo-synthesis, which provides the primary source of energy for cells. Other important activities that occur in plastids include evolution of oxygen, sequestration of carbon, production of starch, synthesis of amino acids, fatty acids, and pigments, and key aspects of sulfur and nitrogen metabolism (Verma and Daniell, 2007). The chloroplast genome consists of a circular double stranded DNA molecule which has self-replicating capability. The size of the chloroplast can differ from 120 to 220kb, arranged in monomeric and circles or in linear molecules (Lilly *et al*., 2001). Plastomes contain two types of genes: (i) photosynthesis-related genes and (ii) genetic system genes such as rRNAs, tRNAs, ribosomal proteins and RNA polymerase subunits. Chloroplast genomes can contain 40 to 50 percent of non-coding spacer regions, introns and regulatory sequences.

Plastids are attractive candidates for the genetic engineering of plants. Since a typical plant mesophyll cell contains approximately 100 chloroplasts, each with about 100 identical genomes, a single gene is represented perhaps 10,000 times within one cell (Bendich, 1987). The copy number of the genes encoded in the two inverted repeat regions of higher plants can reach up to 20,000 copies, resulting in high levels of transgene expression. Foreign proteins have been shown to accumulate over 70 percent of the total soluble protein (Oey *et al*., 2009; Ruhlman *et al*., 2010). Introduction of gene of interest into the chloroplast genome has several advantages over nuclear transformation (Heifetz, 2000; Maliga, 2004; Ruhlman *et al*., 2010, Verma & Daniell, 2007)

These are:

1. The lack of transgene silencing,
2. The absence of position and pleiotrophic effects

3. High level expression of foreign proteins
4. Multiple transgenes can be stacked by linking them together in operons
5. Transgene containment via maternal inheritance

Plastid transformation is based on transgene integration into the plastid DNA via homologous recombination mediated by a RecA-type recombination system between the plastid targeting sequences of the transformation vector and the targeted region of the plastid genome (Cerutti and Jagendorf, 1993). Therefore, site-specific recombination of a transgene into the chloroplast genome eliminates concerns of position effect or effects due to multiple integration events, which frequently occurs in nuclear transgenic plants (Daniell *et al*., 2002). Chloroplasts can also provide an ideal place to accumulate proteins or their biosynthetic products that may be harmful if they were in the cytoplasm (Bogorad, 2000). For example, the expression of xylanase in chloroplast did not cause any cell wall degradation in contrast to nuclear transformation (Leelavathi *et al*., 2003).

Plastid transformation has been accomplished by using particle bombardment and PEG mediated transformation techniques (Figure 1.7). Additionally, DNA was introduced into organelle and isolated chloroplasts by electroporation and microinjection, respectively. Up to now, there are several transgenes, more than 100, expressed in chloroplasts (Bock and Warzecha, 2010). These are related with important agronomical traits as well as industrially valuable biomaterials and therapeutic proteins.

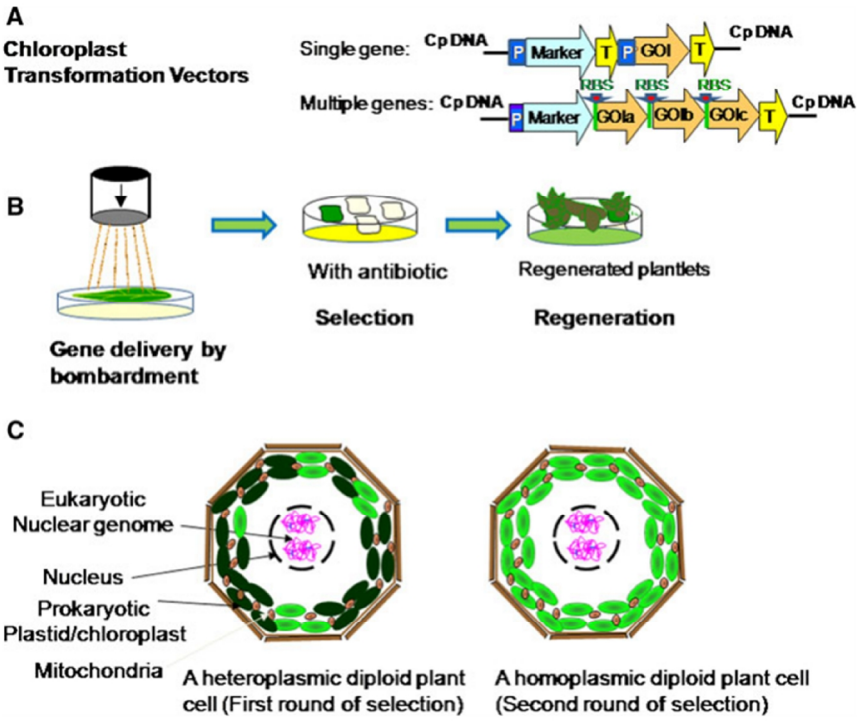

Figure 1. 7.Schematic representation of chloroplast transformation (Taken from Clarke & Daniell, 2011) A. Designing of chloroplast transformation vector B. Transformation event by particle bombardment C.Heteroplasmic diploid cell vs homoplasmic one.

Unfortunately, chloroplast transformation technology is generally applicable for tobacco and a few other dicot crops. Many important crops in the world, such as rice, wheat, maize, sorghum, and barley, are still not responsive to the plastid transformation. There is big hindrance for plastid engineering in cereal crops include the difficulty of expressing transgenes in non-green plastids, where gene expression and regulation systems are highly different from those of chloroplasts.

The well designed chloroplast transformation vector is necessary for enough transgene expression during the chloroplast transformation studies (Daniell *et al*., 2005). Chloroplast transformation vectors consist of two flanking regions containing

1 to 2 kb plastid DNA sequences, with expression cassettes for the selectable marker and for the gene of interest (GOI). These expression cassettes consist of a promoter and 5` and 3` untranslated regulatory regions compatible with plastid gene expression system (Maliga, 2004). Choice of either an endogenous or non-endogenous strong promoters, 5` UTR (untranslated region) and 3` UTR regulatory sequences, species-specific homologous flanking sequences, integration sites for heterologous gene cassette and presence of plastid origin of replication (oriA) have proved important in obtaining increased number of homoplasmic plastid transformants in higher plants (Heifetz, 2000; Verma and Daniell, 2007). Commonly used homologous flanking sequences, promoters, selectable markers, 5` and 3` regulatory sequences and the gene of interest in different crops are shown in Figure 1.8.

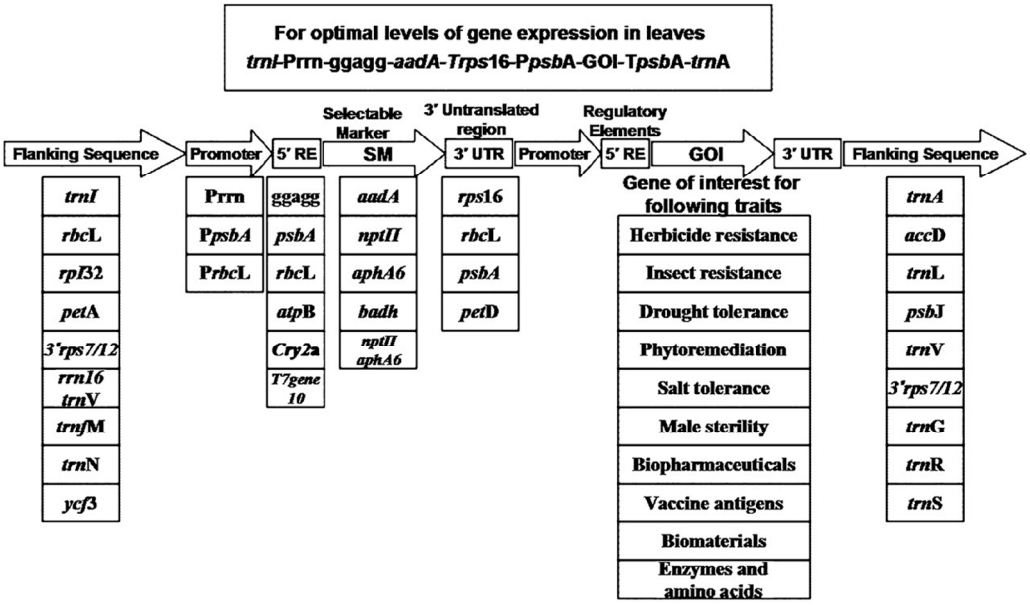

Figure 1. 8.Schematic representation of the chloroplast-specific expression cassette. Map of the chloroplast expression vector shows the integration sites, promoters, selectable marker genes, regulatory elements, and genes of interest (Taken from Verma & Daniell, 2007)

1.4. Aim of the study

The main objective of this study was to develop transgenic wheat and tobacco plants which were resistant to salt stress. Initially four different vectors containing *TaNHX1* and *TaSTR* genes were constructed and then transformed by particle bombardment and *Agrobacterium*-mediated techniques to different explants (immature embryo, immature inflorescence, mature embryo and leaves). Secondly expression and inheritance of the transgene was confirmed by using both molecular, biochemical and physiological analysis.

The other aim of the study was to increase transformation efficiencies, so beyond the particle bombardment and *Agrobacterium*-mediated transformation techniques; chloroplast transformation was also optimized by using tobacco plants.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Plant Material and Plant Tissue Culture Media

In this study, two wheat species *Triticum aestivum (*bread wheat) and *Triticum durum* (durum wheat) and tobacco *Nicotiana tabacum* cv. Petit Havana were used. Immature inflorescences and immature embryos of *Triticum aestivum* cultivar Yüreğir-89 (spring bread wheat) and *Triticum durum* cultivar Kızıltan-91 (winter durum wheat), mature embryo of *Triticum durum* cultivar Mirzabey-2000 (winter durum wheat) were used as an explant for wheat transformation. Leaves of *Nicotiana tabacum* cultivar Petit Havana were used during tobacco transformation experiments. The seeds of Mirzabey-2000 and Kızıltan-91 were obtained from Agricultural Research Institute, Ankara. The seeds of Yüreğir-89 were kindly provided by Çukurova Agricultural Research Institute, Adana. Detailed contents of tissue culture media used for wheat transformation were given in Table 2.1, Table 2.2 and Table 2.3.

Tobacco seeds were germinated on MS medium (MS+ Sucrose (20g/L) and Phytagel (2.8g/L pH: 5.8). Shoot induction from transformed tobacco leaves was achieved by using RMOP medium (4.4 g/l MS, 30xg/l Sucrose, 100mg/l Myoinositol, 2.8 g/l Phytagel, 1mg/BA, 0.1 mg/l NAA, 1mg/l thiamine hydrochloride) supplemented

with selective agent. The resistant shoots were transferred into the MS medium prepared with 1% sucrose without hormones to obtain transgenic tobacco plants.

Table 2.1. Medium compositions for mature embryo based regeneration system

Chemicals	Callus induction medium	Embryogenic callus induction medium	Regeneration medium	Rooting medium
MS (g/l)	4.4	4.4	4.4	2.2
L-Aspartic acid (mg/l)	135	135	135	-
L-Glutamine (mg/l)	150	150	150	-
L-Proline (mg/l)	115	115	115	-
Casein hydrolysate(mg/l)	100	100	100	-
L-Tryptophane (mg/l)	40	40	40	-
Sucrose (g/l)	30	30	30	20
Phytagel (g/l)	2.6	2.6	2.6	2.6
2,4-D (mg/l)	2	2	-	-
Naphtalaacetic acid (mg/l)	-	0.1	-	-
Benzyl adenine (mg/l)	-	0.5	-	-
$AgNO_3$ (mg/l)	-	-	10	-
$CuSO_4$ (mg/l)	-	-	2	-

Table 2.2. Medium compositions for immature inflorescence based regeneration system

Component	Callus Induction medium (/L)	Regeneration medium (/L)	Rooting medium (/L)
MS (g/l)	4.4	2.2	2.2
Sucrose (g/l)	30	30	20
Phytagel (g/l)	2.8	2.8	2.8
2,4-D (mg/l)	2	-	-

Table 2.3. Medium compositions for immature embryo based regeneration system

Component	**Callus Induction medium (/L)**	**Regeneration medium (/L)**	**Rooting medium (/L)**
MS Macro salts (×10)	100 ml	100 ml	100 ml
L7 Micro salts (×1000)	1 ml	1 ml	1 ml
FeNaEDTA (×100)	10 ml	10 ml	10 ml
MS vitamins (×1000)	1 ml	-	-
Vitamins/Inositol (×200)	-	5 ml	5 ml
Inositol	100 mg	100 mg	100 mg
Glutamine	0.5 g	-	-
Casein hydrolysate	100 mg	-	-
MES	1.95 g	-	-
Glucose	-	-	-
Maltose	40 g	30 g	30 g
2,4-D	0.5 mg	0.1 mg	-
Picloram	2.0 mg	-	-
Acetosyringone	-	-	-
Timentin	160 mg	160 mg	160 mg
Zeatin	-	5 mg	-

2.1.2. Bacterial Strains and Plasmids

In transformation studies, *Agrobacterium tumefaciens* strain AGL1 harboring pAL 154-pMAK26 and pAL154-pMAK28 were used. For control experiments, *Agrobacterium tumefaciens* strain AGL1 harboring pAL154 and pAL 156, which contains GUS marker gene was used. The *Agrobacterium* strain including the plasmids (pAL154-pAL156) was donated by Matthew D.Perry and Wendy Harwood from John Innes Centre. The transfer agreement is given in Appendix A. The pPZP201 was kindly provided by Dr. George Liang from Kansas State University. In bombardment studies, pMAK21 and pMAK22 which were harboring mannose and

phosphinothricin selection genes were used, respectively. During the control bombardment experiments of wheat tissues pAHC25 plasmid was used.

2.1.3. Bacterial Growth Media and Culture Conditions

LB medium (Appendix B) was used for the growth of *Escherichia coli* cells during cloning and vector construction studies. MG/L (Appendix B) medium (Tingay *et al*., 1997) was used for the growth of *Agrobacterium tumefaciens* AGL1-pMAK26 and pMAK28 cells.

2.1.4. Chemicals, restriction endonucleases and DNA modifying enzymes

The chemicals used in this study were obtained from Duchefa, Sigma Chemical Company, and Merck Chemical Company, Applichem Chemical Company. All of the solutions were prepared by using distilled water. Restriction endonucleases and DNA modifying enzymes were obtained from Fermentas and New England Biolabs.

2.2. Methods

2.2.1. Generation of salt resistant transgenic wheat plants

In order to develop salt resistant transgenic wheat plants, *Triticum aestivum* sodium hydrogen antiporter (*TaNHX1)* gene which is responsible for the synthesis of sodium/hydrogen antiport protein located on vacuole membrane was cloned from 10 days old Yüreğir-89 seedlings subjected to NaCl (200 mM) for 3 days.

2.2.1.1. Cloning of *TaNHX1* gene from wheat seedlings

TaNHX1 (AY040245) gene was isolated from *Triticum aestivum* cv. Yüreğir-89 leaves by using PCR with gene specific primers including specific restriction sites.

2.2.1.1.1. Primer design

Primers were designed according to *TaNHX1* (AY040245) gene sequence obtained from NCBI database. Forward primer was designed as 5'-CCCGGGCTTATCTTCAGCGAGGATAT-3' which included SmaI restriction site for further cloning. Reverse primer was designed as 5'-CTGCAGATACTGAAGGTTTGTTTGGG-3' which included PstI restriction site for further cloning. For designing primer pairs Gentle and Primer 3 programs were used (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

2.2.1.1.2. Growth of wheat plants

Seeds of Yüreğir-89 were surface sterilized by incubation in 70% ethanol for 30 seconds and sodium hypochlorite for 20 minutes, respectively. Seeds were washed 5 times with sterile distilled water after surface sterilization. Sterilized seeds were transferred to plastic pots covered with cheesecloth as supporting material. Plantlets were grown in ½ strength Hoagland`s solution for 7 days. They were incubated in growth chamber at 25 °C with 16h/8h photoperiod. After this stage, plantlets were subjected to salt stress by using 200mM NaCl solution for 3 days. At the end of stress application period leaf samples were collected and stored in -80 °C freezer for RNA isolation.

2.2.1.1.3. Total RNA isolation

Total RNA was isolated from pre-frozen wheat leaves according to Chomczynski (1993) with slight modification by using TRIzol reagent (Appendix E). All reagents and utensils (mortar and pestle) in this experiment were treated with DEPC (diethyl pyrocarbonate). All the procedure followed during RNA isolation was given Figure 2.1. The quality and concentration of RNA samples were determined by using spectrophotometer. Absorbance of samples in 10 mM Tris-HCl (pH:8.0) were

measured at 260 nm and 280 nm by using Shimadzu UV-Vis Spectrophotometer. The concentrations of samples were calculated by using following formula:
Concentration (µg/µl) = A_{260} X 0.04 X DF, where A_{260} is the absorbance of sample at 260 nm and DF is dilution factor (DF =300, 1 absorbance unit = 0.04 µg/µl RNA). The ratio of OD values at 260 nm and 280 nm (A_{260}/A_{280}) ranged from 1.8 to 2.1, indicating that the purity of RNA was high. In addition to the spectrophotometric analysis, the intactness of RNA samples were checked by running on RNase-free 1% agarose gels prepared with 1X TAE solution at 95V for 40 minutes.

DNase treatment was performed with Fermentas DNase I, in order to clean samples from DNA contamination. 5µg of RNA sample, 1µL of 10X reaction buffer with $MgCl_2$, 1µL of Deoxyribonuclease I (1u/µL) was added into an RNase-free eppendorf tube and finally DEPC-treated water was added to make the final volume of the mixture 10µL. Then, the tube was incubated at 37 °C for 30 minutes. After incubation, 1µL of 25mM EDTA was added into the tube and finally, the tube was incubated at 65 °C for 10 minutes.

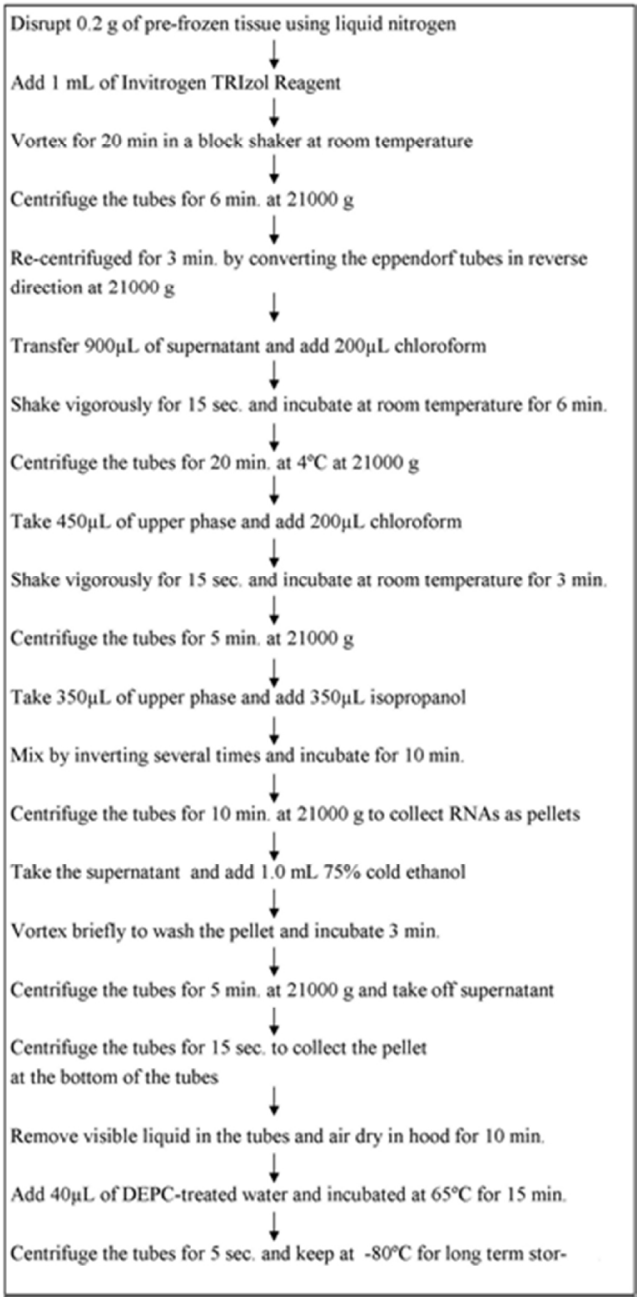

Figure 2. 1. Total RNA isolation method modified from Chomczynski (1993).

2.2.1.1.4. cDNA synthesis

First strand cDNA synthesis was performed on DNase treated 2 µg of total RNA using MMLV reverse transcriptase (Fermentas) with an 1 µl oligo$(dT)_{18}$ primers (0.5 µg/µL) following the standard protocol (Fermentas). After addition of total RNA and primers, total volume was completed to 12µL with DEPC-treated water. All the components were mixed gently and centrifuged for 3-5 seconds in microcentrifuge (Micromax RF, ThermoIEC, USA). The mixture was incubated at 70 °C for 5 minutes and drops were collected with brief centrifugation. The reaction tube was placed onto ice and the following components were added in indicated order: 4 µL of 5X reaction buffer, 1 µL RiboLock™ Ribonuclease Inhibitor (20U/ µL) and 2 µL of 10mM dNTP mix. Then, they were mixed gently, centrifuged for 3-4 seconds and incubated at 37 °C for 5 minutes. After the incubation, 1 µL of RevertAid™ M-MuLV Reverse Transcriptase (200U/ µL) was added and the tubes were incubated at 42 °C for 60 minutes. In order to stop the reaction, the tubes were incubated at 70 °C for 10 minutes and chilled on ice.

2.2.1.1.5. PCR Amplification

Amplification of *TaNHX1* gene was carried out by using *Pfu* DNA Polymerase (Fermentas) and gene specific primers. For this purpose, *TaNHX1* gene specific forward primer including SmaI restriction site designed as 5'-CCCGGGGACCGGCATGGGGCTCGATTTG -3' and *TaNHX1* gene specific reverse primer including PstI restriction site designed as 5'-CTGCAGGCATTTACAAGAAATACACTT -3' by using Primer 3 and Gentle software. In order to optimize PCR conditions, gradient PCR was performed by using different annealing temperature, such as 53.1 °C, 55 °C, 56.9 °C, 58.8 °C, 60.7 °C, 62.6 °C, 64.5 °C. Optimized PCR conditions used for *TaNHX1* amplification are given in Appendix F.

2.2.1.1.6. Purification of the DNA fragments from the gel

PCR products were eluted from agarose gels with GeneMark Gel Elution Kit. PCR band was excised from 1% an agarose gel which was run at 90V for 35 minutes. The gel piece was transferred into eppendorf tube and weight. 1:1 volume of Binding Buffer to the gel slice (volume:weight) was added into the tube and incubated at 65 °C until the gel slice is completely dissolved. Dissolved gel solution was transferred to purification column. Centrifugation was carried out at 13.000xg by using microcentrifuge (Micromax RF, Thermo IEC, USA) for 1 minute and the flow-through was discarded. After this step, 500 µL of binding solution was added into the same column and centrifugation was carried out at 13.000xg for 1 minute. 700 µL of washing solution was added into the column and centrifuged at 13.000xg for 1 minute. Washing step was repeated one more time. The filtrate was discarded and the tube was centrifuged for 5 minutes at 13.000xg. Dried spin column was transferred into a new eppendorf tube and 25 µL of elution solution was applied into the column and incubated at room temperature for 10 minutes. After incubation, PCR product was eluted from column with centrifugation at 13.000xg for 2 minute and stored at -20 °C for further use.

2.2.1.1.7. Subcloning of the DNA fragments into TA cloning vector

PCR product obtained from gel elution was cloned into pJET1.2 vector (Figure 2.2) by using CloneJET™ PCR Cloning Kit (Fermentas) following the manufacturer procedure. Components and volume of the ligation reaction are given in Appendix G. Concentration of gel eluted PCR product was determined by using AlphaSpect µL Spectrophotometer (Alpha Innotech Inc., USA) and 3:1 molar ratio with pJET1.2/blunt was used for ligation. All the reaction components were vortexed briefly and centrifuged for 3-5 seconds. Ligation mixture was incubated at room temperature for 5 minutes. The ligation product was used for transformation into competent *E. coli* cells.

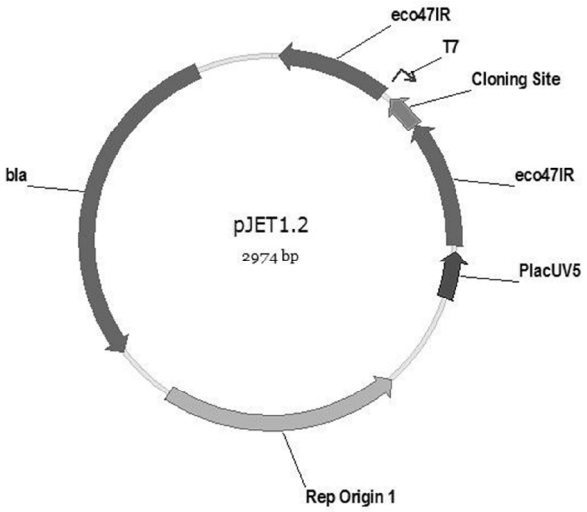

Figure 2. 2. Schematic representation of pJET1.2 vector

2.2.1.1.8. Preparation and transformation of chemically competent *E. coli* cell

Chemically competent *Escherichia coli* cells were prepared by using rubidium chloride. First day, *Escherichia coli* strain Top10 cells were streaked on LB plate and were grown in incubator at 37 °C. Second day, one colony was inoculated into 2.5 ml liquid LB medium and the bacterial culture was grown in shaking incubator at 37 °C by continuous shaking at 200 rpm. Third day, the bacterial culture was transferred into 250ml fresh liquid LB medium. When the bacterial culture reached to A_{600}=0.6-0.7, flask was incubated on ice for 15 minutes. After this step, all the procedure was carried out on ice. The culture was centrifuged at 4000xg for 5 minutes with Sigma centrifuge. After centrifugation, supernatant was discarded and pellet was dissolved in 0.4 volume of transformation buffer 1 (Appendix H). The bacterial culture was incubated on ice for 15 minutes. After incubation cells were

spinned at 4000xg for 5 minutes and supernatant was discarded. The pellet was re-suspended in 0.04 volume of Tfb2 (Appendix H). The cells were immediately transferred into eppendorf tube stored in dry ice and stored in -80 ℃ for further use.

pJET1.2 vector carrying *TaNHX1* gene was transformed into chemically competent *E. coli* cells. For this purpose, 50 µl of competent cells were allowed to thaw. 5µl plasmid DNA was added and incubated on ice for 30 minutes. Samples were subjected to 42 ℃ for 1 minute and they were immediately transferred onto ice and leaved for 3 minutes. After heatshock 950 µl SOC medium (Appendix B) was added onto bacterial culture. The samples were incubated at 37℃ for 50-60 minutes with shaking at 200 rpm. 100 µl of sample was spread onto preheated LB plate with appropriate antibiotics and were grown at 37 ℃ for overnight.

