TRANSFORMATION OF NICOTIANA TABACUM PLANTS WITH Na^+/H^+ ANTIPORTER (AtNHX1) GENE ISOLATED FROM ARABIDOPSIS THALIANA FOR EVALUATION OF SALT TOLERANCE

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ABSTRACT

TRANSFORMATION OF *NICOTIANA TABACUM* PLANTS WITH Na^+/H^+ ANTIPORTER (*AtNHX1*) GENE ISOLATED FROM *ARABIDOPSIS THALIANA* FOR EVALUATION OF SALT TOLERANCE

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Large, membrane-bound vacuoles of plant cells are suitable organelles for the compartmentation of ions. These vacuoles contain Na^+/H^+ antiporters for movement of Na^+ within the organelle in exchange for H^+. They provide an efficient mechanism to prevent the occurance of detrimental outcomes of Na^+ accumulation in the cytosol. Identification of *AtNHX1* gene that confers resistance to salinity by expressing a Na^+/H^+ antiport pump facilitates the understanding of the salt stress tolerance mechanisms of plants.

The aim of the present study was to isolate and clone the *Arabidopsis thaliana AtNHX1* coding sequence for transformation of *Nicotiana tabacum* plants via *Agrobacterium tumefaciens* mediated gene transfer. For this purpose, total RNA was isolated from *Arabidopsis thaliana* plants and cDNA synthesis was performed. *AtNHX1* (1614bp) was amplified by using cDNA of *Arabidopsis* via specific primers. The amplified PCR product was verified by

sequencing. *AtNHX1* coding sequence was cloned into the plant transformation vector pCVB1 and 10 independent putative transgenic tobacco plants were obtained via *Agrobacterium tumefaciens* mediated gene transfer sysytem. Transfer of selected 8 putative transgenic plants to soil provided the regeneration of T_1 seeds. Germination of the seeds under different salt treatments (0, 50, 100, 150, 200, 250 mM NaCl) was observed for evaluating the salt tolerance of transformed plants. The 82% and 60% of the transgenic T_1 seeds were germinated on 150 mM NaCl and 200 mM NaCl containing media, respectively. In contrast the germination percentage of wild type tobacco seeds under 150 mM NaCl and 200 mM NaCl concentrations were 39% and 21%, respectively. The germination rate of the transgenic T_1 seeds were significantly higher (p=0,001) when compared to the control seeds especially under high salt stress conditions (150 and 200 mM NaCl). Taken all together, our results demonstrated that the germination efficiencies and growth of the plants transformed with *AtNHX1* were higher than the wild type tobacco plants under high salt concentrations.

Key words: Salt Stress, *AtNHX1, Arabidopsis thaliana, Nicotiana tabacum,* Na^+/H^+ (pump) antiporter

ÖZ

ARABIDOPSIS THALIANA BİTKİSİNDEN İZOLE EDİLEN Na^+/H^+ ANTIPORTER (*AtNHX1*) GENİNİN *NICOTIANA TABACUM* BİTKİSİNE AKTARILARAK TUZ DİRENCİNİN DEĞERLENDİRİLMESİ

AYSİN, Ferhunde

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Bitki hücreleri sahip oldukları büyük ve kendisini çevreleyen zarıyla bağlantılı kofulları sayesinde iyonların buralarda toplanmasına yapısal olarak oldukça uygundurlar. Bazı bitkiler kofullarında bulunan iyon pompaları sayesinde kolaylaştırılmış difüzyonla protonları dışarı vererek sodyum iyonlarının aktif olarak koful içine alınımını kolaylaştırır. Bu şekilde bitki sodyum iyonlarının zararlı etkilerinden korunmuş olur. Bir Na^+/H^+ iyon pompası kodlayarak bitkilere tuz direnci kazandıran *AtNHX1* geninin tanımlanmasıyla bitkilerdeki tuz toleransı mekanizmaları anlaşılmaya başlamıştır.

Bu çalışmanın amacı *Arabidopsis thaliana* bitkisinde bulunan *AtNHX1* geninin izole edilip klonlandıktan sonra *Agrobacterium tumefaciens* aracılığıyla gen aktarım yöntemi kullanılarak *Nicotiana tabacum* bitkisine

aktarılmasıdır. Bu amaçla *Arabidopsis* bitkisinden RNA izolasyonu yapılmış ve bitkiye ait cDNA sentezlenmiştir. *AtNHX1* (1614bp) cDNA sekansına uygun olarak dizayn edilmiş primerler yardımıyla amplifiye edilmiş ve gen diziliminin doğruluğu sekans analizi yapılarak belirlenmiştir. *XbaI* ve *BamHI* restriksiyon enzimleri kullanılarak yapılan kesim işleminin ardından gen bitki transformasyon vektörüne (pCVB1) klonlanmış ve *Agrobacterium tumefaciens* gen aktarım yöntemi kullanılarak 10 adet transgenik adayı bitki elde edilmiştir. Bu bitkilerden 8 tanesinin toprakta büyütülmesiyle elde edilen tohumların farklı tuz konsantrasyonuna (0, 50, 100, 150, 200, 250 mM NaCl) sahip ortamda çimlendirilmesinin ardından T_1 bitkilerinin tuza karşı direnç geliştirdikleri saptanmıştır. 150 mM NaCl ve 200 mM NaCl içeren ortamda çimlendirilen transgenik tohumların sırasıyla ortalama %82`si % 60`ı çimlenmesine rağmen gen aktarılmamış kontrol bitkilerinin tohumlarının çimlenme oranı ise sırasıyla ortalama %39 ve %21`dir. T_1 tohumlarının farklı tuz konsantrasyonlarında çimlenme miktarlarının istatistiksel olarak analiz edilmesiyle transgenik adayı bitki tohumlarının kontrol bitkilerinin tohumlarına göre daha iyi çimlendiği bulunmuştur ($p=0.001$). Elde edilen bulgular bir bütün olarak değerlendirildiğinde, transgenik bitkilerin kontrol bitkilerine oranla özellikle de yüksek tuz konsantrasyonuna sahip ortamda daha iyi çimlenebilme ve gelişebilme özelliği gösterdiği sonucuna ulaşılmıştır.

Anahtar Kelimeler: Tuz stresi, *AtNHX1, Arabidopsis thaliana, Nicotiana tabacum,* Na^+/H^+ iyon pompası

to my dear family...

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LIST OF ABBREVIATIONS

Ap	Ampicillin Resistance Gene
At	*Arabidopsis thaliana*
ATP	Adenosine Triphosphate
BA	Benzyl Aminopurine
BastaR	Basta Resistance Gene
CaMV35S	Cauliflower Mosaic Virus 35S Promoter
CTAB	Cetyl Trimethyl Ammonium Bromide
DEPC	Diethyl Pyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Tri-Phosphate
EDTA	Ethylene Diamine Tetra Aceticacid
ETOH	Ethanol
LB	Luria-Bertani Broth
MS	Murashige and Skoog
NAA	Naphtalene Acetic Acid
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PPT	Phosphinotricin
rpm	Revolution per Minute
ROS	Reactive Oxygen Species
T-DNA	Transferred DNA
TAE	Tris-Acetate EDTA
TBE	Tris-Borate EDTA
TE	Tris EDTA Buffer
YEB	Yeast Extract Broth

CHAPTER I

INTRODUCTION

1.1. Stress Factors Affecting Plant Growth and Development

Stress is an unfavorable factor that usually stems from the external environment and leads to negative impacts on plant development such as reduction in photosynthetic activity, decrease in productivity and general vigor, or retardation of overall plant growth and development in an inconvenient manner. Plants are exposed to several types of biotic and abiotic stress factors (Figure 1.1, Fujita *et al.*, 2006). Abiotic stress factors comprise water deficit (drought), elevated salt concentration (salinity), extremes of heat and cold (freezing, chilling or heat shock), sudden changes in temperature, lack of oxygen in soil due to flooding, formation of reactive oxygen radicals and exposure to strong light. On the other hand, biotic stresses are infections or competition by other living organisms (Srivastava 2002; Kotuby *et al.*, 1997; Chen and Murata, 2002; Knight and Knight, 2001). Stressed plants are also attacked by pests and pathogens due to the deficient defence mechanisms. Plants have evolved to sense these stresses and to deal with them by means of some evolutionarily conserved mechanisms (Chen and Murata, 2002).

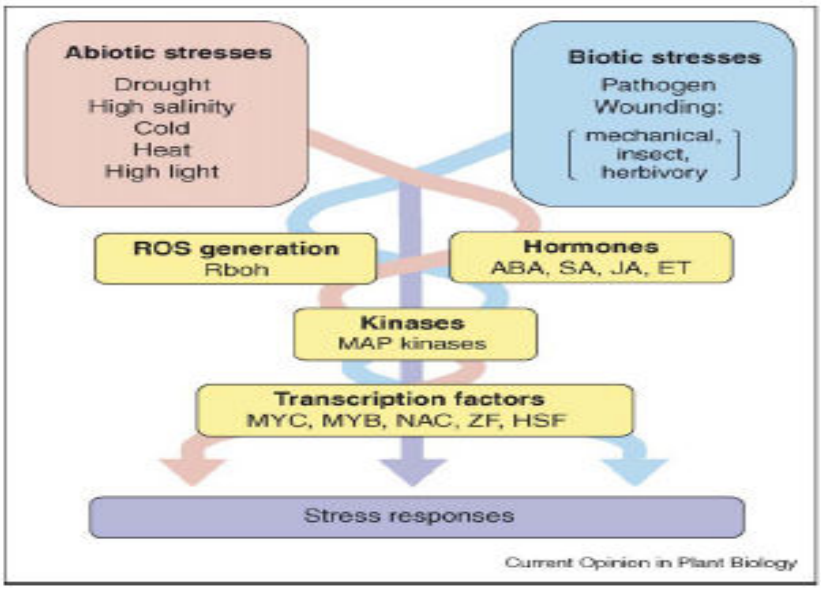


Figure 1.1. Environmental Stress Factors Affecting Plants (Fujita *et al.*, 2006, *Current Opinion in Plant Biology*)

The severity of the stress effect on cellular components depend on the equilibrium between the damage and the repair rate. When the repair degree of a given component is quicker than the rate of damage, no damage can easily be seen. This standard pattern is already present in plants under non-stress conditions. When the repair rate is lower than the damage rate, damage becomes apparent and this situation is generally seen in plants under stress conditions (Chen and Murata, 2002).

1.1.1. Water Stress (Osmotic Stress)

Environmental stresses emerge many different ways, but almost all prevailing stresses show their signs on plant water acquisition mechanisms. Water has important impacts on plants due to its biological roles as a solvent, a transport medium, an electron donor, and an evaporative coolant. Environmental conditions generally impair these important functions. Although plant species differ in their sensitivity and response to the reduction in water

potential caused by drought, low temperature, or high salinity, the encoded capabilities of all plants for stress perception, signaling, and response mechanisms are pretented to be similar (Bohnert *et al.*, 1995; Turco *et al.*, 2004).

Water deficit (Figure 1.2, Bohnert *et al.*, 1995), is the consequence of limited soil moisture level and water loss by transpiration, is the most important stress that plants receive. Lack of water is also a result of the increased soil salt concentrations or freezing temperatures. Salinity diminishes the free water content (water potential) of the soil, thus lowers quantity of water taken by roots from soil and brings about physiological drought. Extremely low temperatures also decrease the free water potential. Ice formation results in loss of intracellular water to the apoplast, causing dehydrative damage. Drought, salinity, and freezing are distinct factors, but both cause dehydration of cells and tissues and accompanying damage to membranes and macromolecules in common (Srivastava, 2002; Xiong and Zhu, 2002).

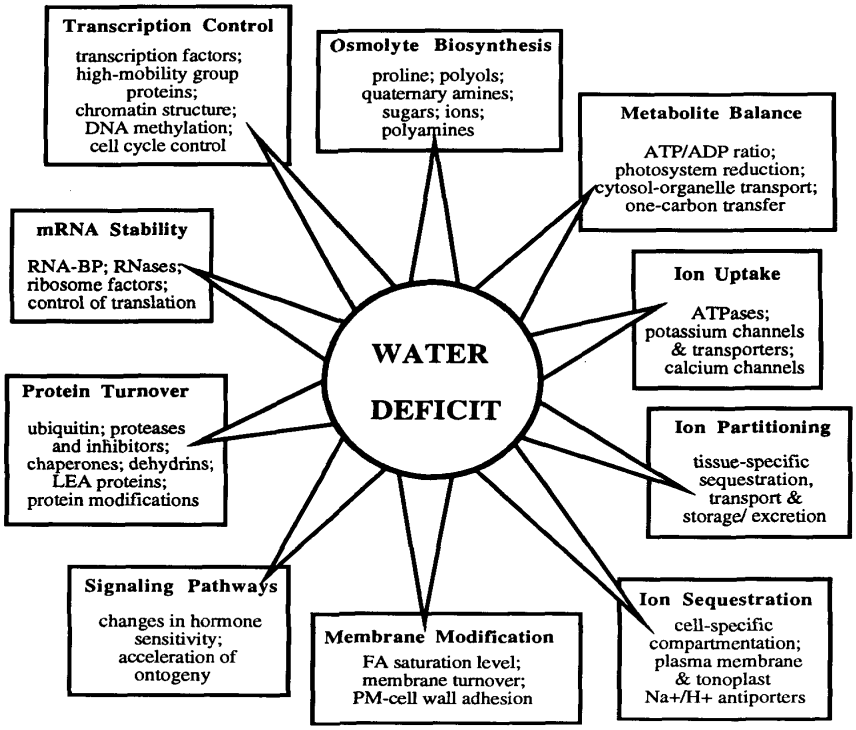


Figure 1.2. Responses against deficiency of water (Bohnert *et al.*, 1995, *The Plant Cell*)

1.2. Soil Salinity

Total amount of soluble salts found in soil is defined as the "soil salinity". So if salinity levels rise, plants take small amount of water from soil, intensifying water stress conditions. Enhanced soil salinity can also lead to nutrient imbalances, end up with the increase in the quantity of elements poisonous to plants and when the level of one salt element-generally sodium (Na^+)-is higher than normal values, water uptake decreases. In many areas, salinization of soil is a limiting factor for plant growth (Kotuby *et al.*, 1997) and salinity also decreases the productivity and limits the agricultural expansion of previously uncultivated lands (Flowers *et al.*,1997).

Salts are general and essential components of soil, and many salts (e.g., nitrates and potassium) are necessary for plant nutrition. Main sources of salts

are mineral weathering, inorganic fertilizers, soil amendments (e.g., gypsum, composts and manures), and irrigation waters. Ice melters used on roads and sidewalks in winter are additional and important causes of landscape soil salinity. The augmentation of any completely soluble elements will enhance soil salinity. Plant growth is inimically affected when soluble salts are found in excessive amounts (Kotuby *et al.*, 1997).

Water-soluble salts are necessary elements of the soils. Essential elements for plant growth and development are absorbed from soil in the form of soluble salts. However, emergence of soil salinity due to excessive deposition of soluble salts result in supression of plant growth and development (Zahran *et al.*, 2007). In arid and semi-arid regions soil salinity is a limiting factor due to poor drainage and/or poor irrigation and these are the most general causes of soil salinization. During absorption of water from soil or evaporation of water, salts remain constant in the soil. Thus, soil becomes saline rich environments (Blaylock, 1994).

