ENGINEERING SALT AND DROUGHT TOLERANCE IN COTTON (*Gossypium hirsutum* L.)

By

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CERTIFICATE

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Dated:

This Effort is
dedicated
To
My teachers.

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List of abbreviations

Acronyms	Full name
2X	Double
35S	35 bases preceding ATG
AtNHX1	*Arabidopsis thaliana* Na^+/H^+ exchanger
ATPase	*Adenosine triphosphatase*
AVP1	*Arabidopsis vacuolar pyrophosphatase*
B5 vitamins	Gamborg's vitamins
C	Callus colonies
CaMV	Cauliflower mosaic virus
CCI	Callus culture induction
cDNA	Complementary DNA
CTAB	Cetyl-trimethyl amino bromide
Ctr	Non-transformed control
DEPC	Diethyl pycarbonate
G.	Gossypium
GUS	Glucuronidase synthase
IRGA	Infra-red Gas analyzer
K^+	Potassium ions
Km	Kanamycin
L.	Lineous
LB	Left border
LB medium	Luria Bertani medium
mM	Milli molar
MPa	Mega pascal
MS	Murashige and Skoog
MS0	MS basal medium
MSK	MS with KNO_3
N	Nitrogen
Na^+	Sodium ions
NHX	Na^+/H^+ exchanger
NOSP	Nopaline synthase promoter
nptII	Neomycin phosphotransferase
NR	Nitrate reductase
OCST	Octopine synthetase terminator
ORFs	Open reading frames
P	Phosphorus
PCR	Polymerase chain reaction
Pn	Photosynthesis
RB	Right border
RWC	Relative water contents
SEM	Scanning electron microscopy
S-medium	Stewart's medium
TE buffer	Tris. EDTA buffer

ABSTRACT

In the modern era of agricultural crop production, commercial applications of crop inputs and industrial activities have increased salt concentrations in our soil and water resources making them toxic for growing crops. During abiotic stresses, plants employ several strategies, among which salt pumping and compartmentalization into intracellular organelles (lumen of vacuole) is a major strategy.

The present study involved the development of transgenic cotton cv. coker-312 tolerant to salt and drought stress. In the first part of the study, silicon carbide whisker mediated cotton calli transformation method was established using *GUS* and AVP1 gene. Fertile transgenic plants were produced from embryogenic calli of four events. PCR and southern hybridization of kanamycin resistant putative *AVP1* embryogenic calli showed the presence and stable integration of transgenes in 1-4 copies in the genome. Results of the salt tolerance study of T_1 *AVP1* transgenic cotton showed significantly greater salt tolerance at 200mM NaCl than non-transformed control plants. Moreover, transgenes segregate in a Mendelian fashion indicated the authenticity of the method.

In the second part of the study, *AtNHX1* gene was cloned under the control of double CaMV 35S promoter and terminator in plant transformation vector pGA482. *Agrobacterium tumefaciens* harboring *AVP1* and *AtNHX1* genes were used to inoculate coker-312 hypocotyls. Putative transgenic kanamycin resistant calli of different events were produced and putative regenerated transgenic plants were obtained. The Overall transformation efficiency was 20-25 %. Regenerated T_0 plants were grown to maturity in the containment for getting selfed T_0 seeds and subsequently T_1 and T_2 seeds. PCR and Southern analyses showed the presence and genomic integration of 1-4 copies of the *AVP1* and *AtNHX1* genes. Northern analyses/cDNA analyses showed transcripts of transgenes in the transgenic plants.

Transgenic plants (T_2) having *AVP1*and *AtNHX1* genes verified by PCR were exposed to salt studies executed with *AVP1* transgenic in both hydroponic as well as in pots while plants having *AtNHX1* gene were exposed only in pots. After 4-6 weeks of germination, NaCl was applied in solutions up to 200 mM. Transgenic plants showed significantly higher accumulation of Na^+, K^+, total free amino acids, proline, total soluble sugars and higher nitrate reductase activities than non-transformed control plants.

A water stress study was performed on *AVP1* T_2 transgenic cotton after two weeks and 8-10 weeks of germination in pots. Stress was applied by withholding water for upto 10 days. Results revealed that transgenic *AVP1* plants have significantly high water contents and low water potentials with less square shedding, retain high number of bolls than non-transformed control plants. Photosynthesis rate was significantly higher in transgenic (*AVP1/AtNHX1*) cotton progenies than non-transformed control plants. Transgenic cotton also showed significantly better growth and yield when compared with non-transformed plants. Fiber analyses by scanning electron microscopy and HV1 indicated that fiber of transgenic plant were healthy with uniform twisting in contrast to weak and shriveled fiber of the non-transformed control plants. Transgenic plants had high fiber strength, length and low mic value than non-transformed control cotton plants respectively. Transgenic germplasm developed in present study might be useful for its application in cotton breeding program aiming salt/drought tolerance in cotton cultivars.

Chapter 1

Introduction and Review of Literature

Sustained agricultural productivity across the world is at risk due to threats from salinity and scarcity of water/soil resources with an ever increasing impact and wide distribution. Salinity impinges significant area of world's arable land (20 %) and ~ 40 % of the irrigated land (Bhatnagar-Muthur *et al.,* 2008).

Similarly, insufficient water is a serious constraint limiting agricultural production. Increasing scarcity of water resources is a global problem leading to drought as a major factor challenging crop production worldwide. Presently, 40 % of global crop produce is contributed by 240 Mha of irrigated lands. It is of paramount importance to enhance the drought tolerance of crops (Lv *et al.,* 2008). Due to the complex and multigenic nature of the abiotic stress tolerance trait, traditional breeding programs are considered insufficient to develop abiotic stress tolerant crop cultivars. In the scenario of high pace of world population increase, increase in food and commercial crops production are urgently required to secure the future food demands a merging of biotechnology into conventional breeding programs to develop salt and drought-tolerant crop varieties.

1.1. Importance of cotton

American cotton (*Gossypium hirsutum* L.) is the political crop with commercial and social importance. Cotton is a heavily traded commodity across 150 countries across the world with economic association of millions of families for their livelihood. It generates economic impact worth of ~500 billion (USA dollars) (Chen *et al.,* 2007). Among 80 countries know for cotton cultivation, Pakistan ranked 4th after China, USA and India.

In Pakistan, cotton is grown over an area of 3 million hectares with a total production of 13.6 million bales of lint with an average yield of 1200 kg/ha (Economic Survey of Pakistan, 2011-12). It provides about 90 % of the raw material to more than 350 textile mills and is the source of more than 60 % of the total foreign exchange earnings. The Cotton crop not only provides livelihood to farmers and workers of ginneries, textile mills and garment factories but also 60 % of edible oil for human

consumption in Pakistan is produced from cotton seed. Whereas, pressed cake/oilseed cake, a by-product of cottonseed serves as an additional food supplement for dairy animals (Chaudhary *et al.,* 2009).

Primarily, cotton is a perennial plant with fair drought tolerance but due to intensive domestication , commercial genotypes are sat and drought prone under field conditions (Gorham, 1996; Naidu *et al.*. 1998). Abiotic stresses like salt and drought affect cotton plants by limiting plant health, growth, fiber yield and lint quality.

1.2. Species of cotton

The Gossypium genus has fifty species in total in which 45 are diploid (2n=2x) and five (2n=4x=52) are tetraploid with disomic inheritance pattern. There are eight groups reported in diploid species having diverse origin. The A, B, E and F genomes have come from African clades (Wendel and Cronn, 2003) as evident by its natural prevalence in Africa and Asia and clade of D genome is natural to the Americas. Other diploid clades of C, G, and K genomes belongs to Australian origin. Cultivated allotetraploids of new world cotton *i.e., Gossypium hirsutum* and *Gossypium barbadense* have evolved from interspecific hybridization of A and D genomes species. Tetraploid cotton *(Gossypium hirsutum* L.) has the progenitors from A genome species like *Gossypium* herbacium (A2) and Gossypium arboreum (A2) with D-genome species *Gossypium* raimondii (D5) (Brubaker *et al.,* 1999).

About 2 million years ago, Genome acquisition and duplication is believed to be arisen during the course of evolution (Wendel and Cronn, 2003), benevolenting five species of allotetraploids. Importantly, species having A genomes were suitable for cultivation aiming to produce fibers for spinning purposes but could not gain wide acceptance by farming community in contrast to the new world cotton having D genomes (Applequist *et al.,* 2001). At present most of the cultivated world cultivated cotton (95 %) belongs to upland American cotton i.e., *Gossypium hirsutum* whereas exceptionally long i.e., Egyptian cotton (*G. barbadense* L.) shares 2% of the total world area (National Cotton Council, http://www.cotton.org.witg 2006). Rational combination of A with D genomes have opened up new horizons useful for improving different cotton characteristics (Jiang *et al.,* 1998; Saha *et al.,* 2006; Yang *et al.,* 2006; Chen *et al.,* 2007).

Genomic characterization of cotton species will open unlimited gene resources of commercial importance.

1.3. Salt and drought problem

Pakistan is located in an arid to semi-arid region with annual rainfall inadequate for crop production. Out of the total cultivated area of 20.00 million ha, about 7 million ha are directly or indirectly salt and drought affected. In Pakistan, drought regime usually results from unfit water resources.

In Pakistan, sodium is the predominant cation in land and water resources at high concentration. Therefore salinity problems are usually associated with drought problems. In Pakistan, more than 0.75 million tube wells are operating and a substantial amount of water is derived from tube well sources for agricultural purposes. About 60 % of the tube wells pump poor quality irrigation water. Therefore, annual land loss due to salinization is about 40000 ha and increase is on pace in the modern era of commercial crop cultivation and production (Mehmood *et al.,* 2009; Ashraf *et al*., 2008).

To combat salinity problem through physical approaches are all difficult and expensive and provide only temporary relief. Among the promising approaches, the growing of salt and drought tolerant plants either developed through conventional breeding or transgenic approaches seems to be a useful and practical approach. These approaches have the potential to improve the plant capability to withstand the abiotic stresses and provide a long run solution to these problems. After the successful deployment of insect resistant transgenic Bt. cotton, the potential of biotechnology is used for the development of salt and drought tolerant plants cotton (Zhu *et al.,* 2011).

Cotton production in Pakistan is facing certain challenges for increase in yield like biotic stresses (insect and cotton leaf curl virus) and abiotic stresses i.e., salt, drought, heat etc. Problems like insect pest attacks have been largely addressed by adoption of Bt cotton varieties (Ali *et al*., 2010; Arshad *et al.,* 2008)). However, problems of salinity and drought are posing as a serious threat to this premium crop. Commercial cotton production has boosted cotton picking yield with a significant increase in water and fertilizer input applications with a likely increase in salt contamination of land and water resources are followed increasing drought stresses in near future.

Biotechnological manipulation of salt and drought tolerance mechanisms would lead to enhanced plant performance with high yield and fiber quality. It will contribute constructive role in cotton based economy of Pakistan by expanding cotton crop to previously inaccesssed areas. To meet growing human population requirements of fiber and feed, modern biotechnological tools should be applied for targeted improvement of cotton genome for a superior yield and quality (Dong, 2012). Plants are equipped with several strategies to cop adverse abiotic stress conditions, among which major one is by concentrating solute contents in lumen of cotton plants. A high vacuolar solute content is accompanied by plants strong ability to conserve water by drawing available moisture under high salt and drought conditions. In present scenario, vacuole is under focus as an alternate compartment by biotechnological tools to introduce tonoplast genes (AVP1/AtNHX1) for enhancement of vacuolar membrane potential for exchange of materials. High salt content in lumen allowed cotton plants to concomitant increase in solute contents by over expression of tonoplast genes (*AVP1/AtNHX1*) facilitated by enhanced tonoplast potential to facilitate transport of extra and waste/excess ions into the vacuoles (Pasapula *et al.,* 2010; He *et al.,* 2005; Shen *et al.,* 2015).

1.4. Effects of salinity on plant organs

Under salt and drought regimes, plants have many overlapping reactions, mechanisms and pathways. As a first indication, plant faces the penalty of disappearing fresh healthy color and declined growth depending upon the plant tolerance potential and severity of the stress. Salt creates cellular dehydration and result in osmotic stress and movement of water from intracellular to extracellular space reducing cytosolic and vacuolar volumes. Under extended regime of salt stress, damage of cellular structures, biological membrane and nucleic acid disruption thereby interfering plant metabolism and pathways negatively (Halliwell and Gutteridge, 1986; Gupta and Huang, 2014). Initial responses to salt and drought stresses include common features except for ionic component. Ionic component is predominant in salt regimes which include (Na^+, $^-$and K^+). These overlapping characteristics include metabolic processes like sudden decline of photosynthesis rate and sudden fluctuation of ABA levels in stressed plants.

In clay soils, improper management of salinity problem leads to accumulation of sodium salt in soils resulting in sodium rich soils. In sodium rich soils, Na^+ has high

affinity for negatively charged particles of clay making swelled and dispersed soil texture making unfit for plant growth (Chinnusamy, *et al.,* 2005). A high NaCl concentration in plant tissues is toxic for growth of glycophytes (Glenn *et al.,* 1999). Radicals alterations due to high NaCl level is dangerous for plant growth due to unusual influx of sodium ions (Blumwald *et al.,* 2000). Plant growth is proportionally suppressed by level of radicals and soluble salts. Ultimate consequences are penalty on plant growth rate, adverse changes in leaf and chloroplast machinery irregularising assimilation of carbohydrates (Miller *et al.,* 2010).

1.4.1. Classical effects of salinity on entire plant

Classically, plants display detrimental effects of high salinity as as heavy penalty on plant growth, reduced yield if continued salinity damage becomes lethal to plant. Leaf tissues with high sodium contents undergo shedding/damaging shortening lifetime impinging crop productivity and ultimately yield. (Munis, 2002).