2.2.1.1.9. Colony PCR and sequencing

In order to confirm the insertion of plasmids into chemically competent *E. coli* cells, colony PCR was carried out with gene specific primers by using a single colony grown on LB plate prepared with Ampicillin (50 mg/ml) antibiotic. Forward and reverse primers were designed as 5'-CGGCCGCGAGCTCTTAACTA-3' and 5'-CATATGGTGGGCAGGTCTCA-3', respectively. After adding all reaction components of PCR into tubes, template DNA was added by picking a colony by the help of pipette tips. Colony PCR conditions and cycling parameters were given in Appendix I. PCR products were loaded on 1% Agarose gel in order to evaluate the insertion of recombinant plasmid into the competent cell. After confirmation of insertion by using agarose gel electrophoreses, PCR positive 5 colonies were selected for sequencing.

2.2.1.1.10. Plasmid isolation

Five colonies produced expected PCR product after colony PCR were grown in liquid LB medium supplemented with ampicillin for plasmid isolation. Plasmid

isolation was performed with Mini Prep Kit (GenMark) by following the standard protocol. Overnight grown bacterial cells were harvested by centrifugation at 15000xg for 2 minutes in microcentrifuge. The supernatant was poured off and pellet was completely resuspended in 200 µl Sol I with vigorous vortexing. After the addition of 200 µl Sol II, the suspension was mixed by inverting 5-6 times. After 5 minutes incubation at room temperature, 200 µl of Solution III was added into the suspension and inverted 5-6 times. The lysate was centrifuged at 15000xg in a microcentrifuge for 5 minutes. The cleared lysate was applied directly to spin column and spinned down for 1 minute. The filtrate in the collection tube was discarded and 700 µl of washing solution was added and spinned for 1 minute. This step was repeated for one more time. In order to remove residual ethanol, centrifugation was performed for 3 minutes at 15000xg. For elution of plasmid DNA, 50 µl of elution solution was applied onto column and was incubated at room temperature for 5 minutes. After incubation, plasmid DNA was eluted by centrifugation at 15000xg for 2 minutes. These plasmids were sent to sequence analysis.

Sequence analyses were done by RefGen Company with ABI 310 Capillary DNA Sequencer by using this eluted PCR product. During sequencing forward sequencing primer (5'-CGACTCACTATAGGGAGAGCGGC-3') and reverse sequencing primer (5'-AAGAACATCGATTTTCCATGGCAG-3') were used. Sequence analysis was done for each sequence in NCBI by blastn search.

2.2.1.2. Vector construction for wheat transformation

2.2.1.2.1. Particle bombardment vectors

All the optimization studies during particle bombardment experiments were performed by using pAHC25 plasmid carrying GUS and BAR marker genes (Appendix J). However, this plasmid was not suitable for subcloning of other genes. So, new plasmids were designed and constructed for particle bombardment of

TaNHX1 gene. The construction of expression vector for particle bombardment was started with PCR cloning of Ubi1 promoter and its intron from pAHC25 plasmid. PCR amplification with Ubi1 specific primers, including NarI and SacI restriction sites, was carried out by using *PfuTurbo® DNA Polymerase* (Stratagene). Forward primer was designed as 5'-GGCGCCGCAGTGCAGCGTGACCCGGTC-3' which is included NarI restriction site for subcloning into PUC19 vector. Likewise, reverse primer was designed as 5'-GAGCTCGTAACACCAAACAACAGGGT-3' which is included SacI restriction site for subcloning into PUC19 vector (Figure 2.3). PCR conditions and cycling parameters were given in Appendix K.

Expected PCR products were eluted from 1% agarose gels with Qiagen Gel Elution Kit by following the standard procedure. PCR band was removed from 1% agarose gel which was run at 90V for 35 minutes. PCR product obtained from gel elution was cloned into pCR4Blunt-Topo vector (Invitrogen-Figure 2.3) following the manufacturer procedure. Resulting vector was transferred to chemically competent *E. coli* cells. In order to confirm the insertion of plasmid into *E. coli* cells, colony PCR was carried out with gene specific primers by using a single colony grown on LB plate prepared with Carbenicillin (100 mg/ml) antibiotic. Forward and reverse primers were designed as 5'-GGCGCCGCAGTGCAGCGTGACCCGGTC-3' and 5'-GAGCTCGTAACACCAAACAACAGGGT-3', respectively. PCR products were loaded on 1% Agarose gel and PCR positive 5 colonies were selected for restriction digestion and sequence analysis.

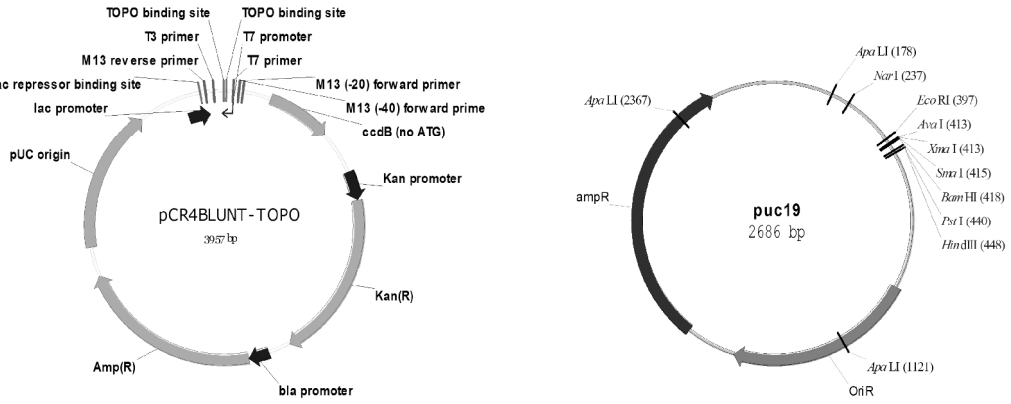

Figure 2. 3. Schematic representation of pCRBlunt-Topo and pUC19 vector

Both pUC19 vector and pCR4Blunt-Topo-Ubi1 vector were digested with NarI and SacI (New England Biolabs). For digestion 5µg plasmid DNAs were used and incubated at 37 °C for 16 hours. After digestion, both plasmids dephosphorylated by using Calf Intestinal Alkaline Phosphatase and the products were loaded to 1% agarose gel. For elution of digested plasmids, desired bands were excised from 1% agarose gels and plasmid DNAs were eluted by using gel elution kit (Qiagen) following the standard procedure.

In order to create an intermediate vector carrying ubi1 promoter and intron, fragments coming from gel elution were ligated by using Quick Ligation Kit (Qiagen). For ligation, 3:1 molar ratio between small fragment (Ubi1+intron) and pUC19 fragment was used. Ligation products were transferred into chemically competent *E. coli* cell with standard protocol. Colony PCR was carried out with colonies grown on LB plates supplemented with 100mg/l Carbenicillin antibiotic by using standard protocol. PCR positive colonies were selected for further cloning.

Same cloning strategy was applied during the cloning of NOS terminator. NOS terminator was produced with PCR by using pAHC25 as a template. Forward primer (AAGCTTTCCCGATCTAGTAACATAGA) with PstI restriction site and reverse primer (AAGCTTTCCCGATCTAGTAACATAGA) with HindIII restriction site were used for amplification of NOS terminator. After all cloning step, an intermediate vector carrying Ubi1 promoter and NOS terminator was produced.

After producing second intermediate vector, *TaNHX1* gene was inserted into this vector. For this purpose, *TaNHX1* gene was produced from pJET1.2-*TaNHX1* vector by using PCR with *PfuTurbo® DNA Polymerase* (Stratagene). Forward primer (GGTACCTCTGCGGCTGCATTGTCTTC) with KpnI restriction site and reverse primer (CTGCAGAAGATGTTGATGGCGACGAT) with PstI restriction site were used for PCR amplification of *TaNHX1* gene. After cloning steps mentioned above pUC19-Ubi1-Nhx1-NOS vector was constructed.

During particle bombardment studies, two types of vectors were used: one of them carrying mannose selection cassette, the other carrying bar selection cassette. In order to clone mannose selection gene (pmi), pPZP201 plasmid (Figure 2.4) was used as a source. pPZP201 and pUC19-Ubi1-Nhx1-NOS was digested with HindIII restriction enzyme. For digestion 5µg plasmid DNAs were used and incubated at 37 °C for 16 hours. After digestion, both plasmids dephosphorylated by using Calf Intestinal Alkaline Phosphatase and the products were loaded to 1% agarose gel. For elution of digested plasmids, desired bands were excised from 1% agarose gels and plasmid DNAs were eluted by using gel elution kit (Qiagen) by following standard procedure mentioned in previous part. Fragments were ligated to each other by using Quick Ligation Kit (Qiagen) by following standard protocol. At the end of all cloning steps, plant expression vector carrying both pmi and *TaNHX1* genes were produced and called as pMAK21 (Figure 2.5).

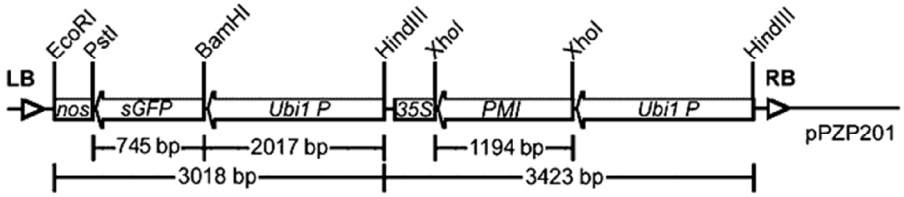

Figure 2. 4. Schematic representation of pPZP201 vector

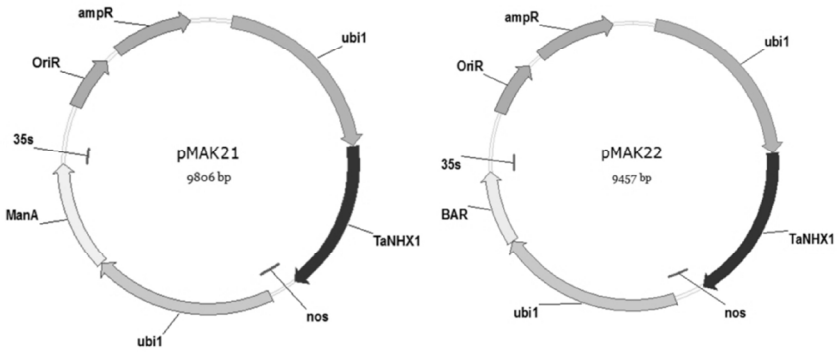

Figure 2. 5. Schematic representation of pMAK21 and pMAK22

The other vector was created by adding bar cassette from pMK2b vector, which was previously constructed (Figure 2.7). This cassette consists of Ubi1 promoter + intron and bar gene and NOS terminator. For digestion 5µg plasmid DNAs were used and incubated at 37 °C for 16 hours. After digestion, this fragment was dephosphorylated by using Calf Intestinal Alkaline Phosphatase and the products were loaded to 1% agarose gel. For elution of digested plasmid, desired band were cut from 1% agarose gels and plasmid DNAs were eluted by using gel elution kit (Qiagen) by following standard procedure mentioned above. After elution, the fragment was filled with DNA Polymerase I Large (Klenow) Fragment (New England Biolabs) for blunting. Blunted fragment were ligated with previously blunted pUC19-Ubi1-Nhx1-NOS fragment by using Quick Ligation Kit (Qiagen) by following the standard protocol. At the end of all cloning steps, plant expression vector carrying both bar and *TaNHX1* genes were produced and named as pMAK22 (Figure 2.5). All the steps belong to vector construction was given in Figure 2.6.

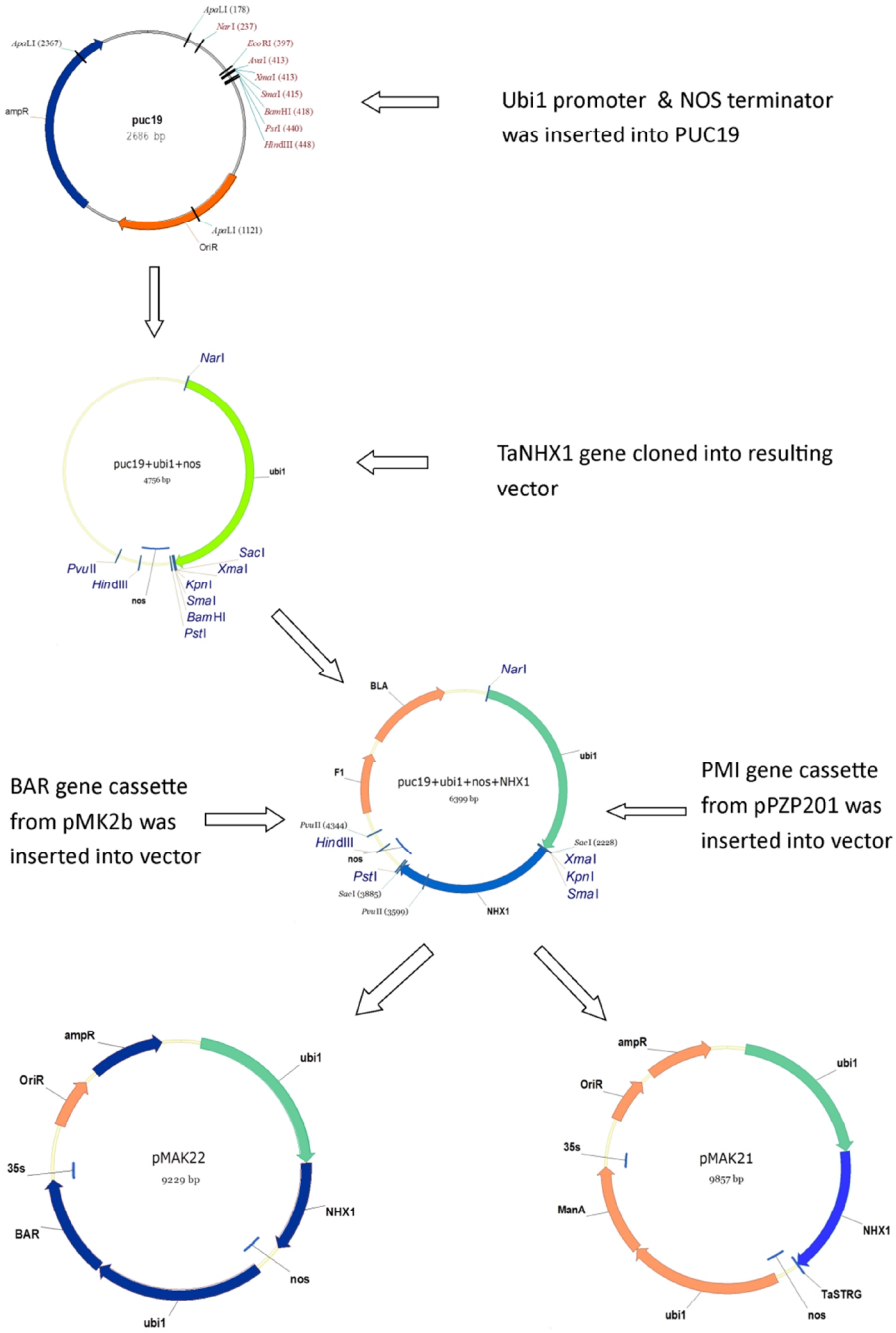

Figure 2. 6. Schematic representation of vector construction for particle bombardment of wheat tissues.

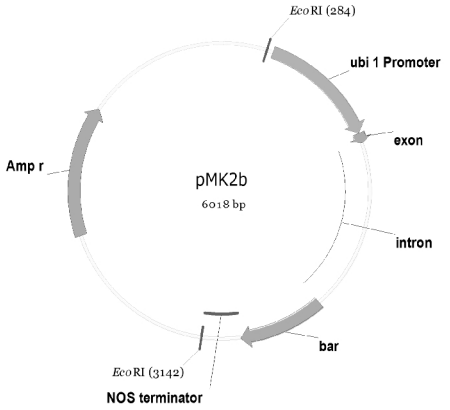

Figure 2. 7. Schematic representation of pMK2b vector

2.2.1.2.2. Preparation of *Agrobacterium*-mediated transformation vectors for wheat transformation

During *Agrobacterium*-mediated transformation wheat tissues, plasmids produced by Gateway Cloning Technology were used. Strategy for Gateway Cloning was given in Figure 2.8.

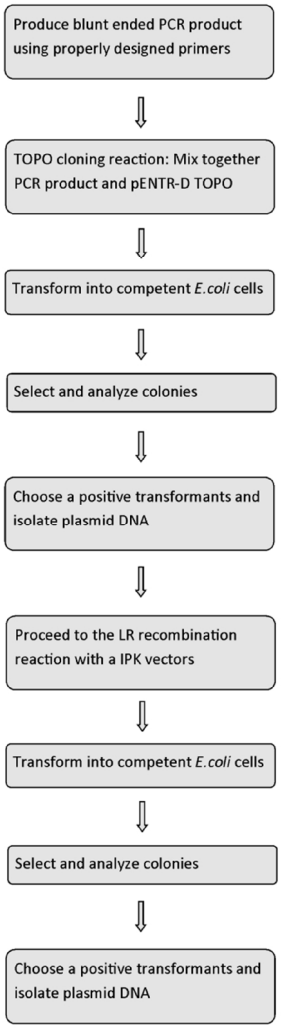

Figure 2. 8. Flowchart for the generation of plant expression vector with Gateway cloning

2.2.1.2.2.1. Amplification of *TaNHX1* Gene

First step of the Gateway cloning technology is the amplification of gene of interest by using PCR. Therefore, in order to clone *TaNHX1* gene into Gateway compatible entry vector, new primers were designed. Forward primer was designed as 5'-

CACCATGGGGCTCGATTTGGGAGCCC-3' including CACC site at upstream region and reverse primer was designed as 5'-TCGACGCGGCCGCGAGCTCT-3'. *TaNHX1* gene was amplified from pJet1.2/TaNHX1 vector with PCR by using Platinum®PfxDNA Polymerase from Invitrogen. PCR conditions and cycling parameters were given in Appendix L.

PCR products were eluted from agarose gels with GeneMark Gel Elution Kit by following the manufacturer protocol. PCR band was removed from 1% agarose gel which was run at 90V for 35 minutes. The gel piece was transferred into eppendorf tube and weight. 1:1 volume of Binding Buffer to the gel slice (volume: weight) was added into the tube and incubated at 65 ºC until the gel slice is completely dissolved. Dissolved gel solution was transferred to purification column. Centrifugation was carried out at 13.000xg for 1 minute and the flow-through was discarded. After this step, 500µL of binding solution was added into the same column and centrifugation was carried out at 13.000xg for 1 minute. 700 µL of washing solution was added into the column and centrifuged at 13.000xg for 1 minute. Washing step was repeated one more time. The filtrate was discarded and the tube was centrifuged for 5 minutes at 13.000xg. Dried spin column was transferred into a new eppendorf tube and 25 µL of elution solution was applied into the column and incubated at room temperature for 10 minutes. After incubation, PCR product was eluted from column with centrifugation at 13.000xg for 2 minute and directly used for ligation reaction with pENTRY-D TOPO vector.

2.2.1.2.3. Cloning of *TaNHX1* Gene into Entry vector

Before cloning of PCR product, concentrations of samples were measured with Nanodrop UV Spectrophotometer (Thermo, US). pENTR™/D-TOPO (Figure 2.9) Cloning Kit (Invitrogen) were used for the insertion of *TaNHX1* gene into Gateway entry clone by using PCR product by following the standard protocol (Figure 2.8). In

cloning reaction 2:1 molar ratio of PCR product:TOPO vector was used. Four microliter of PCR product, one microliter salt solution and one microliter TOPO vector were mixed and incubated at 22 °C for 10 minutes. Ligation products were directly used for competent *E. coli* transformation. In order to confirm the insertion of plasmid into *E. coli* cells, colony PCR was carried out with gene specific primers by using a single colony grown on LB plate prepared with Kanamycine (100mg/ml) antibiotic. Forward and reverse primers for *TaNHX1* were designed as 5'-CGGCCGCGAGCTCTTAACTA-3' and 5'-CATATGGTGGGCAGGTCTCA-3', respectively. Likewise, forward and reverse primers for *TaSTRG* were designed as 5'-CTCCTCTCCTACGCTGCCAT-3' and 5'-AATCGTCTGCCCCACGTCCT-3', respectively. PCR products were loaded on 1% Agarose gel and PCR positive 2 colonies were selected for LR reaction.

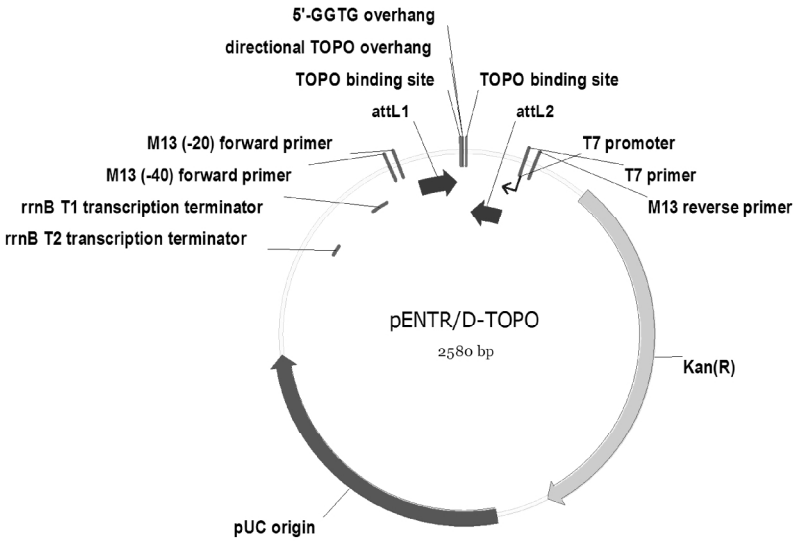

Figure 2. 9. Schematic map of pENTR/D-TOPO

2.2.1.2.4. Cloning of *TaNHX1* Gene into IPKb vectors

Insertion of *TaNHX1* gene into plant expression vector (pIPKb002) was achieved by using Gateway® LR Clonase® enzyme mix by following manufacturer protocol (Invitrogen). IPKb002 vector including ubi1 promoter to control gene of interest and *HptII* antibiotic selection gene was obtained from The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) was used to generate *Agrobacterium*-mediated plant transformation vector (Figure 2.10).

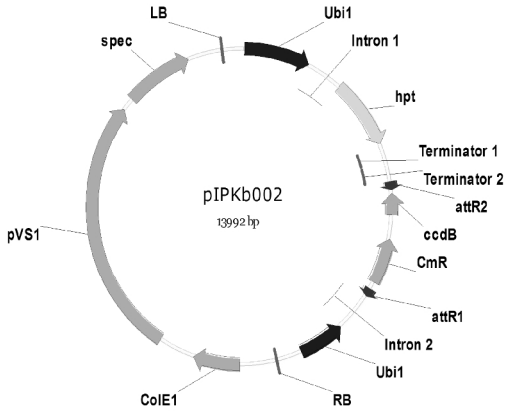

Figure 2. 10. Schematic representation of pIPKb002 vector

After LR reaction, reaction mixture was transferred to chemically competent *E. coli* cells by using previously used standard transformation protocol mentioned in part 2.2.1.1.8. So, pIPKb002-*TaNHX1* was generated and it was named as pMAK26. Although there was a ccdB gene in IPKb vectors to prevent self-ligation, there was a probability to see colonies not containing *TaNHX1*. To select right colonies, colony PCR was carried out by using *NOS* terminator gene and *TaNHX1* gene specific primers. NOS terminator was used to design reverse primer (5'-

TTATCCTAGTTTGCGCGCTA-3') and *TaNHX1* gene specific forward primer (5'-GCTTGTCCTGGGCCTCATCA-3'). Standard colony PCR protocols mentioned in part 2.2.1.1.9 were used with annealing temperature as 55 °C. PCR positive two colonies were selected for plasmid isolation.

2.2.1.2.5. Transformation of IPK vector into *Agrobacterium tumefaciens* strain AGL1

During *Agrobacterium*-mediated transformation, semi empty AGL1 strain was used. This strain was called semi-empty because only helper plasmid (pAL154) was remained after discarding procedure. However, normal *Agrobacterium tumefaciens* AGL1 strain contains both pAL154 and pAL156 at the same time in one cell. In order to insert pMAK26 and pMAK28 into *Agrobacterium* cells, competent *Agrobacterium tumefaciens* AGL1 previously made semi-empty was used to prepare electrocompetent cells.

The procedure was adapted from Mersereau *et al*.,(1990) and Shen and Forde (1989). To make electrocompetent agro cells, semi-empty AGL1 strain was grown in 5 ml liquid LB medium. After inoculation into 500ml of LB medium, they were incubated at 28 °C with vigorous shaking. When the cells were reached to log phase (OD_{550} 0.5-0.8), the culture was chilled by gently swirling in an ice-water bath and the cells were kept at 4 ºC for all further steps. The culture was centrifuged at 4000xg for 10 minutes at 4 ºC in a precold rotor (Sigma 3K). Supernatant was discarded and 10 ml of ice-cold H_2O was added. The cells were gently mixed by using a wide-bore pipette and volume was adjusted to 500 ml with ice-cold H_2O. This step was repeated two times. After the first repeat, the cells were resuspended in a final volume of 250 ml of ice-cold H_2O. After the second repeat, the cells were resuspended in a final volume of 50 ml of ice-cold H_2O. The cells were centrifuged at 4000xg for 10 minutes at 4 ºC in a precold rotor and they were resuspended in 5 ml of 10% (v/v) ice-cold, sterile glycerol. Electrocompetent cells were dispensed as 50 µl aliquots into eppendorf tubes and freezed in dry ice and stored at -80 °C.

2.2.1.3. Transformation of wheat tissues

Wheat transformations were performed with particle bombardment and *Agrobacterium*-mediated transformation by using mature embryo, immature embryo and inflorescence tissues. Regeneration pathway followed after transformation is summarized in Figure 2.12. Same regeneration scheme was applied wheat explants transformed by both *Agrobacterium*-mediated and particle bombardment-mediated.

2.2.1.3.1. Particle bombardment of wheat tissues

Particle bombardment of wheat tissues were carried out by using Obitek Bioloab Gene Transfer system with different tissues, such as mature embryo from *Triticum durum* cv. Mirzabey-2000, immature embryo from *Triticum durum* Kızıltan-91 and *Triticum aestivum* cv. Yüreğir-89, and immature inflorescences from *Triticum durum* Kızıltan-91 and *Triticum aestivum* cv. Yüreğir-89 were used (Figure 2.11).