Calcium (Ca^{+2}), magnesium (Mg^{+2}), sodium (Na^{+}) cations and sulfate (SO_4^{-2}), chlorine (Cl^{-}) anions are the prevailing salts in many saline soils. Elevated Na^{+} levels or the balance among Na^{+}, Ca^{+2} and Mg^{+2} do not keep in the normal values soil crop agriculture can also be effected. The positively charged Na cations interact with the negatively charged clay particles in the soil and soils become sticky when wet, and hard and impermeable when dry (Wentz, 2001).

1.3. Salt stress

Osmotic effect is the general obstructive force in saline environments. In this situation, soluble salts increase the energy of soils to hold water so water is confined in the soil. Thus plants expend more energy to obtain water from soil but plants also require energy for growth, flowering and fruiting. If the energy requirements for water absorbtion increases, plant growth will be interrupted

and growth reduction will occur. As salt concentration increases, water becomes increasingly difficult for the plant to absorb. A plant can really die from water stress or drought in a moist soil if the salt concentration becomes high enough. High soil salinity can also lead to imbalances of nutrients, accumulation of toxic elements to plants and decrease in water infiltration. Sodium ions have detrimental effects on cytosolic enzyme activities, photosynthetic and metabolic activities of plants (Blaylock, 1994).

Salt-affected plants are characterized by dwarf shoots accompanied with dark green leaves which are thicker than normal, leaves are water-storing parts that act as water reservoirs . In woody species, increased soil salinity may cause deprivation of leaves and leaf burn (Kotuby *et al.*, 1997).

1.4. Salt Tolerance of Plants

Plant species show some differences related to their adaptations to the salt-affected soils. Some plants will tolerate high levels of salinity while others can tolerate little or no salinity (Wentz, 2001). Plants can be divided into two groups according to their capacity of living on saline habitats. A ''halophyte '' is a plant that naturally inhabits where it is affected by salinity. Perhaps only 2% of all terrestrial plant species are halophytes. The large majority of plant species are ''glycophytes'' and salinity gives unrecoverable harm to this kind of plants. Halophytes was thought to possess some evolutionarily conserved mechanisms to construct osmotic adjustment; such as accumulation of inorganic salts, mainly NaCl, in the vacuole and accumulation of organic solutes in the cytoplasm. In addition to Na^+ and Cl^- transporters, Na^+ and Cl^- ions can enter the halophyte cells via ion channels and pinocytosis. Na^+ accumulation into vacuoles requires Na^+/H^+ antiporters in the tonoplast and H^+-ATPases to provide energy for sequestration of ions. Tonoplast antiporters are constitutive in halophytes, whereas in salt-tolerant glycophytes they must

be activated by NaCl, and they may be absent from salt-sensitive glycophytes (Glenn *et al.*, 1999).

Salt tolerance is described as the ability of plants to live, grow and develop under salt stress conditions (Wentz, 2001). Salinity tolerance is affected by many factors such as plant and soil type, and changing environmental situations. Their interrelationships also have an impact on salt tolerance. Climatic changes and over-irrigation also influence salinity tolerance. In arid areas, the concentration of the salts raise in the soil solution and depending on that circumstance salt stress increases. Therefore, problems related to salinity are more severe under hot, dry conditions than under cool, moist conditions. Increased irrigation frequency and excess water application more than plant requirements may be needed during drought to reduce salinity stress (Kotuby *et al.*, 1997).

1.4.1. Impacts of Salinity Stress on Plant Growth and Development

Both vacant land`s (7%) and cultivated lands (20%) all over the world suffer from environmental stress factors in particular soil salinity, and salt stress especially limits crop plants` productivity (Flowers *et al.*, 1997). Also one-third of the world's irrigated land is unsuitable for growing crops due to the high levels of salt (Frommer *et al.*, 1999).

Extremely high soil salinity (salt) values reduce the crop yields and usually this reduction might end up with the loss of complete crop. The crop type and the severity of the problem, the impact of the situation varies. Yield reductions or yield losses caused by excessive soil salinity can be prevented by selection or improvement of suitable plants that live on saline environments (Soltanpour and Follett, 1995).

The germination of new seeds is prevented by high soil salinity levels. Plants show similar responses against salinity and drought by prevention of the osmotic activity of the roots where water and nutrient movements take place

the direction of low concentration to high concentration. Therefore, due to the elevated soil salt levels, water and nutrients cannot move into the plant roots. Germinating seedlings are affected from salinity in paralel with the elevated salt levels in the soil. Toxic effects of salinity on plants are a consequence of the high concentration of certain salts in the soil that interferes with the uptake of essential nutrients for healthy growth and also causes nutrient imbalances (Wentz, 2001).

1.4.2. Plant Activities Against Salt Stress

Three interdependent plant activities play important roles to gain salt tolerance (Figure 1.3, Zhu, 2001). First, damage must be attenuated or averted (detoxification). Second, homeostatic conditions must be restored in the new, stressful environment (homeostasis). Third, growth must recommence, remain at a reduced rate (growth control) (Zhu, 2001).

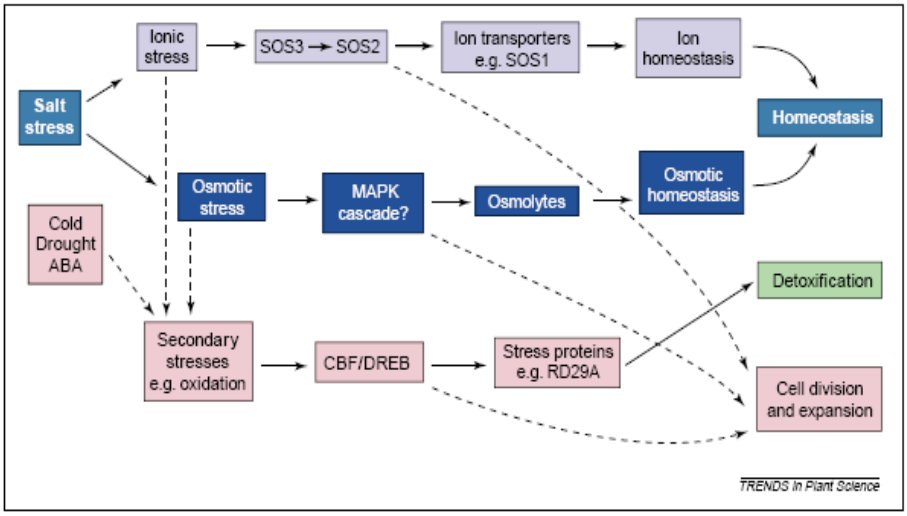


Figure 1.3. Plant Salt Tolerance Activities (Zhu, 2001, *Trends in Plant Science*)

Using tools of recombinant DNA techniques and biotechnology, generation of transgenic plants with modified traits or transfer of a gene with a trait of interest is possible. Acquisition of more information on novel genes, development of new strategies in functional genomics and gene expression profiles in response to several stresses provide a molecular basis for achieving effective stress tolerance through recombinant DNA technology. Therefore, salt tolerance can be established in salt-sensitive plants by the modification of the genes involved in metabolic pathways and mechanisms in transgenic experiments. These genes can be classified into five groups according to their functions:

1) Osmolyte Biosynthesis,
2) Protection of cell integrity,
3) Avoidance form oxidative stress,
4) Transcription factors,
5) Provision of ion homeostasis (Zhu, 2001; Liu and Zhu, 1998; Liu *et al.*, 2000; Chinnusamy *et al.*, 2005).

1.4.2.1 Synthesis of Osmolytes

In plants, the accumulation of compatible osmolytes such as proline (Pro), glycine betaine and sugar alcohols is an effective response against osmotic stress (deficiency of water) (Hanson and Hitz, 1982). These are relatively small, non-toxic compounds that help stabilization of proteins and cellular structures and can increase the osmotic pressure of the cell (Yancey *et al.*, 1982; Fisher, 2006). Increase in osmotic potential with the accumulation of osmotically active compounds in the cytosol provides a balance between the apoplastic solution and the vacuolar lumen (Apse and Blumwald, 2002). Osmolyte production does not interfere with normal biochemical reactions and most of them act as osmoprotectans (osmoprotective compounds) during osmotic stress. Proline accumulates in plants as an osmoprotectant against water stress and salinity in common (Hanson and Hitz, 1982) and also it

renders them tolerant to cold, salt and frost (Gleeson *et al.*, 2005). Reduction in proline content makes plants salt-sensitive (Frommer *et al.*, 1999).

1.4.2.2. Protection of Cell Integrity

Exposure to environmental stresses alters the integrity and composition of the cell wall. Cell wall characteristics like water permeability and elasticity are involved in the maintenance of cell growth during salt stress. Therefore cell wall alterations may be crucial to stress tolerance (Iraki *et al.*, 1989).

1.4.2.3. Oxidative Stress

Salinity propagates an increase in reactive oxygen species (ROS) including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-). ROS generally induce destructive effects on cell metabolism (Borsani *et al.*, 2003; Apse and Blumwald, 2002). Alterations of chloroplast and mitochondrial metabolism during stress lead to the production of this kind of highly reactive molecules. These species cause oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids. Plants generally use enzymes like superoxide dismutase (SOD) for the reduction of adverse effects of ROS and protection against various stresses (Tanaka *et al.*, 1999; Apse and Blumwald, 2002).

1.4.2.4. Regulation of Ion Homeostasis

NaCl is the most common type of salt effecting plant growth and causes retardation in plant development. Increased intracellular concentrations of Na^+ and Cl^- ions show detrimental impacts on cellular systems (Serrano *et al.*, 1999). In addition, the homeostasis of not only Na^+ and Cl^- , but also K^+ and Ca^{+2} ions is disturbed (Serrano *et al.*, 1999; Hasegawa *et al.*, 2000a, b; Navarro, 2000; Chinnusamy *et al.*, 2004). Excessive amounts of sodium and chloride ions have adverse effects on many cellular systems and metabolism (Serrano *et al.*, 1999), therefore, reestablishment of homeostasis is the main requirement for plant survival and growth. Not only excessive uptake but also

insufficient sodium and chloride ion efflux within the plant cells cause reduced plant growth (reduction of photosynthesis and cell expansion) due to deficient osmotic solute production (Seaman, APS 402 Dissertation).

1.5. Ion Homeostasis Mechanisms for Improvement of Salt Tolerance

At present, numerous mechanisms (many cellular, tissue and whole plant adaptations) in plants including ion compartmentalisation, osmotic adaptations, selective transport and uptake of ions and salt excretion are utilized to improve salt tolerance.

Salinity burdens two effective stresses on plant tissues: (1) a water stress due to deficiency of water resulting from the relatively high solute concentrations in the soil; and (2) ion-specific stresses such as salt stress as a result of altered K^+/Na^+ ratios and deleterious Na^+ and Cl^- ion concentrations to plants. Pathways functioning in potassium acquisition also make contribution to sodium uptake due to the similarity between the hydrated ionic radii of sodium and potassium, which makes it difficult to discriminate the two ions by transport proteins. This discrimination problem is also the basis for Na^+ toxicity and the main reason of the altered ion ratios (Blumwald *et al.*, 2000; Apse and Blumwald, 2007; Qi and Spalding, 2004).

Plants tend to maintain a high concentration of K^+ and a low concentration of Na^+ in the cytosol under salinity stress (Zhu, 2003). Sodium efflux and/or sodium compartmentation are the strategies for maintaining a high K^+/Na^+ ratio in the cytosol . The Na^+ intrusion is a passive process: the negative electrical potential difference between the plasma membrane and cytosol (low Na^+ concentrations) assist the movement of Na^+ into the cell. In contrast, Na^+ extrusion and compartmentation are active processes. Na^+/H^+ antiporters mediate the compartmentation of Na^+ within the vacuole and the extrusion of Na^+ from the cell. These processes provide cytosolic Na^+ detoxification and cellular osmotic adjustment that are necessary to tolerate salinity stress (Blumwald *et al.*, 2000; Zhu, 2003).

1.5.1 Sodium Influx, Association with Potassium Uptake

Na^+ influx across the plasma membrane is passive and effllux is active processes accrording to the electrochemical gradient of sodium ions. The extracellular level of Na^+ (relative to the cytosol) increases in connection with the NaCl concentration increases in the surrounding environment. Therefore, Na^+ acts as a competitor of K^+, suggesting that the uptake mechanisms for both cations are similar (Niu *et al.*, 1995).

1.5.2 Sodium Efflux and Vacuolar Compartmentation

Plant cells respond to salt stress by increasing sodium efflux at the plasma cell membrane and sodium accumulation in the vacuole (Borsani *et al.*, 2003). Na^+ and H^+ are the most common ions play important roles in critical events for occurance of indispensable cell functions such as cell bioenergetics and proton concentrations. If concentrations of these two ions are too high or too low, the physiological activities of cells are inhibited and also growth inhibition was observed (Padan *et al.*, 2001; Cheeseman, 1988; Tester and Davenport, 2003).

One of the defense mechanisms used by plants to remain alive under salt stress conditions caused by high NaCl concentration is the removal of Na^+ from the cytoplasm. Na^+/H^+-antiporter proteins localized in plant plasma and vacuolar membranes are involved in this mechanism (Vasekina *et al.*, 2005) (Figure 1.5, Mansour *et al.*, 2003). Taking up of Na^+ within the cytoplasm from external environment and its compartmentation into vacuoles are regulated by the help of active Na^+ flowout from the cytosol across the plasma membrane and vacuolar membrane (Niu *et al.*, 1995). The mechanisms play roles in the inhibition of Na^+ accumulation in the cytoplasm are grouped into three, i.e., restriction of Na^+ intrusion, active Na^+ extrusion and compertmentalization of Na^+ in the vacuoles (Serrano and Navarro, 2001). Compartmentation of Na^+ and $C1^-$ into the vacuoles is an essentia1 mechanism for salt tolerance to keep the ion levels in the cytoplasm at approximately lower ranks and to facilitate

the osmotic adjustment required for cell expansion and maintenance of turgor (Niu *et al.*, 1995). Furthermore, sequestration of Na^+ and Cl^- into the vacuoles contributes to hold the organic solute concentrations in a particular state. Because they function in the establishment of osmotic balance between plants and the salty environment in which they are cultivated by acting as essential osmolytes or osmoprotectans (Xue *et al.*, 2004).

Plant cells possess relatively large vacuoles surrounding by apoplast that provides the connection with the cytoplasm. Therefore, vacuoles are proper organelles for ion accumulation (Xue *et al.*, 2004). The transportation of ions across the plasma membrane and tonoplast is an active process and the required energy is provided by vacuolar and plasma membrane ATPases. Membrane Na^+/H^+ antiporters provide the exchange of sodium and hydrogen ions in opposite directions to achieve the proton gradient within the cell (Mansour *et al.*, 2003). Ion transportation in a direction from the interior toward the exterior in the vacuolar membrane of some plants is produced via antiport pumps. This sodium/proton antiport pump enables the plant to sequester sodium ions in its vacuole, provides an efficient mechanism to prevent the occurance of detrimental outcomes of Na^+ accumulation in the cytosol and maintains osmotic balance by using accumulated Na^+ in the vacuole to drive water into the cells (Xue *et al.*, 2004).