The High sodium in soil reduces biomass of root, shoot and leaf with high root/shoo ration in coton (Meloni *et al.,* 2001). Moreovernce, salt regime onsets and promote senesce resulting in loss or damage of reproductive parts particularly in cotton. In rice salinity can reduce yield up to 45 % by spikelet sterility and seed shriveling (Asch and Woperi, 2001). Salinity was key factor for in causing seed abortion, premature boll opening thereby keeping low plant yield quantitatively and qualitatively (Davidonis *et al.,* 2000). For high adaptability under salt conditions, plants must be equipped with multiple and diverse pathways to avert toxic effects of salinity. Under natural conditions, plants normally sacrificed by loss/abortion ovules and ovules affecting fertility adversely ensuring survival by investing resources into metabolic activities that enables plants to cop salt stress (Lauchli and Graatan, 2007).

1.4.2. Changes in leaf structure

Salt stressed plants have normally thickened leave epidermis, greater length and diameter of palisade cells and spongy cells in cotton, bean and atriplex leaves (Longstreth and Nobel, 1979). Under salt/drought stresss, area of tomato leaf and stomatal conductance is significantly reduced (Romero-Aranda *et al.,* 2001). Thylakoidal structure is disorganized with increased number/size of plastoglobili with reduced starch contents

as revealed by electron microscopic study in tomato plants under salt/NaCl conditions (Hernandez, 1999). Potato plants showed swelling in mesophyll tissues and chloroplasts under severe salt conditions (Mitsuya *et al.,* 2000). Under high salinized conditions, potato plants faced reduction in number and depth of stacks of grana and starch grains in their leaves (Bruns and Hecht-Bucholz, 1990). In Plants of tomato under salt stress, leaves possessed aggregated chloroplasts, disrupted and wrinkled biological membranes without a sign of grana or thylakoid structures when observed under transmission electron microscope (Khavarinejad and Mostofi, 1998). Similar chloroplast showed ultrastructural changes in leaves of salt treated *Eucalyptus* and *Brugeria praviflora* (Keiper *et al.,* 1998; Parida *et al.,* 2003).

1.4.3 Effect of salinity on nitrogen metabolism

Photosynthetic enzyme i.e., nitrate reductase (NR) (EC 1.6.6.1) is responsible for catalyzing reduction reaction of NO_3 to NO_2 and is considered critical factor in nitrogen metabolism (Srivastava, 1990; Lea *et al.,* 1997). Reversible phosphorylation of proteins can shift enzyme activity very rapidly in higher plants. Presence of free Mg^{++} ion leads to interaction of proteins with phosphorylated nitrate reductase. That is why, NR is light sensitive as it switches on during light hours and switches off during dark period. It was believed salt stress affect NR expression and activation adversely even destabilizes under severe conditions (Huber and Kaiser, 1996). Extreme salt stress inhibits plants for uptaking nitrate and declined activity of reductase in leaves (Meloni *et al.,* 2004; Prida and Das, 2004). High levels of Na^+ and $^-$radicals are main factors responsible for reduction of nitrate reductase performance and hindering uptake of NO3 keeping nitrogen contents low in leaves (Silveria *et al.,* 2001; Flores *et al.,* 2000).

Under high salinity conditions, decreased NR activity and reduced nitrate levels directly responsible for a slow plant growth and reduced production of biomass. As an adoptive strategy, salt stressed plants have upregulated NADP specific ICH (isocitrate dehydrogenase), a major enzyme in cytosol serving as skeleton between nitrogen and carbon metabolism in contrast to low activity in roots. Key enzyme of nitrogen metabolism and amino acid biosynthesis i.e., ferredoxin-dependent glutamate synthase becomes inefficient in leaves depending upon the level of stress and duration along with

physiological status and growth stage of the plant as the key determinants (Popova *et al.*, 2002)

1.4.4 Effect of salinity on photosynthesis

During photosynthesis, plants, some bacteria and algae convert light energy into production of organic compounds by physio-chemical process. Photosynthesis plays direct role in plant growth and development. It is highly prone process to abiotic tress and is inhibited by unusual salt and drought regimes thereby reducing photosynthesis rate plant biomass production (Kawasaki *et al.*, 2001). Photosystem II is mainly responsible for response management under disturbed environmental conditions (Baker, 1991), so it has been main area of subject for the study of salt stress effects on PS II. But the results reported that response of PS II photochemistry varied significantly in contrasting manner due to generic characteristics and potential of salt tolerance in plant species. Other studies reported the inhibition of PSII under salt stress (Bongi and Loreto, 1989; Mishra *et al.*, 1991; Masojidek and Hall, 1992; Belkhodja *et al.*, 1994; Everard *et al.*, 1994) and there are also reports reporting no significant adverse effects on PS II (Robinson *et al.*, 1983; Brugnoli and Bjorkman, 1992).

Photosynthesis inhibition may not be necessarily due to salt stress even in some halophytes, it is stimulated by salt stress (Rajesh *et al.*, 1998; Kurban *et al.*, 1999), majority of the reports showed the suppression of photosynthesis under salt stress (Chaudhuri and Choudhuri, 1997; Soussi *et al.*, 1998; AliDinar *et al.*, 1999; Romero-Aranda *et al.*, 2001; Kao *et al.*, 2001). Reduced leaf are is reflected by decrease assimilation of CO2 into carbohydrates (Papp *et al.*, 1983; Munns *et al.*, 2002), improper stomatal opening/closing for gas exchange (Brugnoli and Lauteri, 1991; Ouerghi *et al.*, 2000; Agastian *et al.*, 2000; Parida *et al.*, 2003), mesophyll (Delfine *et al.*, 1998), and the photosynthesis enzyme efficiency (Seemann and Critchley, 1985; Seemann and Sharkey, 1986; Brugnoli and Bjorkman, 1992; Reddy *et al.*, 1992). Extended salt stress impairs the repairing system of PS II by inhibiting of D1proteins synthesis at gene level (Allakhverdiev *et al.*, 2002). Chlorophyll *a*, chlorophyll *b* and total chlorophyll contents were higher in leaves of rice and sunflower under salt stress than under control condition, which agreed with previous observations (Asch *et al.*, 2000; Santo, 2004; Zhen-Hua *et al.*, 2012) and extended stress duration leads to degradation of chlorophyl contents and

imposing significant penalty on plant yield by shedding of leaves, squares and young fruiting bodies.

1.5. Effect of drought on plants

Different plants behave differently to drought stresses for effect on growth, yield component and quality characteristics. As a first sign of indication is reduction in turgor pressure that adversely affect plant growth rate, metabolic activities and its final volume. These observations are peculiar of drought sensitive plants leading to plant leaf wilting, losing fresh appearance, slowing development rate, stem growth, leaf texture and also lowering the stomatal conductance. In water deficient plants, plant photosynthetic activity is badly affected due to interference in carbohydrate metabolism. Under extended drought regimes, photosynthesis decreases with sudden fall of buds and flowers creating the competition between vegetative and reproductive parts for use of carbohydrates. Under drought conditions in cotton, leaf area and development is more susceptible as compared to photosynthesis and any fluctuation in CO2 is mainly responsible for less setting and retention of buds, reproductive parts.

Cotton cultivation requires good quality irrigation water for efficient utilization of water volume unit and nutrients uptake (Radin *et al.*, 1992; Marani *et al.*, 1985). In water deficient soils, there is decrease in availability of nutrients with impaired ability of plants for nutrient uptake which in turn alters the physiochemical composition of xylem sap (Chapin, 1991; Bacon *et al.*, 1998; Schurr and Scukze, 1996). Under semi-drought conditions, xylem pH is decreased and plants ability to uptake nitrate is reduced and leave pH becomes apoplastic (Gollan *et al.*, 1992; Schurr *et al.*, 1992). Other major impacts on drought stressed plants; reduction in leaf water contents, photosynthesis and water use efficiency (Egilla *et al.*, 2005). Alisha *et al.*, (2009) reported that cotton cultivation under arid conditions was marked by low boll setting and less number of monopodial/sympodial branches.

1.6. Mechanisms of abiotic stress tolerance

The adaptability response in plants to abiotic stresses consists of a cascade of processes that require concerted and co-action to alleviate both cellular osmoregularity and ion dis-equilibrium. Consequently plants must be able to produce certain biomass in

saline and droughtful environments. Tolerance and yield stability are difficult to combine but advancement in cell and molecular biology has led to availability of tools for fine tuning of processes to enhance response time efficiency and tolerance potential as compared to original plant species. During the course of evolution, plants were able to be equipped with certain characteristics and strategies to combat the abiotic stresses.

The rich genetic diversity of plants is used to apprehend abiotic stresses (Flowers *et al.,* 1995; Munns, 2002). Unfortunately, most crop plants are salt sensitive or hypersensitive in contrast to halophytes naturally found in saline environments. During the course of evolution, some halophytic plants have been able to develop capacity for accommodation under extreme salinity because of specific anatomical and morphological changes because of very special anatomical and morphological adaptations or avoidance mechanisms (Flowers *et al.,* 1995). Upon exposure to salt and drought stress, plants employ different mechanisms and strategies to combat and avoid toxic effect of salt and drought stresses as follows.

1.6.1. Sodium pumping into vacuoles

Vacuole in plants is the central intracellular organelle occupying 80-95 % of the total cell volume. Due to larger size, it is believed that sequestration of excessive Na^+ into vacuoles is brought about by vacuolar membrane localized Na^+/H^+ exchangers. The source of energy for deriving these Na^+/H^+ antiporters is electrochemical gradient generated by vacuolar propoton pumps. There are three well know H^+-pumps in plant cells, two are of vacuolar type i.e., ATPase (adenosine triphosphatase) and proton pyrophosphatse H^+-pump (Blumwald, 1987; Gaxiola, 1987 and Schroeder, 2004) and third H^+-pumps operated on the plasma membrane. Collectively these three proton pumps are responsible for energizing of biological membranes and facilitate exchange of materials thereby sustaining the process of biological life. The major significance of these pumps is enhance plant ability to push extra and waste Na^+ thereby averting toxic effects of toxicity by keeping low cytosolic level of radicals. By this process, vacuolar lumen would get charged by high level of radicals (Na^+) thus enabling plant to uptake of water and maintain cell turgor pressure under saline water and soil conditions. (Glenn *et al.,* 1999).

Transgenic *Arabidopsis* overexpressing AtNHX1 displayed upregulated Na^+/H^+ exchanging characteristics than wild genotypes. Overexpression of AtNHX1 was associated with high Na^+ contents in vacuole with salt tolerant phenotype than wild counterpart under high salt conditions (Apse *et al*., 1999). These results generated clear evidences about role of Na^+/H^+ antiporters based salt tolerance. Transgenic tomato and canola overexpressing of Na^+/H^+ antiporters generated similar results (Zhang *et al.,* 2001; Zhang and Blumwald, 2001). Upon accumulation of Na^+ in cytoplasm, plants employ two strategies either excluding Na^+ from the cell to the outer space or sequestering sodium into organelle i.e., vacuole by the action of Na^+/H^+ antiporters. (Apse *et al.,* 2007). Plant genomes have wealth of antiporter genes eg., more than 40 genes have been identified which are supposed to encode H^+-couple transporters as homologues of Na^+/H^+ antiporters (Bret *et al.,* 2005; Zhao, *et al.,* 2008). These transporters are categorized into three distinct families i.e., CPA1 (NHX) family has eight members, NhaD HX 28 members and KEA 6 members (Sze *et al.*, 2004). Among 8 members of CPA1 family (*AtNHX1-8*) family, six encode vacuolar/endosomal Na^+/H^+ antiporters (Pardo *et al.,* 2006); Yokoi *et al.,* 2002) and while remaining two plasma membrane specific Na^+/H^+ antiporters (AtNHX1 SOS1 and AtNHX8) have been discovered (Shi *et al.,* 2003). Major functional Na^+/H^+ antiporters AtNHX1 (At5g27150), *AtNHX2 (At3g05030) and AtNHX7/SOS1 (At2g01980)* are well elucidated *(*Yokoi *et al.,* 2002*)*. AtNHX8 (At1g14660) is considered as cell membrane localized Li^+/H^+ antiporter. Firstly, AtNHX3 (At5g5570) was expressed in sugarbeet for enhancement of salt tolerance in transgenic plants.

Further studies prove that AtNHX3 synthesize a protein involved in low K^+ tolerance in *Arabidopsis* (Liu *et al.,* 2008). Similarly, AtNHX4, the At3g06370 homologue was found to contribute in salt adaptive response mechanism. The Arabidopsis mutants for nhx4 gene were evaluated for elevated level of slat stress tolerance. Several heterologous transformation of AtNHX4 in bacterial systems (*E. coli*) whereas heterologous expression of *AtNHX4* in *E. coli strain* BL21 made the transform ants highly sensitive to NaCl (Li *et al.,* 2009). It attracted a lot of attention by different research groups and various NHX homologues were discovered in different plant species (Pardo *et al.,* 2009), and abiotic stress related roles were found by overexpression of

NHX genes in transgenic plants with significant salt tolerance (He *at al.,* 2005; Zhang *et al.,* 2001). During salt stress, tonoplast localized NHX-type Na^+/H^+ antiporters were observed to play a mediating role in the sequestration of Na^+ radicals into lumen so keeping cytoplasmic Na^+ concentration at steady state level and moderating the toxic effects of Na^+ on biological processes.

1.6.2. Sodium extrusion through the plasma membrane

Another arsenal that plants rely is by extruding excess Na^+ out of cell by plasma membrane located Na^+/H^+ antiporters as an adoptive response under salt stress. Evidence generated through physiological and biochemical studies beefed the argument for mediating the Na^+ efflux by Na^+/H^+ antiporters at the plasma membrane level (Dupont, 1992). Barley roots were found to possess a K^+- triggered Na^+ efflux under salt stress(Ratner and Jacoby, 1976). The plasma membrane based changes in pH gradients cereals like barley and wheat showed that similar to antiporter is transferring Na^+/H^+ out of cell cytoplasm (Mennen *et al.,* 1990). Vesicles of wheat plasma membrane possessed antiporters like activity with similar results from tomato and cotton (Allen *et al.,* 1995; Hassidim *et al.,* 1990). Inhibitor of antiporter activity by action of amiloride and Na^+ channel could be achieved in some animal systems (Cuthbert and Shum, 1976; Benos, 1982).