2.2.1.3.1.1. Preparation of plant samples

2.2.1.3.1.1.1. Mature embryo

Intact mature embryo and mature embryo derived 15 days old calli (*Triticum durum* cv. Mirzabey-2000) were used for transformation studies. Mature embryos were aseptically removed from imbibed seeds under stereomicroscope. Seeds were imbibed in distilled sterile water at 4 °C for 16 hours. Before starting to imbibition, seeds were surface sterilized by using 70% ethanol for one minute and 20% NaOCl for 20 minutes, respectively. After surface sterilization seeds were washed 5 times with sterile distilled water (Battal, 2010).

2.2.1.3.1.1.2. Immature embryo

Immature embryos were isolated from wheat plants (*Triticum aestivum*, cultivar Yüreğir-89 and *Triticum durum*, cultivar Kızıltan-91), grown in the greenhouse

under 16h/8h photoperiod. To isolate immature embryos, 14 to 20 days post-anthesis caryopses were surface-sterilized with 20% NaOCl for 20 minutes followed by five times of sterile water. The immature embryos (0.5- 1.5 mm in length) were aseptically removed under a stereomicroscope and placed onto callus induction medium. Forty immature embryos were cultured with the scutellum side up onto the callus induction medium. In addition to intact immature embryos, 10 days old calli were also used for particle bombardment studies.

2.2.1.3.1.1.3. Immature inflorescence

In order to obtain immature inflorescence in the desired stage, tillers containing immature inflorescence were collected prior to the emergence of flag leaf and this period correspond to 50-60 days for Kızıltan-91 and 25-30 days for Yuregir-89 (Kavas *et al*., 2008). Tillers were harvested when inflorescence were generally between 0.5-1.0 cm in length. Surface sterilization was performed by sequentially washing the tillers in 70 % ethanol for 30 seconds and then 20 % sodium hypochlorite for 20 minutes, followed by five rinses in sterile distilled water. All procedure that followed by sodium hypochlorite treatment were conducted under aseptic conditions.

The immature inflorescences were isolated from these tillers under stereomicroscope. Isolated inflorescences were cut into approximately 1mm sections. These pieces were plated on callus induction medium. About 20 pieces were put onto each plate. After sealing, they were incubated in the tissue culture room at 25 °C under dark conditions. Intact inflorescence and 4 weeks old calli were used for transformation.

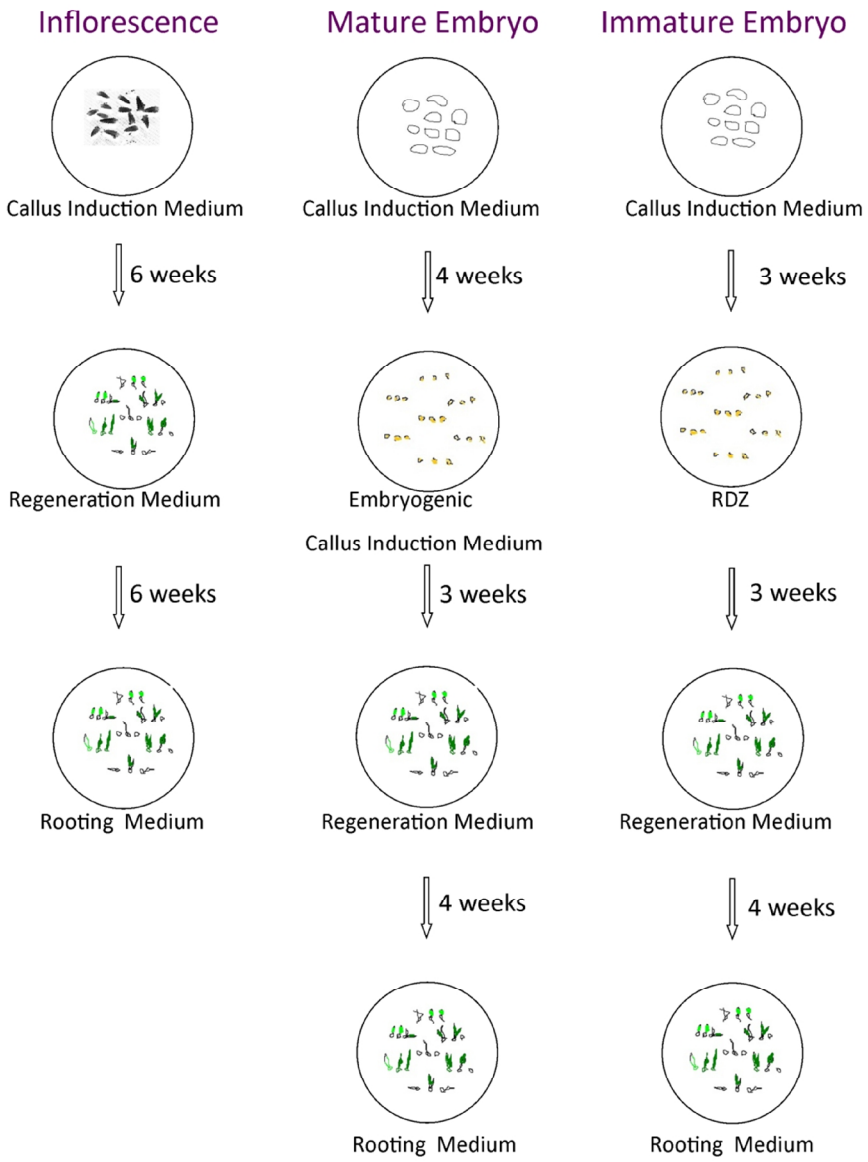

Figure 2. 11. Regeneration pathway of wheat explants

2.2.1.3.1.2. Microprojectile preparation and bombardment

For microprojectile preparation, 5 mg of gold particles was weighed out (1 µm) into a 1.5-mL centrifuge tube and 500 µl of 70% ethanol was added and vortexed for 2-5 minutes. The tube was incubated for 15 minutes at room temperature and a content of the tube was mixed in every 5 minutes. Microparticles were spinned down by using table top microcentrifuge for 5 seconds. Supernatant was discarded and pellet was washed 3 times with 500 µl sterile distilled water. In every washing step, the tube was vortexed for 1 minute and was waited for 1 minute to settle down the microparticles. After last washing, 50 µl of sterile 50% Glycerol was added into tube. Then, 5 µl DNA (1 µg/µL), 20 µl 0.1M spermidine and 50 µl 2.5M $CaCl_2$ were added consecutively and additional three minutes vortexing was done. The tubes were incubated at room temperature for five minutes. Afterwards, the mixture was centrifuged briefly to settle down the gold particles. After discarding the supernatant, 150 µl of 70 % ethanol was added without disturbing the pellet. After spin down, supernatant was discarded and washing step was repeated with absolute ethanol. Finally, gold-DNA mixture was resuspended in 48 µl of 70 % ethanol and 8 µl of mixture was loaded onto microcarrier for bombarding of wheat tissues.

For the optimization of bombardment parameters explants were bombarded with pAHC25 vector including GUS scorable marker gene. While doing optimization different helium pressure, distance between target tissue and microcarrier holder and different particles were tested. Efficiency of bombardment was evaluated by counting blue foci after histochemical GUS assay.

Wheat explants were bombarded by using previously optimized parameters with Obitek Bioloab Gene Transfer System (Figure 2.12). Generally 20-25 explants located center of the plates were bombarded. Samples were incubated on high osmoticum medium, which was produced by addition of 0.4 M of Mannitol into the callus induction medium, 4 hours before bombardment and 16 hours after

bombardment. After high osmoticum period, samples were incubated their callus induction medium in dark condition at 25 °C.

Figure 2. 12. Obitek Bioloab Gene Transfer System

2.2.1.3.1.3. Selection and screening of transformed tissues

Transformed tissues were incubated on high osmoticum medium containing 0.4 M mannitol for 16 hours following the bombardment. After this treatment, 10-15 explants transferred to petri plates containing callus induction medium. In this manner, mature embryos were incubated on callus induction medium for 4 weeks and transferred to embryogenic callus induction medium. About 3-4 weeks incubation on embryogenic callus induction medium, calli were placed onto regeneration medium and selection was started after this stage. Two types of selection agent were used. Mannose (10g/L) and phosphinothricin (4mg/L) were used for transformation of pMAK21 and pMAK22, respectively. Growing shoots were transferred to rooting medium supplemented with selection agent for further selection and rooting. Same selection scheme were applied to immature embryo and immature inflorescence derived calli. Transformed immature embryos were placed

onto callus induction medium and calli were maintained in dark at 25±2 °C for 3 weeks. Then, embryogenic calli were transferred to germination medium (RDZ) in the light. After 3 weeks on germination medium, embryogenic calli were transferred to regeneration medium supplemented with selection agents. The calli derived from immature inflorescence were maintained on callus induction medium for 6 weeks in dark. After this period they were transferred to regeneration medium supplemented with selection agents. Emerging shoots were transferred to rooting medium under same selection pressure. All incubations during transformations were carried out in tissue culture room at 25 ± 1 ºC, 8h/16h photoperiod and PAR of 350 µmol $m^{-2}s^{-1}$.

2.2.1.3.1.4. PCR analysis of putative transgenics

Transgene insertion was evaluated by using PCR analysis with *TaNHX1* gene specific primers. For this purpose genomic DNA was isolated from plant survived in selective medium.

Genomic DNA isolation was performed according to the Saghai-Maroof *et al*., (1984) by using CTAB buffer and the protocols followed during genomic DNA isolation was summarized in Figure 2.13. The purity and concentration of DNA was evaluated by using Nanodrop UV Spectrophotometer (Thermo, USA) and with agarose gel electrophoresis. The isolated DNA was stored at –20 °C for further usage.

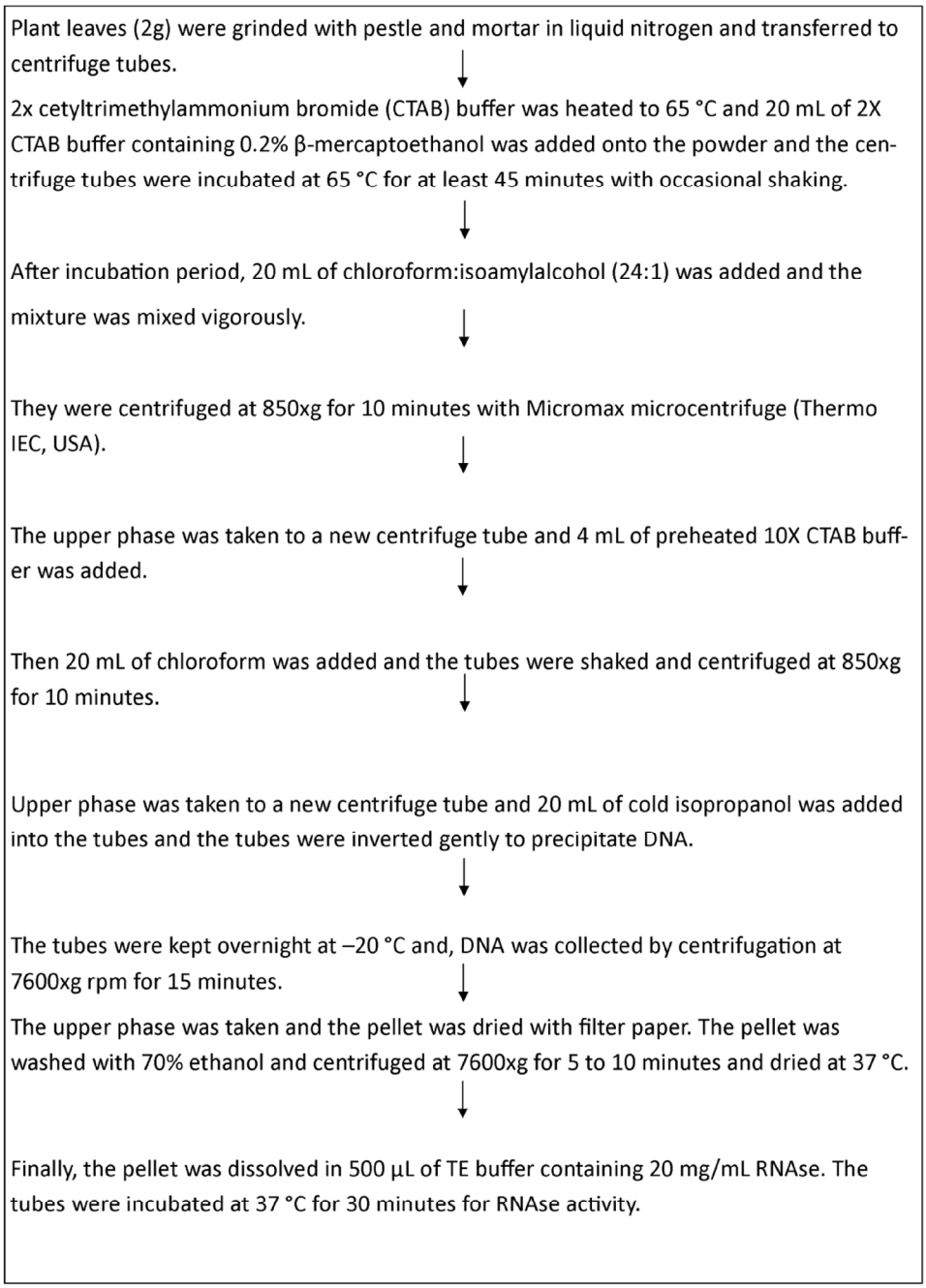

Figure 2. 13. Genomic DNA isolation protocol

2.2.1.3.2. *Agrobacterium*-mediated transformation wheat tissues

Agrobacterium-mediated wheat transformations were carried out by using *Agrobacterium tumefaciens* strain AGL1 harboring pMAK26 plasmid.

2.2.1.3.2.1. Growth of *Agrobacterium tumefaciens* AGL1 and transformation of wheat tissues

Agrobacterium tumefaciens strain AGL1-pMAK26 was grown in MG/L (Tingay *et al*.,, 1997) medium supplemented with carbenicillin (100 mg/L) and spectinomycin (100 mg/L) in shaking incubator (Gallenkamp) at 250 rpm until the cells reached to OD_{600}:1.0-1.2.

Agrobacterium-mediated wheat transformation protocol was adapted from Jones *et al*., (2005) with slight modification. When the cells reached to desired phase (OD_{600}:1.0-1.2), they were spinned down with centrifuge at 4000xg for 15 minutes. Supernatant was removed and the cells were resuspended in half volume of inoculation medium and 0.02% Silwet-77 was added to the suspension. The cell suspension was incubated at 28 °C for 1 hour with continuous shaking at 180 rpm. After incubation, wheat tissues (intact embryos, intact inflorescences and calli) were inoculated with *Agrobacterium tumefaciens* AGL1-pMAK26. Inoculation was carried out on petri plate for 1-2 hours in dark condition by pouring the bacteria over the explants. During transformation, adequate bacterial culture was used to cover completely all the explants. Then, excess bacteria were poured and wheat tissues were transferred to dark incubation box for 3 days co-cultivation period. Three different types of explants such as immature embryo, mature embryo and immature inflorescences were used during *Agrobacterium*-mediated transformation.

2.2.1.3.2.2. Selection and screening of putative transgenic plants

After co-cultivation period, wheat tissues transformed with *Agrobacterium tumefaciens* AGL1 strain carrying *TaNHX1* gene were washed with liquid MS medium supplemented with 160 mg/L Timentin for 1 hour. After washing step, both particle bombarded and *Agrobacterium*-mediated transformed wheat tissues followed same regeneration and selection scheme summarized in Figure 2.11. Two different selection schemes were followed during *Agrobacterium*-mediated wheat transformation. In first selection scheme, 25 mg/L hygromycin was applied as selection agent starting with regeneration period. In second selection scheme, selection period was started with 25 mg/L hygromycin when enough root formation was observed on rooting medium.

2.2.2. Generation of salt resistant transgenic tobacco plants by using *Agrobacterium*-mediated transformation

Salt resistant transgenic tobacco plants were generated by using *Agrobacterium tumefaciens* AGL1 strain harboring pMAK28 carrying *Triticum aestivum* salt tolerant related gene.

2.2.2.1. Custom synthesis and characterization of *TaSTR* gene

Custom synthesis of *Triticum aestivum* salt tolerant related gene (*TaSTRG*-EF599631) was carried out by Genescript Company in USA. The synthesized gene was provided by the company after cloning into *EcoR*V site of pUC57 vector. The gene was designed to include different restriction sites on 5' end (*Kpn*I, *Pac*I, *Asc*I, *Sma*I and *Xma*I) and 3' end (*Not*I, *Pme*I, *Psi*I and *Hind*III) for further cloning. The sequence of custom synthesized gene is given in Appendix D.

For the characterization of *TaSTR* gene, restriction digestion and sequencing were performed. In order to perform restriction digestion, 5µg of plasmid DNAs were digested with *Xma*I, *Xba*I-*Pme*I and *Asc*I enzymes and the reaction mixtures were incubated at 37 °C for 16 hours. Digestions were confirmed by running samples on 1% agarose gel at 75V for 30 minutes and they were visualized by using Gel DocIt 310 (UVP, UK). After verification with enzyme digestion, synthesized *TaSTR* gene were sent to sequence analysis performed by RefGen Company with ABI 310 Capillary DNA Sequencer.

2.2.2.2. Vector construction for *Agrobacterium*-mediated tobacco transformation by using Gateway cloning technology

First step of the Gateway cloning technology is the amplification of gene of interest by using PCR. Therefore, in order to clone *TaSTR* gene into Gateway compatible entry vector, new primers were designed. Forward primer was designed as 5'-CACCATGGAACTCCTCTCCTACGC-3' including CACC site at upstream region of gene. Reverse primer were designed as 5'- GGCCCGGGATCCGATAAGCT-3' for the amplification *TaSTR* gene from customly synthesized and subcloned into pUC57 vector. PCR conditions and cycling parameters were given in Appendix L.

PCR products were eluted from agarose gels with GeneMark Gel Elution Kit. PCR band was removed from 1% agarose gel which was run at 85V for 35 minutes. The gel piece was transferred into eppendorf tube and weight. 1:1 volume of Binding Buffer to the gel slice (volume: weight) was added into the tube and incubated at 65 ºC until the gel slice is completely dissolved. Dissolved gel solution was transferred to purification column. Centrifugation was carried out at 13.000xg for 1 minute and the flow-through was discarded. After this step, 500µL of binding solution was added into the same column and centrifugation was carried out at 13.000xg for 1 minute. 700 µL of washing solution was added into the column and centrifuged at 13.000xg for 1 minute. Washing step was repeated one more time. The filtrate was discarded and the tube was centrifuged for 5 minutes at 13.000xg. Dried spin column

was transferred into a new eppendorf tube and 25 µL of elution solution was applied into the column and incubated at room temperature for 10 minutes. After incubation, PCR product was eluted from column with centrifugation at 13.000xg for 2 minute and directly used for ligation reaction with pENTRY-D TOPO vector.

2.2.2.2.1. Cloning of *TaSTR* Gene into Entry vector

Before cloning of PCR product, concentrations of samples were measured with Nanodrop UV Spectrophotometer (Thermo, US). pENTR™/D-TOPO® Cloning Kit (Invitrogen) were used for the insertion of *TaSTR* gene into Gateway entry clone by using PCR product by following the standard protocol (Figure 2.11). In cloning reaction 2:1 molar ratio of PCR product:TOPO vector was used. Four microliter of PCR product, one microliter salt solution and one microliter TOPO vector were mixed and incubated at 22 °C for 10 minutes. Ligation products were directly used for competent *E. coli* transformation. In order to confirm the insertion of plasmid into *E. coli* cells, colony PCR was carried out with gene specific primers by using a single colony grown on LB plate prepared with Kanamycine (100mg/ml) antibiotic. To amplify *TaSTR* gene by using colony PCR forward and reverse primers were designed as 5'-CTCCTCTCCTACGCTGCCAT-3' and 5'-AATCGTCTGCCCCACGTCCT-3', respectively. PCR products were loaded on 1% Agarose gel and PCR positive 2 colonies were selected for LR reaction.

2.2.2.2.2. Insertion of *TaSTR* gen into IPKb overexpression vectors

Insertion of *TaSTR* gene into plant overexpression vector (pIPKb004) was achieved by using Gateway® LR Clonase® enzyme mix by following the manufacturer protocol (Invitrogen). IPKb004 vector including double 35S promoter to control gene of interest and HptII antibiotic selection gene was obtained from The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) was used to construct *Agrobacterium*-mediated plant transformation vector (Figure 2.14).

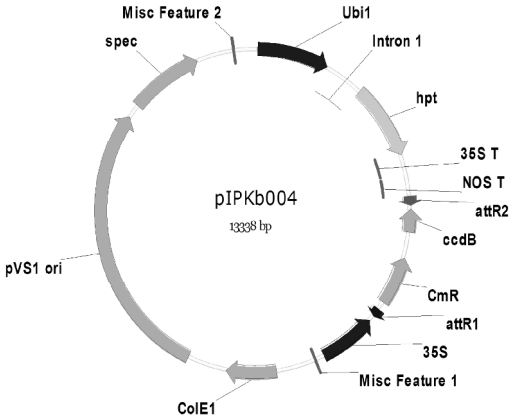

Figure 2. 14. Schematic representation of pIPKb004 vector

After LR reaction, reaction mixture was transferred to chemically competent *E. coli* cells by using previously used standard transformation protocol mentioned in part 2.2.1.1.8. So, pIPKb004-*TaSTR* was generated and it was named as pMAK28. Although there was a ccdB gene in IPKb vectors to prevent self-ligation, there was a probability to see colonies not containing *TaSTRG*. To select right colonies, colony PCR was carried out by using *NOS* terminator gene and *TaSTRG* gene specific primers. NOS terminator was used to design reverse primer (5'-TTATCCTAGTTTGCGCGCTA-3') and *TaSTR* gene specific forward primer (5'-AATCGTCTGCCCCACGTCCT -3'). Standard colony PCR protocols mentioned in part 2.2.1.1.9 were used with annealing temperature as 58 °C. PCR positive two colonies were selected for plasmid isolation.

2.2.2.3. *Agrobacterium*-mediated transformation of tobacco leaves

Tobacco leaves were transformed by using *Agrobacterium tumefaciens* AGL1 strain harboring pMAK28. In this context, *Agrobacterium* cells were grown in MG/L

(Tingay *et al*., 1997) medium supplemented with carbenicillin (100 mg/L) and spectinomycin (100 mg/L) in shaking incubator (Gallenkamp) at 250 rpm until the cells reached to OD_{600}:0.7-0.9. Tobacco leaf discs were inoculated with an overnight culture of AGL1-pMAK28 (OD_{600}:0.7-0.9) for 10 minutes, blotted semidry, and then cocultivated for 2 days on RMOP medium (Svab *et al*., 1990). After co-cultivation, explants were washed with liquid MS medium supplemented with 160mg/L Timentin for 20 minutes. Then, explants were transferred to the RMOP medium containing antibiotics [hygromycin (50 mg/L) and timentin (160mg/L)] for selecting transformed shoots and to restrict the growth of *A. tumefaciens*. Shoots emerged on RMOP medium after 6-8 weeks incubation were transferred to rooting medium supplemented with same antibiotics. Plantlets which were produced enough root were transplanted into the soil.

2.2.2.4. Analysis of putative transgenics

In order to verify the integration, expression and inheritance of the transferred DNA, putative T_1 transgenic plants of tobacco were analyzed by using both molecular and physiological analysis.

2.2.2.4.1. Molecular analysis of putative transgenic tobacco plants

Confirmation of transgene integration into tobacco transformed with *Agrobacterium tumefaciens* AGL1-pMAK28 was carried out with PCR analysis by using genomic DNA isolated from T_1 progeny with *hptII*, *aadA* and *TaSTRG* genes specific primers. The *aadA* primers were used to determine whether there was *Agrobacterium* contamination or not. Primers sequences are given Table 2.4 below. The PCR reactions (25 µl) were denatured at 94 °C for 3 minutes; followed by 30 cycles at 94 °C for 1 minute, 55 °C for 45 seconds, 72 °C for 1 minute for 1 kb, and a final extension cycle of 72 °C for 5 minutes.

Table 2.4. Primer sequences used for screening putative transgenic tobacco plants

aadA forward	TATGGCTCGTGAAGCGGTTATCG
aadA reverse	TTATTTGCCAACTACCTTAGTGATCTCG
hpt forward	CGAAAAGTTCGACAGCGTC
hpt reverse	GGTGTCGTCCATCACAGTTTG
TaSTRG forward	CTCCTCTCCTACGCTGCCAT
TaSTRG reverse	AATCGTCTGCCCCACGTCCT

In order to perform Mendelian segregation analysis seeds belong putative transgenic and wild type tobacco plants were germinated on MS medium supplemented with 50 mg/L Hygromycin after surface sterilization by applying 20% hypochloride for 20 minutes in eppendorf tubes with shaking. The number of surviving plantlets on Hygromycin medium was counted to calculate the Mendelian inheritance pattern after 4 weeks incubation at 25±2 °C with 8h/16h photoperiod. The expected 3:1 ratio of Mendelian inheritance is evaluated by using Chi-square Analysis with $p(\chi^2 \leq 3.841)=0.95$.

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2.2.2.4.2. Physiological analysis of transgenic tobacco plants expressing *TaSTRG*

In order to evaluate the expression of *TaSTR* gene under salt stress conditions, following analysis were performed:

1. Root length
2. Seed germination test under stress conditions
3. MDA assays

Expression of *TaSTR* gene was tested under 150mM, 200mM and 250mM salt stress conditions. In the first part of the experiment, T_0 and control plants were phenotypically compared after 4 weeks stress application. For this purpose, control plants grown in tissue culture conditions and T_0 plantlets regenerated from tobacco leaves were placed into MS without NaCl (control), MS+200mM NaCl and MS+250mM NaCl medium.

In the second part of the study, germination tests were performed by using seeds of T_1 plants and wild type control plants. Seeds of tobacco plants coming from putative transgenic and control plants were surface sterilized as described previously. Surface sterilized seeds were placed on MS without NaCl (control), MS+150mM NaCl and MS+200mM NaCl medium plates for salt stress treatments. The plates were incubated 6 weeks at 25±2 °C with 16 hours/8 hours photoperiod. After salt stress treatments photographs of the plates were taken and surviving plants were counted. Plantlets germinated on MS medium containing 200 mM NaCl were used for root length analysis. For this purpose roots of wild type and transgenic line 8 plantlets were measured and used for statistical analysis.