Vacuolar membrane plays a central role in ion transport to provide cell homeostasis and osmoregulation. Recent researches help to investigate the tonoplast transport mechanisms involved in these key cellular processes. Primary active transporters need high-energy metabolites for their activities at functional and molecular levels. In contrast, both secondary active transporters and also passive transporters` activities have only been defined at functional level. With the increase in our knowledge about the roles of vacuole it is easy to understand the physiology of plant cells.The importance of the tonoplast transporters in sodium accumulation and salt tolerance is stood for existing data and the roles of passive and/or secondary active transporters` activities are

supported by the driving force provided by the primary active transporters have been incorporated into the studies (Barkla and Pantoja, 1996; Marty, 1999).

1.5.2.1. The Role of Na^+/H^+ Antiport for Plant Salt Stress Tolerance

Na^+/H^+ movement across plasma membrane and tonoplast is achieved by ion exchangers or antiporters that transport Na^+ ions out of the cell or sequester them into the vacuole in exchange for H^+. The characterization of tonoplast ion pumps that function in Na^+ influx within the vacuoles is accomplished in various plant species (Blumwald *et al.*, 2000; Padan *et al*., 2001; Qui *et al.*, 2002; Qui *et al.*, 2003; Wang *et al.*, 2003). These transporters play a major role in cellular pH and Na^+ homeostasis (Padan *et al.*, 2001; Wiebe *et al.*, 2001; Horie and Schroeder, 2004, Fukuda *et al.*, 2004a; Wu *et al.*, 2005). Transport mechanisms actively move ions across the tonoplast into the vacuole and removes the potentially harmful ions from the cytosol (Aharon *et al.*, 2003). Genes encoding Na^+/H^+ exchangers have been cloned from bacterial, yeast, animal, and plant cells (Gaxiola *et al.*, 1999; Counillon and Pouyssegur, 2000; Padan *et al.*, 2001). These transporters are composed of a single polypeptide chain with a molecular mass ranging from 56 to 100 kD that have 12 transmembrane portions and the C- and N-terminus of the protein are thought to extend in the direction of the cytoplasm.

In plants, both vauolar and plasma membrane Na^+ /H^+ antiporters function in the removal Na^+ from the cytoplasm to prevent its toxic effects that avert the vital cellular events (Blumwald, 2000; Blumwald *et al.*, 2000; Hasegawa *et al.*, 2000a). Transport of Na^+ into the vacuole via the tonoplast Na^+ /H^+ antiporter is an active process energized by the activity of the V-ATPase and/or the V-PPase and Na^+ accumulation into the vacuole to avoid the cytoplasmic Na^+ toxicity and maintain high cytoplasmic K^+/Na^+ ratio is an efficient mechanism for salinity tolerance. In parallel, vacuolar Na^+ serves as an osmoticum necessary for cellular H_2O homeostasis by facilitation of water uptake from soil. NaCl exposure is predicted to induce the exchange activity of the transport proteins (Garbarino and Dupont, 1988).

1.5.2.2. The Importance of *Arabidopsis* in Stress Response Mechanisms

Arabidopsis is a model system for understanding plant osmotic stress responses. With the complete sequencing of the *Arabidopsis* genome, despite of its glycophytic nature, and the ease of studying stress signalling mechanisms in molecular level at genetic analysis are the most important factors that increase the usage of this model organism (Zhu, 2000). All plants, whether glycophytes or halophytes, appear to have similar machinaries for perception of stress signalling and tolerance. Differences in some regulatory mechanisms or mutations in some key determinants are the main variable factors to specify the salt tolerance and sensitivity. Thus, genetic analysis using the *Arabidopsis* as a model organism continues in a successful manner and contributes to constitution of salt and water stress signalling system applicable to all higher plants (Xiong and Zhu, 2002; Yokoi *et al.*, 2002).

1.5.2.2.1. *Arabidopsis thaliana AtNHX1* Gene

A plant gene from *Arabidopsis thaliana* (*AtNHX1)* homologous to the *Saccharomyces cerevisiae Nhx1* gene product was identified by the completion of its genome-sequencing project (Apse *et al.*, 1999) and shows sequence similarity to mammalian *NHE* Na^+/H^+ exchanger (Counillon and Pouyssegur, 2000), it was also found that there was a functional conservation between yeast and plant endosomal antiporters via using *AtHNX1* gene and *ScNHX1* gene (Quintero *et al.*, 2000). In spite of being a member of glycophytic plants, transgenic tomato plants overexpressing *NHX* gene shows similar characteristics with halophytic plants (Zhang and Blumwald, 2001). *AtHNX1* function may be repressed under normal growth conditions in wild-type plants but overexpression of *AtNHX1* gene provide to gain the salt tolerant characteristic (Apse *et al.*, 1999; Sottosanto *et al.*, 2007). The *Arabidopsis thaliana* vacuolar Na^+/H^+ antiporter *AtNHX1* is a salt tolerance determinant (Yokoi *et al.*, 2002; Sottosanto *et al.*, 2007) and its ion transporter role in maintaining a low cytoplasmic concentration of Na^+ under salt stress was showed in Figure 1.4 (Zhu, 2003) . Salt tolerance was tested in wild type and

transgenic plants overexpressing *AtNHX1*. Wild-type plants displayed progressive chlorosis, reduced leaf size and a general growth inhibition when watered with a NaCl-containing solution. The transgenic plants were unaffected by up to 200mM NaCl and plant development was not compromised. However transgenic plants grown at 300mM NaCl showed the same response like wild-type plants (Apse *et al.*, 1999; He *et al.*, 2005). *AtNHX1* transcription is discovered in both roots and shoots, and salt treatment causes transcriptional increase especially in leaves of transgenic plants (Gaxiola *et al.*, 1999).

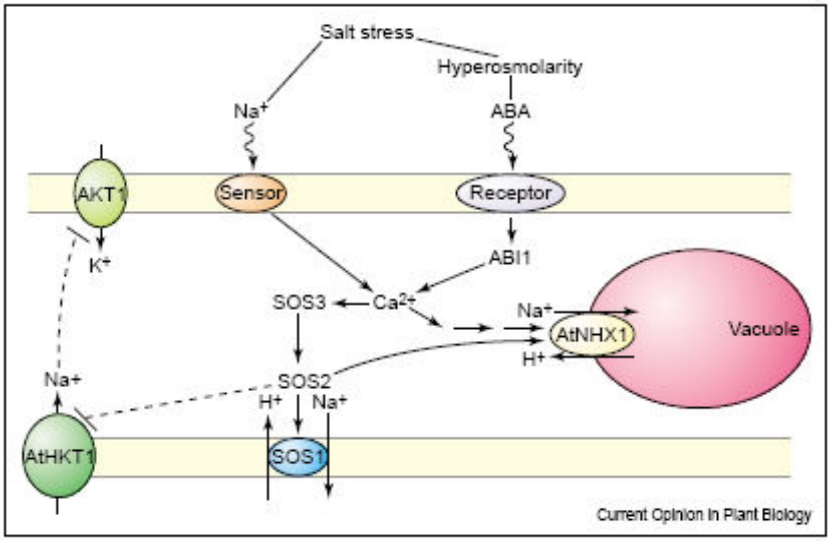


Figure 1.4. The role of *AtNHX1* in salt tolerance mechanisms (Zhu, 2003)

The role of Na^+/H^+ antiporter genes in cell homeostasis during exposure to several stresses was recently illustrated in addition to its roles in developing salt and drought tolerance in plants. Over-expression of the *Arabidopsis thaliana AtNHX1* gene encoding a tonoplast Na^+/H^+ antiporter, enhanced salt tolerance in transgenic *Arabidopsis* plants, which were able to survive 0.2 mol/L NaCl (Wu *et al.*, 2005).

Two H^+-pumps (Figure 1.5, Mansour *et al.*, 2003) named as vacuolar H^+-inorganic pyrophosphatase (V-PPase) and vacuolar H^+-ATPase (VATPase) provide the electrochemical H^+ gradient for transportation of Na^+ from the cytoplasm to vacuoles by the help of tonoplast Na^+/H^+ antiporters. The accumulation Na^+ in the vacuole maintains a higher K^+/Na^+ ratio in the cytoplasm and controls the osmotic balance of the cell with the environment if the plant cells treated with high salt concentrations. In this process, the vacuolar Na^+/H^+ antiporters are thought to play an important role. Salt treatment has been reported to increase the activities and transcript levels of the H^+-pumps (Fukuda *et al.*, 2004b; Shi and Zhu, 2002).

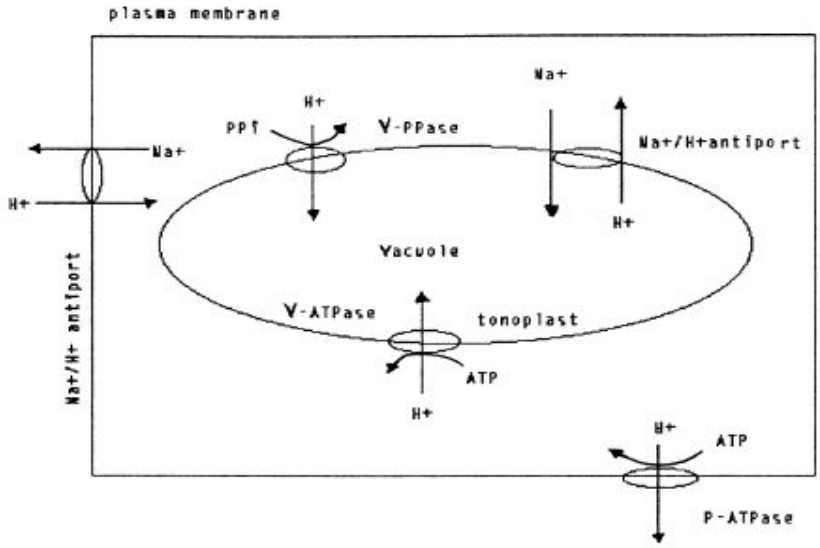


Figure 1.5. Proton pumps and antiporters of plasma membrane and tonoplast (Mansour *et al.*, 2003)

Antiporter activity of the vacuolar membranes has been reported only in plants. The exploration of vacuolar exchangers for salt tolerance was put out in both halophytes and salt-tolerant glycophytes. Plants treated with high salinity accumulate Na^+ in the vacuoles by the exchange activity of the transporters (Fukuda *et al.*, 1999).

1.6. *Agrobacterium tumefaciens* Mediated Gene Transfer to Plants

Agrobacterium tumefaciens is a gram-negative soil plant pathogen used in plant transformation studies. It is one of the most widely used methods to introduce foreign genes into mostly dicotyledonous plant cells. *A. tumefaciens* causes the crown gall disease (tumor formation) (Riva *et al.*, 1998). The bacteria transfers a specific section of its DNA, the transferred DNA (T-DNA), from tumor-inducing (Ti) plasmid located within the bacterium into the nucleus of an infected plant cell (Tempe *et al.*, 1977). After the transfer, the T-DNA is incorporated into the plant genome and is subsequently transcribed (Schell *et al.*, 1979).

1.7. Aim of the Study

The objective of the study is to obtain transgenic tobacco plants by introducing the *AtNHX1* gene encoding a Na^+/H^+ antiporter from *Arabidopsis thaliana* to eliminate the problems related with the soil salinity.

For this purpose, *AtNHX1* coding sequence was cloned from *Arabidopsis thaliana* plants and tobacco plants were transformed with a suitable plant transformation vector carrying the gene of interest. Integration of the gene was verified based on molecular analysis and response of plants under salt stress. Thus, the aim of the study was to construct an initial step to generate salt resistant plants in the future.

CHAPTER II

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Strains and Plasmid pCVB1

Throughout the study *Eschericia coli* (*E. coli*) strain DH5α cells were used as competent cells. EHA105 (Hood, 1993) strain of *Agrobacterium tumefaciens* (*A. tumefaciens*) was used in plant transformation studies. pCVB1 (Appendix A) carrying a cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase (Nos) terminator was used as a vector for the transformation of *Nicotiana tabacum*.

2.1.2. Bacterial Culture Conditions and Growth Media

The complex medium used for *E.coli* DH5α strain was Luria-Bertani Broth (LB) (Appendix B) supplemented with ampicillin at 100mg/L with 1.5% bacterial agar for solid media.The bacterial cultures were incubated at 37°C overnight with vigorous shaking for liquid cultures and the culture plates were incubated at 37°C overnight and stored at 4°C for several weeks for further usage. Yeast Extract Broth (YEB) containing nutrient broth, yeast extract, sucrose and magnesium sulphate (Appendix B) supplemented with rifampicin (20 mg/L) was used to grow *A.tumefaciens* EHA105 strain and 1.5% bacterial agar was also added to media for solid cultures. The bacterial cultures were incubated at 28°C with a 180-200 rpm shaking for 2 days for liquid cultures. A liquid aliquot of the bacterial clone carrying the specific plasmid was also kept at -80°C in 20% glycerol for longer storage.

2.1.3. Plant Material, Plant Tissue Culture Media and Growth Conditions

In the tissue culture and transformation studies, seeds of *Arabidopsis thaliana* ecotype Columbia and *Nicotiana tabacum* L. cultivar Samsun and leaves of *Nicotiana tabacum* were used in the experiments. MS (Murashige and Skoog, 1962) basal media containing MS micro-macro element and vitamins supplemented with 3% sucrose and 0.8% plant agar was used in all plant tissue culture studies (Appendix C). Half-strength MS was used in *Arabidopsis thaliana* tissue culture and full-strength MS was used in tobacco tissue culture studies. Medium was prepared by dissolving the MS and sucrose in distilled water and then the pH was adjusted with NaOH to 5.7-5.8. After addition of plant agar whole medium was sterilized at 121°C for 20 minutes by autoclaving. Freshly prepared filter sterilized (0.2µm pore size) plant growth regulators (naphtalenaceticacid [NAA] and benzylaminopurine [BA]) and antibiotics (ampicillin and cefotaxime/augmentin) were added to the sterile medium. The composition and the use of the media are given in Table 2.1.

All plant tissues were incubated at 24±2°C under fluorescent lights with a 16/8 hour (light/dark) period.

2.1.4. Chemicals and Consumables

The chemicals used in the study were all commercially available from Sigma Chemical Company, Merck Chemical Company, Applichem and Duchefa. The chemicals used in molecular biology studies were ordered from MBI Fermentas.

Table 2.1. The composition and usage of plant tissue culture media (MS: Murashige and Skoog)

Media	Composition	Use
Liquid MS	MS + 3% sucrose (30g/L)	Before transformation provide dilution of *Agrobacterium* culture
MSA	MS + 3% sucrose + 0.8% agar (8gr/L) + 0.1mg/L NAA + 1 mg/L BA	Control leaf disc callus and shoot formation, co-cultivation for *Agrobacterium* treated leaf discs
MSB	MSA + 5 mg/L PPT + 500 mg/L Augmentin	Transformed plant selection and regeneration
MSC	For control shoots, MSB lack plant growth regulators and PPT For transgenic shoots, MSB lack plant growth regulators MSA lack plant growth regulators for germination of surface sterilized seeds	Induction of root formation on regenareted shoots and sub-culturing

2.2. Methods

2.2.1. Growth of *Arabidopsis thaliana*

2.2.1.1. Seed Surface Sterilization of *Arabidopsis thaliana*

Arabidopsis thaliana ecotype Columbia seeds in eppendorf tubes were surface sterilized with 1ml 70% EtOH (ethanol) for 1 minute with continous shaking. Then EtOH was removed and 70% (v/v) sodium hypochloride was added. Seeds were sterilized for 15 minutes with shaking and washed 3-4 times with sterile distilled water. The seeds were incubated in sterile dH_2O at 4°C overnight and germinated on MS medium supplemented with 2% sucrose by using a micropipette. Seeds were grown in short light period (9 hours light, 22°C) and later seedlings were transferred to soil. Explants were irrigated by half strength Hoagland`s E-Medium (Appendix D) containing macro and micro nutrients.