For operation of Na^+/H^+ and cation/proton transporters antiportrs, proton motive force is generated by plasma membrane type (PM-type) H^+-pump i.e., ATPase-pump (Schachtman and Liu, 1999). Intracellular homeostasis is maintained by Na^+ exclusion from the cell by Na^+/H^+ antiporters (Bartels and Nelson, 1994; Zhu, 2001). This phenomenon is energetically expensive due to high demand of energy for operation and is considered a major bottleneck under salinity stresses (Davenport and Tester, 2000). In *Arabidopsis,* several SOS homologs have been discovered and constituted essential component of Na^+ exclusion and operated on cell membrane Na^+/H^+ antiporters making salt tolerance phenomenon in *Arabidopsis* (Zhu, 2003). In Na^+ homeostasis and salt tolerance, Na^+/H^+ involved in SOS pathway possessed twelve trans-membrane domains in N-terminal half and extended hydrophilic C-terminal tail.

The plasma membrane localized role of SOS1 was verified by developing transgenic plants with SOS1-GFP fusion gene and plasma membrane was found to

fluoresce indicating the embedding of SOS1 protein in the plasma membrane (Shi *et al.,* 2003). Salt stress also elicited the signal and is sensed by SOS3 (Li and Zhu, 1998; Ishitani *et al.,* 2000). SOS3 is a serine/threonine kinase and physically interacts with SOS2 (Liu *et al.,* 2000; Halfter *et al.,* 2000). Ca^{++} sensing and signaling leads to SOS2 substrate phosphorylation activity by SOS3. SOS deficient mutants showed inefficient Na^{+}-coupled proton transport in plasma membrane vesicles (Qiu *et al.,* 2002). Moreover, except sos1 mutant, Na^{+}/H^{+} exchange activity was restored in mutant plants with the supplementation with functional SOS2 kinase (Guo *et al.,* 2001; Qiu *et al.,* 2002). All these results support the data stressing the SOS1 as target in SOS signal transduction pathway regulated by SOS2/SOS3 (Zhang *et al.,* 2004). SOS2 is also implicated in the regulation of Na^{+}/H^{+} exchange activity producing an unknown intermediated product (Qiu *et al.,* 2004) and SOS positively regulates the activities of CAX1vacuolar (Ca^{++}/H^{+} exchanger) (Cheng et al, 2004). In SOS signaling pathway, SOS2 is partially regulated by tonoplast cation/proton antiporter activity (Cheng *et al.,* 2004; Qiu et al., 2004, Zhang *et al.,* 2004).

1.6.3. Synthesis or accumulation of osmoprotectants

During salt stress, Na^{+} toxicity is averted either by compartmentalizing it into vacuole or excluding out of cell for avoiding toxic effects of excess Na^{+} ensuring cell turgor for water uptake and growth in saline environment. Salt stressed plants require high osmolytes level which may be achieved either by uptake of soil solutes or by endogenous synthesis of metabolic solutes. But our soil and water resources have considerable amount of Na^{+} and $^{-}$and are toxic to plant cells. Metabolic profile for salt stress is composed of compatible solutes like polyols, amino acids, sugars, betaines and other benign compounds that do not interfere in cellular biochemical and metabolic pathways but creates heavy drain on plant resources compromising on growth rate and yield (Bohnert and Jensen, 1996; Ramanjulu and Bartels, 2002).

The major role of osmoprotectants is for maintenance of lower water potential thereby generating driving force to water uptake (Carpenter *et al.,* 1990). Osmoportectants also serve as chemical chaperones to scavenge free radiclas and plant direct role in stabilizing biological membranes, proteins and enzymes (Akashi *et al.,* 2001; Hare *et al.,* 1998; Bohnert and Shen, 1999; Mcneil *et al.,* 1999; Diamant *et al.,*

2001). Major disadvantage with this strategy of salt tolerance is sacrifice of plants resources for plant survival and results in decrease in plant fertility, ovule abortion and pollen non-functionality due to insufficient resources in reproductive parts of plants (Asch and Wopereis, 2001; Davidonis *et al.,* 2000). There are research findings reporting the success in metabolic engineering to increase osmoprotectants and transgenic plants for enhanced potential of compatible solute synthesis were developed. Transgenic plants for high glycine betaine, sorbitol, betaine, manitol, trehalose and proline have been developed which exhibited significant tolerance to drought, salinity and cold stresses (Men and Churata, 2002). It is thus likely that compatible solute biosynthesis is another important mechanism which enables plants to survive under high salt conditions. Zhang *et al.,* 2009 transformed AhCMO gene into cotton via agrobacterium and observed high glycine (13-26 %) contents and salt tolerance in transgenic cotton plants as compared to non-transformed control plants.

1.6.4. Transcription factors

Plant stress responses are greatly regulated by many transcription factors (TFs) such as activators, repressors and moderators due to their first and early role in expression of environmental stress associated key genes. A series of genes or gene clusters regulated by transcription factors have been identified and their roles in environmental stress management have been proven (Rahaie *et al.,* 2015).

Under abiotic stresses particularly drought stress, role of transcription factors is well elucidated and Arabidopsis thaliana genome wide analysis revealed several targets actively involved during water shortage conditions. Major transcription factors are ABA responsive element binding protein I (AREB1), ABA responsive binding factor 2 (ABF2), dehydration responsive element (DREB) gene, MYB genes, bZIP coding genes, NAC, AP2, C2H2 zinc finger and protein kinases (SNF1, guard cell expressing calcium dependent protein kinases) (Uno *et al.,* 2000; Mare *et al.,* 2004; Sakuma *et al.,* 2006; Rahaie *et al.,* 2015).

NAC transcription factors are widespread in plants (Arabidopsis, rice, wheat, soyabean, and cotton) (Liu et al., 2014). Transcription factors have been transformed into plants of commercial importance such as rice (Ravikumar *et al.,* 2014), chickpea (*Anbazhagan et al.,* 2015), wheat (wang *et al.,* 2006; Chen *et al.,* 2015) and cotton.

Transgenic cotton plants overexpressing SNAC1 transcription factor exhibited vigorous root development than non-transgenic control plants under hydroponic conditions. Similarly, proline contents were higher in tissues of transgenic cotton plants and gave significantly higher yield (Liu et al., 2014). Cotton Dreb transcription factor showed enhanced tolerance against salt, drought and freezing stresses in transgenic wheat (Gao *et al.,* 2009). Similarly, improvement in drought tolerance was observed in transgenic cotton over expressing Dre-binding transcription factor gene. Improved drought tolerance was attributed to high glycine contents in transgenic cotton variety 28 I (Amudha *et al*., 2014).

1.6.5. Role of microRNA

MicroRNAs also known as riboregulators (21-24 nts) were discovered as translational attenuators regulating larval growth and development in *Caenorhabditis elegans* (Lee *et al.,* 1993). MicroRNAs mediate negative regulation of target mRNAS and processed from imperfectly paired hairpin precursors produced from transcriptional units distincting from protein coding genes (Dugas and Bartel, 2004). micRNA regulate target gene expression either by degrading target mRNA or by blocking protein translation through binding with 3UTR of the target mRNA. Initially microRNA roles were identified in developmental timing, cell proliferation and other physiological processes. In plants, microRNAs have been found to play significant role in biotic and abiotic stress responses like oxidative, mineral nutrient deficiency, drought salinity, temperature and abiotic stresses. Studies of microRNA profiling showed the microRNA mediated regulation of plant growth progression and development and are differentially expressed during abiotic stresses. A wealth of microRNA information is coming up in which mRNA is associated with abiotic/biotic stress responses target genes due to the availability of genomic sequences and high-throughout techniques (Kumar, 2014).

In future miRNA-mediated RNA interference would be an effective component of crop improvement programs against biotic/abiotic stresses (Zhou *et al.,* 2013). During drought stress, down regulation of miR 169 was associate with induction of its target nuclear factor YA5 (NF-YA5) which was verified by overexpression in transgenic plants leading to drought sensitive plants with increased water loss compared to standard control plants (Li *et al.,* 2008). Several studies have suggested the potential role of microRNA

and their target in plant response management against abiotic stresses. Drought tolerant transgenic Arabidopsis plants were developed by transforming soybean (Glycine max L.) gene GmNFYA3, a target of microRNA 169 (Ni *et al.,* 2013).

Transgenic tomato over expressing miR 169c exhibited reduced stomata opening, transpiration rate and leaf water loss resulting in drought tolerant phenotype compared with wild plants (Zhang *et al.,* 2011). Similarly, transgenic bent grass overexpressing a rice miR319 was salt and drought tolerant. Overexpression of miR 319 leads to prominent down regulation of four target genes (Zhou *et al.,* 2013). MicroRNA in cotton are well characterized but no report is available on transgenic cotton containing any microRNA genes or target thereof (Zhou and Luo, 2013). Two upland cotton genotypes i.e., salt tolerant Ealistaple7 and salt sensitive Nan Dan Ban Di Da were studied for constructing global map of expressed genes and miRNA. A total of 320 miRNA were identified across the two genotypes during salt stress (Peng *et al.,* 2014).

1.7. Characteristics of Sodium/proton exchangers

Continuous exchange of materials across the biological membranes is abundant, ubiquitous and evolutionary conserved features among microflora on earth. The constant feature of transmembrane transport of Na^+ in exchange for protons is essential for life in all kingdoms of life and has been the subject of extensive research in fundamental research. With application of modern research tools, wealth of genes (200) have been annotated as Na^+/H^+ exchangers in the databases (Brett *et al.,* 2005). In addition to salt and drought tolerance, Na^+/H^+ exchangers serve important role in various physiological processes like cell cycle and division regulation (Pouyssegur *et al.,* 1984; Putney and Braber, 2003), transepithelial Na^+ movement (Zachos *et al.,* 2005), salt tolerance (apse *et al.,* 1999; Moffat, 2002, Shi *et al.,* 2003), vesicle trafficking and biogenesis (Ali *et al.,* 2004; Bowers *et al.,* 2000), and the blue flower coloration in Japanese morning glory (Yamaguchi *et al.,* 2001).

Manipulation of Na^+/H^+ exchangers in plants have huge potential for salt improvement through biotechnology (Moffat, 2002; Shi *et al.,* 2003). Tonoplast Na^+/H^+ antiporter is involved in the transport of Na^+ radicals from cytoplasm of plant cells energized by proton motive force generated but H^+-pumps and this network has been identified as key player in conferring salt tolerance in plants (Blumwald, 2000). First

Na^+/H^+ antiportrer was isolated from Arabidopsis as yeast antiporter (Na^+/H^+ exchanger) homologue and encodes 538 amino acids with near dozen transmembrane domains (Apse *et al.,* 1999; Gaxiola *et al.,* 1999) as well as amiloride-binding domain (Hamada *et al.,* 2001). By immunological methods, co-localization of Arabidopsis NHX1 and Gef1 ¯were detected in prevacuolar compartments in yeast (*Saccharomyces cerevisiae*) (Gaxiola *et al.,* 1999) and prove the role of vacuole in salt tolerance. By topological view, AtNHX1 proteins have 9 transmembrane domains, 3 membrane hydrophobic domains, an N-terminus protruding into cytosol and C-terminal. Topological analysis of-terminal side lumbering in the vacuolar lumen with significant role Na^+/H^+ exchange activity (Figure 1). Deletion analysis of the C-terminus of AtNHX1 protein exhibited higher Na^+/H^+ exchange rate but declined K^+/H^+ exchange arte (Yamaguchi *et al.,* 2003). Vacuolar Na^+/K^+ antiporters encoded by NHX genes are directly involved in the transport of Na^+ into lumen (Nass *et al.,* 1997; Blumwald *et al.,* 2000; Hasegawa *et al.,* 2000). Due to direct role in Na^+ compartmentalization into plant vacuoles, NHX genes are key determinant of salt tolerance (Apse and Blumwald, 2002; Zhu, 2003; Tester and Dvenport, 2003).

After discovery of the NHX genes for salt tolerance, research efforts were directed towards other plant species and twenty four NHX homologues were identified in fourteen different species of plants. In response to NaCl stress, NHX genes were invariably induced and characterized by overexpression for salt tolerance in transgenic plants. Different NHX genes were transformed into plants and transgenic plants overexpressing NHX genes were found highly salt tolerant as compared to wild type plants. NHX transgenic model and agronomic crops plants were developed such as Arabidopsis, canola, tobacco, wheat, tomato and rice (Apse *et al.,* 1999; Fukuda *et al.,* 1999; Gaxiola *et al.,* 1999, Zhang and Blumwald 2001, Zhang *et al.,* 2001, Ohta *et al.,* 2002, Fukuda *et al.,* 2004, Wu *et al.,* 2004, Moghaieb *et al.,* 2014; Xue *et al.,* 2004, Yarra *et al.,* 2012. In the absence of AtNHX1 function, Arabidopsis plant were not able to develop root system and seedling establishment along with altered leaf development indicative of the role of NHX genes not only in salinity tolerance but also in leaf development (Apse *et al.,* 2003). Transgenic plants co-overexpressing further improved the salt and drought tolerance not only than nontransformed control but also than either of

AtNHX1 or AVP1 transgenic cotton plants (Shen *et al.*, 2014). These studies are of the opinion that multigene strategies are required to play their role under their different mechanism thereby contributing in additive manner determing plant adaptability response under diverse condition of different stressful conditions particularly abiotics stresses. With the advancement in modern molecular biology and its merging with bioinformatics to handle ever growing data, its processing and interpretation are the milestones t achieve pyramided genotype for commercial adventures. That is why, present literature trend is rich in addressing multiple nature problems of plants and exploring their solution by array genes rather limiting to one or few genes.