In addition to these analyses, MDA assay was carried out. Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content according to the method of Ohkawa *et al*., (1979). Fresh shoot tissues collected from plantlets germinated on 200 mM NaCl for 4 weeks were weighed as 0.2 g and homogenized with liquid nitrogen by the addition of 1 ml of 5% trichloroacetic acid (TCA). The homogenates were transferred to tubes and centrifuged at 13000xg for 15 minutes at room temperature with Microfuge 22R Centrifuge (Beckman Coulter, USA). Freshly prepared 0.5% thiobarbituric acid (TBA) in 20% TCA and supernatant in equal volumes were put into eppendorf tubes and incubated for 25 min at 96 °C. The tubes were placed in ice bath and then centrifuged at 9000xg for 5 min. Absorbance of the supernatant was determined at 532 nm and the correction for non-specific turbidity was performed by subtracting the absorbance at 600 nm. MDA contents were calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

2.2.3. Generation of transplastomic tobacco plants

2.2.3.1. Vector construction for chloroplast transformation of tobacco leaves

In this study, two different chloroplast transformation vectors were constructed. One of them was pJF29::aadA::xyl10B (Kim *et al*., 2010) and the other was pMAK16. In chloroplast transformation of tobacco studies only pJF29::aadA::xyl10B construct was used, pMAK16 was prepared for further transformation studies. In order to generate pJF29::aadA::xyl10B, Xyl10B was amplified by using PCR and cloned under the control of both rice prrn promoter and tobacco prrn promoter after sequence analysis. The first fourteen amino acids from green fluorescent protein (GFP) was subcloned downstream of the start codon and the 3'UTR of the tobacco rps16 gene. For the generation of final chloroplast transformation vector (pJF29::aadA::xyl10B) expression cassette carrying xyl10B was inserted into the pJF29 vector carrying the aadA gene controlling by tobacco prrn promoter and 3'UTR of the tobacco psbA. The rbcL/accD genes (Svab and Maliga, 1993) used as flanking sequence provide site specific integration by homologous recombination between pJF29::aadA::xyl10B and chloroplast genome (Figure 2.15).

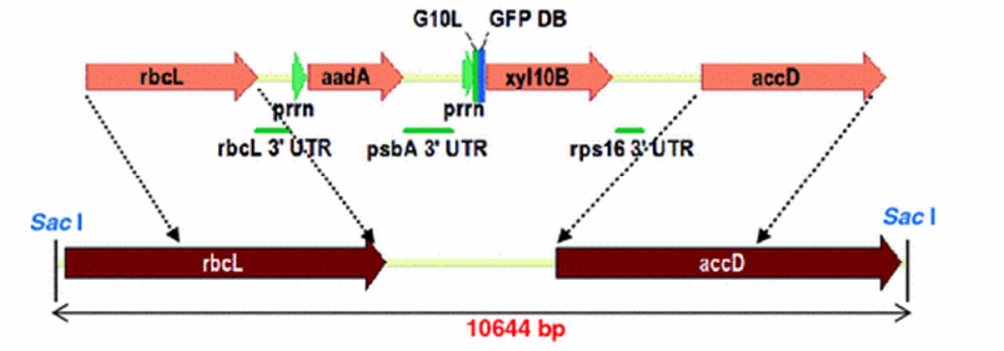

Figure 2. 15. Schematic representation of tobacco chloroplast transformation vector

In order to decrease unintended recombination between chloroplast transformation vector and chloroplast genome, the new vector was designed and named as pMAK16 (Figure 2.16).

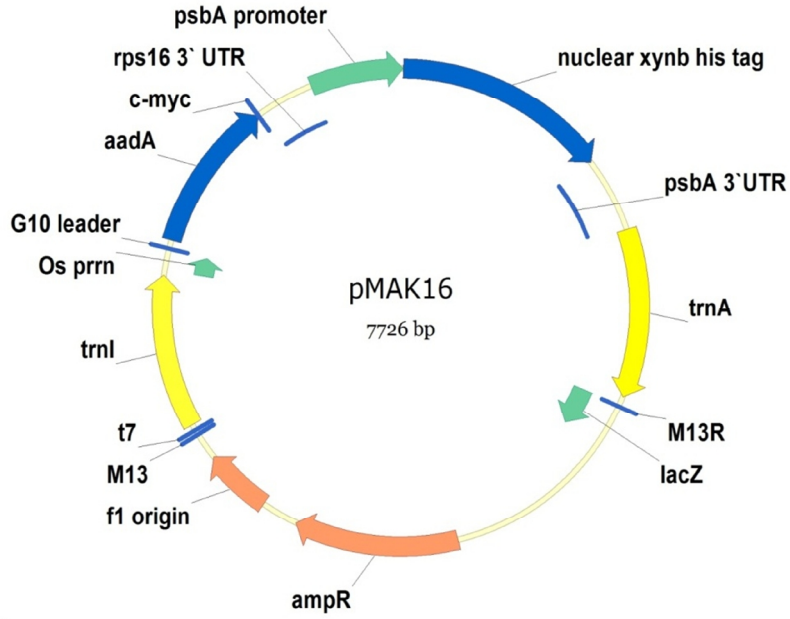

Figure 2. 16. Tobocco chloroplast transformation vector

Components belong to new vector and their source is given in Table 2.5 below. Each component was cloned into chloroplast transformation vector by standard cloning protocol.

Table 2.5. Components of new chloroplast transformation vector

Vector Elements	**Species**	**Size (bp)**
prrn promoter	*O. sativa*	111
psbA promoter	*O. sativa*	472
rps16 3` UTR	*O. sativa*	279
psbA 3` UTR	*G. max*	359
G10 leader	*Lambda T7*	42
GFP2 (14aa)	*A. victoria*	45

2.2.3.2. Microprojectile preparation and bombardment of tobacco plants

For microprojectile preparation, 30 mg of gold particles was weighed out (0.6 μm) into a 1.5-mL centrifuge tube and 1 mL of 70% ethanol was added and vortexed for 2-5 minutes. The tube was incubated for 15 minutes at room temperature and a content of the tube was mixed in every 5 minutes. Microparticles were spinned down by using table top centrifuge for 5 seconds. Supernatant was discarded and pellet was washed 3 times with 1ml sterile distilled water. In every washing step, the tube was vortexed for 1 minute and was waited for 1 minute to settle down the microparticles. After last washing, 500μl of sterile 50% Glycerol was added into tube and the mixture was allocated into eppendorf as 30μl and was stored in -20 °C (Kim *et al*., 2010).

Before transformation, the previously prepared gold particles that were stored at –20 °C were vortexed until they were completely resuspended. 30 μl of plasmid DNA (5μg) was added and vortexed for 1 minute. While vortexing, 20 μl 0.1M spermidine

and 50 µl 2.5 M $CaCl_2$were added consecutively and additional one minute vortexing was done. The mixture was centrifuged briefly to settle down the gold particles. After discarding the supernatant, 250 µl of absolute ethanol was added without disturbing the pellet. After spin down, supernatant was discarded and 80 µl of absolute ethanol was added. Gold-DNA mixture was ready and 10 µl of mixture was loaded onto microcarrier.

2.2.3.3. Selection and screening of transplastomic tobacco plants

Tobacco leaves transformed with particle bombardment were selected on RMOP medium supplemented with 500 mg/L spectinomycin for 7-10 weeks. In selection of second and third cycle plant both spectinomycin (500 mg/L) and streptomycin (500 mg/L) were used together to select transformed tobacco cells. Transgene integration into chloroplast genome was evaluated by PCR and southern blot analysis with genomic DNA of transplastomic plants. The gene specific and chloroplast genome specific primers were designed as aadA forward, aadA reverse, xyl10B forward, xyl10B reverse, Tch forward, and Tch reverse (Figure 2.17). The PCR conditions and cycling parameters were given in Appendix M. The PCR products were analyzed by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining.

Radioactive labeling system was used for southern blot analysis and procedure was summarized in Figure 2.18.

Figure 2. 17. Schematic representation of chloroplast genome and transformation vector

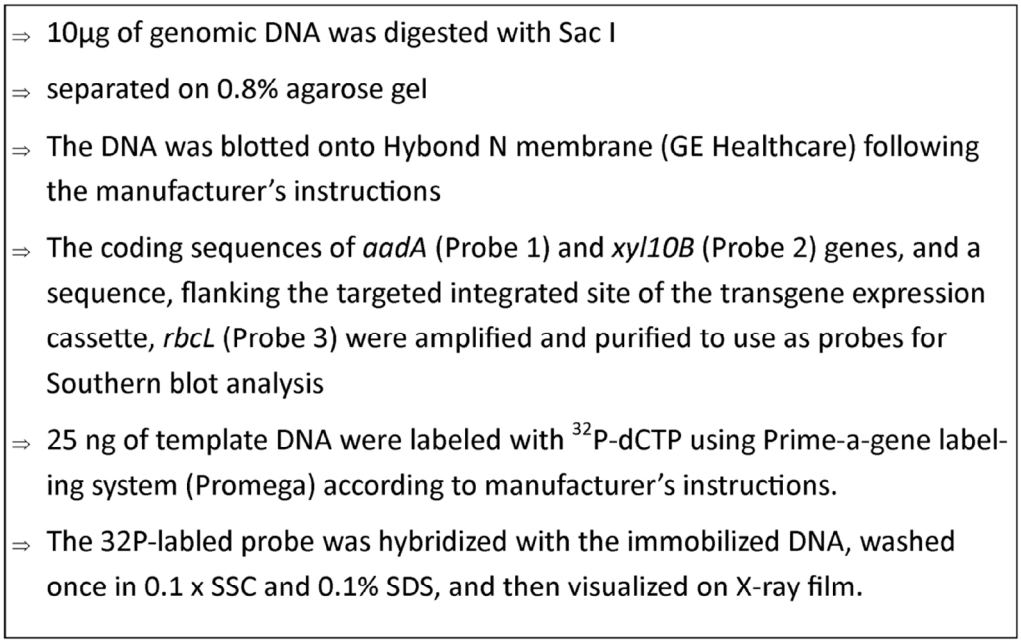

Figure 2. 18. Southern blot analysis with radioactive labeling

2.2.4. Statistical analysis

All of the statistical analyses were carried out by using Minitab Statistical Software. Analysis of variance (ANOVA) was used to investigate the relationship between a response variable and one or more independent variables.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Salt resistant transgenic wheat plants

3.1.1. Total RNA isolation and PCR Cloning of *TaNHX1* gene

In order to clone *TaNHX1* gene, total RNA was isolated from 10 days old *Triticum aestivum* cv. Yüreğir-89 wheat leaves subjected to salt stress (200mM NaCl) for 3 days according to Chomczynski (1993) by using TRIzol reagent. The quality and quantity of RNA samples were evaluated by using Shimadzu UV-Vis Spectrophotometer (Table 3.1).

Table 3. 1. Absorbance values and calculated concentrations of RNA samples

RNA samples	**A_{260}**	**A_{280}**	**A_{260}/ A_{280}**	**Concentrations (µg/µL)**
1	0.077	0.043	1.79	0.92
2	0.065	0.037	1.75	0.78
3	0.087	0.047	1.82	1.04

The quality of RNA samples were also checked by running on RNase-free 1% agarose gels prepared with 1X TAE solution at 100V for 40 minutes. Observation of 28S rRNA, 23S rRNA and 18S rRNA bands in the gels clarified the integrity of RNA samples (Figure 3.1). Any kind of DNA contamination might be observed as bands in the wells or any smear formation above the rRNA bands.

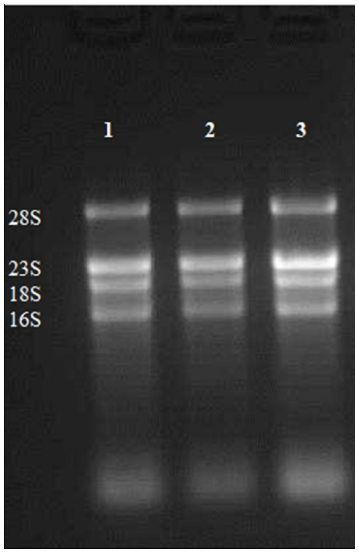

Figure 3. 1. Agarose gel (1 %) electrophoresis of total RNA samples isolated from 200mM NaCl-treated Yüreğir-89 leaves for 3 days after 7 days of normal growth. Lanes 1, 2 and 3 represent different total RNA samples.

Two microgram of purified RNA sample was used for the first strand cDNA synthesis. cDNA synthesis was carried out by using oligo (dT)18 primer following the manufacturer protocols. Synthesized cDNA sample was used as a template for PCR.

In order to optimize the annealing temperature, gradient PCR was carried out by using cDNA. Amplification of *TaNHX1* gene was checked with different annealing

temperatures, such as 53.1 °C, 55 °C, 56.9 °C, 58.8 °C, 60.7 °C, 62.6 °C and 64.5 °C (Figure 3.2). As a result of gradient PCR, 65 °C was determined as an annealing temperature for PCR amplification of *TaNHX1* gene. Therefore, the open reading frame of *TaNHX1* gene, 1641bp, was amplified by using optimized PCR conditions with Pfu DNA polymerase (Fermentas) from five different cDNA sample (Figure 3.3).

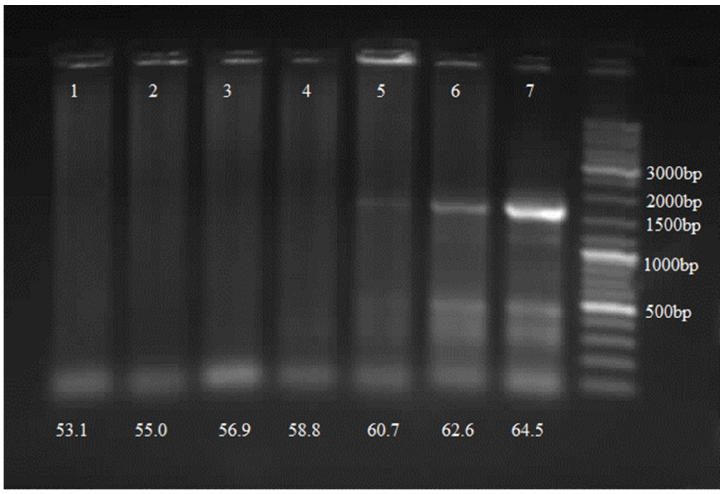

Figure 3. 2. Agarose gel (1%) electrophoresis of *TaNHX1* amplified with gradient PCR. Each lane represents the different annealing temperature (53.1 °C, 55 °C, 56.9 °C, 58.8 °C, 60.7 °C, 62.6 °C and 64.5 °C).

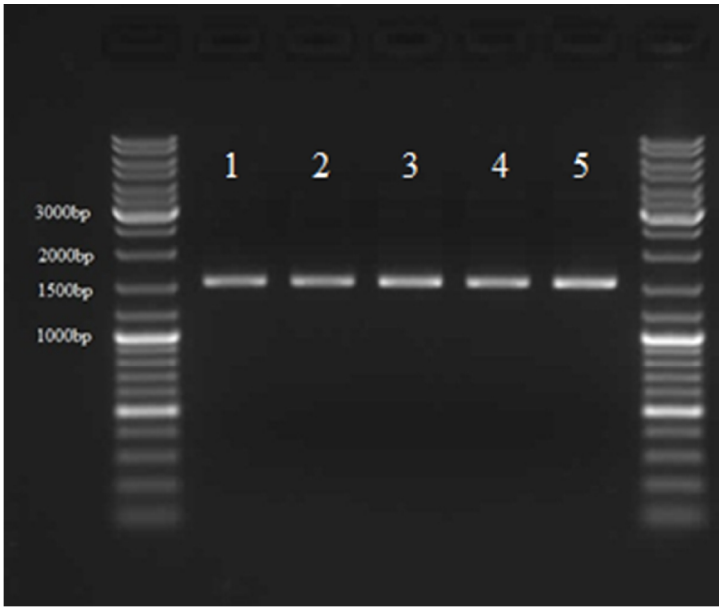

Figure 3. 3. Agarose gel (1%) electrophoresis of PCR amplified *TaNHX1*gene. Each lane represent the usage of different cDNA template.

Expected PCR band was eluted from 1 % agarose gel by using standard protocol given in part 2.2.1.1.6. Eluted DNA was used for the ligation in a 3:1 molar ratio with pJET1.2/blunt with standard PCR product cloning protocol. The product of ligation was directly used for transformation of chemically competent *E. coli* cells. In order to find correct ligation products, colony PCR was carried out by using colonies grown on LB medium supplemented with 50 mg/L Ampicillin. Fifteen colonies were selected for PCR analysis and they were checked with gene specific primers (Figure 3.4). Although there was a *eco47IR* gene (lethal gene) to prevent self-ligation, only five colonies produced expected bands (1653 bp) with gene specific primers and they were selected for plasmid isolation. After plasmid isolation, five plasmids were sent to the sequence analysis. According to the sequence analysis, error free plasmid was selected for further cloning.

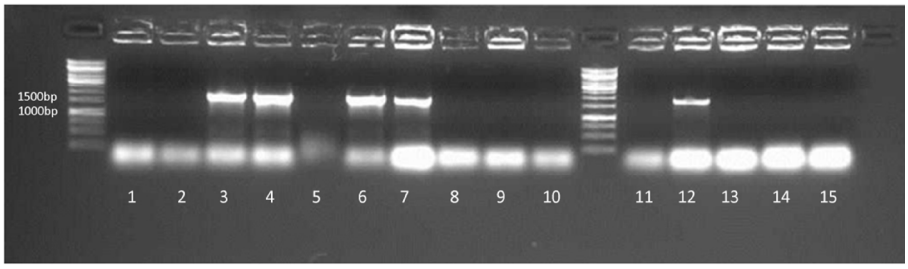

Figure 3. 4. Agarose gel (1%) electrophoresis of product of colony PCR. Each lane indicate different colony used for amplification of *TaNHX1* gene.

3.1.2. Vector construction for particle bombardment and *Agrobacterium*-mediated transformation

Vectors used in particle bombardment of wheat tissues were generated by conventional cloning technique including standard enzyme digestion and ligation. However, *Agrobacterium*-mediated transformation vector was generated by using Gateway cloning technology and pIKB004 plant overexpression vector was used as destination vector for LR reaction.

3.1.2.1. Monocot expression vector for particle bombardment experiments

Two types of particle bombardment transformation vectors were prepared by conventional cloning techniques. One of them was carrying ManA (pmi) gene for mannose selection and called as pMAK21. The other one was called as pMAK22 which was included bar gene for phosphinothricin selection. These vectors were constructed onto pUC19 plasmid. Cloning studies was summarized as follow.

- PCR amplification of Ubi1 promoter with *Nar*I-*Sac*I restriction sites (Figure 3.5A). PCR product was about 1991bp and transferred into pCR4Blunt-Topo vector.
- PCR amplification of NOS terminator with *Pst*I-*Hind*III restriction sites (Figure 3.5A). PCR product was about 257bp and transferred into pCR4Blunt-Topo vector.
- Digestion of both pUC19 and pCR4Blunt-Topo-Ubi1 with *Nar*I-*Sac*I and dephosphorylation with CIP (Figure 3.5B).
- Ligation of two fragments with quick ligation kit.

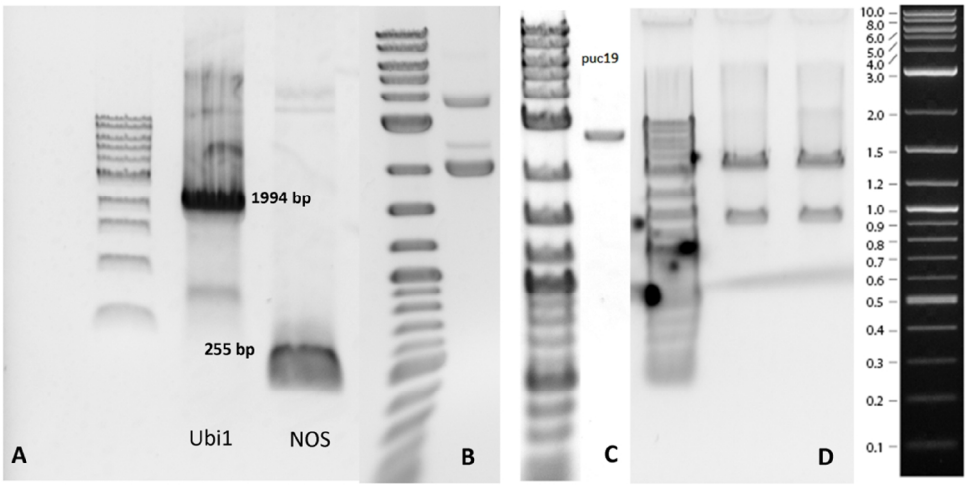

Figure 3. 5. Agarose gel (1%) electrophorese patterns of vector constructs A. PCR amplification pattern of Ubi1 promoter and NOS terminator B. Digestion of PCR4-Ubi1 vector with *Nar*I-*Sac*I (expected size 3957-1991bp) C. Digestion of pUC19 with *Nar*I-*Sac*I (expected size 2516-120bp) D. Test digestion of pUC19-Ubi1-NOS vector with *Sal*I (expected size 1416bp-3340bp)

Maize ubiquitin (Ubi1) promoter and its first intron and exon segments were produced from pAHC25 (Christensen and Quail, 1996) with specific primer including *Nar*I and *Sac*I restriction sites. In second step, NOS terminator from pCR4-Blunt-Topo vector created with *Pst*I-*Hind*III restriction sites was inserted into pUC19-Ubi1 vector generated in first step. Resulting vector was checked with *Sal*I digestion (Figure 3.5D). In third step, *TaNHX1* gene was amplified by *PfuTurbo*® *DNA Polymerase* with gene specific primer including *Kpn*I and *Pst*I sites (Figure 3.6A). PCR product was ligated with pCR4-Blunt-Topo and transferred into *E. coli*. For the insertion of *TaNHX1* into intermediate vector generated in previous step, both vectors were digested with *Kpn*I and *Pst*I enzymes (Figure 3.6B) and fragments were ligated to each other with standard protocol after dephosphorylation with CIP. After standard protocols, such as *E. coli* transformation and colony PCR, resulting vector was controlled with *Eco*RI enzyme digestion (Figure 3.6C). At the end of this step, third intermediate vector carrying *TaNHX1* cassette was generated.

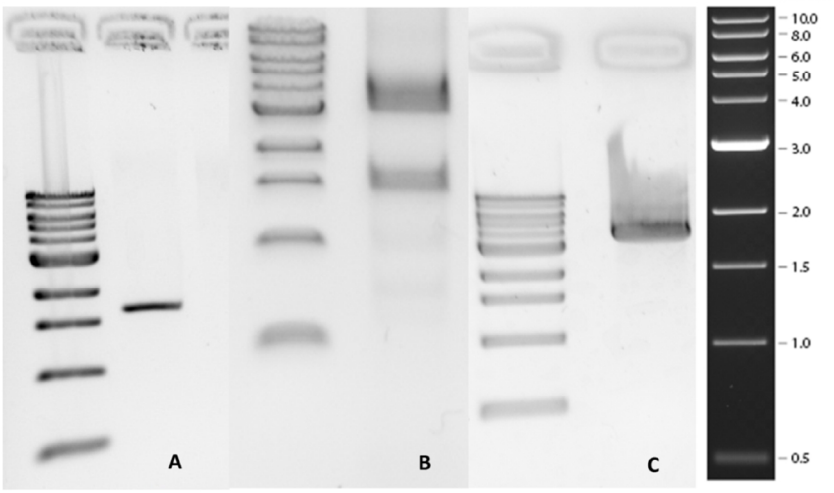

Figure 3. 6. Agarose gel (1 %) electrophoresis of T*aNHX1* gene amplification and its digestion A. *TaNHX1* amplification with PCR by using Pfu Polymerase B. Digestion pattern of pCR4-Blunt-Topo-*TaNHX1* with *Kpn*I-*Pst*I enzymes (expected size 1690bp-3957bp) C. Test digestion of pUC19-Ubi1-NOS-*TaNHX1* vector with EcoRI enzyme.

Finally, mannose (ubi1-pmi-35s) and bar (ubi1-bar-Nos) selection cassettes were inserted independently and they were called as pMAK21 and pMAK22, respectively. PMI expression cassette consist of ubi1 promoter, pmi gene and 35S promoter was isolated from pPZP201 vector (Gao *et al*., 2005) with *Hind*III digestion. After gel elution and dephosphorylation step, mannose selection cassette was inserted into third intermediate vector digested with *Hind*III. In order to generate final transformation vector carrying bar gene, bar gene expression cassette was released from pMK2b vector with *EcoR*I digestion. Following the gel elution, fragment ends filled up with Klenow Fragment by using standard protocol to produce blunt end fragment. Before ligation reaction third intermediate vector also digested with *Hind*III and blunted with Klenow fragment. After dephosphorylation with CIP, two fragments were ligated onto each other. Final vectors were digested with *Age*I (pMAK21) and *BamH*I (pMAK22) in order confirm whether there was desired ligation or not (Figure 3.7).

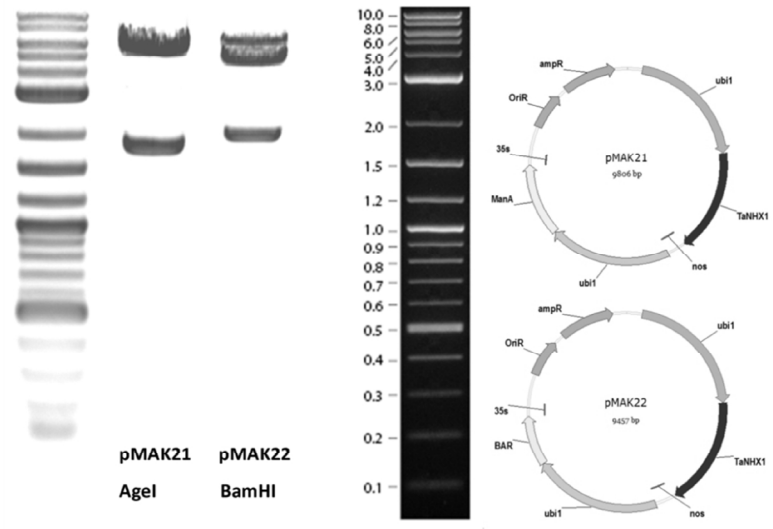

Figure 3. 7. Agarose gel (1 %) electrophoreses results of test digestion of final vectors with *Age*I and *BamH*I restriction enzymes.

3.1.2.2. Generation of overexpression vector for *Agrobacterium*-mediated transformation of wheat

Monocot and dicot binary expression vectors were constructed based pIPKb vectors (Himmelbach *et al*., 2007) by using Gateway cloning technology. Gateway® recombination cloning technology circumvents traditional restriction enzyme based cloning limitations, enabling researcher to access virtually any expression system All the basic steps used in Gateway Cloning Technology were summarized in Figure 2.8 in previous chapter.

3.1.2.2.1. Amplification of *TaNHX1* gene

The first step of Gateway cloning was inserting gene of interest into the Gateway. That is, inserting gene of interest into entry clone. For this purpose, *TaNHX1* gene was amplified with specific primers. During designing primers, CACC nucleotides were added to forward primers for both genes to provide directional insertion into the entry vector. PCR reactions were carried out by using Platinium Pfx DNA polymerase. Forward primer (5'-CACCATGGGGCTCGATTTGGGAGCCC-3') and reverse primer (5'-TCGACGCGGCCGCGAGCTCT-3') produced a PCR product around 1664 bp from *TaNHX1* template (Figure 3.8).