2.2.2. Total RNA Isolation from *Arabidopsis thaliana*

All the materials (mortars, pestles, eppendorf tubes and tips), solutions and distilled water used in RNA extraction procedure were treated with DEPC (diethyl pyrocarbonate) and autoclaved. Freshly weighed 0.1 gram of leaf samples were ground in liquid nitrogen (grinding step was crucial) to obtain a fine powder and the half of the eppendorf tubes were filled with the tissue powder, put into liquid nitrogen. 1ml of TRI (Trizol) reagent (Appendix E) was added for each tube for 0.1 g of starting material. Until all plant material was dissolved fully (at least 10 minutes), samples were incubated at room temperature with occasional shaking. Each 1ml of TRI reagent 0.2 ml of chloroform was added to samples and centrifugated at 13000 rpm (revolution per minute) for 5 minutes in a microcentrifuge. Then the upper phase was transferred into clean eppendorfs and equal volume of isopropanol was added onto it. After incubation at room temperature for 10 minutes, RNA was precipitated by centrifugation at 13000 rpm for 15 minutes in a

microcentrifuge. The supernatant was discarded and the pellet was washed with 70% ethanol before drying at 65°C for few minutes. Pellet was dissolved by vortexing and heating at 65°C in 300 µL DEPC-treated water. 30 µL DEPC-treated 3M NaAcetate and 150 µL Tris equilibrated phenol were added subsequently and mixed with occasional shaking for 3 minutes. Following addition of 150 µL chloroform, whole content was centrifugated at 13000 rpm for 5 minutes in a microcentrifuge and upper phase was taken into new eppendorfs. 1 volume of isopropanol was added, the tubes were mixed by inverting and incubated at room temperature for 10 minutes. Then RNA was pelleted by centrifugation at 13000 rpm in a microcentrifuge for 15 minutes and the supernatant was removed. The pellet was washed with 70% ethanol for 3 minutes. Another centrifugation step at 13000 rpm in a microcentrifuge for 5 minutes was carried out and the supernatants were discarded again. The pellets were dried at 65°C and finally 40 µL DEPC-treated water was added to each tube to dissolve the pellet. RNA was kept at -80°C for further use.

2.2.2.1. Spectroscopic Quantification of RNA

The RNA concentration was quantified by using absorption of light at 260 nm and 280 nm (A_{260}/A_{280}) on a spectrophotometer. After measuring the absorbance at 260 nm and 280 nm, the RNA concentration was calculated by using the following formula:

[RNA] (µg/mL)= 40 µg/mL (one absorbance unit) X Dilution Factor X A_{260}

The integrity of RNA was tested and visualized by agarose gel electrophoresis.

2.2.2.2. First Strand cDNA Synthesis

Fermentas RevertAid™ First Strand cDNA Synthesis Kit was used for synthesis of first strand cDNA from RNA template. The first strand of cDNA was directly used as a template in following PCR reactions. The kit contains Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase,

recombinant RiboLock™ Ribonuclease Inhibitor which is fully compatible with reverse transcription reaction, oligo$(dT)_{18}$ primers which anneals selectively on the poly(A) tail of mRNA and 5X reaction buffer. The reaction conditions are given in Table 2.2.

Table 2.2. Reaction Conditions for First Strand cDNA Synthesis

Reagents	**Volume**
Total RNA (0.1-5µg)	18 µL
Oligo$(dT)_{18}$ Primer (0.5µg/µL)	2 µL
DEPC-treated Water	4 µL
5x Reaction Buffer	8 µL
RiboLock Ribonuclease Inhibitor (20u/µL)	2 µL
10Mm dNTP mix	4 µL
(M-MuLV) reverse transcriptase (200u/µL)	2 µL
Total Volume	40 µL

2.2.2.3. PCR Analysis for Amplification of *AtNHX1* from *Arabidopsis*

Two specific primers were used in PCR amplification studies (Table 2.3) to obtain *AtNHX1* cDNA. Primers were designed with *XbaI* and *BamHI* cut sites respectively for enabling the cloning according to the sequence of *Arabidopsis thaliana* obtained from the NCBI database. The template DNA used in PCR reactions was the first strand cDNA from *A. thaliana*.

PCR was carried out in a total volume of 50 µL, optimized PCR conditions and PCR program used in amplification studies are given in Table 2.4 and Table 2.5)

Table 2.3. Specific primers for *AtNHX1* coding sequence.

AtNHX1-sense	5'-TGCTCTAGAGCAATGTTGGATTCTCTAGTGTCG-3'
AtNHX1-antisense	5'-CGCGGATCCGCGTCAAGCCTTACTAAGATC-3'

Table 2.4. Optimized PCR Conditions for Taq DNA Polymerase

10X Rxn. Buffer	5 µL
2.5 mM dNTP	5 µL
10 µM Primer F	4 µL
10 µM Primer R	5 µL
Sterile dH_2O	24.5 µL
25 mM $MgCl_2$	2.5 µL
Tag DNA Polymerase	1.5 µL
Template DNA	2.5 µL
Total	50 µL

Table 2.5. Conditions for PCR Cycles

Initial Denaturation	3 min at 94°C
Denaturation	30 sec at 94°C
Primer Annealing	30 sec at 55°C (35 cycles)
Extension	90 sec at 72°C
Final Extension	5 min at 72°C

2.2.2.3.1. Agarose Gel Electrophoresis

In the course of the study, DNA molecules were visualized on agarose gels with different concentrations ranging from 0.6-1% according to the purpose. Gel solutions were prepared with the help of 0.5-1X TBE or TAE buffers (Appendix F). 0.4g agarose was dissolved in 50mL of 0.5X TBE or 1X TAE electrophoresis buffer via heating in microwave oven to prepare 0.8% gel. Then the solution was cooled down around 50°C and 3 µL ethidium bromide (EtBr) (6mg/mL) was added. Electrophresis tray with appropriate comb to obtain wells was prepared and gel solution was poured without forming bubbles. The gel was solidified at room temperature, 0.5X TBE buffer was poured carefully and the comb was removed.

The samples were prepared by mixing with 6X loading buffer (1X final concentration) and were loaded into the wells. A DNA ladder (molecular size marker) was applied into a seperate well. Afterwards, the tank was connected to a power supply and the gel was run under constant voltage of 50-60 volts for 45-60 minutes. Then the gel was visualized under UV light and photographed. The PCR amplified DNA fragments were isolated from the agarose gel via using Gene-Mad DNA Purification Kit and verification of the amplicons was detected with sequence analysis at Iontek company, İstanbul.

2.2.3 Transformation Studies for Bacterial Cells

The plasmid pCVB1 was first transformed into *E. coli* DH5α cells. Colonies were selected on ampicillin containing LB-agar medium. To provide *Agrobacterium* mediated gene transfer to tobacco, this vector was then transferred to *Agrobacterium* EHA105 strain.

2.2.3.1 Competent *E. coli* Preparation with $CaCl_2$

E. coli DH5α cells, taken from glycerol stock, were streaked on LB agar plates and cultured overnight at 37°C. Then, a single colony was transferred to 50 mL liquid LB medium in a 250 mL flask and incubated at 37°C overnight. Next morning 300 µL overnight grown culture was taken and inoculated in 50 mL of LB. The culture was grown 2-2.5 hours at 37°C with vigorous shaking at 300 rpm until OD_{600} reached to 0.6. After that, the culture was divided into two sterile prechilled centrifuge tubes and were incubated on ice for 10 minutes. Following centrifugation at 4000 rpm for 10 minutes at 4°C in a microcentrifuge was carried out, the pellet was suspended in 1 mL of 10 mM ice cold, filter sterilized $CaCl_2$ by vortexing. Then the solution was recentrifuged at 3000 rpm for 10 minutes at 4°C in a microcentrifuge and the pellet in each tube was resuspended with 1 mL of 75 mM filter sterilized $CaCl_2$. Prepared competent cells were divided into sterile eppendorf tubes in 100 µL aliquots and were immediately chilled in liquid nitrogen. The frozen competent cells were stored at -80°C for few months without detectable loss in their competencies.

2.2.3.2 Competent *Agrobacterium tumefaciens* Preparation

YEB agar plates were prepared to make *A. tumefaciens* EHA105 streak culture and cells were grown 2 days at 28°C. After colony formation, a single colony was picked and transferred to 50 mL YEB medium. Bacterial solution was incubated overnight at 28°C with vigorous shaking. Then the cells were chilled on ice for 10 minutes and were spun down by centrifugation at 4000g for 10 minutes. The supernatant was discarded and 10 mL of 0.15 M ice-cold $CaCl_2$ was used to resusped the pellet. Bacterial cell suspension was centrifuged at 4000g for 10 minutes at 4°C and these last two steps were repeated. The supernatant was removed and cells were resuspended in 1 mL of ice-cold 20 mM $CaCl_2$. Suspension was divided into sterile eppendorf tubes in 100 µL aliquots by freezing in liquid nitrogen. The competent cells were stored at -80°C for further use.

2.2.4. *E. coli* Transformation with the Plasmid pCVB1

Frozen competent *E. coli* cells were thawed on ice and 5-20 µL (at least 10 ng) plasmid pCVB1 was added for transformation. The plasmid contains strong, constitutive 35S promoter from cauliflower mosaic virus, ampicillin resistance gene for bacterial selection and PPT resistance for plant selection. The cells were incubated in ice for 45 minutes by following heat shock with incubation at 42°C for 90 seconds to enhance the entrance of plasmid into the cells. Then the cells were again incubated on ice for 5 minutes and at the end of the period 900 µL liquid LB was added to complete the volume to 1 mL with 45 minutes incubation at 37°C with vigorous shaking. This application allowed bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid. The cells were centrifuged at 3000 rpm for 3 minutes in a microcentrifuge, 900 µL supernatant was removed and the pellet was dissolved in the remaining 100 µL. Finally transformed bacteria were spread onto the ampicillin containing agar plates for overnight incubation at 37°C. After the colonies were visible the plates were stored at 4°C for further use.

2.2.4.1 Mini Scale Plasmid Isolation from *E. coli*

With the purpose of future use, the plasmid pCVB1 was isolated. A single colony of *E. coli* transformed with the plasmid was taken from the selective agar plate with the use of a sterile toothpick. It was inoculated in a sterile falcon tube containing 3 mL LB including ampicillin and the culture was grown at 37°C overnight with continous shaking. Bacterial culture was divided into sterile eppendorfs and centrifuged at 13000 rpm for 30 seconds in a microcentrifuge. The pellet was suspended in 100 µL of Sol I (Appendix G) by vortexing and 200 µL Sol II (Appendix G) was added. This suspension was inverted several times to obtain a clear mixture and incubated on ice for 5 minutes. After the addition of 150 µL of Sol III (Appendix G) the suspension was mixed gently and incubated on ice for 10 minutes. Addition of 450 µL phenol-chloroform-isoamyl alcohol (25:24:1) maintained the separation of cell debris and choromosomal DNA in different phases and the mixture was

centrifuged at 13000 rpm in a microcentrifuge for 10 minutes, the upper phase was collected into new eppendorf tubes. Following precipitation of plasmid DNA with equal volume of isopropanol at -20°C for 30 minutes the plasmid DNA was obtained in the pellet via centrifugation at 13000 rpm for 10 minutes. After washing the pellet with 70% ethanol the centrifugation step at 13000 rpm was repeated for 5 minutes in a microcentrifuge and supernatant was discarded. The pellet was dried comletely and dissolved in 50 µL TE buffer (Appendix G) containing RNAse (20µg/mL). For RNAse activity the solution was incubated at 37°C for 30 minutes and plasmid DNA was stored at -20°C until usage.

2.2.5. Restriction Enzyme Digestion

Both the plasmid DNA and purified PCR product were exposed to enzymatic digestion. The digestion mixture contained;

- DNA (6 µg/ µL plasmid DNA, 60 ng/ µL PCR product)
- Restriction endonucleases (*XbaI* and *BamHI* [10u/ µg DNA])
- 10X restriction endonuclease cutting buffer (10X Tango buffer) (1X)
- Sterile distelled water

The mixtures were then incubated at specific temperatures according to the enzymes used for few hours or overnight. In order to determine whether the plasmid DNA was cut, so it was purified from gel, the sample was loaded on a 1% agarose gel as described in section 2.2.2.3.1 and run by using 0.5X TAE buffer. After visualization under UV light, the expected band corresponding to molecular size marker was cut and put into an eppendorf tube. By using Gene-Mad DNA purification Kit the isolation of the fragment was completed. The isolation of digested PCR product was prepared in the same manner.

2.2.5.1. DNA Insert Ligation into Plasmid DNA

Isolated digested DNA fragment was ligated into the digested plasmid as described below;

- Vector DNA (50-100 ng)
- Insert DNA Fragment (10-100 ng)
- 10X Ligation Buffer (1X)
- DNA Ligase (T_4 DNA ligase from Fermentas-200u [1u/ μL] (1-2u)
- Deionized water

The ligation reaction mixture was incubated at 22°C for 1 hour and the activation of DNA ligase was stopped by heating the solution at 65°C for 10 minutes. Ligation mixture was used to transform the competent *E. coli* cells and plasmid isolation was performed by using Gene-Mad Plasmid Isolation Kit. Then plasmids were checked by PCR using specific primers for the presence of the desired gene and visualized on agarose gel as described earlier. The DNA fragment was isolated from the gel and sequence analysis was preformed to confirm the presence of error free DNA sequence of full-length *AtNHX1* cDNA. Following sequence analysis, isolated plasmids were used in *Agrobacterium* transformation.

2.2.5.2. Transformation of Competent *Agrobacterium tumefaciens* Cells

The vector pCVB1 was transferred to *A. tumefaciens* by direct transformation method. 1μg of plasmid (maximum 5μL) was added to competent cells and was mixed well. The bacterial solution was incubated on ice for 30 minutes and frozen in liquid nitrogen for 5 minutes. The heat shock was applied at 37°C for 25 minutes. After the heat shock, the cells were suspended in 1 mL of sterile YEB medium and grown at 28°C for 3 hours with vigorous shaking at 150 rpm. At the end of the incubation period bacterial culture was spread onto selective YEB agar plates containing ampicillin and rifampicin. The plates were incubated at 28°C for 2-3 days until colony formation and stored at 4°C for further use.