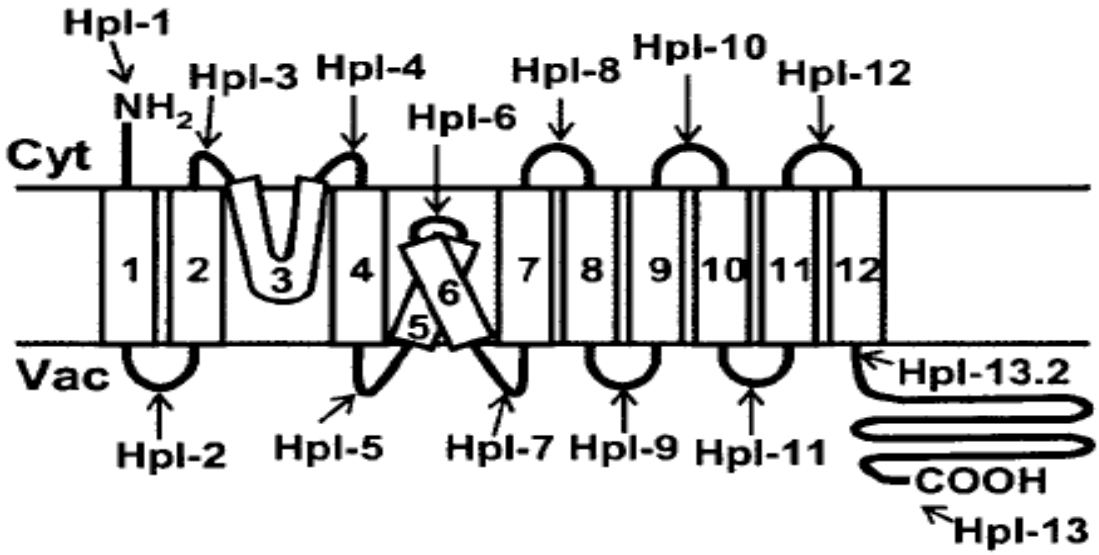


Figure 1.1 Proposed topological model of *AtNHX1***.** The boxes indicate putative transmembrane domains, and the arrows (HpI-1 to -13.2) indicate the positions of 3XHA-tag insertion in pHPI-1 to -13.2, respectively.

1.8. Characteristics of H^+-pumps

Using modern biotechnological tools and bioinformatics approaches, important biological processes have been elucidated indicating their diverse and multifunction nature working in consortia and overlapping manner to regulate the adaptation response in plants. With modern biotechnological tools, it was found that plant vacuoles are the prime target to be studied for salt and drought tolerance. Being largest intracellular organelle, it possesses the potential to confine extra and waste material by the tonoplast action of H^+-pumps and Na^+/H^+ exchangers.

Plant vacuoles have tonoplast localized H^+-pumps responsible for generation of proton electrochemical gradient and create have tonoplast bound H^+-pumps which functions as primary transducers in living cells. During photosynthesis, plant cells interconvert light, chemical and electrical energy by using proton motive force generated H^+-pumps. By generation and maintenance of transmembrane proton motive force, H^+-pumps serve as electric potential generation system and energize the transport of materials and solutes on tonoplast in particular and other biological membranes in general. Due to significant multitude of biological pathways and reactions energetically mediated by ATP, primary translocation and interconversion of ATP have been identified as powerhouse of usable energy in biological cells. Another fact is that plants have another major H^+-pump i.e., inorganic pyrophosphatase substrated H^+-pyrophosphatase (H^+-PPase). Both of these

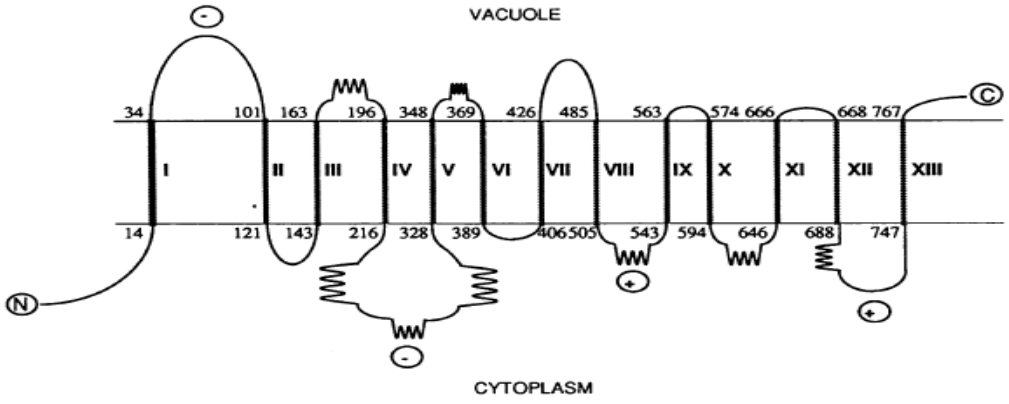


Figure 1.2 Topographic model of Arabidopsis tonoplast H^+-PPase amino acid sequence. Values above the median show hydrophilic segments; values below the median represent hydrophobic segments. Numbers I through XIII represent the putative membrane-spanning segments depicted in the tentative topographic model of the tonoplast H^+-PPase. The structures indicated are ⋀⋀, α-helix; -, random coil; ⊖,clusters of negative charge; ⊕ , clusters of positive charge; N, amino terminus; and C, carboxyl terminus.

tonoplast H^+-pumps, sponsor energy to catalyze electrogenic H^+-translocation for inwards movement from cytoplasm into vacuolar lumen but operation of H^+-PPase is directly on use of PPi as substrate source (Figure 1.3); Rea *et al.*, 1992). The tonoplast localized H^+-PPas (EC 3.6.1.1) has the molecular weight (Mr) 64.500 to 73.000 and MgPPi binding subunit of H^+-PPase ranges between 1 % and 10 % of total vacuole membrane protein and purified pyrophosphatase is highly active as verified by high turnover number between 50 and 100S depending on origin and method of preparation. Similarly, it has high substrate (MgPPi)-

binding subunit of the H^+-PPase and constitutes between 1 % and 10 % of total vacuolar membrane protein. When these characteristics considered on account of vacuole size, bioenergetics impact of H^+-PPase is potentially great (Sarafian *et al.,* 1992).

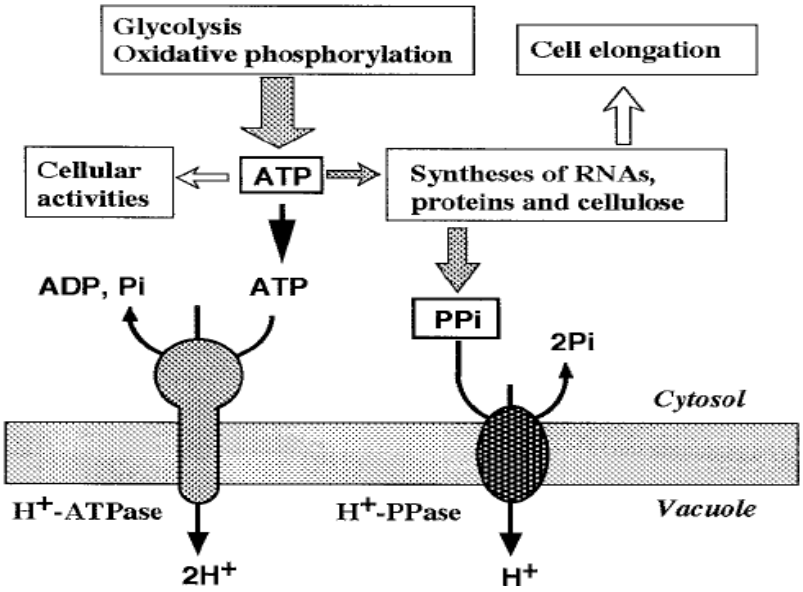


Figure 1.3 PPi production and vacuolar membrane H^+-ATPase and H^+-PPase. PPi is supplied as a by-product of biosynthesis of macromolecules such as RNA, proteins and cellulose synthesis in elongating cells.

Vacuole organelle is considered as the central and largest H^+ storage house. And its acidification is accomplished by two types of H^+-pumps systems: H^+-ATPase and H^+-PPase. The acidification of the *Arabidopsis* vacuole is carried out by two systems: the vacuolar H^+-ATPase and the vacuolar H^+-pyrophosphatase. The vacuolar H^+-ATPase (EC 3.6.1.1) is encoded by multigene families and is a complex of a multisubunits (400-600 KDa). These subunits are encoded by at least 26 genes and are translocated (Sze *et al.*, 2002). On the other hand, H^+-PPase is single subunit protein and its three homologs have been identified i.e., *AVP1-AVP3* (Drozdowicz and Rea, 2001). This diversity in structure and energy requirements of vacuolar H^+-ATPase and H^+-PPase offer plants the comprehensive biochemical and regulatory potential allowing the generation of proton electrochemical gradient under different growth conditions.

Many genes have been identified in Arabidopsis and yeast that encode secondary transporters energized by PEG (Anraku, 1996; Serrano and Alonso, 2001. Unraveling the roles of these secondary transporters is initial stage implicated in plant growth in

response to different environmental stresses of salinity and under drought stresses. By sequencing and bioinformatics tools, about five hundred co-transporters have been discovered and majority of these co-transporters are tonoplast specific.and relied directly/indirectly on PEG for their operation. It dignifies the role PEG across tonoplast for regulation through manipulation through biotechnological tools and engineering the activity of H^+-pumps across the vacuolar membrane may be promising strategy for coordinating and regulating plants response under abiotic stresses.

1.9. Cotton biotechnology

Due to versatile and premium crop, it has been major for biotechnological research and several agronomic traits for applied research and fundamental research has been carried out. Since the beginning of cotton biotechnology in 1970, cotton has under gone under biotechnological improvement throughout the world. Cotton biotechnology marks its entry into cotton improvement through tissue culture techniques and most of the improvement were brought in the cotton genome and diversity through transformation techniques. Since the end of previous century, cotton sector has gained boom due to adoption of transgenic cotton varieties developed by modern biotechnology like somatic culture, protoplast culture, shoot tip culture, somatic hybridization and genetic engineering (Zhu *et al.,* 2011).

1.9.1. Cotton tissue culture

Initially, cotton tissue culture was carried out by in-vitor culturing of isolated plant organs, tissues, cells and protoplasts in an artificial medium for subsequent regeneration into complete plants anther and pollen culture (Qin and Liu, 2006). Establishment of cotton tissue culture provide platform for cotton improvement through genetic engineering so cotton somatic embryogenesis and regeneration into fertile plants is the foremost requirement in cotton genetic engineering.

Well streamline tissue culture system is an open secret for genetic transformation of cotton (Firoozabady and DeBoer, 1993). There was rapid improvement in cotton tissue culture and somatic culture after first report on induction of cotton calli from upland cotton and now systems for cotton somatic culture, (Chen *et al.,* 1987), anther culture

(Zhang *et al.,* 1998) and protoplast culture (Zhang *et al.,* 1992) has been optimized opening opportunities for cotton improvement through genetic engineering.

1.9.1.1. Cotton stem-tip culture

An efficient and reproducible transformation and regeneration system is the corner stone in biotechnological improvement of cotton and a lot of fundamental were under taken regarding cotton tissue culture and transformation. Cotton shoot meristem is a group of actively dividing cells in meristem regions which is highly totipotent to form a complete plant let with minimum variation. Limitation of normal tissue culture like length culture time, high frequency of somatic embryogenesis, lower regeneration and sterility in regenerated plants can be avoided by using stem tip culture. Promising transformation system in cotton by using stem tip culture as explants along with parallel transformation techniques such as microparticle bombardment and agrobacterium mediated gene delivery has veen developed (Wu *et al.,* 2009; Zhou *et al.,* 2009).

Response of twenty two cotton varieties of shoot apices from in-vitro germinated seedlings were evaluated on simple MS mediun without addition of any hormone and different growth pattern in different varieties were observed. Induction of roots and shoots were observed in all varieties and plantlets were shifted to pots with specific composition of soil, sand and peat moss. These regenerated plantlets grow as normal and entered reproductive phase with good fertility (Rashid *et al.,* 2004). Shoot apexes have been used as explant successfully for cotton (Gould *et al.,* 1991; Wilkins *et al.,* 2004), petunia (Ulian *et al.,* 1998), sunflower (Schrammeijier *et al.,* 1990). Bazargani *et al* (2011) obtained multiple shoots from shoot apex of two cotton cultivars (Sahel and Veramin) (*G. hirsutum* L.). Shoot apex is being utilized as a tool for rapid micropropagation of genotypes.

1.9.1.2. Cotton somatic culture

Cotton is notorious in their response for callus induction, somatic embryogenesis, delayed regeneration, abnormality of regenerated plantlets and slow growth to reach reproductive phase. A landmark achievement was witnessed in history of cotton improvement when induce cotton calli showed somatic embryogenesis and produced fertile plants. But still cotton tissue culture was an expensive job long duration of tissue

culture timeframe, lower frequency of somatic embryogenesis and higher frequency of abnormal embryos. Cotton calli was reported for the first time in wild species *G. davidsonii* and successfully regenerated plants (Liu, 1983). Later on other research results were published reporting the induction of callus from cultivated species including *G. arboretum, G. hirsutum, G. raimondii, G. gossypiodes* and *G. davdsonii*. (Wu *et al.*, 1988; Tan and Qian, 1988; Lu and Xia 1991). Media composition and culture conditions were further optimized for improvement in somatic embryogenesis and plant RI 13 (Feng *et al.,* 1998; Zhang *et al.,* 2009).