PCR products were eluted from agarose gel by using standard protocol mentioned in part 2.2.1.1.6 and inserted into pENTR-D-TOPO PCR cloning vector according to the manufacturer protocols. After ligation, products were transferred into chemically competent *E. coli* cells. The cells were incubated at 37 °C for overnight on LB plates supplemented with 100mg/L Kanamycine. In order to select right colonies carrying the gene of interest, colony PCR was conducted with gene specific primers. As a result of *TaNHX1* colony PCR, desired PCR band (1664bp) was obtained from five colonies out of ten (Figure 3.9).

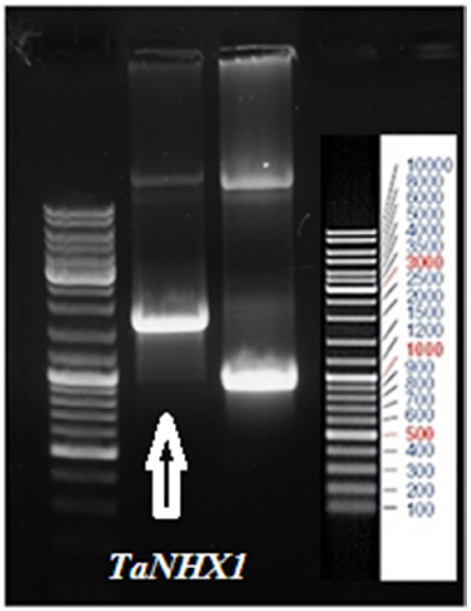

Figure 3. 8. Agarose gel (1%) electrophorese patterns of PCR amplification of *TaNHX1(1664bp)* gene with Gateway compatible primers.

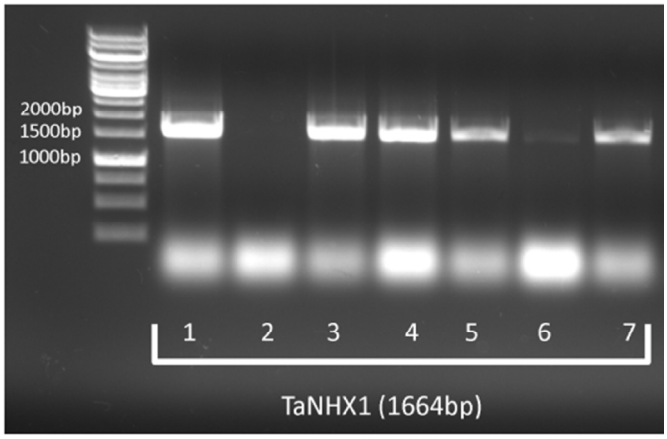

Figure 3. 9. Agarose gel (1 %) electrophoresis of *TaNHX1* amplification after colony PCR *(seven colonies)*. Each lane represent independent colony used for PCR.

One colony was selected from PCR positive colonies from each of transformation event to isolate plasmid. After plasmid isolation, these plasmids were used for sequencing. After confirmation of sequence of *TaNHX1* gene, isolated plasmids were used to LR reaction with IPKB overexpression vectors to produce final transformation vectors. In this study, pIPKB002 overexpression vector including maize Ubi1 promoter used as a destination vector for LR reaction which was final step of Gateway cloning. Therefore, LR reaction was performed by using pENTR-D-TOPO-*TaNHX1* with pIPKB002 to generate final overexpression vectors for *Agrobacterium*-mediated transformation of wheat. Recombination products were transformed into chemically competent *E. coli* cells and they were grown in LB medium supplemented with Spectinomycin (100 mg/L) for overnight. In order to find right colonies, colony PCR was carried out by using individual colonies grown in selective media with *TaNHX1 and NOS* gene specific primers. For this purpose NOS terminator was used to design reverse primer (5'-TTATCCTAGTTTGCGCGCTA-3') and *TaNHX1* gene was used to design forward primer (5'-GCTTGTCCTGGGCCTCATCA-3') and colony PCR was carried out by picking up individual colony (Figure 3.10).

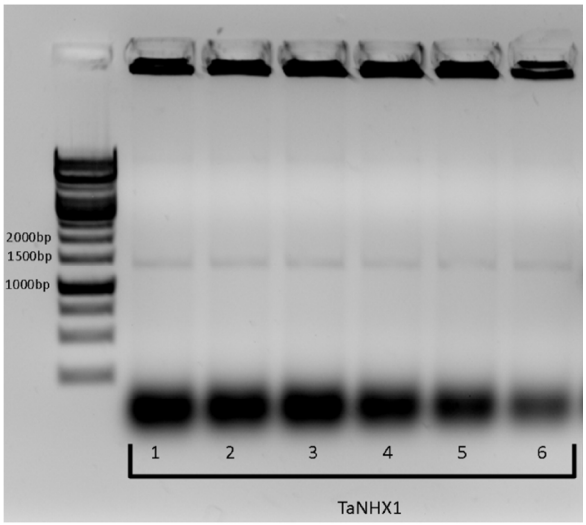

Figure 3. 10. Agarose gel (1 %) electrophoresis belongs to amplification of *TaNHX1* with colony PCR. Each lane indicates the individual colony used for amplification.

3.1.2.3. Transformation of overexpression vector into *Agrobacterium tumefaciens* AGL1 strain

Overexpression vector (pMAK26) was transferred into *Agrobacterium tumefaciens* AGL1 strain. In this study, semi empty AGL1 strain, not including pAL156 plasmid and including pAL154 plasmid, was used. pAL156 plasmid was discarded after seven cycle of growth in liquid LB medium supplemented with Carbenicillin (100 mg/L). After seven passages, plasmid isolation was carried out by using *Agrobacterium* culture. PCR was carried out by using these plasmids with ubi1 promoter primers, since pAL156 has maize ubi1 promoter. There was no band with these primers. Another confirmation PCR was made with Komari fragment primers, since there is 15.2 kb Komari fragment in pAL154 vector. Komari fragment primers were produced expected size of PCR product around 1000bp (Figure 3.11). Overexpression vectors were transferred into electrocompetent *Agrobacterium* cells by using electroporation. *Agrobacterium* cells carrying overexpression vectors were selected on LB plates supplemented with Carbenicillin (100 mg/L), Tetracycline (5 mg/L) and Spectinomycin (50 mg/L).

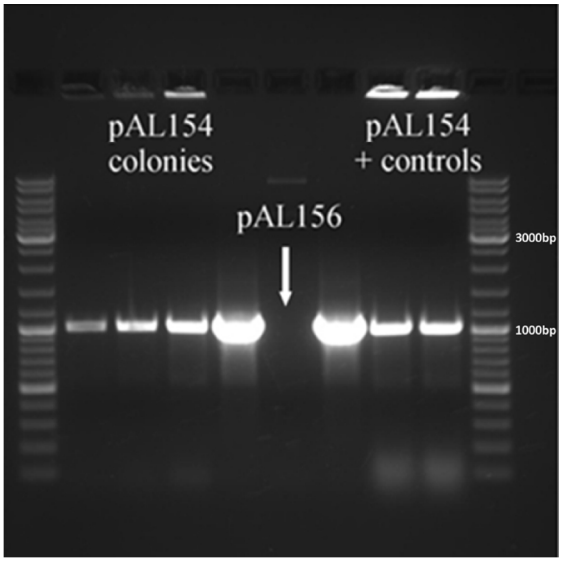

Figure 3. 11. Agarose gel (1 %) electrophoresis belong to amplification of Komari fragment.

3.1.3. Particle bombardment experiments in wheat tissues

In this study, three different wheat tissues were used for transformation of *TaNHX1*. In order to increase the chance of obtaining transgenic wheat plants, mature embryo of Mirzabey-2000, immature embryo of Kızıltan-91 and Yüreğir-89 and immature inflorescence of Kızıltan-91 and Yüreğir-89 were used as an explant (Figure 3.12). Since, there are several studies showing advantages of each of explants in literature. Customarily, immature embryo derived-calli have been accepted as the best explant for in vitro regeneration in wheat, but in recent year, mature embryo derived tissues have also been verified for successful regeneration and transformation studies (Patnaik *et al*., 2006; Zale *et al*., 2004).

There are physical parameters such as helium pressure, the negative value of vacuum pressure, distance between target tissues and particle sources and chemical parameters such as chemical reagent (spermidine, $CaCl_2$, glycerol) used for

precipitation and coating of DNA and biological parameters such as type of explant must be optimized to obtain transgenic plants by using particle bombardment (Fadeev *et al*., 2006). Therefore, we started to particle bombardment experiments of wheat by optimization of these parameters.

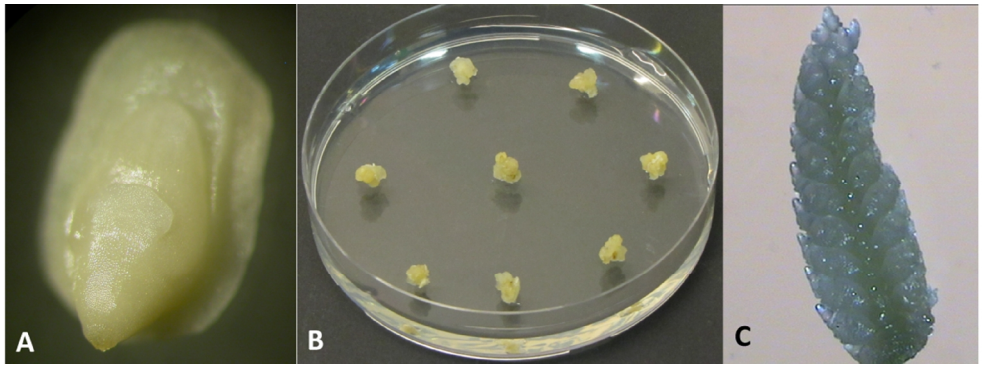

Figure 3. 12. Explants used in wheat transformation A. Immature embryo of Kiziltan-91 B. Mature embryo derived calli of Mirzabey-2000 C. Immature inflorescence of Yüreğir-89.

Since there was no *uidA* (GUS) gene in our constructs (pMAK21-pMAK22), optimization studies were conducted by using pAHC25 (Christensen & Quail, 1996). Effectiveness of bombardment was evaluated with histochemical GUS assay by counting blue spots shown in Figure 3.13 (Jefferson, 1987). During bombardment studies, distance between target tissue and microcarrier, amount of gas pressure and type of microcarrier were optimized.

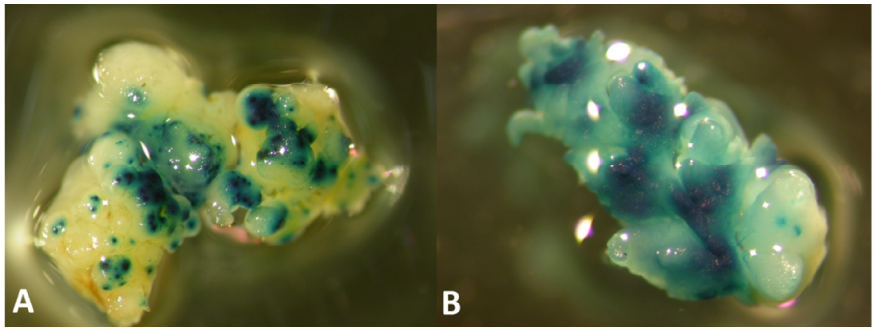

Figure 3. 13. Bombarded wheat tissues after histochemical GUS assay A. Calli derived from immature inflorescence of Yüreğir-89 B. Intact immature inflorescence of Kiziltan-91

Three different microcarrier, tungsten M-17 (1.1 µm diameter), tungsten M-25 (1.7 µm diameter) and gold particles (1.0 µm diameter) were used for the optimization of particle bombardment parameters (Figure 3.14). Immature inflorescence derived 4 weeks old calli belong to Kızıltan-91 and Yüreğir-89 were used as an explant. Each shot was performed by using about 300 ng microcarrier with 30 bar helium gas. According to histochemical GUS assay results, there was no significant difference between different microcarrier when 4 weeks old Yüreğir-89 calli were used as an explant. However, the transformation rate of tungsten M-17 was significantly higher than other particles when particle bombardment experiments were carried out by using 4 weeks old Kızıltan-91 calli.

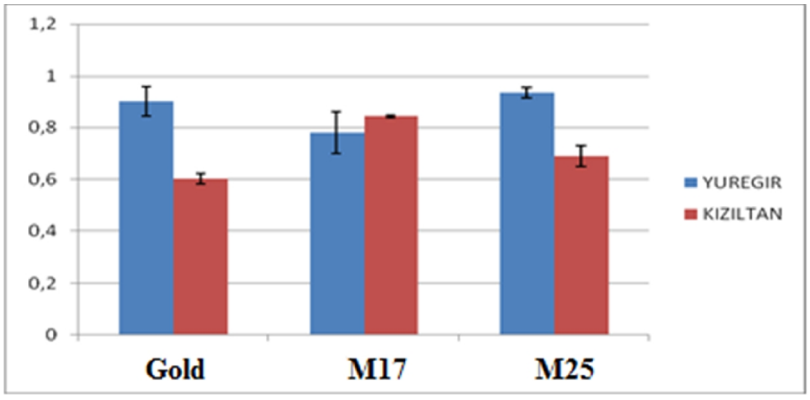

Figure 3. 14. Percentage of blue spot carrying calli transformed with different microcarrier by using 4 weeks old Yüreğir-89 and Kızıltan-91 calli . Tungsten M-17 (1.1 µm diameter), tungsten M-25 (1.7 µm diameter) and gold particles (1.0 µm diameter) were used.

For the optimization of helium pressure amount, 4 weeks old immature inflorescence derived Kızıltan-91 calli were bombarded with single shot and double shot by using 25 bar, 30 bar, 35 bar and 40 bar helium. According to histochemical GUS assay, explants bombarded with 30 and 35 bar of helium showed best transformation rates for both single shot and double shot (Figure 3.15). In addition to these parameters, distance between microcarrier holder and target tissues were also optimized and 8 cm was found best distance in terms of transformation efficiency.

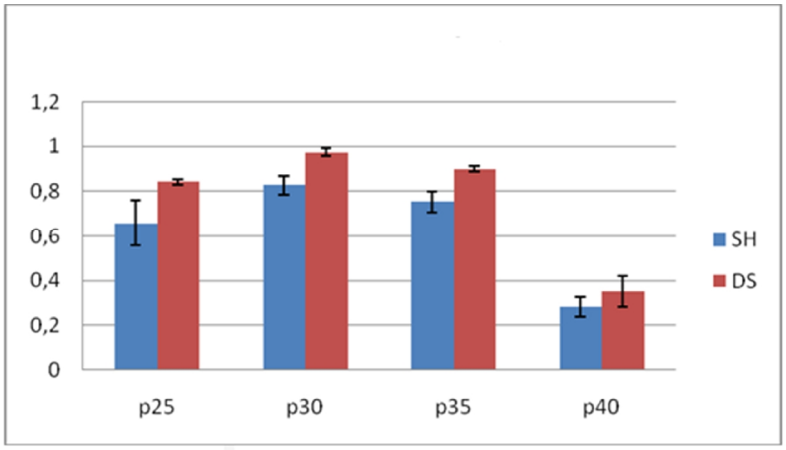

Figure 3. 15. Percentage of blue spot carrying calli transformed with different helium pressure p: helium pressure (bar) SH: single shot DS: double shot

After optimization of particle bombardment related parameters, particle bombardment experiments were carried out by using pMAK21 (mannose selection) and pMAK22 (ppt selection) plasmids carrying *TaNHX1* gene under the control of ubi1 promoter. In this manner, totally 8960 explants were bombarded with 1µm gold particles (Table 3.2). Intact mature embryo and mature embryo derived 15 days old calli were used for bombardment experiments. After high osmoticum period (16 hour after bombardment) period at dark condition, explants were transferred to callus induction medium and they were incubated at dark for 4 weeks. Then, they were transferred to embryogenic callus induction medium for around 3-4 weeks incubation. After incubation they were transferred to regeneration medium. Intact immature embryo and immature embryo derived 10 days old calli used during particle bombardment experiments. After high osmoticum period (16 hour after bombardment) period at dark condition, explants were transferred to callus induction medium and they were incubated at dark for 4 weeks. Then, they were transferred to embryogenic callus induction medium for around 3-4 weeks incubation. After incubation they were transferred to regeneration medium. In addition to the mature and immature embryo, immature inflorescence also was used for particle

bombardment. The calli derived from immature inflorescence were maintained on callus induction medium for 6 weeks in dark after high osmoticum period (16 hours after bombardment). After this period they were transferred to regeneration medium.

Two different selection schemes were tested during particle bombardment of wheat tissues. In first selection scheme, selection was started at callus induction period with 3 mg/L L-PPT and increased to 4 mg/L L-PPT during rooting stage. In second selection scheme, selection was started with rooting stage with 3 mg/L L-PPT and increased to 4 mg/L L-PPT during further rooting stage. As seen in Figure 3.16, selection was started at regeneration stage with 3mg/L PPT and was kept in this medium for 3 weeks (Figure 3.16A) and increased to 4 mg/L PPT at rooting stage (Figure 3.16B).

Table 3. 2. Summary of particle bombardment studies on wheat tissues

Explant type	**Cultivar**	**Number of explant**
Mature embryo	Mirzabey-2000	2540
Mature embryo derived callus	Mirzabey-2000	1980
Immature embryo	Yüreğir-89	1610
	Kızıltan-91	980
Immature embryo derived callus	Yüreğir-89	500
	Kızıltan-91	400
Immature inflorescences	Yüreğir-89	500
	Kızıltan-91	450
		8960 TOTAL

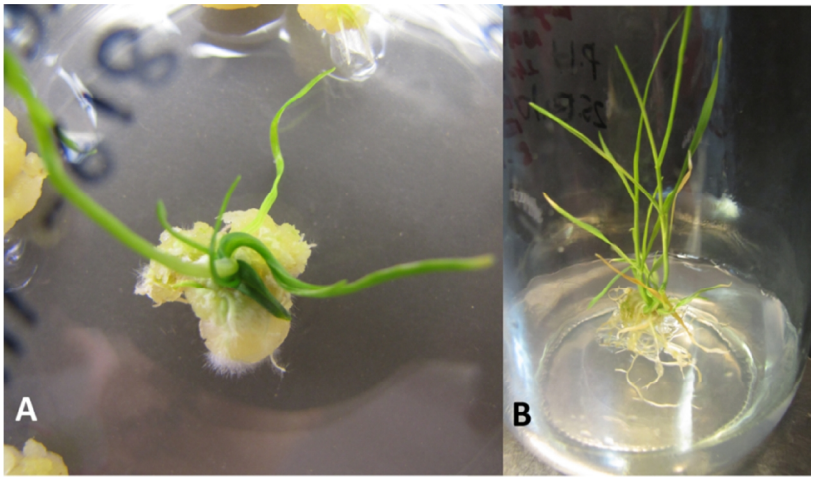

Figure 3. 16. Application of first selection scheme during regeneration and rooting stage A. Seven weeks old regenerated callus (Mature embryo derived-Mirzabey-2000) in 3mg/L PPT B. 10 weeks old plantlets (Mature embryo derived-Mirzabey-2000) in 4mg/L PPT at rooting stage

It was observed that 3mg/L PPT was not effective for selection of transformed tissues while application of second selection scheme, because they were growing and rooting in normal way (Figure 3.17A). However, when they were transferred to fresh rooting medium supplemented with 4mg/L PPT (Figure 3.17B), plants were started to die and after 6 weeks of incubation they were completely death.

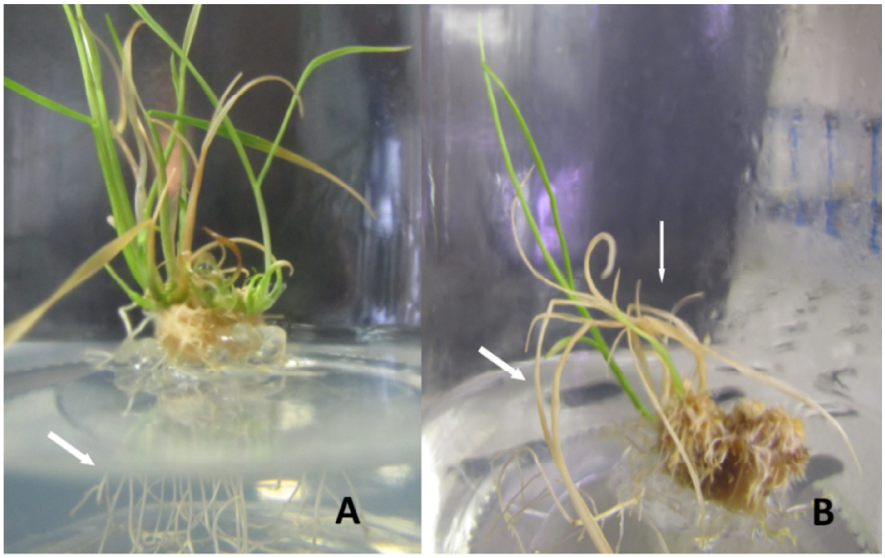

Figure 3. 17. Selection on rooting stage with mature embryo of Mirzabey-2000 A. Normal root formation in rooting medium supplemented with 3mg/L PPT B. Selection with 4 mg/L PPT

There were a few plants survived in rooting medium supplemented with 3 mg/L L-PPT for 8 weeks and they were used for PCR analysis of transgene integration. For this purpose genomic DNA was isolated by using standard protocol summarized in Figure 2.16. The PCR reactions (50 µl) were denatured at 94 °C for 5 minutes; followed by 30 cycles at 94 °C for 1 minute, 55 °C for 30 seconds, 72 °C for 2 minutes, and a final extension cycle of 72 °C for 5 minutes. However, amplification of *TaNHX1* was not observed when PCR products were loaded into 1% agarose gel (Figure 3.18).

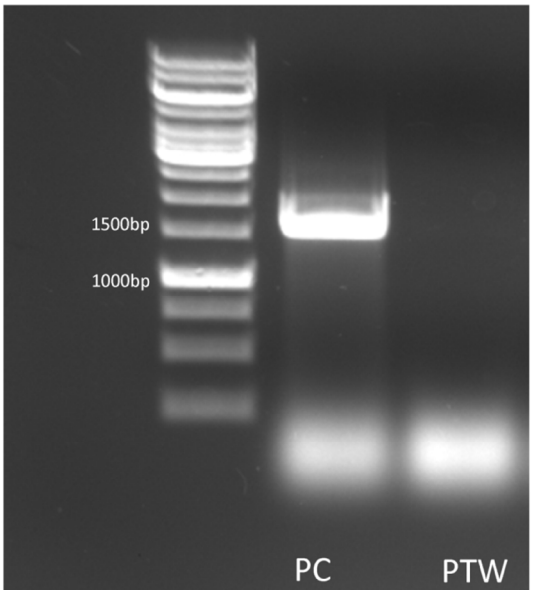

Figure 3. 18. Agarose gel (1%) electrophorese patterns of amplification of *TaNHX1* from genomic DNA of wild type plant (PC) and putative transgenic wheat plant (PTW)

After several trials, we couldn't obtain any transgenic plants from particle bombardment of wheat tissues. There were several factors affecting the particle bombardment effectiveness. Spermidine was the most important one. Since, if the DNA was not effectively protected by spermidine, the integration of transgene is not possible because of nuclease attack. In order to get rid of this problem, fresh spermidine was started to use after preliminary studies. Maybe the most important factor affecting the efficiency of transformation was related with device itself. According to our results, we can say that our device couldn't provide effective pressure and velocity for integration of genomic DNA to the nucleus.

3.1.4. *Agrobacterium*-mediated transformation of wheat tissues

In this study, semi empty *Agrobacterium tumefaciens* AGL1 strain was used for *Agrobacterium*-mediated transformation. AGL1 was the most widely used strain of

Agrobacterium in cereal transformation because of hypervirulance property (He *et al*., 2010, Zale *et al*., 2009, Zhao *et al*., 2011). Original AGL1 includes two plasmid, one overexpression and the other is helper plasmid provides extra virulence because of Komari fragment (Wu *et al*., 2003). Our plasmid (pMAK26) was inserted into *Agrobacterium* cells carrying only helper plasmid (pAL154) included Komari fragment to provide hyper virulence by electroporation. In *Agrobacterium*-mediated transformation studies, mature embryo of Mirzabey-2000, immature embryo of Kızıltan-91 and Yüreğir-89 and immature inflorescence of Kızıltan-91 and Yüreğir-89 were used. In this manner, totally 5650 explants were used during transformation studies (Table 3.3).

Table 3. 3. Explant types of different wheat varieties used for *Agrobacterium*-mediated transformation

Explant type	**Cultivar**	**Number of explant**
Mature embryo	Mirzabey-2000	1320
Mature embryo derived callus	Mirzabey-2000	850
Immature embryo	Yüreğir-89	1220
	Kızıltan-91	680
Immature embryo derived callus	Yüreğir-89	560
	Kızıltan-91	490
Immature inflorescences	Yüreğir-89	320
	Kızıltan-91	210
		5650 TOTAL

Agrobacterium cells were grown in MG/L medium supplemented with spectinomycin (100 mg/L) and carbenicillin (100 mg/L) for overnight. When they reached to OD_{600}:0.8-1.2, they were precipitated and dissolved in inoculation medium supplemented with 0.01% Silwet-77. Explants were incubated with cells for 1 hour and excess bacteria were poured. Explants incubated on co-cultivation medium at dark for 3 days (Figure 3.19). After washing with liquid MS medium supplemented with 160 mg/L Timentin for 1 hour, they were transferred to callus induction medium. For regeneration, rooting and selection, same approach was applied with particle bombardment experiments. Selection was applied in two ways, the one during regeneration (Figure 3.20) and the other after root formation stage with hygromycine (25 mg/L).

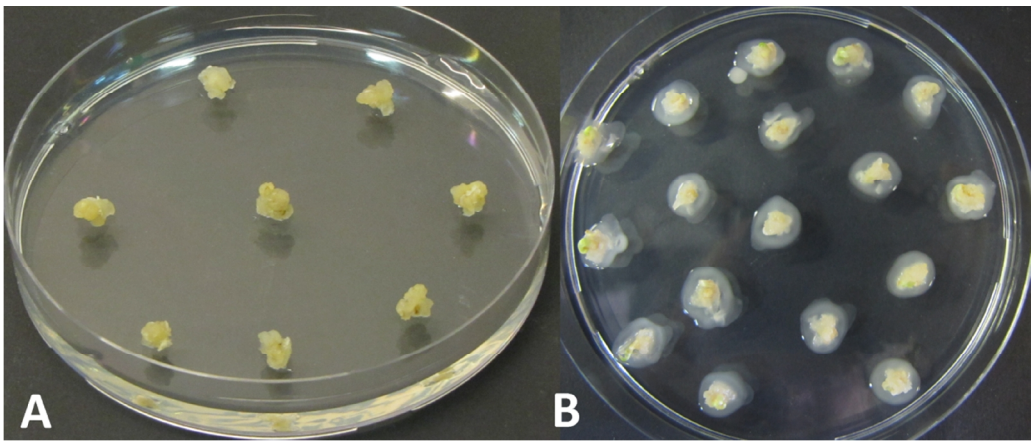

Figure 3. 19. Calli before and after co-cultivation with bacteria A. 10 days old immature embryo derived calli of Yüreğir-89 B. 10 days old immature embryo derived calli after co-cultivation

The first selection scheme was started with 25 mg/L hygromycin during regeneration period (Figure 3.20) and continued with same selection pressure during rooting stage. In second selection scheme, selection was applied after enough root formation.