2.2.5.3. *Agrobacterium tumefaciens* Mediated Transformation of *Nicotiana tabacum*

Nicotiana tabacum plants were grown in sterile jars on MS medium at 24°C. *Agrobacterium* cells transformed with the plasmid was grown overnight in 250 mL YEB medium containing 100mg/L rifampicin and 100mg/L ampicillin at 28°C with 200 rpm shaking. Following dilution of the culture 10 times with liquid MS, sterile leaves were cut aseptically into leaf discs and put into the diluted bacterial solution. The cell suspension was incubated 10 minutes to let the infection of leaf discs. 8-10 leaf discs were put in a seperate liquid MS solution without the bacteria to use as a control. After incubation the leaf discs were washed with liquid MS to get rid of bacterial cells and blotted on sterile paper. Then the leaf discs were placed on MSA (MS agar + 1mg/L BA + 0.1mg/L NAA) containing petri plates and incubated at 22°C (climate room) at dark (in a box) for 48 hours. After two days of co-cultivation the explants were washed with liquid MS supplemented with 500mg/L Augmentin for one hour with continous shaking on a shaker with rpm not more than 100 for elimination of *Agrobacterium* cells. The leaves were dried by blotting and transferred to petri plates containing MSB (MSA + 500mg/L Augmentin + 100mg/L Ampicillin + selection agent (PPT [5mg/L]) for selection and regeneration. Plates were incubated at 25°C with 16 hours light, 8 hours dark period and refreshed every 10 days. Following callus formation after 2-3 weeks, when shoots were large enough to be cut they were transferrred into jars containing MSC medium during 6-8 weeks period to induce root formation. Plantelets were transferred to soil and grown in the growth chamber at 24±1°C with 16 hours light and 8 hours dark periods. After the emergence of the flower buds, plants were covered with nylon bags to prevent cross-pollination. Seeds of putative transgenic plants were collected when they dried completely.

2.2.6. Analysis of Putative T_0 Transgenic Tobacco Plants

The verification of the integration and expression of the transferred DNA, transformed plants were analyzed by regeneration test under salt stress and PCR analysis.

2.2.6.1. Regeneration Tests Under Salt Stress

Leaf samples from control and putative transgenic plants were tested for their ability of callus formation and shoot initiation on simple MSB containing different concentrations of salt (0 mM for control, 50 mM, 100 mM, 150 mM and 200 mM NaCl). After a month later, the photographs of the plates were taken and the regeneration was scored by weighing the samples.

2.2.6.2. PCR Analysis

Genomic DNA of independent lines of putative transgenic and control plants were isolated and amplifed by specific primers for *AtNHX1* cDNA. Thus, screening of putative transgenic lines was done by PCR.

2.2.6.2.1. Genomic DNA Isolation

A modified CTAB (Hexadecyl-trimethyl amonium bromide) Plant Genomic DNA Isolation Method based on Saghai-Maroof et al., 1984 was used for DNA extraction. 0.5-2 grams of plant material was ground to a fine powder in mortar by using liquid nitrogen and transferred to sterile centrifuge tubes. 20 mL of pre-heated 2X CTAB Buffer (Appendix H) containing 0.2% β-mercaptoethanol was added onto the powder and the tubes were incubated at 65°C for 45 minutes with occasional shaking. At the end of the period, 20 mL of chloroform:isoamylalcohol (24:1) was put into tubes and tubes were mixed vigorously. After centrifugation at 3000 rpm for 10 minutes in a microcentrifuge, the supernatant was taken to new sterile centrifuge tubes and 4 mL of pre-heated 5X CTAB Buffer (Appendix H) and 20 mL of chloroform were added into tubes respectively. Then the tubes were shaked and cenrifuged

at 3000 rpm for 10 minutes in a microcentrifuge. The upper phase was taken to new sterile centrifuge tubes and with addition of 20 mL cold isopropanol by inverting the tubes gently provided the precipitation of DNA. The tubes were kept overnight at -20°C, after incubation period DNA was collected in the pellet via centrifugation at 9000 rpm for 15 minutes in a microcentrifuge. The upper phase was removed and pelleted DNA was washed with 70% cold ethanol. An additional centrifugation step was performed at 9000 rpm for 10 minutes in a microcentrifuge and the pellet was completely dried at 37°C. Finally, the pellet was dissolved in 500 µL TE Buffer supplemented with RNAse (20mg/mL) and the tubes were incubated at 37°C for 30 minutes. The purity and concentration of DNA was determined by spectrophotometry and agarose gel electrophoresis. The prepared DNA was stored at -20°C for further use.

2.2.6.2.2 PCR Conditions

The isolated DNA from both putative transgenic plants and also control plants were analyzed with PCR for the presence of *AtNHX1* cDNA. The optimized PCR conditions and PCR cycling conditions for amplification are given in Table 2.6 and Table 2.7, respectively. According to the DNA concentration 100 ng of DNA was used for PCR.

Table 2.6. Optimized PCR conditions for amplification of *AtNHX1* from plant genomic DNA

10X PCR Buffer for Pfu	2.5 µL
dNTP (2 mM)	2.5 µL
10 µM Primer F	2 µL
10 µM Primer R	2 µL
dH_2O	12-X µL
Pfu DNA Polymerase (5000u/ mL)	0.2 µL
Template DNA (1µg)	X µL
Total volume	25 µL

Table 2.7. PCR Cycling Conditions for Genomic DNA

Initial Denaturation	5 min at 95°C
Denaturation	1 min at 95°C
Primer Annealing	1 min at 55°C (35 cycles)
Extension	3 min at 72°C
Final Extension	5 min at 72°C

Amplification products were run on 1% agarose gel in TAE buffer under constant voltage of 75V and the gel was visualized under UV.

2.2.7. Analysis of Tobacco T_1 Progeny by Seed Germination Test Under Salt Stress

Seeds of putative transgenic and control tobacco plants were surface sterilized, germinated on MS without NaCl for control and MS+50, MS+100, MS+150, MS+200, MS+250 mM NaCl containing petri plates for salt stress. The plates were incubated at 25±2°C. The photographs of the plates were taken, surviving plants were counted and were statistically analyzed.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Results

3.1.1. Cloning of the *AtNHX1*

Arabidopsis thaliana cDNA was used as a template for amplification of the coding sequence of the *AtNHX1*. PCR was carried out by using specific primers as described earlier (section 2.2.2.3). Total RNA was isolated by using Trizol reagent from *Arabidopsis thaliana*. Isolated samples were loaded into the wells and agarose gel electrophoresis was performed to check the quality of RNA (Figure 3.1). Then, cDNA synthesis was performed.

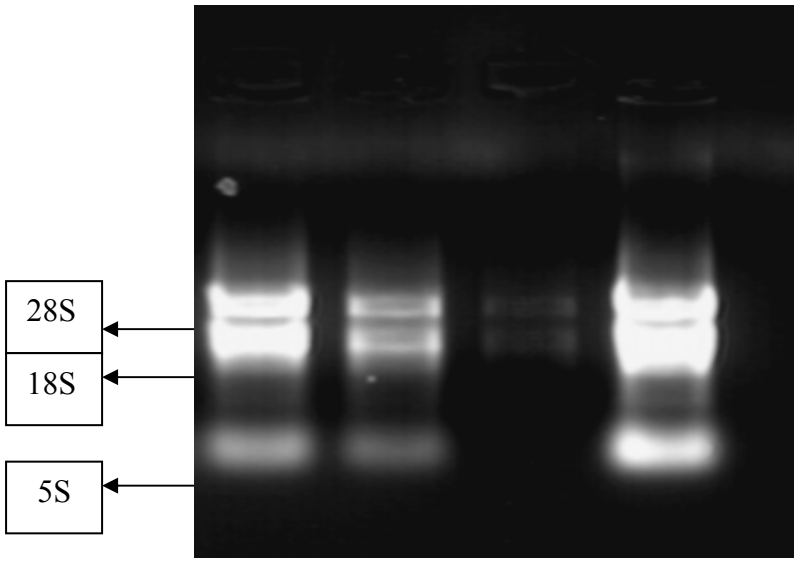


Figure 3.1. Results of RNA Gel Electrophoresis (RNAs were isolated from whole plant parts of *Arabidopsis thaliana* grown in jars. Isolated RNAs were loaded on 1% agarose gel and the three bands seen on the photogaph of the gel indicated the 28S, 16S and 5S ribosomal RNA bands.)

By using synthesized cDNA, PCR analysis was done for amplification of the *AtNHX1* coding sequence (1614bp). The amplified fragments were observed on agarose gel and photographed. The PCR results are shown in Figure 3.2. The PCR amplified *AtNHX1* coding sequence was extracted from the agarose gel.

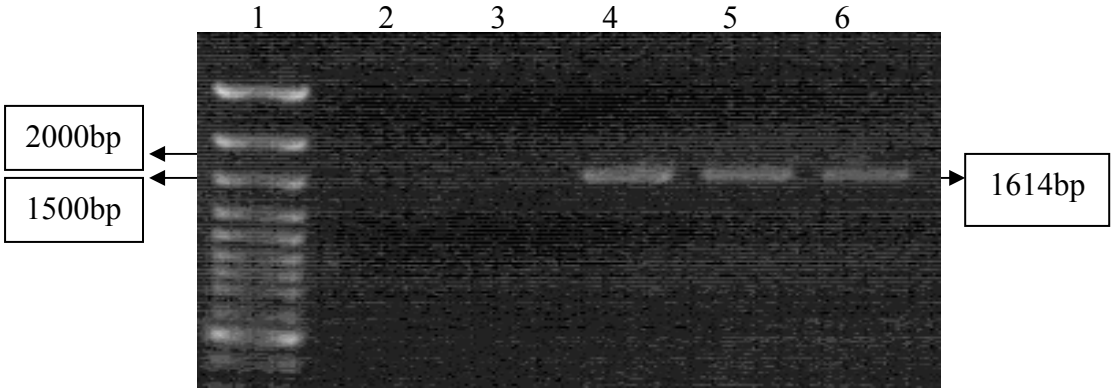


Figure 3.2. Agarose gel electrophoresis results of PCR amplified *AtNHX1* (Lane 1: GeneRuler™ 100bp DNA Ladder Plus, Lane 2: Blank, Lane 3: Empty lane and Lane 4-6: PCR amplified *AtNHX1*. The expected size of the amplicon was 1614 bp.)

3.1.2. Sequence Analysis of *AtNHX1*

The sequence analysis of PCR products were done at Iontek Company. Sequence analysis showed that an error free full-length *AtNHX1* was obtained after PCR amplification. The 1614bp coding region of *AtNHX1* was amplified and purified from the gel. This region codes for a 538 amino acid protein.

```
   1  atatttctct ctctactgtg tttagaaatt ggaatcttct ctcttctcta tctccctct
  61  tcttaaaaag ggaccgtaca cgtctctctc tatttccagt aaaaaatcga aatttcgtat
 121  aatttcctca gtcccgtaat tttctccttt tttttcttcc ccaattcctt caattttcga
 181  attcgcctct ctgtttcgtt cctcgtagac gaagaagaag aagaatctca ggttttagct
 241  ttcgaagctt ccaaaatttt gaattttgat cttctgggct cttttgtaaa tcagactgaa
 301  gatatttaga ttacccagaa gttgttcaag gaatggtttc agtggacagc acggaaagat
 361  aaaagagact ttttttttcca gattttgctg atccaaaatc tgaatagttg ttcatgttct
 421  tggatcaaat ctggaaagag gaagtttgtt ggatctagaa gaagataaca atgttggatt
 481  ctctagtgtc gaaactgcct tcgttatcga catctgatca cgcttctgtg gttgcgttga
 541  atctctttgt tgcacttctt tgtgcttgta ttgttcttgg tcatcttttg gaagagaata
 601  gatggatgaa cgaatccatc accgccttgt tgattgggct aggcactggt gttaccattt
 661  tgttgattag taaaggaaaa agctcgcatc ttctcgtctt tagtgaagat cttttcttca
 721  tatatctttt gccacccatt atattcaatg cagggtttca agtaaaaaag aagcagtttt
 781  tccgcaattt cgtgactatt atgctttttg gtgctgttgg gactattatt tcttgcacaa
 841  tcatatctct aggtgtaaca cagttcttta agaagttgga cattggaacc tttgacttgg
 901  gtgattatct tgctattggt gccatatttg ctgcaacaga ttcagtatgt acactgcagg
 961  ttctgaatca agacgagaca cctttgcttt acagtcttgt attcggagag ggtgttgtga
1021 atgatgcaac gtcagttgtg gtcttcaacg cgattcagag ctttgatctc actcacctaa
1081 accacgaagc tgcttttcat cttcttggaa acttcttgta tttgtttctc ctaagtacct
1141 tgcttggtgc tgcaaccggt ctgataagtg cgtatgttat caagaagcta tactttggaa
1201 ggcactcaac tgaccgagag gttgccctta tgatgcttat ggcgtatctt tcttatatgc
1261 ttgctgagct tttcgacttg agcggtatcc tcactgtgtt tttctgtggt attgtgatgt
1321 cccattacac atggcacaat gtaacggaga gctcaagaat aacaacaaag catacctttg
1381 caactttgtc atttcttgcg gagacattta ttttcttgta tgttggaatg gatgccttgg
1441 acattgacaa gtggagatcc gtgagtgaca caccgggaac atcgatcgca gtgagctcaa
1501 tcctaatggg tctggtcatg gttggaagag cagcgttcgt ctttccgtta tcgtttctat
1561 ctaacttagc caagaagaat caaagcgaga aaatcaactt taacatgcag gttgtgattt
1621 ggtggtctgg tctcatgaga ggtgctgtat ctatggctct tgcatacaac aagtttacaa
1681 gggccgggca cacagatgta cgcgggaatg caatcatgat cacgagtacg ataactgtct
1741 gtctttttag cacagtggtg tttggtatgc tgaccaaacc actcataagc tacctattac
1801 cgcaccagaa cgccaccacg agcatgttat ctgatgacaa caccccaaaa tccatacata
1861 tccctttgtt ggaccaagac tcgttcattg agccttcagg gaaccacaat gtgcctcggc
1921 ctgacagtat acgtggcttc ttgacacggc ccactcgaac cgtgcattac tactggagac
1981 aatttgatga ctccttcatg cgacccgtct ttggaggtcg tggctttgta ccctttgttc
2041 caggttctcc aactgagaga aaccctcctg atcttagtaa ggcttgaggg taacgtggaa
2101 gaaaagcttt gatttttttt ggtagaaaag ggtgattcaa attatgcttt tgtgtaaatt
2161 atccatttgt aatattgttt gtgaggacag aaatctgtcc taacgttttg agagcagaaa
2221 gcaaaacatg gcaactttga agtgtttgat tgatgtatgt aattatattc atatttgttt
2281 tgttgtaaca caaactacac atttgtttat gttttgaatt tggtttttgc ttcg
```

Figure 3.3. Sequence of *AtNHX1* cDNA (The sequence between 471-2085 pointed out the coding sequence of the gene. atg: stands for the start codon, tga: stands for the stop codon)

3.1.3. Cloning of *AtNHX1* in pCVB1

The PCR product was purified from the gel and cleaved with restriction enzymes (*XbaI* and *BamHI*) that these restriction cut sites were also found on the pCVB1 plant transformation vector (Double digestion was performed by using Fermentas 1X Tango buffer and one unit of both enzymes in 50µl total volume of reaction mixture. Reaction mixture was incubated at 37°C overnight).

Digested plasmid DNA (Figure 3.4) and PCR products were purified for the ligation reaction by using Fermentas T_4 DNA ligase (For ligation reaction; one unit of T_4 DNA ligase, 100ng vector DNA, 19.05ng of insert DNA and 1X ligation buffer were mixed in 20 µl reaction mixture and it was incubated overnight at 22°C).