Somatic embryogenesis and plant regeneration from eight cotton species including three cultivated species, *G. hirsutum, G. barbadense,* and *G. arboreum* and many other cultivars/germplasms of commercial cultivars have been obtained (Cui *et al.,* 2001). For optimizing explant type, different explants like hypocotyls, cotyledons, petiole, leaf radicle and stem have been used in cotton tissue culture among which hypocotyls proved best explant in the induction of viable callus, good somatic embryogenesis and regeneration. Despite all these developments, callus induction, somatic embryogenesis and plantlet regeneration is still challenging and genotype dependent. Genotypes having efficient regeneration response are viable plant materials for introduction of foreign genes in transgenic plants and serve as source of genetic variation in cotton breeding program. Among the various constraints, somatic embryogenesis and regeneration sytem are still challenging factors in cotton tissue culture restricting the application of biotechnology for cotton improvement.

Good somatic embryogenesis and regeneration potential is the genuine requirement for transgene delivery by any transformation method. An efficient cotton tissue culture protocol has the characteristic to regenerate plant from a single cell. Tissue culture protocols reporting somatic embryogenesis and regeneration have been developed in most of major crops species (Evan and Sharp 1981). Tissue culture of soybean and cotton proved tedious for somatic embryogenesis and regeneration (Scowcroft, 1984). Moreover, regeneration of a plantlet from a unique stem cell provides information for investigation of meristem formation in embryogenesis research and plant totipotency (Imin *et al.,* 2005). Coker and Coker derived lines were first among cotton genotypes which showed viable response for call somatic embryogenesis and plantlet regeneration

(Shoemaker *et al.,* 1986). Coker-201 showed very strong commitment for callus somatic embryogenesis and regeneration (Gang *et al.,* 1996).

An efficient and rapid somatic embryogenesis response was observed within short time (3 months) followed slow for GSCs and very poor for non-Coker cultivars (Firoozabady and DeBoer, 1993). In addition, the effect of environmental conditions, media regimes, growth regulators and different treatments of produced calli were studied and optimized protocols were developed. Later on regeneration of Chinese cotton cultivars such as Simian 3 and Zhingmian 12 were also obtained (Zhang *et al.,* 1996). Embryo formation was obtained by establishing cotton cell suspension cultures in wild cotton species (*G. klotzschianum*) but regenerated plantlets were abnormal by stem and leaves (Price and Smith 1979; Finer and Smith 1984). These studies were based on somatic embryogenesis and plant regeneration from embryogenic calli cultures of *G. klotzschianum*. Cell suspension cultures of thirty eight cotton genotypes were evaluated for regeneration of plants and classified as high, moderate, low and non-embryogenic (Trolinder and Xhixian, 1989).

1.9.1.3. Protoplast culture and somatic hybridization

Good traits from distant parent's species can be combined into one genotype by somatic hybridization via protoplast fusion otherwise transfers of some characteristics are difficult through traditional breeding program. Protoplast culture was used for regeneration of plantlets in upland cotton (She *et al.,* 1989). Protoplast cultures were produced and showed somatic embryogenesis in more than twenty genotypes such as Xinhai 3, Xinhai 6, Xinhai 7, 282, K252 Junhai 1 and Giza 70 etc. Regeneration of cotton plantlets was obtained from protoplast cultures of Lumian 6 cotton cultivar.after several months (Wang 1998). Similarly, Lv *et al.* (1999) was able to regenerate plants from protoplast of upland cotton cultivar var. Coker 201.

Protoplasts can be used as explants in plant transformation techniques and proved best for direct delivery of transgene (Potrykus, 1991) without relying on biological and physical methods of DNA delivery. In fact protoplst cells are devoid of any cell wall barrier and plasma membrane is directly used for transfer of DNA based information. Despite of all these developments, system from protoplast to plant is still empirical and is challenging task in the application of somatic hybridization. Protoplast based tissue

culture and plant regeneration system could not gain wide acceptance and initial findings were based on attempts to culture cotton protoplasts which multiplied into micro colonies or callus without plant regeneration (Finer and Smith, 1982; Saka *et al.,* 1987). Feeder layer cells were also used to promote protoplast-to-regeneration system in cotton (Peeters *et al.,* 1994).

1.9.1.4. Cotton anther culture and haploid breeding

Production of haploid plants is required to develop homozygous plant population by doubling chromosome to get stable diploid plants. Importantly, duration of breeding can be reduced greatly using haploid breeding methods. Plant population developed through doubled haploids is considered a valuable population in theoretical research of plant hereditary and QTL mapping. Moreover, differentiation of cotton pollens or microspores can be studied for understanding of somatic embryogenesis mechanism.

Reports on cotton anther culture are limited, process and morphological characteristics of embryogenesis and organ differentiation in anther derived calli culture of *G. klotzschianum.* Anther calli showed differentiated embryoids, adventitious buds, and recovery of haploid plants (Zhang *et al.,* 1996). Unluckily, no report is available on anther culture of upland cotton. In upland cotton, anther culture is associated with problem of division of the pollens/microspores inside the anther. Important factors which determine the efficient of anther culture are genotypes, developing time of anther, medium and different growth regulators. It was considered that the cotton anther culture and producing of cotton haploid lines are valuable for fundamental research of cotton biotechnology especially for the study of differentiation of cotton pollens or microspores and understanding the mechanism of somatic embryo formation thereby deepening the knowledge on cotton somatic embryogenesis.

1.9.2. Factors affecting cotton tissue culture and transformation

1.9.2.1. Genotypes and explant types

Cotton tissue culture is tedious due to stubborn behavior of majority of cultivars towards somatic embryogenesis and regeneration response. Up till now, cotton tissue culture is highly genotype specific and genotype spectrum is narrow as regeneration is

only found in small number of cotton lines (Firoozabady *et al.,* 1993; Trolinder and Xhixian, 1989; Wilkins *et al.,* 2004).

The trait of embryogenesis in cotton has low inheritance and varies from cultivar to from seed lot to seed lot (Gawel 1990; Kumar, 1998, Mishra *et al.,* 2003). Moreover, pedigree analysis showed that Coker-100W is a common ancestor of most of the Coker method cotton transformation and regeneratable cotton lines particularly Coker-312-17, Coker-312-5A, GC510, DP6166, Cascot 2910. Mar 10 and DP lines. Some cotton lines have intermediate tissue culture response as their calli entered embryogenic phase but don't exhibit regeneration of magnitude to that of Coker 312 (Trolinder and Hhixian, 1989; Firoozabady *et al.,* 1993). Optimization of culture conditions for callus induction, and somatic embryogenesis expanded the diversity of genotypes that can be successfully transformed and regenerated such as Acala and Coker cotton (Mishra *et al.,* 2003). Different explant types have been used from a variety of tissues such as leave, cotyledons and hypocotyls (Hashmi *et al.,* 2011; Zhang *et al.,* 2009). However, normally somatic embryogenesis occurs from seedling hypocotyls and immature leaves whereas mature explants tissues produce non-embryogenic callus (Trolinder and Goodin, 1998). High gossypol contents in explant tissues are considered as major problem and produce calli of poor texture. Although there are studies reporting the use of different explants but still now only hypocotyl explants are widel used in cotton tissue culture and transformation for fundamental and applied research.

1.9.2.2. Culture conditions

Cotton tissue culture is multistep, lengthy and laborious work making it an expensive adventure of cotton transformation and regeneration of transgenic plants. So it requires very precise tissue culture conditions during the course of its tissue culture steps, transformation and regeneration of transgenic plants. Cotton seed germination requires dark conditions at 30C and within 72 hrs 98 % germination is obtained. Germinated seeds require high light conditions 2000 lux to facilitate fast growth of newly developed coleoptiles.

Agro-inoculation gave maximum transformation efficiency when it was carried out at 25 °C and co-cultivation was given at 25°C in low light conditions. After co-

cultivation, explant tissue is cultured on callus induction medium at 28 °C at 65 % relative humidity and 16/8 hrs photoperiod having 1600 lux light conditions. Any deviation from these conditions interfered with the efficiency in tissue culture and transformation particularly calli cultures are highly prone to these conditions, any change in these culture conditions, calli cultures lost their embryogenesis, if these conditions continued, embryogenesis is lost irreversibly. Normally a temperature-controlled growth room maintained a temperature of 26-28 °C with even distribution of heating and cooling is required (Jin *et al.,* 2005; Wilkins *et al.,* 2004). Light study was conducted by using six light sources on the micropropagation of cotton shoot apex. Significant root activity, starch, sucrose and soluble sugar contents were observed in cultures under red LED. O red/blue light proved ideal for growth of upland cotton cultures (Li *et al.,* 2010). Optimized transformation protocols are required to accelerated the science of gne delivery for fundamental and applied research so that improvement of cotton varieties may be realized in efficient manner.

1.9.2.3. Culture/subculture time

Cotton tissue culture is considered an expensive adventure at it requires multiple subcultures over several months. So culture/subculture intervals are key factors in the smooth running of cotton tissue culture system.

For efficient callus induction and somatic embryogenesis, strict schedule of culture subculture time intervals is very critical for proper callus growth rate, somatic embryogenesis and plant regeneration (Zhang *et al.,* 1992). Depending on initial material, subculture time varies from three to six week (Zhang *et al.,* 1992). Extended subculture time on solid medium leads to nutrient, moisture and air starvation stress on calli and embryo viability may become poor with difficulty of regeneration. Subculture strategy based on "solid-liquid alternative culture" rotation is considered useful for maintaining viability for long duration (Zhang *et al.,* 1992).Wang *et al.* (1994) reported that embryogenic calli cultures of upland cotton changed from light green or gray loose particles into yellowish compacted lumps form and embryogenesis increased with increasing of subculture period but further delay decrease the embryogenesis (Asad *et al.,* 2008). For most of cotton cultivars, studies on culture time is at empirical stage and is highly desired for optimization if efficient cultures are objectives in cotton tissue culture

and transformation for delivery of transgenes as it will reduce the occurrence of somatic variation in calli cultures.

1.9.2.4. Transformation method

Transformation efficiencies is profoundly affected by several factors, such DNA delivery methods like *Agrobacterium tumefaciens,* biolistic delivery and silicon carbide fiber mediated gene deliveries (Asad *et al.* 2008). Among these, *Agrobacterium* is the major delivery tool and depends on strain, type of growth medium, temperature/duration of co-cultivation, relative humidity/lighting, and size of explants co-cultivated and even the type of gene construct (Hood *et al.,* 1993). The most widely used Agrobacterium commercial strain is LBA4404 and other super virulent Agrobacterium strains such as EHA105$(Kn)^S$, EHA101 $(kn)^R$ GV and A281 that work well have been reported (Ooms *et al.,* 1982; Stelly *et al.,* 1989; Mishra *et al.,* 2003; Wilkins *et al.,* 2004).

1.9.2.5. Callus induction

During cotton tissue culture, callus induction is major step for high frequency of embryogenic calli and plant regeneration. During the course of repeated subculturing, cotton calli is marked by different colors, texture, and growth rate which are important for efficient somatic embryogenesis and transgenic plant regeneration. Cotton tissue culture is hampered significantly by failure of callus to form embryogenic calli, embryo regeneration and lack of root/shoot development (Wilkins *et al.,* 2004; Mishra *et al.,* 2003).

Immature embryos can be recovered from hard, compact, dark green or white calli but usually it is non-embryogenic. Ideal calli cultures are or cream to light green color and good quality of callus show the presence of red pigmentation due to anthocyanin accumulation. Another useful feature of cotton calli is its loose and granular texture giving it a soft and beady appearance and is readily dispersed in liquid culture. Nonembryogenic calli cells are small, cytoplasmically dense, highly vacuolated. In hypocotyl based transformation by agrobacterium, calli size should be about of pea size as calli is gaining momentum for multiplication and has high survival rate as a response of community (Mishra *et al.,* 2003).

1.9.2.6. Media composition

Cotton tissue culture is heavily dependent hormone inclusion and combination in callus induction medium. In most cases, callus induction medium is composed of MS salts (Murashige and Skoog, 1962) along with 2, 4-D as Auxin and kinetin as cytokinins (Sunilkumar and Rathore, 2001; Rajaeskaran 1996; Trolinder and Goodin, 1988). It was also shown that 2, 4-D is more effective than NAA for production of high quality callus and other studies reported that medium containing MS salts supplemented with myo-inosito 100mg l-1, B5 vitamins (Thiamine HCl 1mg l^{-1}, nicotininc acids 1mgl^{-1}, pyridoxine 0.75 mg l^{-1}), MgCl2 0.75 g l^{-1}, glucose 30 g $l^{-1,}$ NAA (10.7 µM) and 2, 4-D (0.2 µM) with pH adjusted at 5.8 before addition of phytagel 2.5g l^{-1} was designated as MCIM . MCIM medium were found extremely useful to form regenerating Coker and Acala genotypes (Mishra *et al.*, 2003).

The main factors that influence the cotton tissue culture includes type of regulator used (Trolinder and Goodin, 1988; Sun *et al.*, 2006), sugar types (Ishii *et al.*, 2004), culture medium (Popelka and Altpeter, 2001) and genotypes (Sebrook and Doughlas 2001). A lot of studies for *Gossypium* callus initiation and maintenance have been carried by several laboratories (Michel *et al.*, 2008; Zouzou *et al.*, 2000; Sun *et al.*, 2006; Xie *et al.*, 2007).

1.9.3. Genetic engineering of cotton

Cotton is at forefront among commercial crops and has been domesticated by traditional approaches for its agronomic improvement. The productivity of cotton is under threat by pests such as weeds, insects, disease and abiotic factors. Traditional breeding has met with insufficient success to meet the challenges of cotton production and genetic engineering of cotton has come to play viable role to meet the challenges of cotton crop in modern system of crop production.

A journey started some years ago, this is nineteenth the consecutive successful year of transgenic crop adoption. The transgenic cotton crop which was grown on small area has now surpassed all conventional cotton cultivars and helped growers with better crop management gaining significant return to growers (Chakravarthy *et al,* 2014). Transgenic crops are now invariably grown particularly in twenty seven countries on 180 M hac. and reducing cost of crop production with high yield and net return (ISAAA,

2014). It is foreseen that cisgenic transgenic plants will overtake the market replacing conventional varieties of commercial crops.