As shown in Figure 3.21, regeneration was achieved without selection pressure and then explants transferred to rooting medium not supplemented with hygromycin. Explants were kept in this medium about 4-6 weeks and selection was started in jar with 25mg/L Hygromycin. Selection was continued with 6 weeks in these jars. Unfortunately, all the plants died at the end of this period (Figure 3.22).

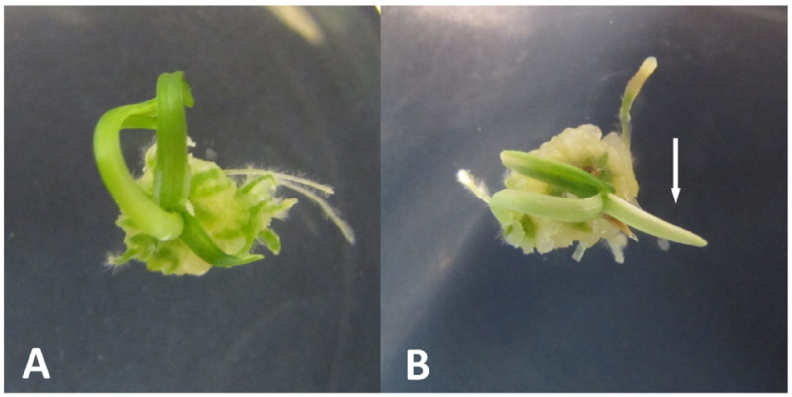

Figure 3. 20. Application of first selection scheme with 8 weeks old regenerated callus by using hygromycin (25mg/L). A. 8 weeks old regenerated calli from immature embryo Kızıltan-91 B. Selection with hygromycin (25mg/L) after 10 days.

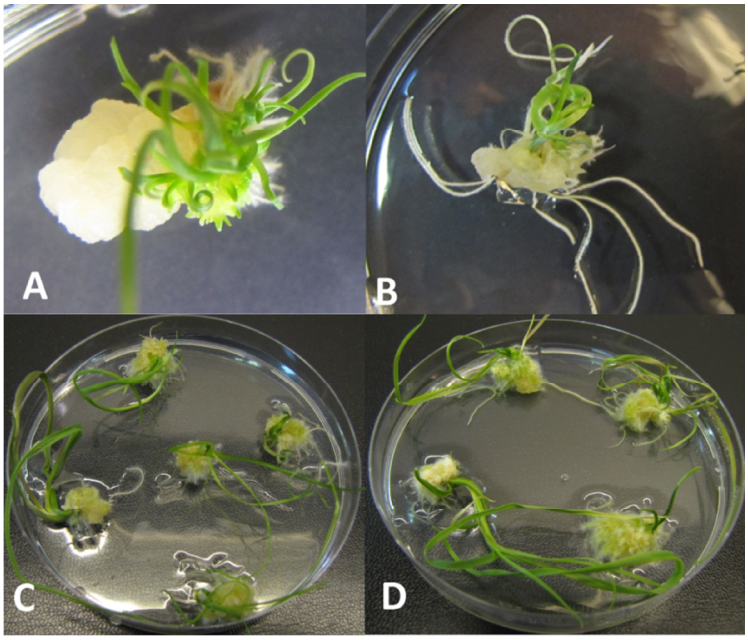

Figure 3. 21. Application of second selection scheme with 25 mg/L hygromycin A. Regenerated callus derived from immature embryo (Kiziltan-91) B. Rooted plantlets from Yüreğir-89 immature embryo C. Plantlets incubated in rooting medium for 4 weeks (Kiziltan-91) D. Plantlets incubated in rooting medium for 4 weeks (Yüreğir-89)

As a consequence of our *Agrobacterium*-mediated transformation studies, we couldn't obtain any transgenic wheat plants. We observed that our explants didn't amenable to Agro infection. In literature, many attempts have been made to establish efficient regeneration and transformation systems for wheat by using *Agrobacterium tumefaciens*, but most protocols have been developed with spring wheat 'Bobwhite' as a model system, which has limited agronomic qualities (Chauhan *et al*., 2007). There is big disadvantage of *Agrobacterium tumefaciens*, which is genotype dependence. Because of this drawback, *Agrobacterium*-mediated genetic transformation of cereals has been largely confined to particular genotypes that combine the amenability to gene transfer with adequate regeneration potential. After

our studies, we concluded that our cultivars used in this study didn't meet these criteria by showing the recalcitrance to *Agrobacterium*-mediated transformation.

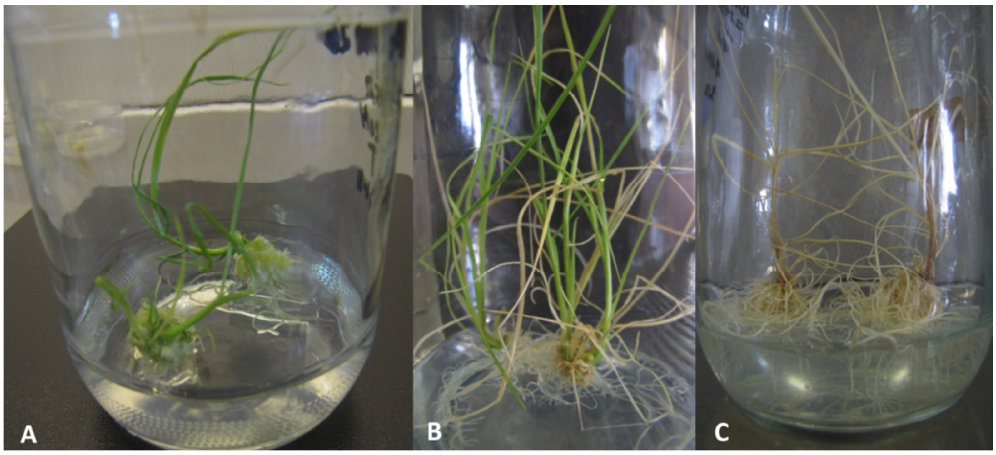

Figure 3. 22. Immature embryo derived plants (Yüreğir-89) effected by 25mg/L Hygromcin A. Two weeks after hygromycin selection B. Six weeks after hygromycin selection

3.2. Salt resistant transgenic tobacco plants

Salt resistant transgenic tobacco plants were generated by using *Triticum aestivum* salt tolerance related gene which was isolated from mutant wheat variety (Zhou *et al*. ,2009).

3.2.1. Synthesis and test digestion of *TaSTR* gene

Triticum aestivum salt tolerance related gene (*TaSTRG*-913 bp) was customly synthesized by Genescript Company in USA. Synthesized *TaSTR* gene was provided

after cloning into EcoRV site of pUC57 vector. To confirm the synthesis of the gene, restriction digestion of pUC57-*TaSTRG* and sequencing were performed. Restriction digestion was performed with AscI, XmaI and XbaI-PmeI enzymes. When digestion products of these enzyme run on agarose gel (1 %), one band around 3650 bp from AscI digestion and two bands around 950-2700 bp from XmaI digestion and two bands around 930-2720 bp from XbaI-PmeI double digestion were observed as expected (Figure 3.5 and Figure 3.6).

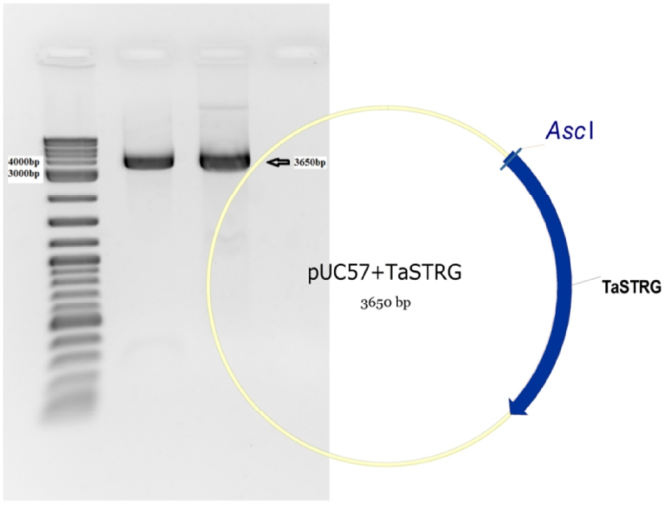

Figure 3. 23. Agarose gel (1%) electrophorese patterns of digestion pUC57+*TaSTRG* vector with AscI

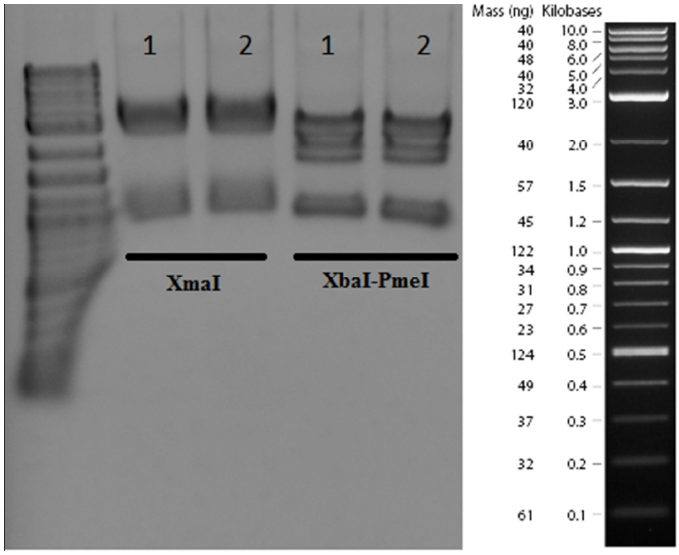

Figure 3. 24. Agarose gel electrophorese patterns of digestion of pUC57-*TaSTRG* vector on 1% Agarose gel. First two lane indicate XmaI digestion and second two lane indicate XbaI-PmeI digestion of pUC57-TaSTRG plasmid.

3.2.2. Vector construction with Gateway cloning technology

Gateway cloning technology was used to generate plant overexpression vector carrying *TaSTRG* for *Agrobacterium*-mediated transformation of tobacco leaves. Same cloning strategy was followed with the generation of *Agrobacterium*-mediated wheat transformation vector which was generated by using Gateway cloning technology (part 3.1.2.2). The first step of Gateway cloning was consisting of insertion of gene of interest into the Gateway Entry clone. For this purpose, *TaSTR* gene was amplified with specific primers. During designing primers, CACC nucleotides were added to forward primers for both genes to provide directional insertion into the entry vector. PCR reactions were carried out by using Platinum Pfx DNA polymerase. Forward primer (5'-CACCATGGAACTCCTCTCCTACGC-3') and reverse primer (5'- GGCCCGGGATCCGATAAGCT-3') were used to produce *TaSTR* gene which was expected around 951bp (Figure 3.25).

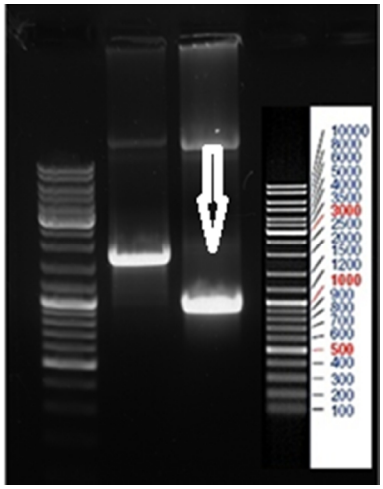

Figure 3. 25. Agarose gel (1%) electrophoreses patterns of PCR amplification of *TaSTRG (951bp)* gene with Gateway compatible primers.

PCR products were extracted from agarose gel by using standard protocol mentioned in part 2.2.1.1.6 and inserted into pENTR-D-TOPO PCR cloning vector according to the manufacturer protocols. After ligation, products were transferred into chemically competent *E. coli* cells. The cells were incubated at 37 °C for overnight on LB plates supplemented with 100mg/L Kanamycine. In order to select right colonies carrying the gene of interest, colony PCR was conducted with gene specific primers. As a result of *TaSTRG* colony PCR, desired PCR band (951bp) was obtained from one colony out of ten (Figure 3.26).

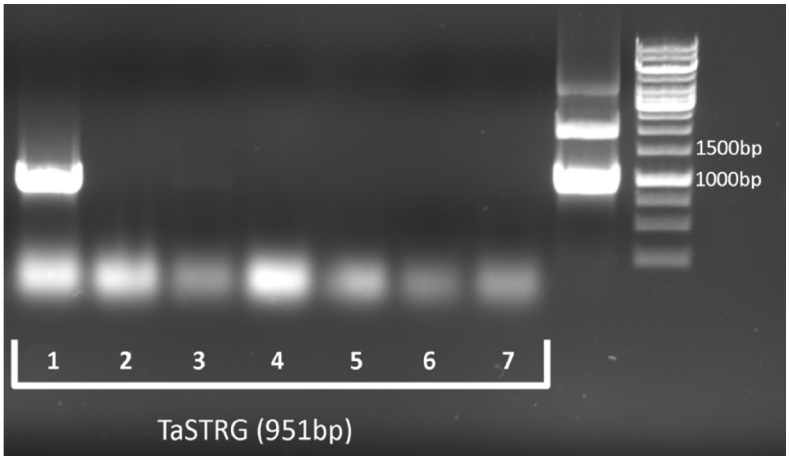

Figure 3. 26. Agarose gel (1 %) electrophoresis of *TaSTRG* amplification after colony PCR *(seven colonies)*. Each lane represent independent colony used for PCR.

One colony was selected from PCR positive colonies from each of transformation event to isolate plasmid. After plasmid isolation, these plasmids were used for sequencing. After confirmation of sequence of *TaSTR* gene, isolated plasmids were used to LR reaction with IPKB overexpression vector to produce final transformation vectors. For tobacco transformation, pIPKB004 overexpression vector including double strength 35S promoter used as a destination vector for LR reaction which was final step of Gateway cloning. Therefore, LR reaction was performed by using pENTR-D-TOPO-*TaSTRG* with pIPKB004 to generate final overexpression vectors for *Agrobacterium*-mediated transformation of wheat. Recombination products were transformed into chemically competent *E. coli* cells and they were grown in LB medium supplemented with Spectinomycin (100mg/L) for overnight. In order to find right colonies, colony PCR was carried out by using individual colonies grown in selective media with *TaSTRG and NOS* gene specific primers. For this purpose NOS terminator was used to design reverse primer (5'-TTATCCTAGTTTGCGCGCTA-3') and *TaNHX1* gene was used to design forward primer (5'-CTCCTCTCCTACGCTGCCAT-3') and colony PCR was carried out by picking up individual colony (Figure 3.27).

Final plant overexpression vector obtained from LR reaction was named as pMAK28 and transferred to electrocompetent *Agrobacterium tumefaciens* AGL1 strain by using standard protocol mentioned in part 3.1.2.3.

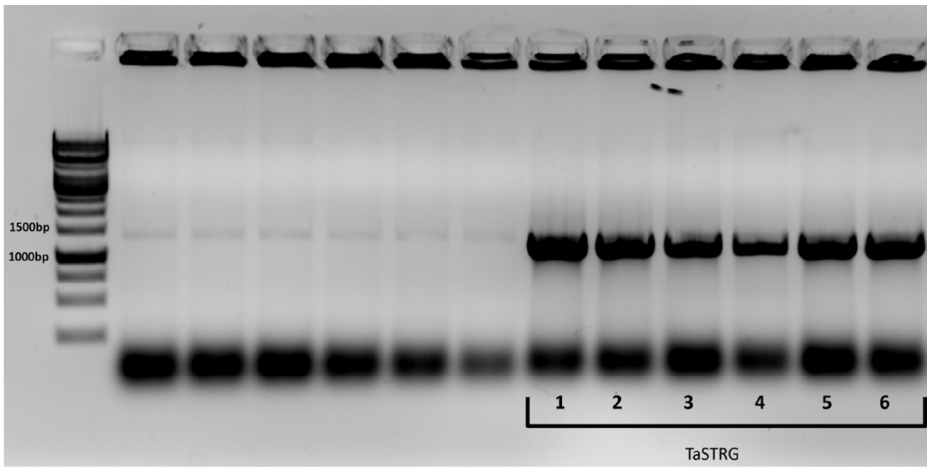

Figure 3. 27. Agarose gel (1 %) electrophoresis belong to amplification of *TaSTRG* with colony PCR. Each lane indicates the individual colony used for amplification.

3.2.3. *Agrobacterium*-mediated transformation of tobacco leaves

The leaves collected from four weeks old tobacco plants were used for the *Agrobacterium*- mediated transformation. Tobacco leaves were transformed with pMAK28-TaSTRG plasmid containing hygromycine selection gene (*hptII*) as a selection marker gene. Prior to start selection of transformed tissues, effective hygromycin concentration required for selection was determined. In literature, there was several study used hygromycin selection in tobacco transformation. Generally effective dose of hygromycin can vary from 30 mg/L to 50 mg/L (Mette *et al*.,2000). In present study, 50 mg/L hygromycin was applied to wild type tobacco plant grown

in tissue culture to test the effect of selective agent. At the end of 10 days, wild type plant was completely died (Figure 3.28). Therefore, this determined effective dose of selection was used during further studies.

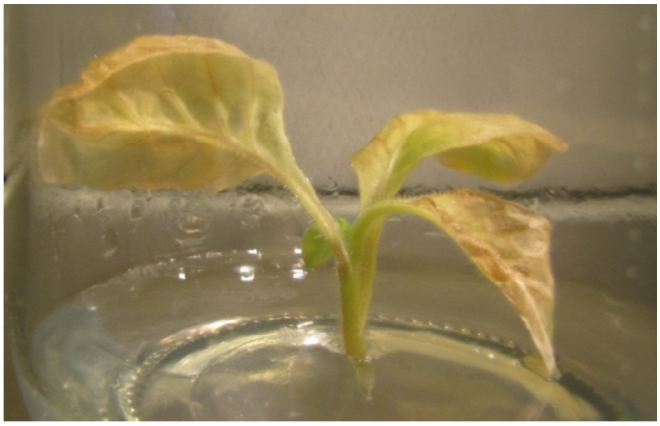

Figure 3. 28. Determination of hygromycin lethal dose with wild type tobacco plant

The putative transgenic plants were regenerated on RMOP (Svab and Maliga, 1993) medium containing 50 mg/L hygromycin, 1mg/L BA and 0.1 mg/L NAA. 4-5 weeks after the transformation shoots started to emerge. Control plants did not initiate any shoots and they died after 10 days (Figure 3.29A). On the average, each disk transformed with pIPKB004-TaSTRG (pMAK28) produced 4 shoots that can be transferred to root initiation media (Figure 3.29B). These shoots were cut from their bases and transferred to rooting media containing 50 mg/L hygromycin (Figure 3.30). Further selection and subculturing on hygromycin containing medium gave rise to 22 individual lines that were transformed by *TaSTR* gene.

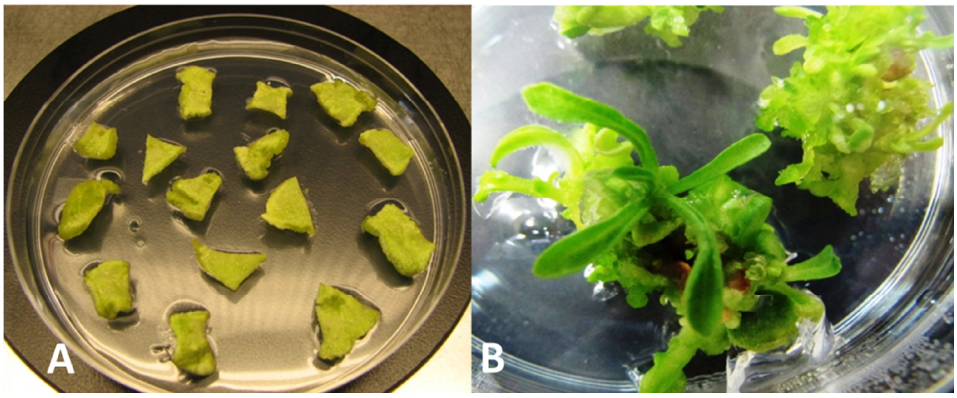

Figure 3. 29. Regeneration of tobacco in RMOP medium supplemented with 50mg/L hygromycine A. Control *Nicotiana tabacum* leaf disks on hygromycin (50mg/L) selection B. Putative transgenic plantlets on selective medium

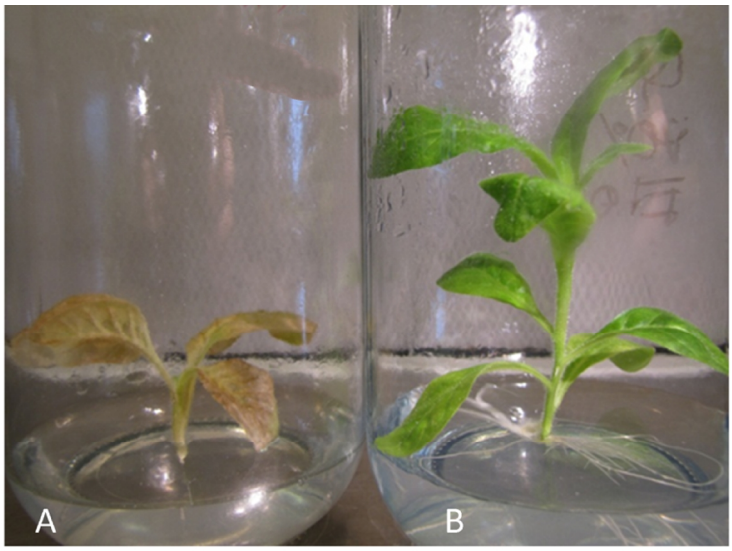

Figure 3. 30. Rooting medium supplemented with Hygromycin (50 mg/L) A. Wild Type Plant B. T_0 plant transformed by pMAK28.

After development of enough roots, usually took 4-6 weeks, plants were transplanted into the soil and further growth was achieved in greenhouse with 8h/16h photoperiod at 25±3 °C (Figure 3.31). Prior to flowering plants were covered with plastic bags to prevent cross-pollination. T_1 generation was obtained from all putative transgenic lines (Figure 3.32). All the seeds over putative transgenic tobacco plants were collected after maturation and kept in eppendorf tubes for further analysis.

Figure 3. 31. The growth of a putative transgenic tobacco in soil

Figure 3. 32. Flowering of putative transgenic tobacco plants

3.2.4. Analysis of T_1 putative transgenic tobacco expressing *TaSTRG*

T_1 generation of transgenic tobacco plants expressing *TaSTRG* was obtained by collecting the seeds from T_0 plants and germinating them in 50 mg/L hygromycin containing medium. The plants were later transferred to jars with selective rooting media (Figure 3.33). The rooted plants were transferred to soil and grown to maturity in green house. After self-pollination, flowering and capsule formation, T_2 seeds were collected.

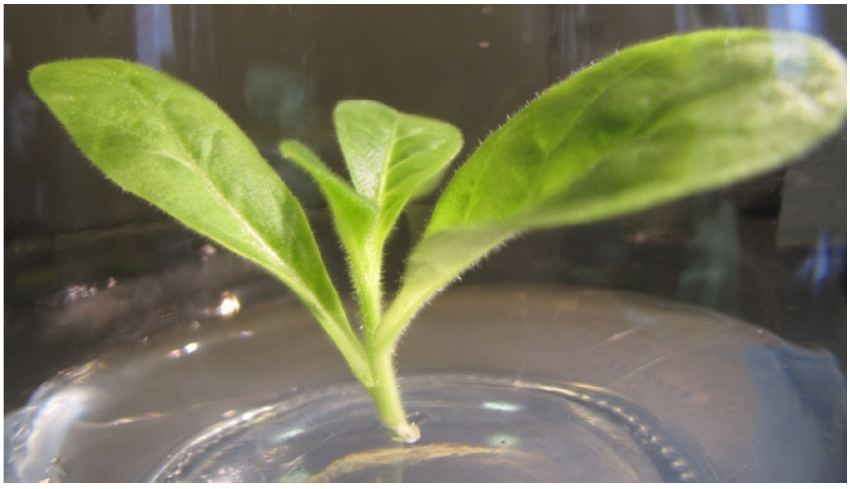

Figure 3. 33. The growth of T_1 tobacco plant containing *TaSTRG* on 50 mg/L Hygromycin containing media

3.2.4.1. Molecular analysis

Molecular analysis of T_1 transgenic plants was carried out by using Mendelian inheritance analysis and PCR. In order to perform these analyses, T_1 seeds were germinated in selective media containing 50 mg/L hygromycine.

3.2.4.1.1. Mendelian inheritance analysis

Phenotypic ratio for hygromycine resistant and sensitive seedlings were evaluated by counting the number of survivors on selective media after 4 weeks incubation. In segregation analysis only PCR positive transgenic lines were used. For each line, the experiment was repeated three times by certain number of seeds and the cumulative results are given in Table 3.4. The expected 3:1 ratio of Mendelian inheritance is evaluated by using Chi-square Analysis with $p(\chi^2 \leq 3.841)=0.95$.

Chi-square analysis indicated that most of the progeny segregated in a Mendelian fashion (3:1). However, Line 9 showed non-mendelian segregation ratio (Table 3.4).

Table 3. 4. Mendelian inheritance analysis of T_1 progeny of tobacco

Lines	Total seed numbers	Germinated seed numbers	χ^2	$p(\chi^2$ 0.05)	Decision
WT	78	0	260	NA	Reject
1	90	75	3.33	<0.05	Fail to reject
2	92	78	3.52	<0.05	Fail to reject
3	94	76	1.7	<0.05	Fail to reject
5	80	67	3.26	<0.05	Fail to reject
6	102	83	2.20	<0.05	Fail to reject
8	110	88	1.46	<0.05	Fail to reject
9	100	88	9.01	>0.05	Reject

3.2.4.1.2. PCR analysis

In order to confirm presence of transgenes, PCR analyses were performed with *HptII* gene specific and *TaSTR* gene specific primers. PCR analyses were carried by using 100ng genomic DNA isolated with CTAB method. In this study, 22 putative transgenic lines were produced, but 9 lines were selected for PCR analysis. Initial PCR analyses were carried out by using *TaSTR* gene specific primers. It is expected to obtain the PCR product about 900 bp with these primers. The results of PCR showed the presence of an amplified fragment belonging to *TaSTRG* in 7 selected independent transgenic lines. Such a fragment was not observed in wild type plants and in Line 4 and Line 7 (Figure 3.34). Therefore, line 1, line 2, line 3, line 5, line 6, line 8 and line 9 were selected for further PCR and other analysis. Second PCR analyses were performed with hygromycin phosphotransferase II gene (*HptII*) specific primers. In this step, only positive lines confirmed with PCR by using *TaSTRG* primers were selected for analysis. Amplification of *HptII* gene was observed on agarose gel from all selected lines other than line 9 (Figure 3.35). Although the presence of hygromycin resistant plants can provide sufficient evidence that a regenerated plant is transformed with introduced DNA, escapes that represent partial or no transfer may be present within the population of primary transformants (Worrall, 1998). So this PCR negative line was not a big surprise for us.