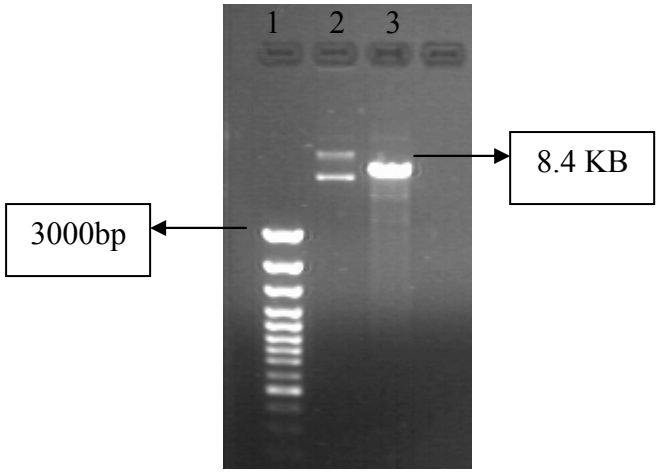


Figure 3.4. Agarose gel electrophoresis result of *XbaI* and *BamHI* digestion of the plasmid pCVB1 (Lane 1: GeneRuler™ 100bp DNA Ladder Plus, Lane 2: Uncut plasmid (+Control), Lane 3: Double digestion with *XbaI* and *BamHI*-cut plasmid (Plasmid was seen in linear form))

After digestion reactions, ligation of *AtNHX1* insert between the *XbaI* and *BamHI* sites of pCVB1 was carried out overnight and plasmids were used to transform *E. coli* cells. Following transformation *E. coli*, positive colonies that grew on ampicillin containing plates were tested for the presence of the insert by PCR and the DNA fragment in question was digested with the two resriction enzymes. Plasmid isolation was done for selection of positive colonies (Figure 3.5). Selected positive colonies were confirmed to carry the gene by PCR analysis after plasmid isolation (Figure 3.6) and restriction digestion (Figure 3.7).

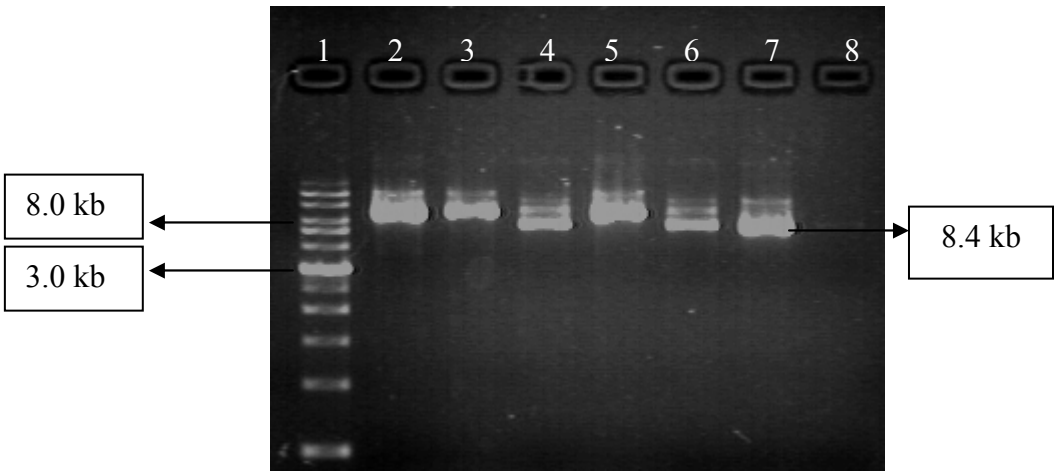


Figure 3.5. Plasmid isolation after transformation (Lane 1: Gene-KB DNA ladder, Lane 2,3-5: Putative plasmids containing *AtNHX1* coding sequence, Lane 4,6-7: Untransformed plasmidss (8.4 kb) , Lane 8: Empty plasmid (control))

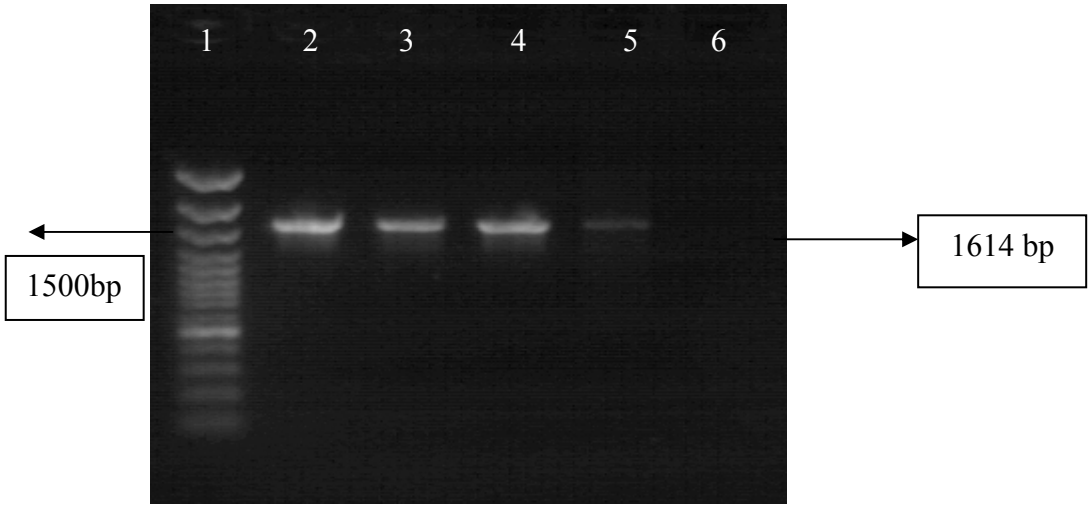


Figure 3.6. Analysis of pCVB1 for the presence of *AtNHX1* by PCR confirmation (Lane 1: GeneRuler™ 100bp DNA Ladder Plus, Lane 2-4: PCR amplification of *AtNHX1*, Lane 5: Amplified PCR product as positive control, Lane 6: (-) Control).

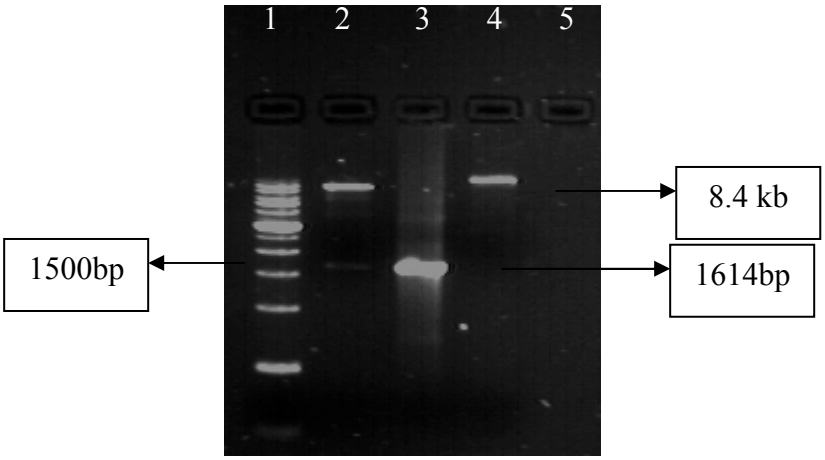


Figure 3.7. Agarose gel electrophoresis result of *XbaI* and *BamHI* digested plasmid (Lane 1: Gene-KB DNA ruler, Lane 2: Digested plasmid containing *AtNHX1* (The expected band belonging to *AtNHX1* was seen on the gel photograph), Lane 3: Gel extracted PCR product for positive control, Lane 4: Uncut plasmid).

Plasmids isolated from the positive colonies was analyzed for the presence of the gene by sequence analysis performed at Iontek Company, İstanbul. For this reason, two pairs of extra internal primers were designed (Table 3.1). According to the sequence analysis full-length *AtNHX1* was cloned into plasmid pCVB1.

Table 3.1. Primer sequences used in sequence analysis

Plasmid f-1 Plasmid f-2	5'- TGATTATCTTGCTATTGGTGCCATA -3' 5'- TAAGGAAGTTCATTTCATTTGGAGAGGACC -3'
Plasmid r-1 Plasmid r-2	5'- TCGTACTCGTGATCATGATTGCATT -3' 5'- ATCTAGCTGGCCGTACGTTCGACTA -3'

3.1.4. Transformation and Selection of Plants

Tobacco leaves were transformed with pCVB1 containing *AtNHX1* by *Agrobacterium* mediated leaf disk gene transformation method. *Agrobacterium tumefaciens* EHA105 strain was used for direct transformation of plants. After the selection on ampicillin and rifampicin containing plates, plasmids from positive colonies were isolated and used in PCR studies for verification of the presence of the gene of interest (Figure 3.8).

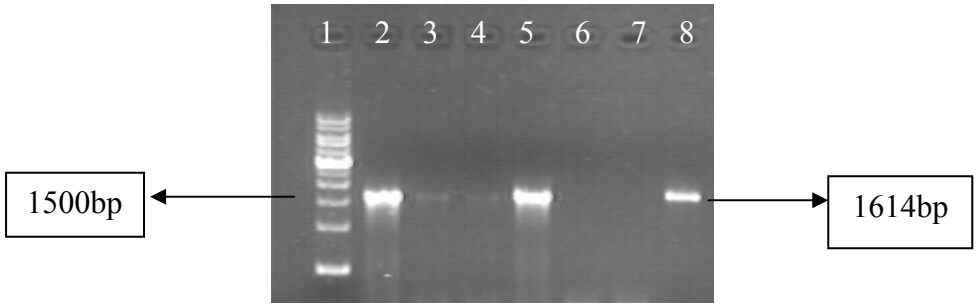
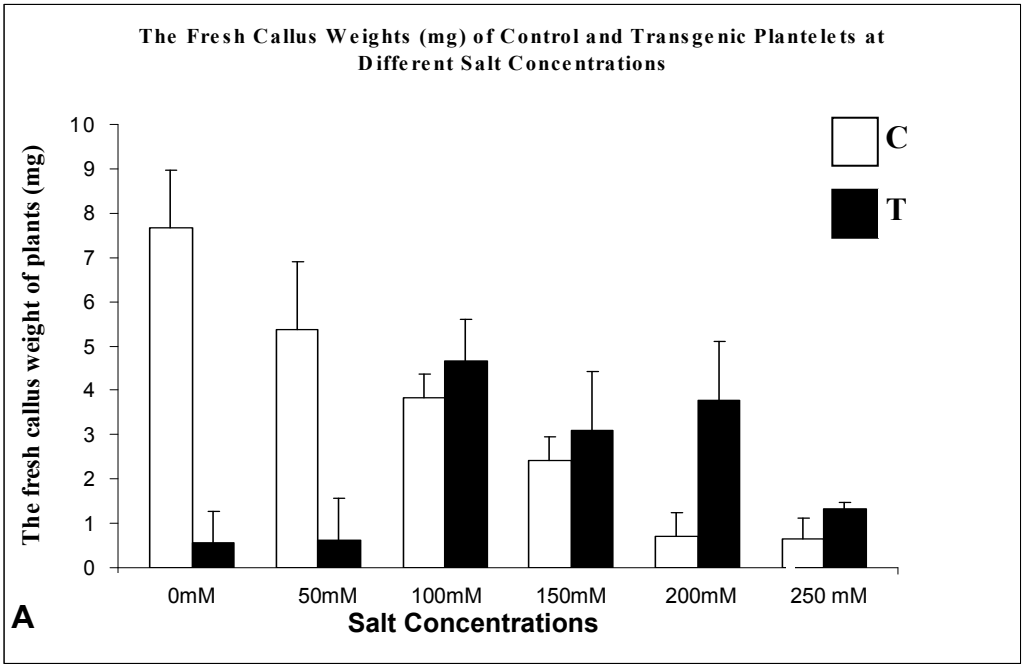


Figure 3.8. Agarose gel results of PCR amplified gene product after plasmid isolation from *Agrobacterium tumefaciens* (Lane 1: Gene-KB DNA ruler, Lane 2,3,4 and 5: amplified PCR products of *AtNHX1* from isolated plasmids, Lane 6: (-) control, Lane 7: PCR amplification by using plasmid from an untransformed colony , Lane 8: *AtNHX1* for positive control)

3.1.5. Regeneration Test Under Salt Stress

The leaves of tobacco were used for *Agrobacterium*-mediated transformation. The leaves were almost at the same age (6 weeks) and grown at the same conditions. The putative transgenic plants were regenerated on MSB medium containing 5mg/L PPT and different concentrations of NaCl (0, 50, 100, 150, 200, 250 mM NaCl). Following callus formation, the regeneration efficiency of control and transgenic plants on salt containing media were assessed by weighing the fresh callus weights (Figure 3.9 and Figure 3.10).



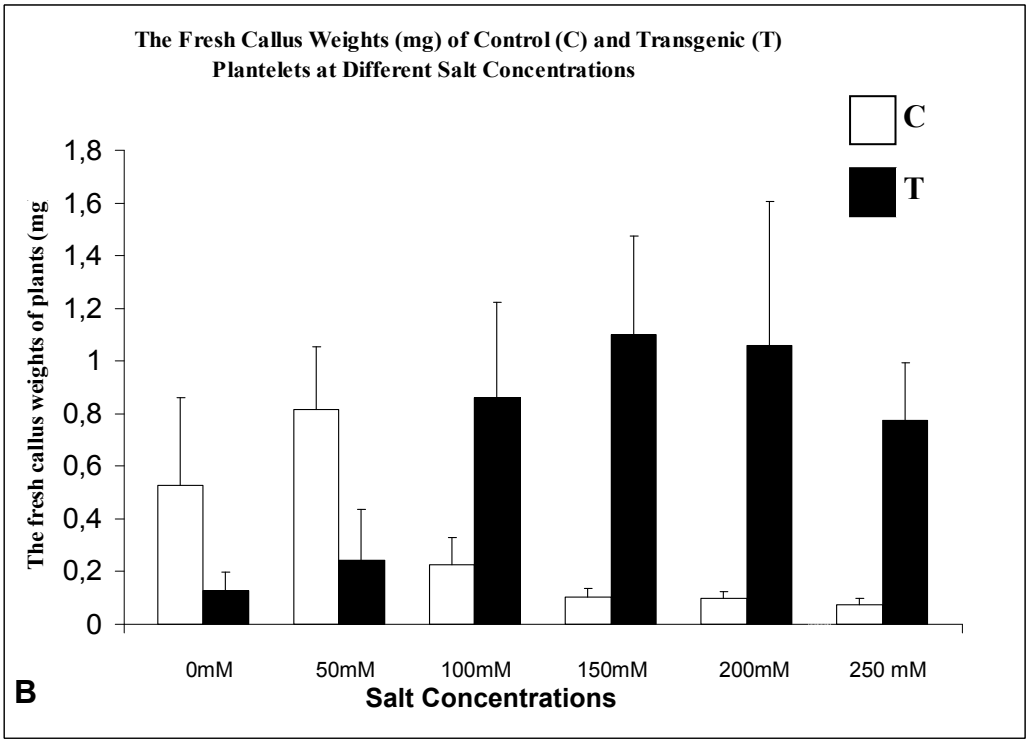


Figure 3.9. Regeneration ability of control and transgenic plantelets on different salt concentrations based on the fresh callus weight (A: Graph belongs to 3 weeks old plants, B: Graph belongs to 6 weeks old plants).