1.9.3.1. Herbicide resistant cotton

Cotton crop is infested by various weeds and cause loss qualitatively and quantitatively. Weed invade cotton field and compete with cotton plant for moisture, nutrients, space, sunlight, provides shelter to insect/disease pathogens and interfere irrigation water and interculture/harvest practices (Rajput *et al.,* 2008)

Transgenic glufosinate cotton was developed by transforming bar gene and significant herbicide tolerance was achieved (Keller *et al.,* 1997) which then commercialized in USA in 2000. A multiple gene expression cassette containing GNA and Bar genes was introduced into cultivars "699", "24" of uplant cotton and herbicide tolerance level was improved in cotton (Guo *et al.,* 1999). Herbicidal gene bar and salinity tolerance gene BADH was delivered into cotton by method of pollen tube pathway (Jiang *et al.,* 2007). Activity of acetolactate synthase (ALS) can be inhibited by sulfonylurea and imidazolinone allowing the accumulation of α-ketoglutarate to a toxic level leading to plant death. Sulfonylurea herbicide resistance was developed in cotton Coker 312 germplasm i.e., 19-51a by transforming chlorsulfuron-resistant gene (als). For expanding plant potential against drought and weed problems, transgenic cotton Lumian 19 was developed (Lian *et al.,* 2008).

Glyphosate is all weed killer herbicide and is effective against all types of vegetation. It is consistence performance of glyphosate that 90 % of soybean, 30 % of maize fields, and 75 % of cotton were produced by controlling weeds with application of glyphosate in USA (ISAAA, 2014). Monsanto is the pioneer in isolating the 5-enolpyruvylshikimate-3-phosphate synthase gene (EPSPS) from the Agrobacterium var. CP4 which was later used to develop glyphosate tolerance by overexpressing in cotton Coker 312. In addition to agronomic trait, glyphosate resistance gene is also used as selection marker in the selection of transgenic tissues for delivery of transgenes (Zhao *et al.,* 2006). Third generation transgenic cotton containing CP4-EPSPS with insecticidal bt genes was developed and is considered to address the multi problems associated with cotton production (Zhang, 2015). Herbicide resistance is global's top popular trait in 12 major crops by 336 events (Rhodora *et al.,* 2015) as it offers sustained and vialble options

for economic control of cotton field weeds there by allowing better use of space, moisture and nutrients by cotton plant.

1.9.3.2. Insect resistant cotton

Agricultural crop production has been revolutionized by introducing insect resistant transgenic crops (Shelton *et al.,* 2002). Natural host plant resistance to insects is encoded as quantitative trait involving several loci making efforts for insect resistance slow and difficult. Engineering insect resistance has become major success story by applying plant genetic engineering technology to cotton, maize for managing against lepidopteran and coleopteran caterpillars (Gatehouse, 2008). Gene cloning and transformation techniques have generated unlimited gene pool (endogenous, exogenous or synthetic genes) which is above all barriers normally associated with conventional breeding efforts and it is now straight forward to choose desirable gene from a growing list of insecticidal genes and insert into plant genomes conferring insect resistance (Benedict, 1994).

Cotton was transformed with insecticidal API gene and insect resistance was developed. Double insect resistance expression vector was developed by stacking bt Cry1Ac genes with Bt29k and proteinase inhibitor B (API-B) (Wu *et al.,* 2003; 2011). Commercial line of transgenic cotton cultivar Ji321 was developed by Agrobacterium method of cotton transformation. A high resistance to bollworm and aphid was obtained in transgenic germplasm over expressing a double insecticidal genes (BT+GNA) and express stably in subsequent generation (Lu *et al.,* 2004). A triple gene construct containing BT+CpTI+GNA was transformed into cotton via agrobacterium and transgenic cotton plants containing triple genes were regenerated and inherited to next generations (Wu *et al.,* 2008). Leelavathi *et al.,* (2004) transformed *CryIIAc* into cotton and observed insect mortality. Li *et al.,* (2009) transformed multiple genes i.e., Bt (*Bacillus thuringiensis*) and cowpea trypsin inhibitor, bar gene for glufosinate tolerance, keratin and fibroin for fiber improvement in two Chinese cotton varieties i.e., Simian 3 and WC. Significant insect resistance in transgenic cotton was achieved by overexpression of TVip3A (Wu *et al.,* 2011).

1.9.3.3. Disease resistant transgenic cotton

Cotton production is problematic due to notorious disease of leaf curling and has caused significant drift on efforts directed towards improving cotton yield. This disease is caused by whitefly-transmitted begomovirus and is still without any stern control measure. Hashmi *et al.,* 2011 transformed Coker-312 with different *AC1* (rep gene) versions via *Agrobacterium* and developed 5′ and 3′tAC1 transgenic cotton lines showing reproducible tolerance against cotton leaf curl disease upto T_2 generation. In addition to CLCuD, cotton is also attacked by Fusarium Wilt and Verticillium Wilt. Fungal resistant gene was transformed into cotton and transgenic cotton plants overexpressing Rs-F1 mycellium growth and spore germination of the verticillium Kleb (Zuo *et al.,* 2002).

NDR1 and PR1 fungal resistance genes were introduced into upland cotton and Verticillium wilt resistance in transgenic cotton was much better in transgenic cotton plants than non-transformed control. Sohrab *et al.* (2014) transformed antisense βC1 gene into cotton and CLCuV resistant transgenic cotton plant were developed.

1.9.3.4. Improvement of cotton fiber quality

Cotton is well known for source of silver fiber and it is fiber which makes it "White Gold" due to premium fiber product. Transformation of cotton for transgenic improvement of cotton fibers quality is comparativerly neglected area as most of the research is focused to address biotic and abiotic stress related factors. Few reports are available which showed the use of modern biotechnology on cotton fiber quality improvement. Sucrose biosynthesis pathway was engineered by transforming sucrose phosphate synthase (SPS) gene which is involved in catalyzing fructose-6-phosphate into sucrose. SPS gene was isolated from spinach and transformed into cotton.

Transgenic SPS cotton plants exhibited higher SPS activity in leaf and fiber cells than non-transgenic control plants (Haigler *et al.,* 2007). During 15-19 night hrs, fiber characteristics like seed cotton weight, fiber maturity, and fiber strength from transgenic cotton plants were superior quality than that of non-transformed control plants. Keratin fiber gene isolated from rabbit was also introduced into cotton SGK321 and improvement in fiber quality was observed without significant impact on staple length and strength (wang *et al.,* 2002). Later, cotton cultivar Sumian 16 was also transformed with rabbit

keratin gene under regulatory role of fiber specific E6 promoter and three lines of transgenic plants were developed as verified by molecular techniques (Zhang *et al.*, 2004). Fiber characteristics of rabbit-keratin transgenic cotton was improved especially fiber strength. Fiber strength of transgenic cotton plants was increased by overexpression of spider silk protein gene ADF3 by gene gun delivery technology (Huang *et al.*, 2004). Li *et al.* (2004) transformed acsA and acsB genes via vacuum infiltration and fiber improvement was achieved in transgenic cotton plants.

Cotton seed is second largest source of edible and animal feed. Cotton seed was improved using inverted repeat gene silencing technique targeting two seed specific fatty acid desaturase genes i.e., ghSAD-1and ghFAD2-1 and increase in levels of stearic acid and oleic acid contents were observed whereas palmatic acid contents were reduced (Liu *et al.*, 2000).

1.9.3.5. Improvement of salt/drought tolerance

Cotton is primarily a glycophytic plant with moderate salt and drought tolerance. After commercial domestication, cotton is grown as annual plant and its cultivation has undergone intensive use of inputs in the form of water, fertilizers and pesticides etc. Transgenic plants are need of the time with transgenes for abiotic stress tolerance.

Development of salt and drought tolerant cotton is the need of time and will be essential in future. Cotton was transformed with different abiotic stress related genes with the aim to improve the tolerance against salt and drought stresses in marginal lands (Table 1.1). Asad et al, (2008) transformed AVP1-H+ gene into cotton Coker-312 via silicon carbide fiber and regenerated salt tolerant AVP1 transgenic plants. He et al. (2005) transformed Arabidopsis isolated AtNHX1 gene in cotton and observed improvement in photosynthetic performance in transgenic lines than their non-transformed counterparts under salinity stress. There is increasing concern about the use of tonoplast specific genes as major single dominant gene as literature is being saturated with scientific reports documenting promising role tonoplast specific genes to confine waste or undesired radicls from cytosol into vacuolar lumen.

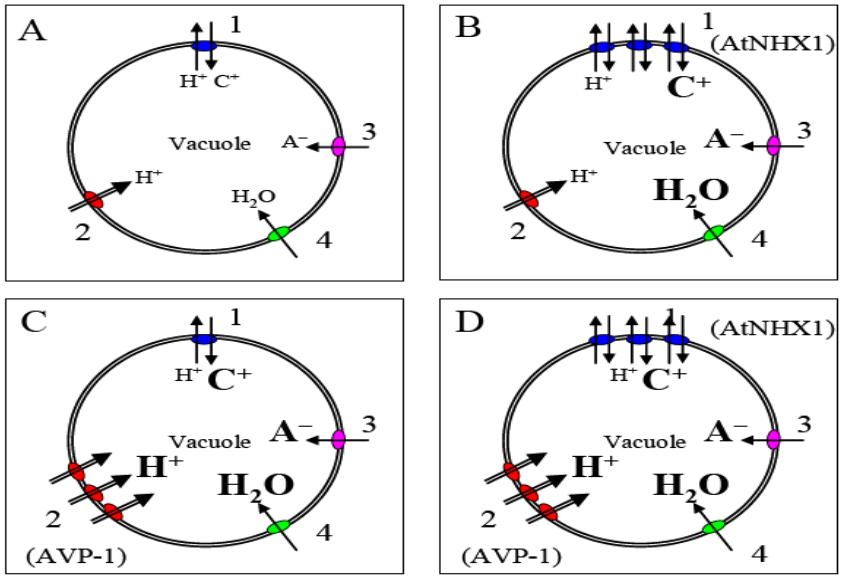


Figure 1.4 Model of proton pumps (H^+-pump) and antiporters found in the plant vacuole membrane. A) H^+-ATPase (1) and H^+-PPase (2) transport protons (H^+) into lumen of vacuole. Organic and inorganic anions (A-) enter the vacuole via channels (4) to achieve electroneutrality, allowing the generation of a pH-gradient. The proton electrochemical gradient (PEG) energizes secondary active accumulation of organic and inorganic cations into the vacuole via H^+/cation antiporters (3) with osmotically driven water uptake. B) Transgenic plants with overexpressed cation/H^+ antiporters (1). C) Transgenic plants with over expressed *AVP1* H^+-PPase. D)Transgenic plants with *AVP1/AtNHX1* stacking in vacuolar membrane sequester higher amounts of cations through the utilization of the existing PEG generated via the two H^+-pumps (1 & 2) and stable expression of AVP1 in transgenic plants have high PEG. This altered PEG is used to increase the transport activities across the vacuolar membrane.

1.10 Rational for creating transgenic cotton with higher salt and drought tolerance

Abundant, cheap and good quality water commodity access is diminishing due to contamination of water resources by ever increasing human population and commercial activities. It is believed that human civilization will clash on issue s of water in the future and water is going to be life line for the nations. Morever, salinity problem is worsening with commercialization of agronomic crops (Roy et al., 2014; Kuppu et al., 2012; Yu et al., 2015).

Therefore, the major challenge which scientists will face is the problem of water shortage and elevating salt to a level formidable to crops with present genetic potential.

Therefore, cotton research efforts are geared up by transforming salt and drought conferring genes through gene delivery techniques to make cotton sowing possible on

Table 1.1 Transformation of cotton with tonoplast genes (*AVP1* & *AtNHX1*) and transcription factors against abiotic stresses

Gene Origin	Promoter	Morphological impacts	Reference
Arabidopsis (*AVP1*)	2x35S	More vigorous growth, large root system	Pasapulla *et al.,* 2010
Arabidopsis (*AVP1*)	2x35S	Improved salt tolerance, early and quick seed germination	Asad *et al.,* 2008
Thallungiella hallophiella (*TAVP1*)	35S	High growth rate, high melondialdehyde contents, high salt tolerance	Lv *et al.,* 2008
Arabidopsis AtNHX1	35S	High proline contents, high photosynthetic performance	He *et al.,* 2005
Arabidopsis AVP1/AtNHX1	35S	Further improvement of salt drought tolerance than either gene containing transgenic cotton	Shen *et al.,* 2014
SNACI (rice) transcription factor	35S	Highly drought tolerant, vigorous root system, high proline contents, high boll number and high yield.	Liu *et al.,* 2014
AtDREB1A	35S	High drought tolerance, proline contents, vigorous growth and high chlorophyll contents	Amudha *et al.,* 2014

marginal and water scared conditions. Such developments in these aspects of food and fiber production will have an enormous impact on the economies arid, semi-arid and marginal lands around the world.

After exploring natural genetic variation for improvement of abiotic stress tolerance by conventional breeding, genetic engineering has emerged as an alternate option to meet the future challenges for abiotic stress tolerance (Table 1.1). One approach to boost salt- and drought tolerance in plants is to raise solute contents in the lumen of vacuoles of plant cells (osmotic adjustment), therefore elevating the vacuolar osmotic

pressure accompanied with decrease in water potential, which would allow water movement from soil into plant root cells. Two approaches have been used to increase solute contents in plant vacuoles. The first approach involves increasing the activity of a vacuolar sodium/proton (Na^+/H^+) antiporter that mediates the exchange of cytosolic Na^+ for vacuolar H^+ (Apse *et al.*, 1999, Blumwald *et al.*, 2001). The second approach involves increasing the activity of a H^+ pump on the vacuolar membrane to move more H^+ into the vacuoles, therefore generating a higher proton electrochemical gradient (PEG) that can be used to energize secondary transporters including vacuolar Na^+/H^+ antiporters (Gaxiola *et al*., 2002). Both approaches enhance Na^+ accumulation in the vacuoles and reduce the potential of Na^+ toxicity in the cytoplasm, leading to higher salt and drought tolerance in transgenic plants.