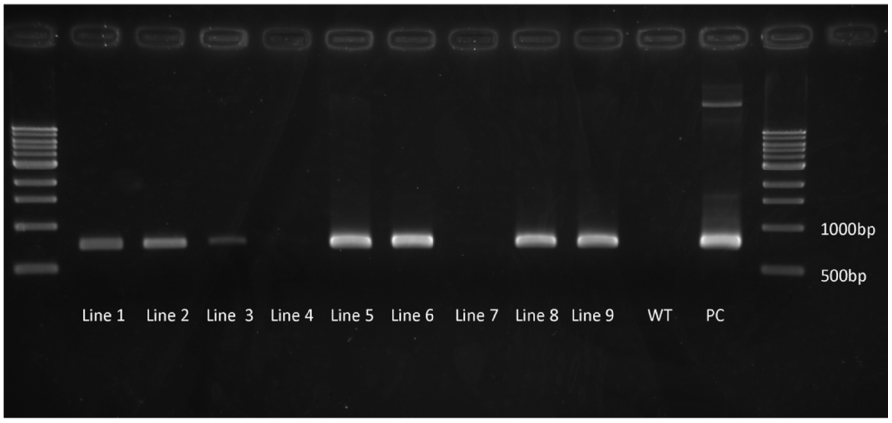

Figure 3. 34. PCR amplification of the *TaSTR* gene in T_1 transgenic tobacco

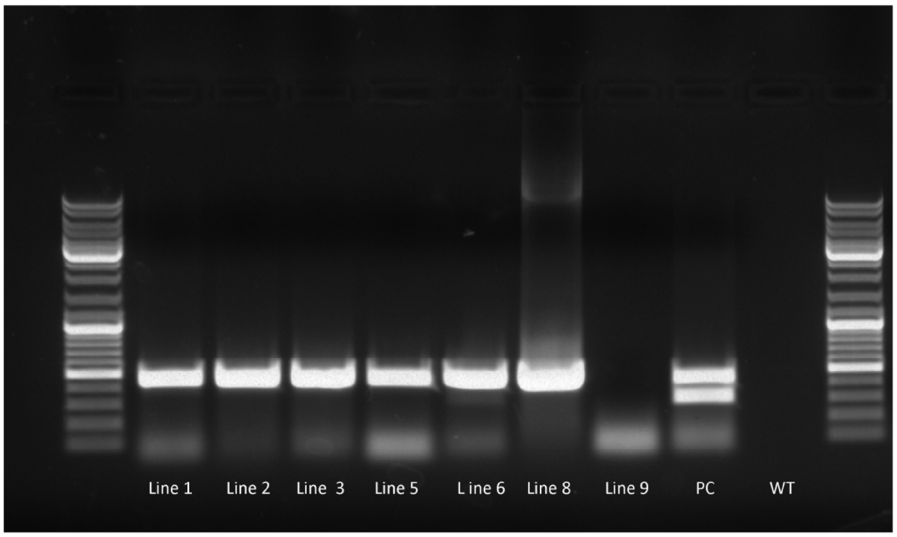

Figure 3. 35. PCR amplification of the hptII gene in T_1 transgenic tobacco (495 bp)

3.2.4.2. Physiological and biochemical analysis of transgenic tobacco plants

The expression of *TaSTR* gene in putative T_0 transgenic tobacco plants was evaluated by using rooting medium supplemented with 200 mM NaCl and 250 mM NaCl. T_0 plant and tissue culture grown wild type plant were cultured in these medium for 4 weeks and photograph were taken from each plants. As seen in Figure 3.36A-D, putative transgenic plants could resist the high concentration of salt and wild type plant couldn`t. In addition to the normal growth, also they produced normal root.

Figure 3. 36. Expression analysis of T_0 plants under 200mM NaCl (A-T_0, B-Wild Type) and 250mM NaCl (C-Wild Type, D-T_0)

The self-pollinated seeds of independent transgenic lines were surface sterilized with 20% sodium hypochloride in a centrifuge tubes for 20 minutes and the seeds were grown in a medium containing 50mg/L hygromycin for 4 weeks (Figure 3.37). After this period surviving plantlets were counted and this data were used in Mendelian segregation analysis. Leaf samples from control and putative transgenic plants were tested for their ability of callus formation and shoot initiation on selective RMOP media supplemented with 50mg/L hygromycin, 1mg/L BA and 0.1mg/L NAA. As shown in Figure 3.38, leaf sample from wild type plant didn't produce any callus and shoot under selection pressure.

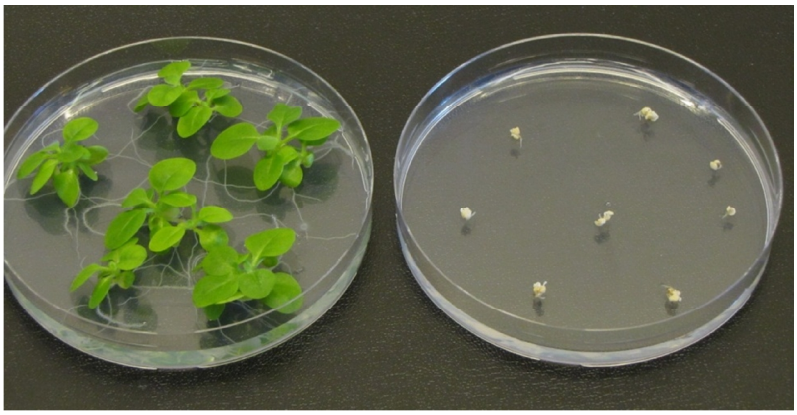

Figure 3. 37. Germination test with MS medium containing 50mg/L Hygromycin

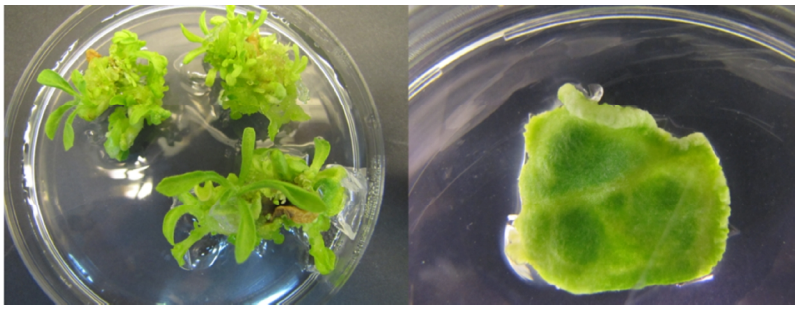

Figure 3. 38. Regeneration test by using 50mg/L Hygromycin

Another evidence showing the effect of salt stress in plants was the length of root. To measure root length of transgenic and wild type plants, seeds were germinated on MS medium and MS medium with 200mM NaCl for 6 weeks (Figure 3.40). Data were collected from only line 8 and control plants and totally 30 plants with triplicate were used. According to our results, wild type plants had significantly short root length when compared to transgenic line 8 under salt stress (Figure 3.39). This result correlated with literature also (Xu *et al*., 2010). Since, they also observed that both the height and root length of transgenic plants were higher than those of the control plants after exposure to 0.8% NaCl. Actually there were very high standard errors of mean in our study. It was very difficult to separate the root as intact from medium and this reason could be explain this error. As a consequence, long root length of *TaSTRG* expressed transgenic plants were a good evidence for the function of *TaSTR* gene under salt stress.

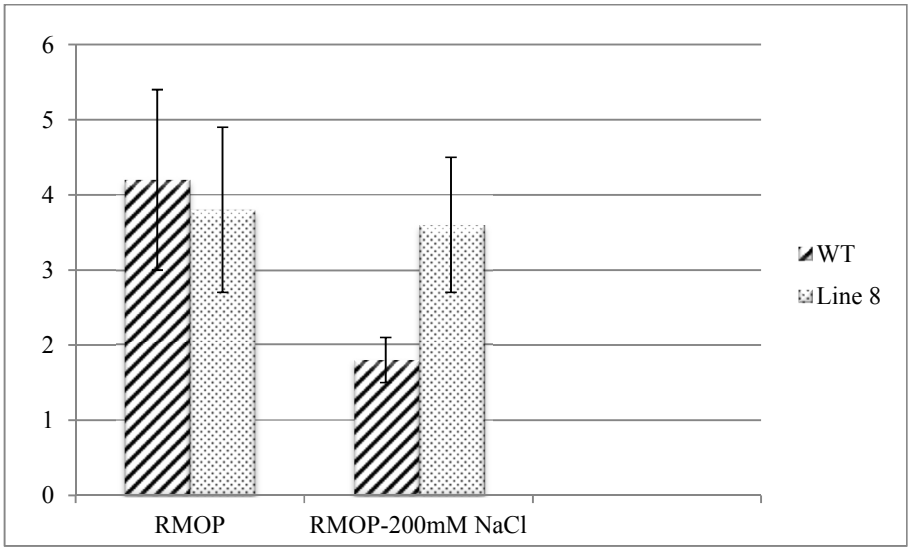

Figure 3. 39. Root length under normal and salt stress conditions

Figure 3. 40. Root length of transgenic and wild type plants (6 weeks old) germinated on MS medium supplemented with 200mM NaCl

The seeds coming from individual transgenic lines that express *TaSTR* gene at different levels were germinated on media containing 150mM NaCl and 200mM NaCl. The seedlings were grown in these media for 6 weeks. There is no significant difference between transgenic lines in terms of germination on media containing 150mM NaCl. However, wild type plants have lower germination rate than transgenic counterparts (Figure 3.41).

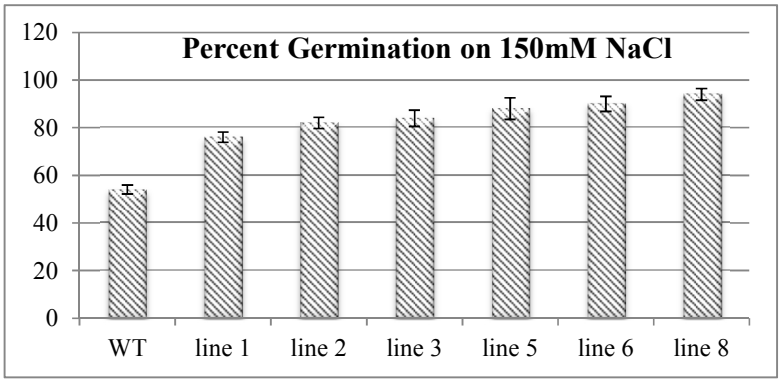

Figure 3. 41.Percent germination of control and transgenic tobacco plants under 150 mM NaCl treatment

The differences observed in 200mM NaCl containing medium were much. Except line 5, all of the transgenic lines had higher germination rates when compared to control lines. The best responding lines to salt stress were observed to be lines 3 and lines 8 with germination percentages of 76% and 79% on 200mM NaCl containing medium, respectively (Figure 3.42 and Figure 3.43). Germination rates of wild type and line 5 significantly lower than other transgenic lines (Figure 3.43).

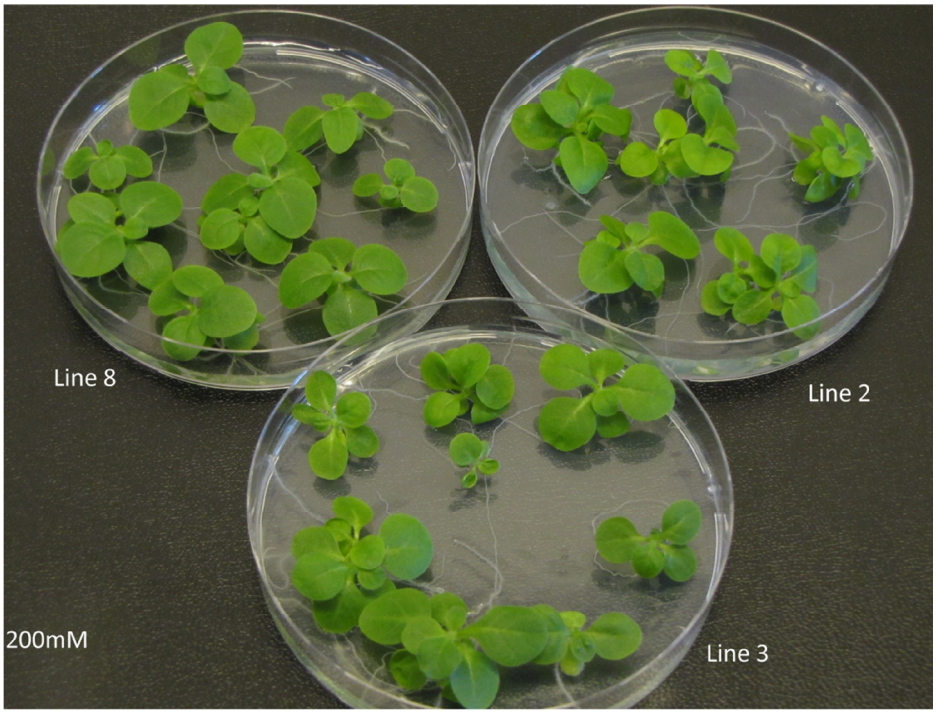

Figure 3. 42. Photographs transgenic tobacco plants under 200mM NaCl stress after 6 weeks of treatment

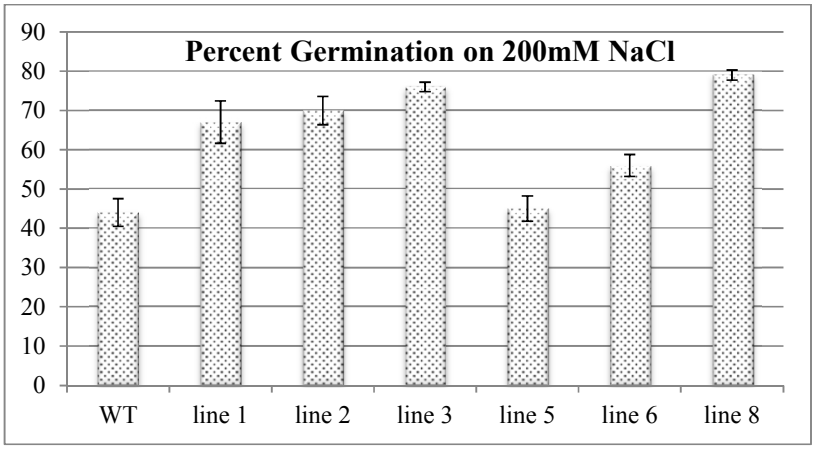

Figure 3. 43. Percent germination of control and transgenic tobacco plants under 200 mM NaCl treatment

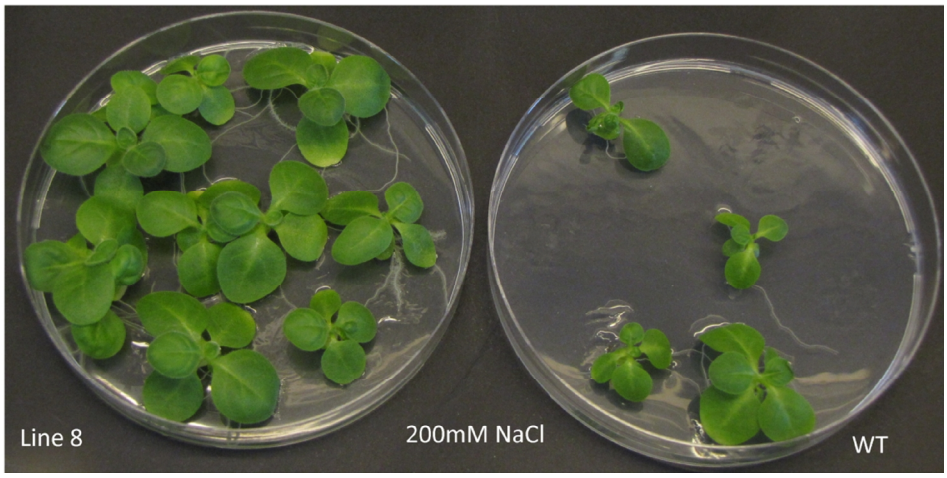

Figure 3. 44. Photographs of control and transgenic tobacco plants under 200mM NaCl stress after 6 weeks of treatment

3.2.4.2.1. MDA assay

Expression of *TaSTRG* was evaluated with MDA assay. Abiotic stresses conditions such as high salt concentration cause to the accumulation of reactive oxygen species that have detrimental effects on plant growth and survival. One of the effects of these radicals is the peroxidation of lipids. MDA is a byproduct of lipid peroxidation and thus a representative of membrane damage occurring under stress conditions. So, MDA level indicates the amount of lipid peroxidation in a tissue and can give an idea about the free radical damage that occurred in the cell. For this purpose, line 2 and line 8 were selected since they were phenotypically best lines under salt stress in preliminary studies. Seeds belong to transgenic lines and wilt type plant were germinated on MS medium supplemented with 200mM NaCl and 250mM NaCl for 6 weeks. According to MDA results, transgenic lines were less affected than control plants under salt stress, since they produced fewer MDA than control plants. There was significantly difference between control and wild types plants at both salt concentrations (Figure 3.45). However, there was no difference between transgenic lines under two different salt stress conditions. Selcuk (2004) and Xu *et al*.,(2010) also reported that transgenic tobacco lines expressing *STO* and *LbVAH* genes, had significantly lower MDA amount when compared to control plants under salt stress conditions.

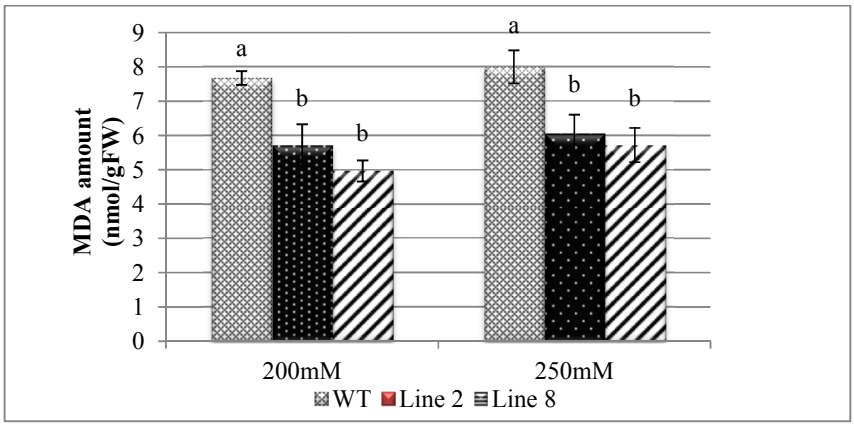

Figure 3. 45. MDA amount under salt stress

So all these results suggest that the salt tolerance of *TaSTRG*-transformed tobacco plants was improved compared with that of wild type plants. The role of *TaSTRG* was evaluated in rice by Zhou *et al*., (2009) under salt stress. Their results were correlated with our results because they showed that the expression of *TaSTRG* was strongly related with salt tolerance in rice. Therefore *TaSTRG* was a good candidate to generate salt resistant transgenic wheat plants in future.

3.3. Homotransplastomic tobacco plants

3.3.1. Chloroplast transformation vector

In this study modified PJF29-1 vector was used during chloroplast transformation. Xyl10B cassette from pJK101 was amplified with PCR and inserted into pJF29-1. Later, tobacco prrn promoter upstream of the Xyl10B cassette was replaced with rice prrn promoter. Activity of Xyl10B was tested with Western blot analysis by using *E. coli* cells carrying pJF29:Xyl10B-rps16 3'-UTR. Expression of *Xyl10B* was observed on blotting membrane (Figure 3.46).

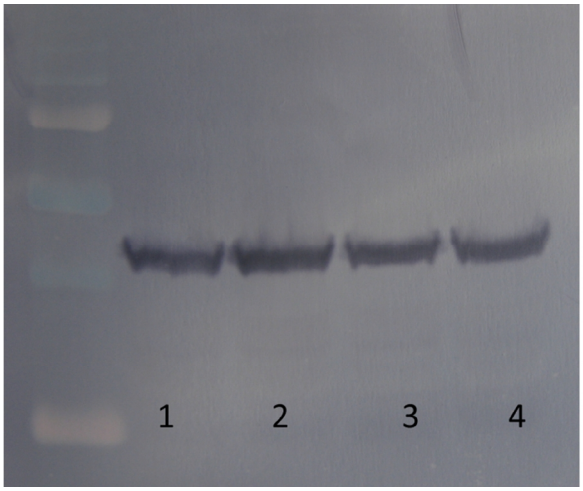

Figure 3. 46. Western blotting with *E. coli* proteins

In order to decrease unintended recombination with chloroplast genome and chloroplast transformation vector, new transformation vector was constructed. In new vector, all the promoters and terminators were selected from foreign species other than tobacco. In this context prrn promoter from rice (111bp), rps16 3'UTR from rice and psbA 3'UTR from soybean were synthesized by Genescript Company. psbA promoter was amplified with PCR by using Pfu Polymerase from rice genomic DNA (Figure 3.47). Also, trnA and trnI flanking sequence were used in new vector instead of rbcL and aacD region. For this purpose trnA (878bp) and trnI (800bp) was amplified from genomic DNA of tobacco with PCR.

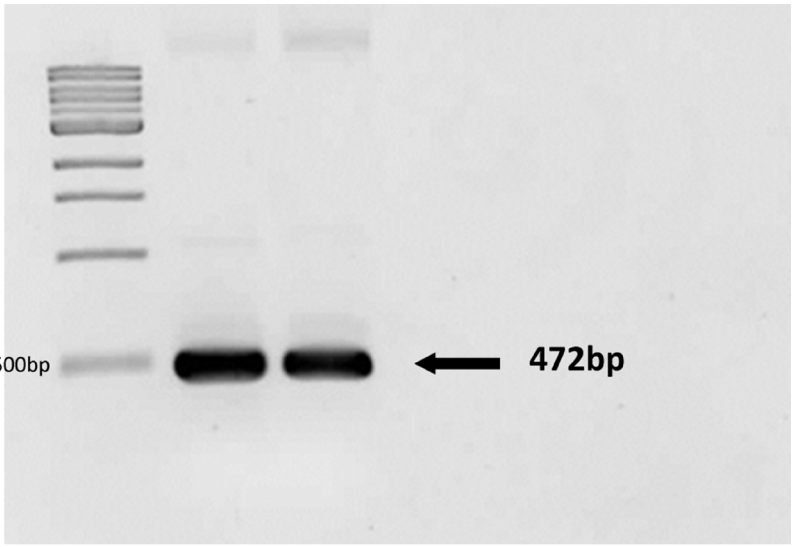

Figure 3. 47. Amplification of psbA promoter from rice genomic DNA

After production of components, the construction of new chloroplast transformation vector was started based on pWF401B and pWF211B. The main step was summarized at below.

- psbA 3`UTR in pWF401b was replaced with rice psbA 3' UTR by cutting BspHI and PsiI
- prrn promoter in pWF401b was replaced with psbA promoter with pmeI and NcoI digestion
- rps16 3`utr in pWF211b was replaced rps16 3`utr from rice with psiI and ascI digestion
- the new expression cassette constructed in previous step (prrn promoter-aadA-rps16 3`utr) was inserted into modified pWF401B
- Finally trnA and trnI amplified by PCR were inserted into resulting vector and final transformation vector was called as pMAK16.

3.3.2. Particle bombardment of tobacco leaves

Chloroplast transformations were carried out by using 4 weeks old tobacco leaves with particle bombardment (Bio-Rad PDS1000) in Department of Agronomy at University of Florida. Gold particles (0.6 μm) were coated with 5μg chloroplast transformation vector with two different coating agents, spermidine and protamine. In this study, modified pJF29 chloroplast transformation vector was used for the introduction of *Xyl10B* gene into the chloroplast genome. Prior to start chloroplast transformation study, tobacco prrn promoter of the pJF29 vector was replaced with rice prrn promoter. Therefore, during chloroplast transformation studies two types of pJF29 vectors, one of them had rice prrn promoter and the other one had tobacco prrn promoter at upstream of the *Xyl10B* gene were used. Totally 70 plates were bombarded with different constructs and coating agents in 4 independent experiment (Table 3.5). After biolistic delivery of the transgene, putative transplastomic tobacco plants were selected and regenerated in medium containing 500 mg/L spectinomycin They were cut into about 5x5mm piece and incubated on RMOP medium with subculturing at 2 weeks intervals (Figure 3.48A).

Table 3. 5. Chloroplast transformation experiments

Transformation number	**Coating agent**	**Plasmid**	**# of bombarded plates**
1	spermidine	pJF29 Tobacco prrn	6
	protamine		6
2	spermidine	pJF29 Tobacco prrn	5
	protamine		5
3	spermidine	pJF29 Rice prrn	10
	protamine		10
4	spermidine	pJF29 Tobacco prrn	5
		pJF29 Rice prrn	5
	protamine	pJF29 Tobacco prrn	4
		pJF29 Rice prrn	4

About 7-10 weeks after bombardment shoots were started to emerge on selection medium (Figure 3.48B). Emerging shoots were transferred into rooting medium (Figure 3.48C). In order to eliminate non-transformed chloroplasts, second cycle of selection was started with leaves of first cycle plants. Following two subsequent cycles of plant regeneration from leaf explants on 500 mg/L streptomycin and 500 mg/L spectinomycin containing media putative homotransplastomic plants were generated (Arai *et al*., 2004).