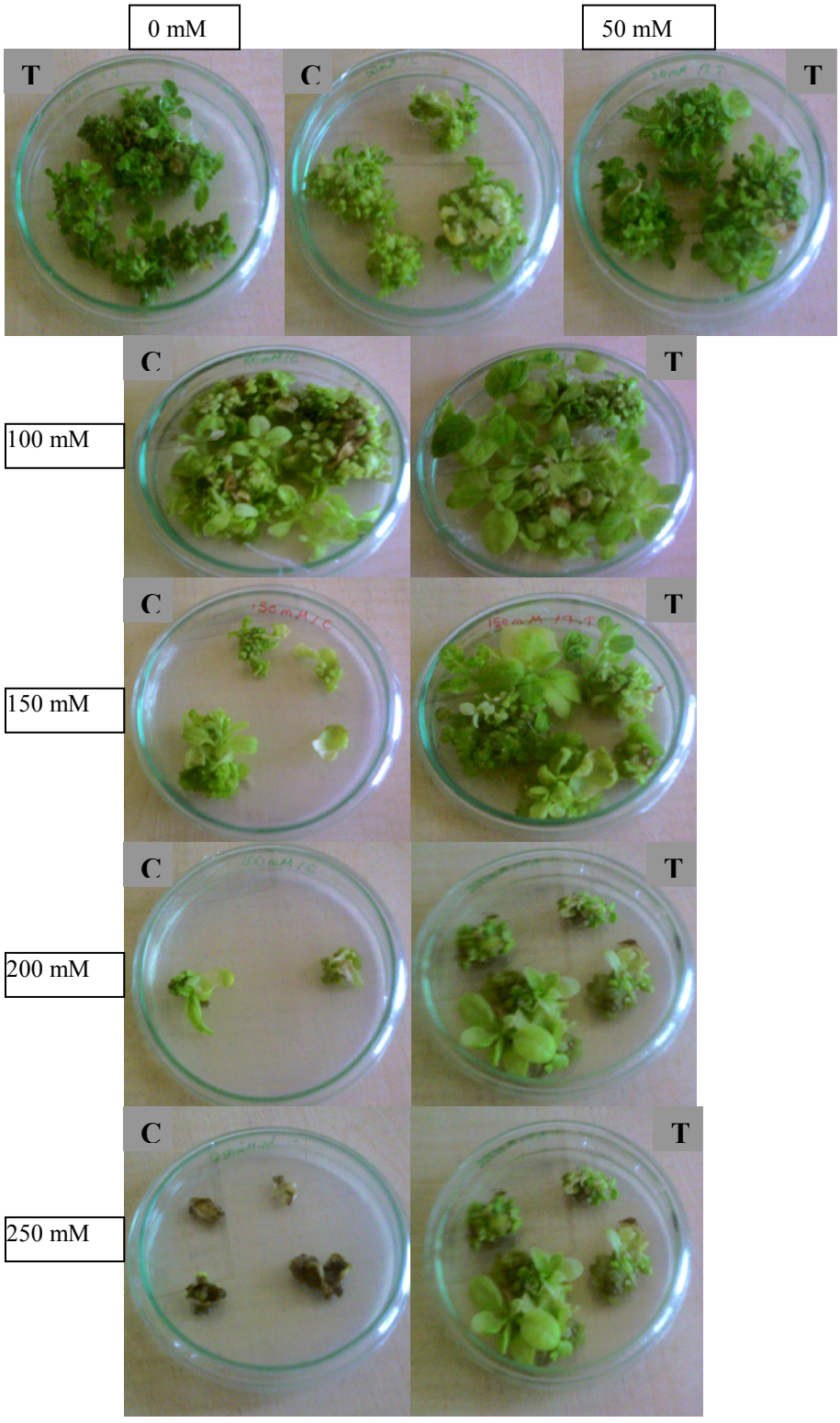


Figure 3.10. 3 weeks old control and transformed tobacco leaf disks on different concentrations of salt containing plates (plantelets on the left are the control ones, plantelets on the right are the transgenic ones) (C: Control, T:Transgenic)

The regeneration ability of transgenic plants carrying *AtNHX1* was higher than the control ones. It was observed that 150 mM NaCl concentration was enough to kill the non-transformed wild type plants within 4 weeks. The produced shoots of transformed plants were cut from their bases and transferred to MSC root inducing media containing 5 mg/L PPT. Root formation was observed in jars within 2-3 weeks.

3.1.6. Development in Soil

The regenerated plantelets were transferred from jars to soil. Transfer of explants to soil provided the regeneration of T_1 seeds and germination of the seeds in salt containing media confirmed the evaluation of salt tolerance of transformed plants. Due to sensitivity of plantelets, they were kept in growth chamber at 25°C with 70% humidity at a photoperiod of 16 hours light 8 hours dark for 20 days before taken to the greenhouse. The control plants reached the size to produce seeds quicker than the transgenic ones. Figure 3.11 shows the development of three different putative transgenic lines in the greenhouse.

Figure 3.11. The growth of putative transgenic plants in the greenhouse conditions

3.1.7. PCR Analysis Results of Putative T_0 Transgenic Tobacco Plants

By using isolated genomic DNA, PCR analysis was made for verification of the presence of T-DNA in T_0 putative transgenic plants. PCR was performed with specific primers to check the presence of *AtNHX1*. The amplified fragments were observed on agarose gel and photographed. The PCR results of 7 transgenic lines are shown in Figure 3.12. There was a band of expected size for the *AtNHX1*. The same band was absent in control plants.

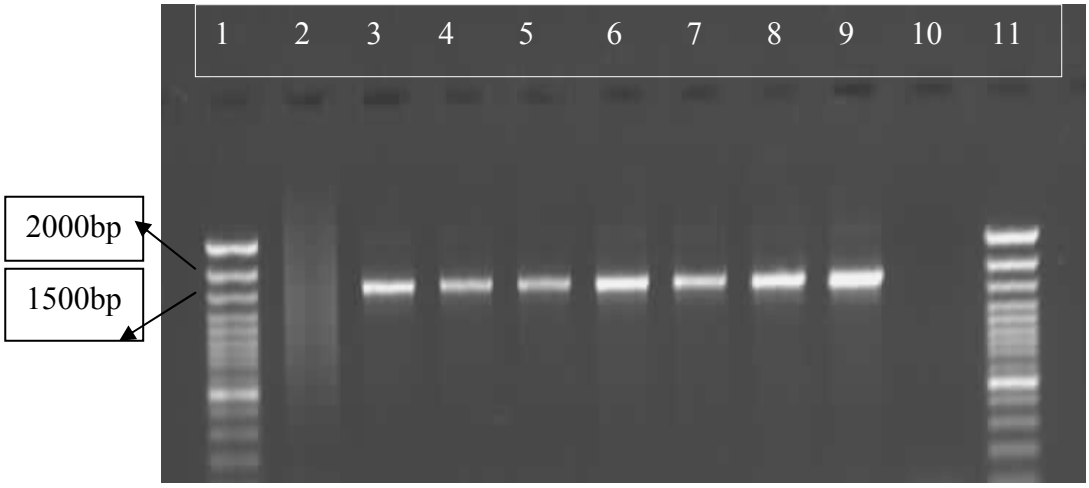


Figure 3.12. PCR analysis of putative T_0 transgenic tobacco plants using specific primers for *AtNHX1* (Lane 1: GeneRuler™ 100bp DNA Ladder Plus, Lane 2: Control Tobacco , Lane 3: T.L1, Lane 4: T.L2, Lane 5: T.L3, Lane 6: T.L4, Lane 7: T.L6, Lane 8: T.L7, Lane 9: T.L8, Lane 10: Blank (-Control) and Lane 11: GeneRuler™ 100bp DNA Ladder Plus. The expected size of the PCR product was 1614 bp).

3.1.8. Analysis of T_1 Transgenic Plants Under Salt Stress

The collected seeds of independent putative transgenic lines and non-transformed control plants were germinated on MS petri plates containing different salt concentrations (0, 50, 100, 150, 200, 250 mM NaCl). These concentration values were selected based on the previous studies (Zhang *et al.*, 2001; He *et al.*, 2005; Zhang and Blumwald, 2001). The seedlings were grown in these media for 3 and 6 weeks (Figure 3.13 and Figure 3.14). The number of germinated seeds were counted and were analyzed statistically via performing paired-samples t test for 3 weeks germinated T_1 seeds.

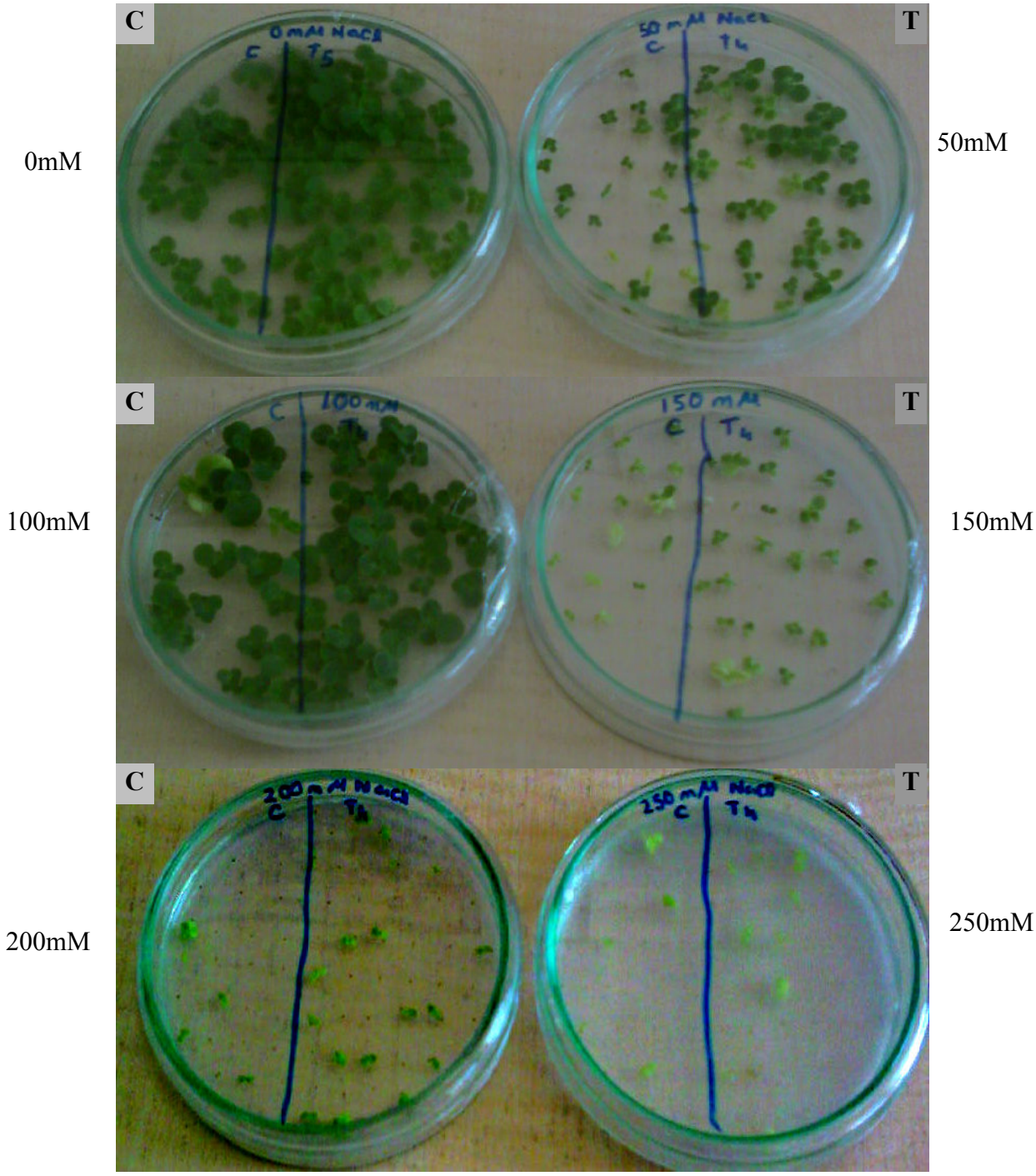


Figure 3.13. Results of T_1 progeny growth of 3 weeks old control and transgenic plants on NaCl selective medium (C: Control, T: Transgenic)

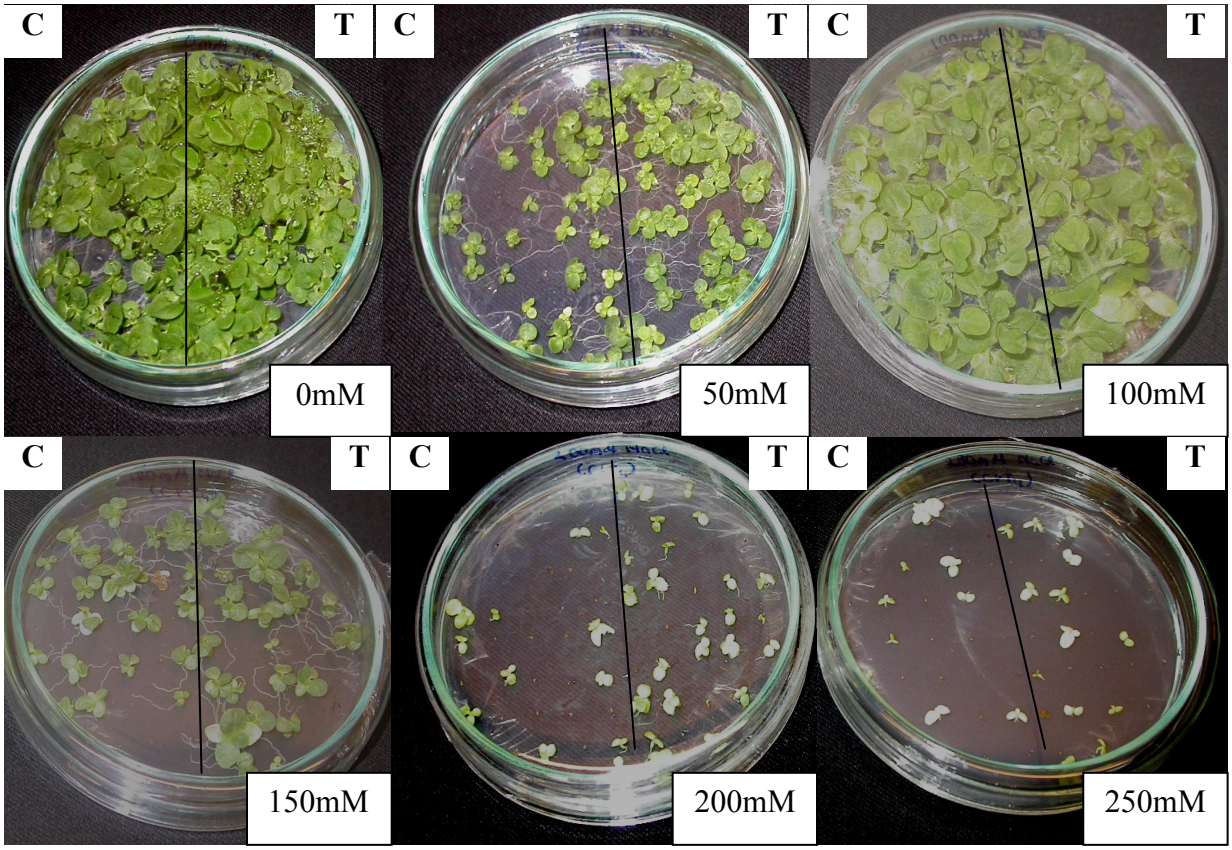


Figure 3.14. Results of T_1 progeny growth of 6 weeks old control and transgenic plants on NaCl selective medium (Left side of the plates contain untransformed control seeds and right side contain transgenic seeds).