1.11. Goals of the study

Therefore, the main goals of present study were;

1. Development of transformation method of cotton embryogenic calli with *GUS* reporter and *AVP1* genes using silicon carbide whiskers.
2. Cloning of *AtNHX1* into plant transformation vector pGA482*AtNHX1*
3. Transformation of cotton (*Gossypium hirsutum L.*) coker-312 with *AVP1* and *AtNHX1* genes via *Agrobacterium*.
4. Molecular analysis of *AVP1* and *AtNHX1* transgenic cotton
5. Salt and drought study of *AVP1* and *AtNHX1* transgenic cotton plants.

Chapter 2

Silicon Carbide Whisker Mediated Transformation of Cotton

2.1. Introduction

Cotton is produced commercially in the temperate and tropical regions of more than 50 countries as an important source of fibers, cottonseed oil, and livestock feed. The productivity of cotton is adversely affected by abiotic stresses (salt/drought) and biotic stresses (viral/fungal infections, insect predation). For this reason efforts are being made to improve productivity by genetically modifying cotton to enhance abiotic stress tolerance, fiber quality and increase the protein content of cottonseed oil. Transformation of cotton remains a bottleneck to the introduction of new traits (Wilkins *et al.,* 2004).

Various protocols have been explored for the transformation of cotton, including meristem transformation (Gould *et al.,* 1991) particle bombardment (Rajasekaran *et al.,* 2000) and *Agrobacterium*-mediated transformation (Firoozabady *et al.,* 1987). The meristem transformation protocols, although genotype-independent, are extremely laborious and generate a high frequency of chimeras (Satyavathi *et al.,* 2002). A large number of meristems have to be transformed and, if the cultivar is not amenable to induction of multiple shoots and axillary branching, the transformed status of the plants can be analyzed only in the next generation. Accessible meristematic cells are limited and regenerable cells are not abundant leading to a low number of explants (Sunilkumar *et al.,* 2001).

Particle gun procedures involving somatic embryogenesis utilize embryogenic cell suspensions for bombardments, are expensive, requiring continuous supply of consumables, and generate multiple insertions. Both Coker and Acala cotton varieties have been transformed using this physical procedure (Rajasekaran *et al.,* 2000). Similarly, *Agrobacterium*-mediated transformation of cotton is expensive, lengthy, and requires sophisticated equipment as well as good sterile techniques (Wilkins *et al.,* 2004). Whisker-mediated gene transfer, by virtue of its simplicity and potential for up-scaling, is an attractive means of delivering DNA into cells (Muzino *et al.,* 2004). Whiskers have been used to transform cells of the alga, *Chlamydomonas reinhardtii* (Dunahay *et al.,* 1993), as well as suspension culture cells of maize (Petolino *et al.,* 2000), several forage

grass species (Dalton *et al.,* 1997, rice (Komatsu *et al.,* 2006), and soybean (Khalafalla *et al.,* 2006). Callus derived from immature or mature embryos are routinely transformed using *Agrobacterium* for cereals and cotton. The advantage of using callus as a source of explant for transformation is evident from the fact that it allowed delivery of transgenes into cells or group of cells committed for regeneration in several species of monocots and dicots. Here, we employ silicon carbide-mediated transformation of cotton using callus (variety Coker-312) as a constant source of explant for transformation. Our results indicate that embryogenic callus can be transformed using silicon whiskers and the regenerating embryogenic line, once established, can be routinely sub-cultured and used for multiple transformations via whiskers with shortened culture period, with higher number of transformation events into regenerants as well.

The present procedure is economical, rapid, and less laborious than other conventional transformation procedures, which use hypocotyls or cotyledonary leaves as explants for transformation and require more than one year to transform hypocotyls and regenerate transgenic plants. Here we report the production of fertile transgenic cotton plants using the silicon carbide whisker transformation method within four months. This presents the first report of the transformation of any cotton species using the silicon carbide whisker-mediated approach.

2.2. Materials and Methods

2.2.1. Plasmid materials

Two different plasmids were used in the present study i.e., the plasmid pGreen0029 a contained reporter (*GUS*) gene and an *nptII* gene, they encode *glucuronidase, neomycin phosphotransferase II*, conferred GUS expression and resistance to kanamycin respectively (Figure 2.1a). The plasmid pRG229 is a courtesy of Dr. Roberto Gaxiola (Department of Plant Science, University of Connecticut, USA) contained the *Arabidopsis* vacuolar pyrophosphatase proton pump (*AVP1-*H^+) gene which encodes a tonoplast specific protein involved in sequestering of H^+ into vacuole lumens (Figure 2.1 b). Both of the *GUS* and *AVP1* genes were under regulatory controls of 35S promoter with duplicated enhancers (DE35S) of the cauliflower mosaic virus and polyadenylation region from the nopaline synthase terminators (NOST).

Plasmid DNA for inoculation were prepared by alkaline lysis described by Sambrook (1989) as follows; ten *E. coli* colonies of transconjugants (*AVP1*) were individually grown in 2 ml of LB liquid medium containing 50 µg ml^{-1} spectinomycin. The mini plasmid isolation procedure was carried for isolation of plasmid. In each 1.5 ml eppendorf tube, an individually grown overnight culture was added and spun at 14000 rpm for 10 minutes. 1 ml of cell pellet was resuspended in 200 µl of buffer (25 mM Tris, 10 mM EDTA, pH 8.0). The cells were lysed by adding 200 µl of lyses buffer (0.2 N NaOH, 1 % (w/v) SDS) and mixed the contents by inverting the tubes. The reaction was neutralized by adding 200 µl of 3 M potassium acetate and mixed by inverting the tubes five times. The tubes were centrifuged at 12000 rpm for 10 minutes. The supernatants were transferred to fresh eppendorf tubes and plasmids were precipitated by adding 1200 µl of ethanol. The tubes were incubated at –20 ^oC for 30 min and centrifuged at 12, 000 g for 10 minutes. Supernatant was discarded and the plasmid pellets were washed with 70 % (v/v) ethanol and air dried for 10 min and dissolved in 20 µl of water. Prior to the transformation, supercoiled plasmid DNA was diluted to 1 µg $µl^{-1}$ in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

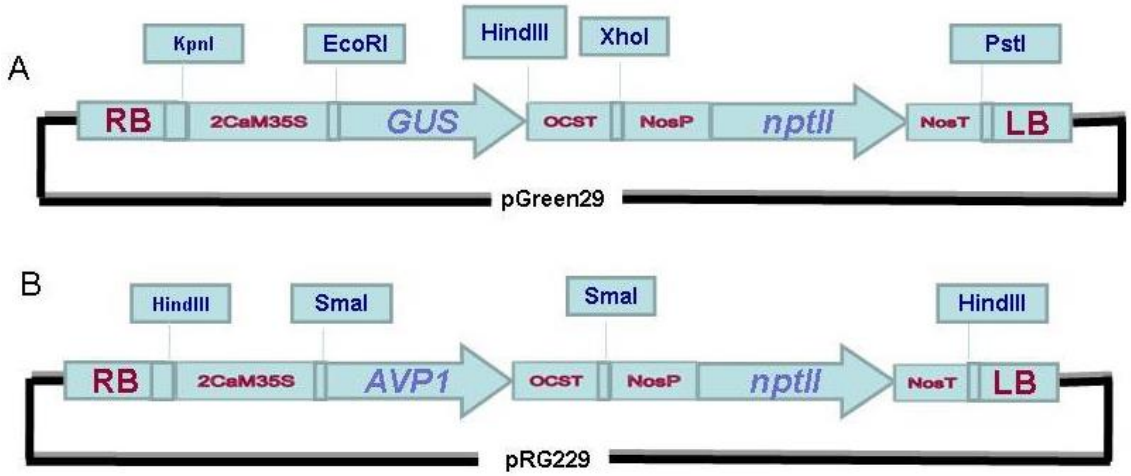


Figure 2.1 Schematic presentation of plasmid maps A) pGreen0029 (*GUS*), B) pRG229 (*AVP1*) used in transformation experiments. 2×35S; double enhancer of 35S promoter of cauliflower mosaic virus, *GUS*; Glucuronidase, *AVP1*; *Arabidopsis vacuolar pyrophosphatase I*, OCS; octopine synthase terminator, NOSP; nopaline synthase promoter, *nptII*; *neomycin phosphotransferase II* gene, NOST; nopaline synthase terminator. The position of unique and important restriction sites used in cloning and molecular analyses in pGreen0029 (*GUS*) and pRG229 (*AVP1*).

2.2.2. Production of embryogenic callus

Cotton (Coker-312) seeds were delinted with concentrated H_2SO_4 for 3 minutes and thoroughly washed with tap water. Delinted seeds were then sterilized with 0.1 % (w/v) $HgCl_2$, 0.1 % SDS (w/v) solution for 10 min, and rinsed four times with sterile distilled water. Sterile seeds were placed in 30×120 mm test tubes containing 5 ml MS0 (4.3 g l^{-1} MS basal medium, 30 g l^{-1} sucrose, 2.5 % phytagel (w/v) (Murashige and Skoog, 1962) medium for germination, as described previously (Wilkins *et al*., 2004). Seeds were cultured at 28 °C in the dark for three days and then transferred to a growth room maintained at 28°C with a 14:10 hour day: night photoperiod with light provided by cool-white fluorescent lamps. Cotton hypocotyl explants obtained from 7–8 days old seedlings were cultured on callus culture induction (CCI) medium referred as MSB1 containing 4.3 g l^{-1} MS salts; 0.1 mg l^{-1} 2,4-D; 0.5 mg l^{-1} kinetin; 30 g l^{-1} glucose; 1.60 g l^{-1} $MgCl_2$ $6H_2O$; 0.1 g l^{-1} myoinositol and B5 vitamins (10 mg l^{-1} thiamine HCl, 10 mg l^{-1} nicotinic acid and 1 mg l^{-1} pyridoxine HCl as described by Gamborg *et al*. (1986).

Cotton calli were obtained within four weeks on MSB1 and the induced calli were

transferred to MSB2-medium (4.3 g l^{-1} MS salt, 30 g l^{-1} glucose, 1.60 g l^{-1} $MgCl_2.6H_2O$ and 1.80 g l^{-1} KNO_3). The embryogenic callus was obtained within six weeks on MSB2 medium.

2.2.3. Preparation of silicon carbide whiskers and DNA delivery

Silicon carbide whiskers were prepared by adding approximately 50 mg of dry whiskers (Alfa Aesar, Avocado Organics) into a pre-weighed 2.0 ml eppendorf tubes, wrapped in two layers of aluminum foil, placed them into a glass jar and autoclaved at 120°C for 20 minutes. Approximately 250 mg of whiskers were transferred into a 5 ml MSB1 liquid medium in a 50 ml plastic tube containing 1 mg ml^{-1} plasmid DNA in the tube. The tubes were then vortexed for 2 minutes. Approximately 2 g of embryogenic calli were added to the tube containing the silicon carbide whiskers and 1 mg ml^{-1} plasmid. The tubes were capped and vortexed for 2 minutes. Experimental controls used were replicated samples lacking either whiskers or DNA.

2.2.4. Effect of calli and sub-culturing interval

Effect of callus type and number of days after subcultures on transformation efficiencies were determined by treating embryogenic and non-embryogenic callus at 6, 12, 18, and 24 days after subcultures. The transgenic status of calli was assessed either by histochemical *GUS* assay or PCR analysis. After 12 week of gene delivery, emerged km resistant calli that were assayed for *GUS* expression and transgene presence. The number of kanamycin resistant calli that emerged from embryogenic and non-embryogenic transformed calli at given time intervals were counted.

2.2.5. Selection for stable transformants

Samples of cotton embryogenic/non-embryogenic calli treated with silicon carbide whiskers, to deliver pGreen0029 (*GUS*), pRG229 (*AVP1*) and control samples (without-whisker or DNA), were transferred into disposable petriplates which contained MSB1 mediums having five different kanamycin regimes (20, 30, 40, 50, 60 mg l^{-1}). Two weeks later, cells from a single treatment were divided into two–three equal portions, and each portion was evenly distributed in a thin layer over a fresh MSB1 medium solidified with 0.25 % (w/v) gelrite (Scott Laboratories, West Warwich, RI) containing different

kanamycin concentrations. Transformed cotton callus cells were transferred to fresh selection medium after two week intervals. Individual colonies were transferred directly onto kanamycin containing MSB1 selection medium and sub-cultured at regular intervals. Kanamycin-resistant callus colonies were placed directly onto the MSB2 selection medium for further proliferation and plants were regenerated via somatic embryogenesis.

2.2.6. Histochemical GUS assays

Histochemical analysis of GUS activity was conducted essentially as described by Jefferson *et al* (1987). Transient GUS activity was assayed in embryos transiently, and callus 6 weeks after whisker treatment by incubating at 37 °C for 10–12 h in GUS substrate (1 mM 5-bromo-4-chloro-3-indolyl-β-D glucuronide cyclohexyl ammonium salt (X-Glue), 100 mM sodium phosphate (pH 7.0), 0.1 % (w/v) Triton X-100).

2.2.7. Southern hybridization analysis

2.2.7.1. Isolation of genomic DNA

Genomic DNA was isolated from the putative T_0 and T_1 cotton plant leaves using the method of Iqbal *et al*. (1997). Just 20ml of 2X (CTAB) (2 % cetyltriethylmethylammonium bromide (w/v); 100 mM Tris (pH 8.0); 20 mM, EDTA (pH 8.0),1.4 M NaCl, 1 % PVP (polyvinylpyrolidone) and 100µl of 2-mecraptoethanol was pipetted in 50ml falcon tube and incubated at 65 °C . One gram of fresh leaves of transgenic cotton plants were ground in liquid nitrogen, transferred to the tube and 15 ml of hot 2X CTAB solution were poured into ground powder, suspended and incubated at 65 °C for 30 minutes. Cool down at room temperature, equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently. Tubes were centrifuged at 4000×g for 10 min at room temp. In the supernatant 0.6 volume of cold iso-propanol (two third volume) was added, mixed and placed at -20 ^{o}C for 30 minutes. Tube was placed at room temperature for a while and centrifuged at 4000 rpm for 20 minutes. DNA pellet was washed with 70 % ethanol, allowed to air dry, dissolved in 1 ml TE.