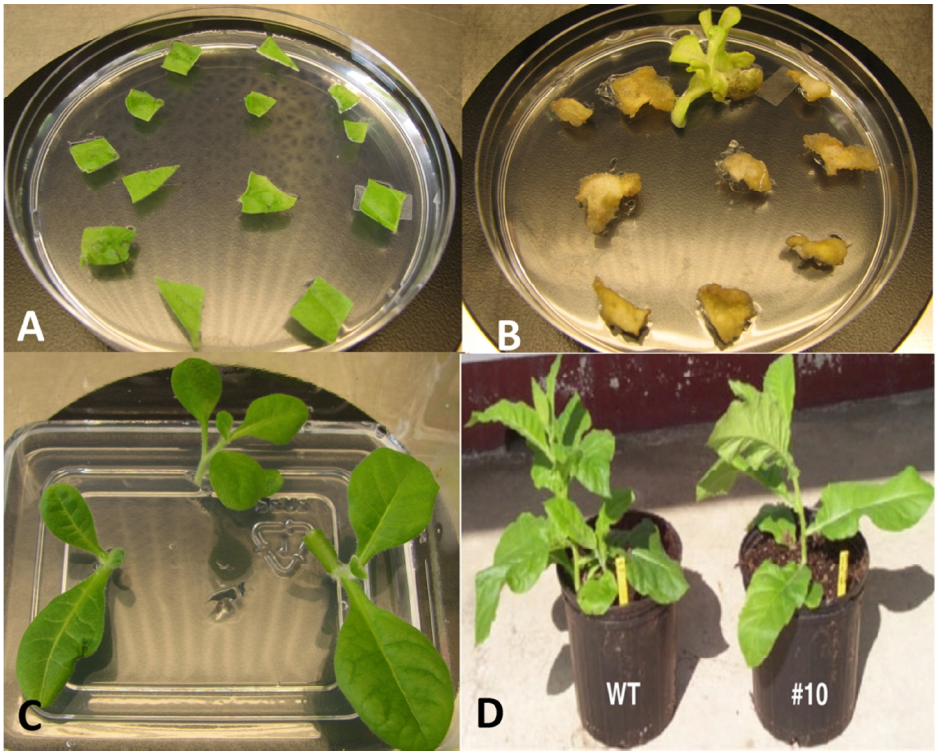

Figure 3. 48. Chloroplast transformation of tobacco leaves A. Transformed tobacco leaves incubated on RMOP medium B. Regenerated tobacco shoots after 7 weeks incubation on RMOP medium C. First cycle transgenic tobacco plants on rooting medium D. Growing wild type and transgenic tobacco plants after 3 weeks in soil.

3.3.3. Molecular analysis of homotransplastomic tobacco plants

Transgene integration into chloroplast genome was evaluated by using PCR and Southern blot analysis.

3.3.3.1. PCR analysis

In preliminary PCR analysis of first cycle tobacco plants, *aadA* gene specific primers (AaDF-AaDR) were used to screen the transgenic tobacco lines. In this study, 14 independent putative transgenic lines surviving in spectinomycin (500 mg/L) were

obtained (Table 3.6). According to the first PCR analysis, 9 lines produced expected bands with *aadA* specific primers. In second PCR analysis, this time *Xyl10B* gene specific primers were used. However, in this PCR analysis only line 1, 3, 10 and 11 were produced expected size. This results could be explained with unintended recombination, because unintended recombination and truncated insertion events was observed frequently during chloroplast transformation. Therefore, line 1, 3, 10 and 11 were used during further studies.

Table 3. 6. PCR analysis results of putative transgenic tobacco lines

Line	Coating reagent	Promoter	PCR result
1.	Spermidine	Rice prrn	+
2.	Spermidine	Tobacco prrn	-
3.	Spermidine	Rice prrn	+
4.	Spermidine	Tobacco prrn	-
5.	Spermidine	Tobacco prrn	-
6.	Spermidine	Tobacco prrn	-
7.	Protamine	Tobacco prrn	-
8.	Spermidine	Rice prrn	+
9.	Spermidine	Tobacco prrn	+
10.	Spermidine	Rice prrn	+
11.	Protamine	Rice prrn	+
12.	Spermidine	Tobacco prrn	+
13.	Spermidine	Rice prrn	+
14.	Protamine	Rice prrn	+

In further PCR analysis of transplastomic tobacco plants, 4 different primer pairs were used to confirm site directed integration. These are forward and reverse primers of Xyl10B (Xyl10BF-Xyl10BR), forward and reverse primers of aadA (AaDF-AaDR), TchF-AadR and Xyl10BF-TchR (Figure 3.49A). PCR using gene specific xyl10B (Figure 3.49B) and aadA (Figure 3.49C) primers confirmed the presence of these transgenes in the genomic DNA extract of transplastomic lines 1, 3, 10 and 11 in contrast to wild type (WT). In order to evaluate the transgene integration into the targeted integration site, tobacco chloroplast genome specific primers were designed to anneal to the upstream region of the rbcL targeted recombination site (TchF, Figure 3.49D) and the downstream region of the accD targeted recombination site (TchR, Figure 3.49E). A fragment of approximately 2.7 kb was amplified from transplastomic lines 1, 3, 10 and 11 via PCR (Figure 3.49D) using TchF/aadAR and an approximately 2.7 kb fragment was amplified from transplastomic lines 1, 3, 10 and 11 (Figure 3.49E) using TchR/Xyl10BF primers in contrast to wild type. These amplification products are consistent with the expected amplification products following integration of the transgene expression cassettes between the targeted rbcL and accD genes in the tobacco chloroplast genome.

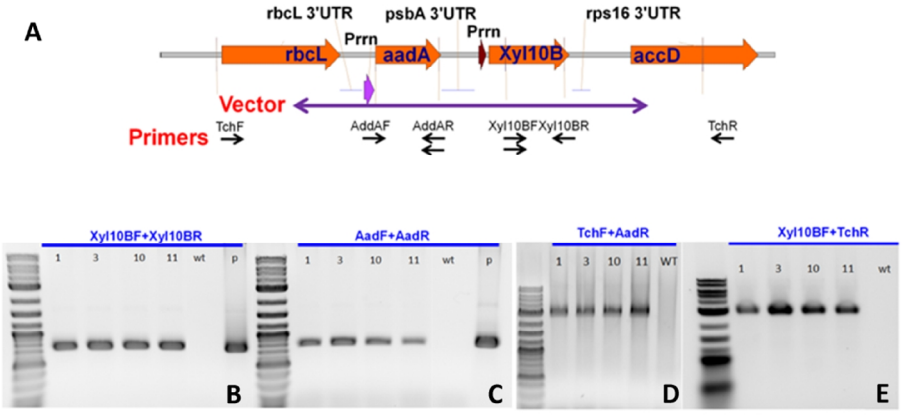

Figure 3. 49. Confirmation of transplastomic line by using PCR

3.3.3.2. Southern blot analysis

Transplastomic plants were also analyzed by Southern blot analysis. Genomic DNA was isolated from both transgenic tobacco leaves and wild type plant leaves. 10 µg of genomic were digested by using Sac I enzyme,because there was no SacI site in chloroplast transformation vector pJF29::aadA::xyl10B. However one Sac I site in each flanking region the wild type tobacco chloroplast genome within a distance of 10,644 nucleotides (Figure 3.49A). It was expected that integration of Xyl10B and aadA expression cassettes into the targeted integration site increase the size of Sac I digestion product to 13,613 nucleotides. ^{32}P-labeled probes (aadA, Xyl10B and rbcL) were hybridized with digestion. All transgenic lines (1, 10, 11) showed a hybridization product of approximately 14 kb in size with the aadA (Figure 3.50A) and xyl10B (Figure 3.50B). In contrast to transgenic lines, there was no hybridization product for wild type control plants. This results verify the integration of aadA and xyl10B genes into the tobacco genome. Hybridization with the rbcL probe (P3) also displayed a signal of approximately 14 kb in size from transplastomic plants 1, 10, and 11 and a signal in the wild type of approximately 10.5 kb. This result is consistent with the integration of the transgene expression cassettes into the targeted integration site. The 10.5 kb hybridization signal representing the wild type chloroplast genome fragment was absent in transplastomic plants suggesting a homotransplastomic status of these plants (Figure 3.50C)".

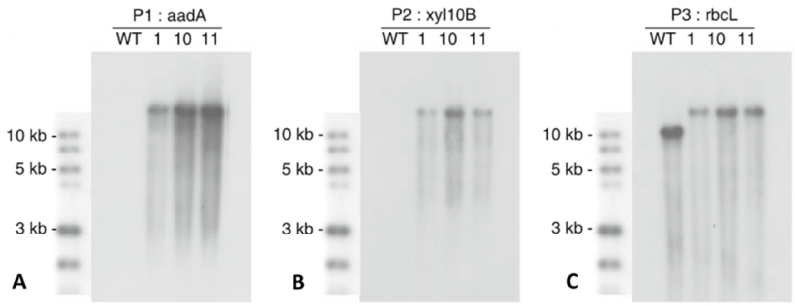

Figure 3. 50. Southern blot analysis of transplastomic tobacco plants (1, 10, 11) and wild type (WT)

As a result of chloroplast transformation studies, we can conclude that chloroplasts are attractive compartment for the expression of foreign proteins. So, they are good candidate to develop salt resistant transgenic wheat. The chloroplast transformation of wheat has been generally accepted as difficult due to lack of sufficient selection and regeneration system. But there is a new study showing the staple chloroplast transformation of wheat (Cui *et al*.,2011).

CHAPTER 4

CONCLUSION

In this study we aimed to generate salt resistant transgenic wheat and tobacco plants by using *TaNHX1* and *TaSTR* genes, respectively. For this purpose monocot and dicot overexpression vectors developed for both particle bombardment experiments and *Agrobacterium*-mediated gene transfer studies. In addition to the nuclear transformation, chloroplast transformation of tobacco was achieved by using particle bombardment.

In order to generate salt resistant transgenic wheat plants, *TaNHX1* gene was isolated from *Triticum aestivum* cv, Yüreğir-89. After cloning of *TaNHX1* gene, three different vectors were developed to use wheat transformation studies, both particle bombardment and *Agrobacterium*-mediated gene transfer experiments. During particle bombardment of wheat tissues, two types of overexpression vectors namely pMAK21 and pMAK22 were developed by using conventional restriction digestion and ligation methods. Wheat particle bombardment experiments were carried out by using immature embryo, immature embryo derived calli, mature embryo, mature embryo derived calli and immature inflorescence tissues by using Obitek Biolab Gene Transfer System. In this context totally 8960 explants were bombarded with two different selection constructs namely pMAK21 and pMAK22. Mannose selection was applied by using 10xg/L mannose during regeneration stage and phosphinotricin selection was applied during regeneration and rooting stage with 3-4mg/L L-PPT. Only two plantlets survived in 3 mg/L L-PPT after 8 weeks incubation and these plantlets were used for screening of transgene with PCR

analysis. Unfortunately, there was no amplification when PCR analysis was performed by using *TaNHX1* gene specific primers with genomic DNA isolated from survived plantlets. As a result of particle bombardment of wheat tissues, there was no staple transformation event after several trials.

Agrobacterium-mediated wheat transformations were carried out by using *Agrobacterium tumefaciens* AGL1 strain harboring pMAK26 carrying *TaNHX1*gene. Overexpression vector (pMAK26) used in *Agrobacterium*-mediated transformation was generated by using Gateway Cloning Technology. *Agrobacterium*-mediated transformation of wheat tissues were carried out by using immature embryo, immature embryo derived calli, mature embryo, mature embryo derived calli and immature inflorescence tissues with *Agrobacterium tumefaciens* semi empty AGL1 strain. In this manner, totally 5650 explants were used for *Agrobacterium*-mediated transformation of wheat tissues. Selection was applied by using Hygromycin (25mg/L) during regeneration and rooting stage. As a result of *Agrobacterium*-mediated transformation of wheat tissues, there was no staple transformation event. Although there was no staple transformation event, *TaNHX1* gene was isolated from Turkish cultivars for the first time.

In second part of the study salt resistant transgenic tobacco plants were obtained from *Agrobacterium*-mediated transformation by using AGL1 strain carrying *Triticum aestivum* salt tolerance related gene (*TaSTRG*). Presence of transgenes was confirmed with PCR analysis by using genomic DNA isolated from T_1 progeny. PCR results showed that lines; 1, 2, 3, 5, 6 and 8 genomes have both *hptII* and *TaSTR* genes. T_1 progeny showed segregation of the transgenes in a typical Mendelian fashion in most of the plants. Expression of *TaSTRG* in tobacco was evaluated by physiological and biochemical analysis, such as germination test, root length and MDA analysis. According to germination test under 200 mM NaCl stress, wild type plants, line 5 and line 6 showed significantly lower germination rate when compared to other transgenic lines. Also malondialdehyde content of transgenic lines (line 2 and line 8) were significantly lower than wild type plant under 200 mM NaCl

and 250 mM NaCl salt stress conditions. Therefore, results of physiological assay showed that salt tolerance of *TaSTRG*-transformed tobacco plants was improved when compared to wild type plants.

In the third part of study, chloroplast transformation of tobacco was carried out by using 4 weeks old tobacco leaves. Two types of chloroplast transformation vectors were used in this experiment. One of them was modified pJF29 and the other one was pMAK16. All the chloroplast transformation studies were performed with modified pJF29 harboring *Xyl10B* gene. pMAK16 were designed for further transformation to decrease the unintended recombination. Chloroplast transformation vector pJF29 was inserted into chloroplast genome by using particle bombardment technique and transformed chloroplast cells were selected by using 500 mg/L Spectinomycin. In order to decrease heteroplasmy, two additional cycle of selection were performed by using both 500 mg/L Spectinomycin and 500 mg/L Streptomycin. Stable integration of transgenes and homoplasmy were confirmed with PCR and Southern blotting. PCR and Southern blot analysis showed that homotransplastomic tobacco lines were produced during bombardment studies.

The studies to be conducted in the future would include:

i. Wheat transformation will be carried out with different cultivars.
ii. *TaSTR* gene will transferred into the wheat tissues to develop salt resistant transgenic wheat plants.
iii. Expression analysis of *TaNHX1* under salt stress will be evaluated by using qPCR.
iv. Chloroplast transformation of wheat will be carried out to develop salt resistant transgenic plants.

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APPENDIX A

TRANSFER AGREEMENT FOR THE PLASMIDS AND BACTERIA

MATERIAL TRANSFER AGREEMENT FOR RESEARCH-ONLY PURPOSES

Huseyin Avni Öktem, an employee of Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey, ('the Recipient') wishes to obtain certain tangible materials and/or information from Wendy Harwood & Matthew Perry of the John Innes Centre as described on the reverse side ('the Materials') for the sole purpose of conducting the research specified on the reverse side ('the Research'). The Recipient acknowledges that all rights to the Materials, whether directly or indirectly enclosed therein as well as extracts, replications, summaries, or derivatives thereof, are the sole property of the John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK ('JIC') and warrants not to use the Materials for any form of commercial exploitation howsoever.

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1. Not to transfer or distribute any part of the Materials or any extracts, replications, summaries, or derivatives thereof to any third party howsoever.
2. Not to use any part of the Materials or any extracts, replications, summaries, or derivatives thereof for any other purpose than the Research.
3. Not to disclose any information whatsoever with regards to the Material and use thereof, without the prior written approval of JIC.
4. To acknowledge the contribution of [Wendy Harwood & Matthew Perry / JIC / the Biotechnology and Biological Sciences Research Council (BBSRC)] in any publication that may result from use of the Materials.
5. To hold harmless JIC and its governors, officers, employees and agents from any and all liabilities or claims brought by third parties resulting from the transfer to and use of the Materials by the Recipient.
6. This Agreement is personal to the Recipient and not capable of assignment.
7. This Agreement is subject to English Law and exclusive interpretation by the English Courts

Please, have (an) authorized officer(s) of the Middle East Technical University signify the Recipients acceptance of the above by signing and dating two copies of this Agreement and return both copies to Mary Anderson, Contracts Manager, John Innes Centre, Norwich Research Park, Colney, Norwich, NR4 7UH, UK. Upon receipt of two completed and executed copies of this Agreement the Materials will be then be sent to the Recipient.

On behalf of and for Middle East Technical University Date: March 26, 2003	Huseyin Avni Öktem Date: March 25, 2003
Signature: [signature]	[signature]
Name (print): AHMET ACAR	HÜSEYIN AVNI ÖKTEM
Title: PROF. DR., VICE-PRESIDENT	PROF. DR.

LIST OF THE MATERIALS

Plasmid pAL156, developed in the Crop Genetics department by D. Lonsdale.
pAL156 is an *Agrobacterium tumefaciens* binary vector carrying the BAR selectable marker and the GUS reporter genes.

Plasmid pAL154, developed in the Crop Genetics department by D. Lonsdale.
pAL154 is an *Agrobacterium tumefaciens* binary vector; a derivative of pSoup carrying the Kumari fragment.

DESCRIPTION OF THE RESEARCH

The material will be used in the optimisation of *Agrobacterium*-mediated transformation conditions for local wheat cultivars. Experiments will be conducted on mature & immature embryos, immature inflorescence and callus explants.

APPENDIX B

BACTERIAL CULTURE MEDIA

MG/L Medium (1L)

Mannitol	5 g
Yeast extract	2.5 g
Glutamic acid	1 g
$MgSO_4.7H_2O$	0.1 g
KH_2PO_4	0.25 g
NaCl	0.25 g
Tryptone	5 g

The pH of the medium was adjusted to 7.0. After autoclaving the medium at 121°C for 20 minutes, sterile Biotin was added to the medium (final concentration is 1 μg/L).

LB Medium (1L)

Bacto-Tryptone	10xg
Bacto-yeast extract	5 g
NaCl	10xg

SOC Medium (1L)

Bacto-tryptone	20xg
Bacto-yeast extract	5 g
NaCl	0.5 g
1M KCl	2.5 ml

Adjust pH to 7.0 with 10N NaOH, autoclave to sterilize, add 20 ml of sterile 1 M glucose immediately before use

APPENDIX C

SEQUENCE INFORMATION OF *TaNHX1* GENE

5’-

tcgcctcgcatccagactccagacctttctctcctctcctctcccccacacccctctccagatccccgcggactgtcaagcagctg
aatctctcgtccggcccaggctgccggaggccgggggagaccgggatccgccgaggtggcgaccggcatggggctcgatttgg
gagccctcgctctcaagtacaccgggctggcggtgtcggaccacgactccatcgtcgccatcaacatcttcatcgcgctgctctgcg
gctgcattgtcttcggccacctgctcgaggggaaccgctgggtcaatgagtccaccaccgcgcttgtcctgggcctcatcaccggtg
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tgacagagaagttgctatcatgatactcatggcatacctttcgtatatgctgtcaatgctgctggatctgagtggcattctaaccgtgttctt
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gcaagtgtatttcttgtaaatgctcagatatcactcagttttgctcttgggattctttcggtgtattatcgctatttgttggtgtatattgtgcag
ataatttctacaggaccatgtacgctgtgtagagttttgggagccattcaagatgtacaattaatctgtaaattaatctcgttcaaaaaaaa
aaaaaaaaaaaaaa-3’

APPENDIX D

SEQUENCE INFORMATION OF TaSTR GENE

5'-

cccgggccttggggcgcgccggtaccttaattaaatggaactcctctcctacgctgccatgtgcctctcgctggcgtttctt
ctccattcctacgccgcggcgacggcgccaatcggcatgcttgagcgggagaccaagcagcaaatcatagcgagcatc
ccgccgcacgggcaggagaaccccgtgctattcctaacgtcgccgtccggcaagtacgcggcgcacttcatgcgcagc
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taggaatcggagttgcactgggtgtcagctctgccactgtcatggccctcggtatctgagcggccgcgtttaaacactagtt
tataaaagctt-3'

APPENDIX E

The PREPARATION of TRIZOL REAGENT

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

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

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

APPENDIX F

PCR CONDITIONS AND CYCLING PARAMETERS FOR *TaNHX1*

PCR conditions for *TaNHX1* amplification

Pfu Buffer with $MgSO_4$ (10x)	5µL
Forward primer (10µM)	4µL
Reverse primer (10µM)	4µL
dNTPmix (2mM)	5µL
Template cDNA	3µL
Pfu polymerase (2.5 u/µl)	1µL
Nuclease-free water	28µL
Total	50µL

PCR cycle parameters for *TaNHX1* amplification

Step	Temperature °C	Time	Number of cycles
Initial denaturation	94	5 min	1
Denaturation	94	1 min	35
Annealing	63	30 sec	
Extension	72	4 min	
Final extension	72	7 min	1

APPENDIX G

COMPONENTS AND VOLUME OF LIGATION REACTION

Component	**Volume**
2X Reaction Buffer	10µL
PCR product	3µL
pJET1.2/blunt Cloning Vector (50 ng/µL)	1µL
Nuclease-free water	5µL
T4 DNA Ligase	1µL
Total volume	20µL

APPENDIX H

COMPOSITION OF BUFFER TO PREPARE CHEMICALLY COMPETENT *E.COLI* CELLS

Composition of Transformation buffer 1

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	2.0xg	50 mM
Glycerol	30 ml	15% v/v

For 200ml. pH:5.8 and filter sterilized.

Composition of Transformation buffer 2

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

For 100ml. pH 6.5 with dilute NaOH and Filter Sterilize.

APPENDIX I

COLONY PCR CONDITIONS AND CYCLING PARAMETERS

PCR conditions for colony PCR

Taq Polymerase Buffer (10x)	2µL
Forward primer (10µM)	1µL
Reverse primer (10µM)	1µL
dNTPmix (2mM)	2µL
$MgCl_2$(25mM)	2µL
Taq polymerase (2.5 u/µl)	0,5µL
Nuclease-free water	11,5µL
Total	20µL

Cycling parameter for colony PCR

Step	**Temperature °C**	**Time**	**Number of cycles**
Initial denaturation	95	3 min	1
Denaturation	94	30sec	25
Annealing	50	30 sec	
Extension	72	1 min	
Final extension	72	5 min	1

APPENDIX J

SCHEMATIC MAP OF pAHC25

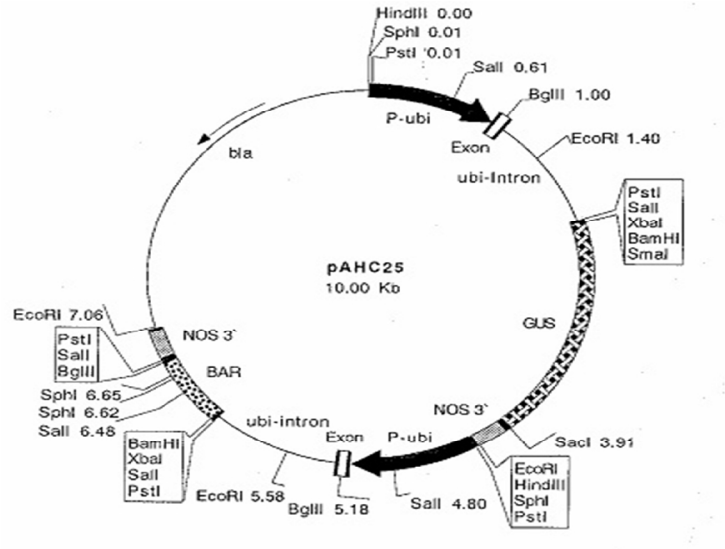

Figure J. 1. Map of pAHC25

APPENDIX K

PCR CONDITIONS AND CYCLING PARAMETERS OF UBI1 CLONING

PCR conditions for Ubi 1 cloning

Pfu DNA polymerase reaction buffer (10x)	5µL
Forward primer (10µM)	1µL
Reverse primer (10µM)	1µL
dNTPmix (2.5mM)	8µL
Template cDNA (564ng/ul)	1µL
Pfu DNA polymerase (2.5 u/µl)	0,5µL
Nuclease-free water	33,5µL
Total	50µL

Cycling parameters for Ubi1 cloning

Step	**Temperature °C**	**Time**	**Number of cycles**
Initial denaturation	94	4 min	1
Denaturation	94	1min	30
Annealing	60	45 sec	
Extension	72	2 min	
Final extension	72	10 in	1

APPENDIX L

PCR CONDITIONS AND CYCLING PARAMETERS FOR *TANHX1* AND *TaSTRG* IN GATEWAY CLONING

PCR conditions for *TaNHX1* and *TaSTR* gene cloning

Pfx Amplification Buffer (10x)	5µL
$MgSO_4$(50mM)	1µL
Forward primer (10µM)	4µL
Reverse primer (10µM)	4µL
dNTPmix (10mM)	1.5µL
Template DNA (pJET1.2/NHX1-pUC57-*TaSTRG*)	3µL
Pfx polymerase (2.5 u/µl)	1µL
Nuclease-free water	30,5µL
Total	50µL

Cycling parameters for *TaNHX1* and *TaSTR* gene cloning

Step	**Temperature °C**	**Time**	**Number of cycles**
Initial denaturation	94	3 min	1
Denaturation	94	30 sec	
Annealing	60	30 sec	
Extension	68	2 min(*TaNHX1*) 1 min(TsSTRG)	30
Final extension	72	7 min	1

APPENDIX M

PCR CONDITIONS AND CYCLING PARAMETERS FOR SCREENING OF TRANSPLASTOMIC PLANTS

PCR conditions for *TaNHX1* and *TaSTR* gene cloning

Buffer (10x)	2.5 µL
$MgCl_2$(50 mM)	1.25 µL
Forward primer (10 µM)	1 µL
Reverse primer (10 µM)	1 µL
dNTPmix (2.5 mM)	0.5 µL
Genomic DNA (20 ng/µL)	1 µL
Stratagene DNA polymerase (2.5 u/µl)	0.25 µL
Nuclease-free water	17.5 µL
Total	25 µL

Cycling parameters for *TaNHX1* and *TaSTR* gene cloning

Step	**Temperature °C**	**Time**	**Number of cycles**
Initial denaturation	94	3 min	1
Denaturation	94	1 min	30
Annealing	60	45 sec	
Extension	72	1 min 1 kb	
Final extension	72	4 min	1

VITA

PERSONAL INFORMATION

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Degree	Program	University	Year
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Master Thesis :

Optimization of tissue culture, regeneration and *Agrobacterium*-mediated transformation parameters in winter wheat cultivars

Ph.D Thesis:

Development of Salt Resistant Transgenic Plants by Using *TaNHX1* and *TaSTR* Genes

Work Experience:

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Research Assistant	METU Department of Biology	2002-2011

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PUBLICATIONS:

1. **M. KAVAS, H.A. ÖKTEM and M. YÜCEL** "Factors affecting plant regeneration from immature inflorescence of two winter wheat cultivars " Biologia Plantarum 52 (4): 621-626, 2008.
2. **U.Ç. AKÇAY, O. ERCAN, M. KAVAS, L. YILDIZ, C. YILMAZ, H.A. ÖKTEM, M. YÜCEL** " Drought-induced oxidative damage and antioxidant responses in peanut (*Arachis hypogaea* L.) seedlings " Plant Growth Regul (2010) 61:21–28
3. **JAE Y KIM, MUSA KAVAS, WALID M FOUAD, GUANG NONG, JAMES F PRESTON, FREDY ALTPETER** "Production of hyperthermostable GH10 xylanase *Xyl10B* from *Thermotoga maritima* in transplastomic plants enables complete hydrolysis of methylglucuronoxylan to fermentable sugars for biofuel production" Plant Molecular Biology (2011) 76 :357-369

BOOK: Bayrac AT, Baloglu MC, Kalemtas G, Kavas M,(2007) Genetically Modified Organisms, MetuPress, 2007