According to the paired-samples t test, the germination efficiency and growth rate of T_1 seeds were higher than the control ones especially under high salt concentrations (150, 200 and 250 mM NaCl). For 150 mM NaCl, the p value was 0,001 and for 200 mM NaCl, the p value was also 0,001. These results were statistically significant for the germination efficiencies of transgenic T_1 seeds.

3.2. Discussion:

AtNHX1 gene is the most generally studied plant vacuolar Na^+/H^+ antiporter (Apse *et al.*,1999; Gaxiola *et al.*,1999; Blumwald *et al.*, 2000). Regulation of this Na^+/H^+ antiporter's expression by salt stress is necessary for gaining of salt tolerant characteristics (Shi and Zhu, 2002). The expression of *AtNHX1* gene in cotton, brassica and tomato plants under salt conditions (He *et al.*, 2005; Zhang *et al.*, 2001; Zhang and Blumwald, 2001) and as well as over-expression of the gene in *Arabidopsis* plants (Apse *et al.*, 1999; Sottosanto *et al.*, 2007; Shi *et al.*,2003) were analyzed and it was found that vacuolar Na^+/H^+ antiporter activity enhances Na^+ compartmentation into vacuoles. Also isolation of Na^+/H^+ antiporters from different plants confers salt tolerance (Ohta *et al.*, 2002; Wu *et al.*, 2005).

For these reasons, in the first part of the study, the isolation of error free full-length *AtNHX1* from *Arabidopsis* was carried out. *AtNHX1* was amplified by using cDNA of *Arabidopsis* via specific primers and cloning into pCVB1 plant transformation vector containing ampicillin resistance gene for bacterial selection and *PPT* gene for plant selection.

Tobacco is generally used as a model organism for plant transformation studies, development and evaluation of stress tolerance mechanisms. So it was adopted as a model system in this study. Hansen and Wright, 1999 pointed out that target tissues used in transformation studies, the type of DNA delivery system, selection possibilities and derivation of fertile transgenic plants were the main basis of the transformation studies. According to these criteria *Agrobacterium*-mediated transformation method seemed to be the most suitable method for plant transformation studies. Therefore, throughout the study EHA105 strain of *Agrobacterium* was used as a delivery system. Sterile leaf disks of tobacco were transformed with *Agrobacterium*, putative transgenic plants were obtained and callus formation and regeneration under different salt concentrations (0, 50, 100, 150, 200 and 250 mM NaCl) were evaluated (Figure 3.9 and Figure 3.10). For 150 mM NaCl concentration, after

the transformation, shoots were seen in 2-3 weeks whereas control plants did not initiate any roots. Control plants started to die after 3 weeks. As it was suggested by Xue *et al.*, 2004, Wu *et al.,* 2005 and Zhang *et al.,* 2001 the Na^+/H^+ antiporter activity increases in connection with the increase in salt concentration up to 200 mM NaCl. For concentrations higher than 150 mM NaCl, untransformed plants did not initiate roots and started to die sooner whereas transgenics were able to form proper callus and roots up to 200 mM NaCl. In aggrement with the results of the previous studies (Sottosanto *et al.*, 2004; Gaxiola *et al.,* 1999; Sottosanto *et al.*, 2007), low salt concentrations lead to some retardations in the growth and callus formation of transgenic plants. This might be due to the low expression of the gene at low salt concentrations, expression of this protein may have created a burden for the cell and also Na^+/H^+ antiporters may function in different physiological processes as proposed by Yokoi *et al.,* 2002. So the plants may have grown slowly in response to this extra burden. Also, *AtNHX1* gene codes for a Na^+/H^+ antiporter that pumps H^+ ions within the cytoplasm of the cell in exchange of Na^+ ions. This situation decreases the pH of the cytoplasm, the acidity of the cell increases to the extent of that and it may also cause growth retardations especially under low salt concentrations due to the low induction of the gene. However, according to Figure 3.9, the plants were growing normally depending on the increase in the salt concentrations of 100 mM, 150 mM and 200 mM. At the same time the growth of the control plants started to slow down sharply. At the concentration of 250 mM both transgenic and control plants were badly affected within weeks (Figure 3.10).

Transfer of explants to soil provided the regeneration of T_1 seeds and germination of the seeds in salt containing media confirmed the evaluation of salt tolerance of transformed plants. Both control and transgenic seeds were germinated on medium containing no NaCl. With the increase in salt concentration the germination rate of the control seeds were reduced gradually. Both T_1 seeds and control seeds were germinated on the medium containing no NaCl at hundred percent. The 82% and 60% of the T_1 seeds were germinated at

the concentration of 150 and 200 mM NaCl respectively. However, the germination percentages of untransformed tobacco seeds at 150 and 200 mM NaCl were 39% and 21%. The results showed the increased salt tolerance of transgenic line compared to the control within weeks (Figure 3.13 and Figure 3.14). The regeneration ability of transgenic tobacco seeds were higher than the control ones especially on high concentrations (150 mM, 200 mM, 250 mM) of salt containing media both in 3 weeks old and 6 weeks old plants.

A pair sample t-test was conducted to evaluate the impact of gradually increased salt concentrations on the germination efficieny of the putative transgenic T_1 seeds and control seeds. There was not statistically significant difference between the germination efficiency of the transgenic and control groups at 0 mM salt concentration (p=0,256). On the other hand, at 50, 100, 150, 200 and 250 mM salt concentrations, the germination efficiencies of the putative transgenic T_1 seeds were statistically higher than the control seeds (p_{50}=0,003, p_{100}=0,013, p_{150}=0,001, p_{200}=0,001 and p_{250}=0,001). All the results obtained from the analysis supported that transgenic tobacco plants live under saline conditions by the help of the activity of *AtNHX1* compared to control ones.

CHAPTER IV

CONCLUSION

In this study, we aimed to isolate the coding sequence of *AtNHX1* coding for a Na^+/H^+ ion transporter that functions in salt stress tolerance from *Arabidopsis thaliana* and introduced it to *Nicotiana tabacum* cv. Samsun for evaluation of salt tolerance. For the purpose of obtaining salt tolerant transgenic tobacco plants, coding sequence of *AtNHX1* was originally isolated from *A. thaliana*, amplified and used in the transformation of model plant (tobacco).

Sequence analysis of PCR products indicated that the full-length *AtNHX1* cDNA was isolated and introduced into tobacco plants. In transformation studies, *AtNHX1* was transferred to tobacco via *Agrobacterium*-mediated transformation method. The expression of the gene was performed under the control of CaMV35S (35S Cauliflower Mosaic virus) constitutive promoter. When the transformation was completed, explants were grown in selective media containing 5mg/L PPT. At the end of the transformation studies 10 independent putative transgenic lines were obtained. Eight of these lines were transplanted to soil to achieve the generation (T_1 generation) of *AtNHX1* transformants.

The analysis included the regeneration tests on media containing different concentrations of salt (NaCl), fresh weight determinations and molecular analysis such as PCR. Our results showed that the regeneration abilities and germination efficiencies of transgenic plants versus wild type tobacco plants under salt stress conditions were higher especially in high salt concentrations (150 mM, 200 mM NaCl). Under low salt conditions (no salt, 50 mM NaCl) control plants tend to grow easily and rapidly than transgenic ones. These results showed similarities between previous studies and ours that

expression of the Na^+/H^+ antiporter gene even under no/low salt stress conditions may be creating a burden for the cells. Thus, growth retardations were observed under low salt concentrations.

Further studies will include bioassays with T_1 plants and analysis of T_2 progenies under salt stress conditions. Southern Blot Hybridization and Northern Blot Hybridization of transgenic plants will be performed to analyse the presence and the expression of the gene. The ultimate goal of this study would be the improvement of yield quality and the inrease in yield production of various plant species via introduction of the *AtNHX1*.

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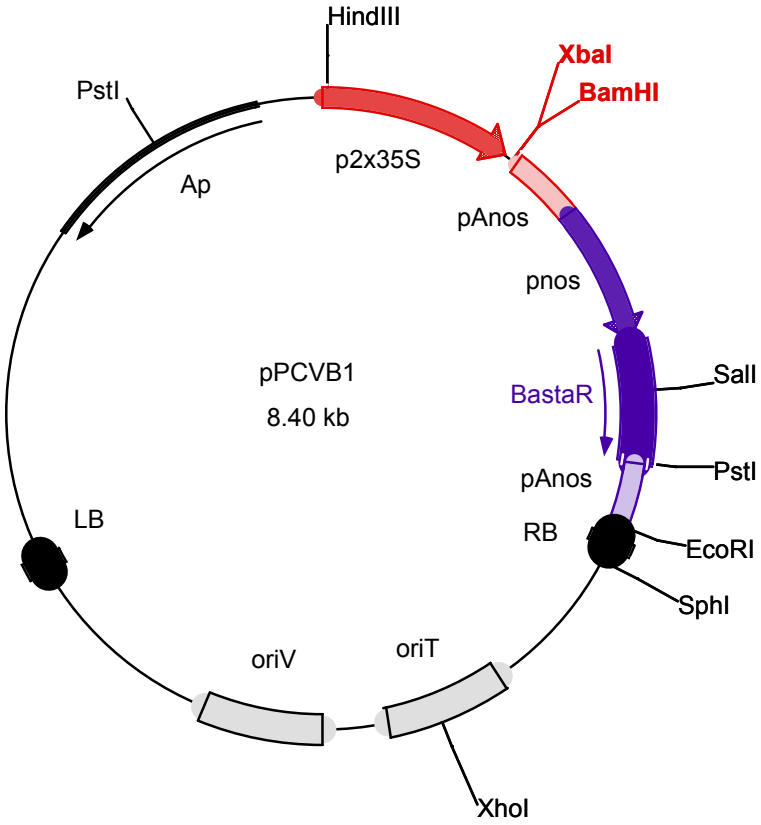
APPENDIX A

MAP of THE PLASMID pCVB1



APPENDIX B

BACTERIAL CULTURE MEDIA

Yeast Extract Broth Medium (YEB) (1lt)

Nutrient Broth	13,5 gr
Yeast Extract	1 gr
Sucrose	5 gr
$MgSO_4.7H_2O$	2 mM

Add 15 gr bacterial agar if solid media is required. (pH: 7.2)

Luria-Bertani Broth (LB) (1lt)

Bacto tryptone	10 gr
Yeast extract	5 gr
NaCl	10 gr
1N NaOH	1 ml

Add 15 gr bacterial agar if solid media is required. (pH: 7.4)

APPENDIX C

PLANT TISSUE CULTURE MEDIA COMPONENTS

Table C.1. Composition of Plant Tissue Culture Media

Sigma Plant Cell Cultures	**M5519 (**With the macro- and micronutrients, and vitamins)
Components	**mg/L**
Ammonium Nitrate	1650
Boric Acid	6.2
Calcium Chloride anhydrous	332.2
Cobalt Chloride.$6H_2O$	0.025
Cupric Sulfate.$5H_2O$	0.025
Na_2EDTA	37.26
Ferrous Sulfate.$7H_2O$	27.8
Magnesium Sulfate	180.7
Magnesium Sulfate.H_2O	16.9
Molybdic Acid.$2H_2O$	0.25
Potassium Iodide	0.83
Potassium Nitrate	1900
Potassium Phosphate monobasic	170
Zinc Sulfate.$7H_2O$	8.6
Organics	
Agar	_
Glycine	2
Myoinositol	100
Nicotinic Acid	0.5
Pyrodoxine.HCl	0.5
Sucrose	_
Thiamine.HCl	0.1
Grams of Powder (1 liter)	4.4

APPENDIX D

HOAGLAND'S-E MEDIUM

Table D.1. Preparation of macronutrient solution and media

COMPOSITION	STOCK SOLUTION	USE (ml/L)
$MgSO_4.7H_2O$	24.6 g/100 mL	1.0 mL
$Ca(NO_3)_2.4H_2O$	23.6 g/100 mL	2.3 mL
KH_2PO_4	13.6 g/100 mL	0.5 mL
KNO_3	10.1 g/100 mL	2.5 mL
Micronutrients	See table D.2	0.5 mL
FeEDTA	See table D.3	20.0 mL

Table D.2. Preparation of micronutrient stock solutions

MICRONUTRIENT	STOCK SOLUTION
H_3BO_3	2.86 g/L
$MnCl_2.4H_2O$	1.82 g/L
$ZnSO_4.7H_2O$	0.22g/L
$Na_2MoO_4.2H_2O$	0.09 g/L
$CuSO_4.5H_2O$	0.09 g/L

Table D.3. Preparation of FeEDTA Stock Solution

ADDITION	STOCK SOLUTION
$FeCl_3.6H_2O$	0.121 g / 250 mL
EDTA	0.375 g / 250 mL

Adjust the pH of the medium to 5.8. Autoclave the medium. (FeEDTA addition to the medium should be performed after autoclaving).

APPENDIX E

TRI REAGENT PREPARATION

TRI (Trizol) Reagent (110mL):

23.7 g guanidine thiocyanate

12.5 mL 100 mM Na-citrate buffer (pH 7.0)

Add DEPC-dH_2O to 45 mL, dissolve all solid material

Add 715 µl 35 % sarcosyl

Add 340 µl β-mercaptoethanol

Bring volume to 50 mL with DEPC- dH_2O

Autoclave

Add 50 mL water saturated phenol

Add 10 mL 2 M KOAc (pH 4.8)

APPENDIX F

ELECTROPHORESIS BUFFER PREPARATION

10X TBE (Tris Borate) Buffer (1L):

Tris-base 108 g

Boric acid 55 g

0.5M EDTA (pH: 8.0) 40 mL

All the components are mixed and the volume is completed to 1 L with dH_2O.

50X TAE (Tris-acetate) Buffer (1 L):

Tris-base 242 g

Glacial acetic acid 57.1 mL

0.5 M EDTA (pH: 8.0) 100 mL

All the components are mixed and the volume is adjusted to 1 L with dH_2O.

APPENDIX G

PLASMID ISOLATION SOLUTIONS

Sol I for 10 mL:

50 mM glucose 0.292 g
10 mM Tris Cl pH 8.0 500 µl (from 1M Tris Cl pH 8.0)
1 mM EDTA pH 8.0 100 µl (from 0,5 M EDTA pH 8.0)
Must be autoclaved and ice-cold before use.

Sol II for 10 mL:

0.2 N NaOH 200 µl (from 10 N NaOH solution)
1 % SDS 500 µl (from 20% SDS solution)
Must be freshly prepared before use.

Sol III:

4 V 5 M potassium acetate
1 V 10 M acetic acid
Must be autoclaved and ice-cold before use.

APPENDIX H

CTAB DNA ISOLATION SOLUTIONS

2X CTAB (Hexadecyl-trimethyl amonium bromide) Extraction Solution (100 mL):

CTAB	2 g
1 M Tris.Cl (pH: 8.0)	10 mL
0.5 M EDTA (pH: 8.0)	4 mL
5 M NaCl	28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.

5X CTAB Extraction Solution (100 mL):

CTAB	5 g
1 M Tris.Cl (pH: 8.0)	10 mL
0.5 M EDTA (pH: 8.0)	4 mL
5 M NaCl	28 mL

All are dissolved in dH_2O and volume is completed to 100 mL.