One microliter of RNase (10 mg ml^{-1}) was added to DNA and the tube was incubated at 37 ^{o}C for 15 minutes. DNA was then precipitated with 1/10 volume of Na-acetate (3 M) and 0.6 volume of cold isopropanol, placed at- 20 ^{o}C for 30 minutes,

centrifuged, washed with 70 % (v/v) ethanol , air dried and finally the pellet was dissolved in 1ml TE. Optical density (OD) was measured at 260 and 280 nm by using UV/VIS spectrophotometer. An OD260 of 1 corresponds to a dsDNA concentration of 50 mg ml^{-1}. The ratio 260/280 indicates the purity of DNA and should be between 1.8 -2.0 (Sambrook *et al.*, 1989)

2.2.7.2. Digestion and separation of DNA samples on agarose gel

Genomic DNA (10-15 µg) of transgenic and non-transgenic lines were isolated using CTAB method and digested overnight with *Sma*I/*Eco*RI enzymes. To this amount of DNA, 10 µl of 10X reaction buffer and 2 µl of *Sma*I/*Eco*R1 (10 U/µl) were added and placed for overnight incubation at 37°C.

2.2.7.3. DNA transfer to Nylon-membrane (capillary transfer method)

The overnight digested samples were size fractionated by electrophoresis on 1 % agarose gel under low voltage (20-25 volts). Prior to transfer, the DNA in the gel was depurinated by submerging the gel in 0.25 N HCl with shaking for 20 minutes at room temperature. The gel was thoroughly rinsed with sterile double distilled water. Then the DNA in the gel was denatured by submerging the gel twice in denaturation solution (0.5 N NaOH, 1.5 M NaCl) for 15 minutes at room temperature with gentle shaking. The gel was rinsed with sterile double distilled water. After denaturation, the gel was submerged twice in neutralization solution (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.5) for 15 minutes at room temperature with gentle shaking.

The gel was equilibrated for at least 10 minutes in 10X SSC (3 M NaCl, 300 mM sodium citrate, pH 7.0). DNA was overnight transferred on nylon membrane by capillary method in 10X SSC transfer buffer. The membrane was removed and washed with 2X SSC. The DNA was air dried for 2 hours and crosslinked by exposing the membrane in a UV cross linker (CL-1000 ultra voilet crosslinker-UVP, Cambridge, England) at 120 mJ/cm^2 energy for 2 minutes.

2.2.7.4. Synthesis of radio-labeled [α^{32}P] dATP probes

The *AVP*1 specific gene fragment was amplified from plasmid and purified by using CONCERT^{TM} Rapid Gel Extraction system (GIBCO BRL, California, USA)

according to supplier's instructions. The purified product of specific gene was labeled by using Rad-Primed labeling kit (GIBCO-BRL, USA) following manufacturer's instructions. The probe was prepared in 50 µl final reaction volume. First, 25 ng of template DNA (PCR purified product) was taken in microfuge tube and 20 µl of sterile ddH2O was added to it. Denaturation of this template DNA was done by placing microfuge tube in boiling water (95°C) for 5 minutes. After that, microfuge tube was placed immediately on ice. Then 1 µl of each dGTP, dCTP, dTTP, 20 µl of 2.5X random primer solution/buffer and 5 µl of radio-labeled [$\boldsymbol{\alpha}^{32}$P] dATP mixture were added. The reaction mixture was mixed briefly and 1 µl of Klenow fragment was added. The Reaction mixture was mixed again gently and centrifugation was done for 30 seconds. The reaction mixture was incubated at 37°C for 10 minutes. Finally, 5 µl of stop buffer was added to the reaction mixture to terminate the reaction.

2.2.7.5. Prehybridization of the blot

Membranes containing DNA were prehybridized (Sambrook *et al*., 1989) in hybridization tubes to block the non-specific binding of the probe. Each tube containing 20 ml of pre-warmed (68 °C) pre-hybridization buffer (125 mM sodium phosphate buffer pH 7.2, 250 mM sodium chloride, 7 % (v/v) SDS). Prehybridization was carried out at 55 °C for at least one hour in a hybridization oven (Hybaid Limited, Kingsclere, Hampshire, UK).

2.2.7.6. Hybridization of the blot

The corresponding radio-labeled probe was denatured by placing in boiling water bath for five minutes and chilled quickly on ice. The denatured probe was carefully added to 10 ml of pre-warmed pre-hybridization buffer and mixed by inversion to form the hybridization solution. Immediately, hybridization solution containing radio-labeled probe was added to hybridization tubes containing pre-hybridized membranes very carefully. Hybridization with AVP1 specific probe was carriedout in hybridization oven at 58 °C overnight.

2.2.7.7. Washing and developing of the radioactive probed blot after hybridization

The membrane was submerged in a tray containing 100 ml of 2X SSC and 0.5 % (w/v) SDS at room temperature for 5 minutes. Then the membrane was transferred in fresh tray containing low stringency buffer (2X SSC, 0.1 % SDS) and incubated at room temperature for 15 minutes with occasional gentle agitation. After that the blot was transferred to tray containing 0.1X SSC, 0.5 % (w/v) SDS and incubated for 30-60 minutes at 37 °C in hybridization oven with gentle shaking. Solution was replaced with fresh 0.1X SSC + 0.5 % SDS solution and the tray was incubated at 55 °C for 30-60 minutes in hybridization oven. The amount of radioactivity on the blot was monitored. Briefly, the blot was washed with 0.1X SSC at room temperature. The excess of the buffer was gently removed and blot was wrapped in Saran wrap and exposed to phosphor-imaging screen for 24 h at -80°C. Finally, the phosphor-imaging screen was developed in phosphor imager.Typhoon 8600 (Amersham Pharmacia Biotech, California, USA) and the image displaying hybridization signals were retrieved.

2.2.8. Salt treatment

After Southern hybridization analysis of transformed calli, plants were regenerated via somatic embryogenesis and grown to maturity. Mature seeds were harvested from T_0 regenerated cotton plants and T_1 progenies were sown. After kanamycin screening of *AVP1* transgenic plants, salt treatment was administered by watering the plants with 200 mM NaCl solution supplemented with 10 mM KNO_3 and 5 mM $Ca(NO3)_2$ solution for 25 days as described by He *et al*. (2006). Salt tolerance was assessed by measuring growth rate and plant health in comparison with non-transgenic control plants.

2.2.9. Inheritance of transgenes

To study the transgene inheritance pattern, screening of T_1, T_2, and T_3 segregating progeny was accomplished by applying seven to nine day old cotton seedlings with kanamycin (10 µg) on the upper surface of cotyledonary leaf with cotton swab. The response to kanamycin was recorded after 72 h. Microsoft Excel TM was used for graphical presentation of data. Chi-square test was used to study the segregation pattern of transgenes from T_0 to subsequent T_1, T_2, and T_3 generations.

For transformation efficiency calculation, almost 44 embryogenic callus masses (six petri dishes, approximately 6–7 masses per dish) were used for each treatment, in

which triplicate were included. Frequency of kanamycin resistant calluses was evaluated after selection for eight to ten weeks.

2.3. Results

2.3.1. Transformation of cotton calli and molecular analysis

Calli inductions were achieved within 6-8 week and multiplied within 3-4 week. Multiplied callus was transferred to embryogenic medium (MSB2) and embryogenesis was achieved within 6 week, Non-embryogenic and embryogenic calli was separated and maintained on embryogenic medium (MSB2) as continuous source of embryogenic calli (Figure 2.2 a, b).

Initial experiment was conducted with a reporter gene (*GUS*) to assess the validity of the current method by studying the transient GUS expression (Figure 2.2 c, d), in the treated samples. Cotton calli treated with silicon carbide whiskers and pGreen0029, pRG229 exhibited Km^r callus colonies within 6–8 weeks. Control calli (no DNA or whiskers) did not express GUS activity. An average of 50–60 kanamycin resistant calli were observed per vortexed sample of cotton embryogenic calli treated with pGreen0029 + fibers following approximately six to eight weeks of selection (Figure 2.2 d, e) giving a transformation efficiency of 94 % (Table 3.1). Km^r callus was not produced in the control samples even after several weeks of selection. Cotton calli treated with pGreen0029 (*GUS*) in the absence of whiskers did not express GUS activity even after 8 weeks of culture (Figure 2.2 f, left), presumably because the calli did not receive the foreign DNA. However, cotton calli cells treated with pGreen 0029 (*GUS*) and silicon fibers transiently expressed *GUS* activity in treated embryos (Figure 2.2c) and also in the transformed calli after 8 weeks of selection at an average rate of 50–60 colonies per sample (Figure 2.2d).

The growth or development of putative transgenic calli colonies were not observed in any of the controls. An average of 50–60 putative km^r cotton calli were observed per sample treated with pGreen0029 (*GUS*) + fibers following 6–8 weeks of selection. After the validity of this procedure, another DNA construct, i.e. pRG229 was transformed, which possessed the *AVP1* gene which has been shown to confer the salt tolerance in transgenic plants (Gaxiola *et al.*, 2001, 2002). Approximately same pattern of putative transgenic calli formation was observed on MSB1 selection medium in embryogenic calli treated with pRG229 + fibers (Figure 2.2e) after 6–8 weeks of selection. Figure 2.3a displays the *AVP1* gene integration in the higher molecular weight

DNA when *Hin*dIII restricted genomic DNA was hybridized with *AVP1*-specific probe. Full length and intact *AVP1* expression cassette was released from pRG229 by *Hin*dIII restriction. Therefore, intensity of signals is proportional to number of copies. DNA hybridization signals of varying intensity were observed. DNA isolated from un-treated or non-transgenic calli did not hybridize with the *AVP1* probe (Figure 2.3 lane 3). From this blot, it is possible to assess the intactness as well as copy number of the integrated gene in the nuclear DNA isolated from C1, C2, C3, C4, and C5 (lanes 4–8) calli colonies sample.

The southern analysis of blots probed with 3.0 Kbp *AVP1* expression cassette has shown not only intact copies of *AVP1* expression cassette but also in one or more number of copies. Unpredicted restriction fragment sizes of *AVP1* expression cassette were also present of either lower or larger sizes showing random *AVP1* integration only in higher molecular weight genomic DNA. The different DNA restriction pattern (Figure 2.3 a) of each colony indicated that C1, C2, C3, C4, and C5 calli arose from different transgene integration events showing copy numbers 2–4. Figure 2.3 b shows the number of transgenes in a transgenic event in four copies in the genomic DNA digested with *Pst*I, which cuts the expression cassette DNA at unique site releasing a DNA fragment containing transgene and a part of genomic DNA (lane 1, Figure 2.3 b) and total uncut genomic DNA (lane 2, Figure 2.3 a).

2.3.2. Effect of callus type and days after subculture

The explant type and days after subculture were significant factors in affecting the emergence of callus colonies. At all intervals after subcultures, embryogenic calli surpassed non-embryogenic calli by giving the higher percentage of transgenic calli. The stage of explant optimum for transformation by the current method after subculture was obtained by treating the cotton calli obtained at 6, 12, 18, and 24 days after subculture. For either callus explant type, calli at day 18 have shown an optimum number of kanamycin resistant calli colonies formation which decreased at early or delayed stage of the subculture period (Figure 2.4 a). Extended subculture intervals (24 days) or soon after subculture time (6 days) produced comparatively low number of emergence of kanamycin resistant callus colonies.

2.3.3. Effect of antibiotic concentration

For the emergence of transgenic calli, antibiotic selection played a pivotal role when different kanamycin antibiotic concentrations (40-60) were used. It was observed that kanamycin concentration in MSB1 medium containing 50 mg l^{-1} kanamycin profoundly affected the development of transgenic calli. At kanamycin 50mg l^{-1}, growth of non-transgenic embryogenic callus was inhibited by 95 %, whereas 60 mg l^{-1} kanamycin resulted in total cessation of growth followed by death within three to four weeks. For reliable selection at calli stage on MSB1 medium, 50 mg l^{-1} kanamycin was optimally required. There were excessive escapes when lower concentration of 30 or 40 mg l^{-1} were used (Figure 2.4 b). Whereas higher concentration resulted in less number of calli colonies showing high frequency of transgenic calli induction than no transgenic calli with slowed proliferation (Figure 2.4 b). From these experiments, it was concluded that selection at callus stage, 50 mg l^{-1} kanamycin was required at callusing phase, which is reduced to 25 mg l^{-1} when transferred to MSB2 (embryo induction) medium to allow fair embryogenesis and plant regeneration.

2.3.4. Salt treatment of T_1 transgenic cotton plants

To test whether the transgenic cotton plants over-expressing the *AVP1*-H^+ pump gene over-expressing cotton were more tolerant to salt stress in comparison to the non-transgenic plants, four T_1 transgenic lines were analyzed for their salt tolerance in glass pots. The transgenic plants were first screened for kanamycin resistance by applying kanamycin (100mg) on cotyledons (Figure 2.2 k) and resistant ones were allowed to grow until three weeks old. The seedlings were then watered with 100 mM of NaCl for the first and second week and 200 mM for the third and fourth weeks. Under continued increased salt concentration to 200 mM, the phenotypic differences between wild type and *AVP1*-expressing cotton plant became apparent after second week from the start of treatment. Growth of wild-type plants were severely inhibited by salt treatment, whereas no adverse effect of salt treatment was observed in *AVP1* over-expressing cotton plants and they showed significantly higher growth than the wild-type control plants (Figure. 2.2 l).

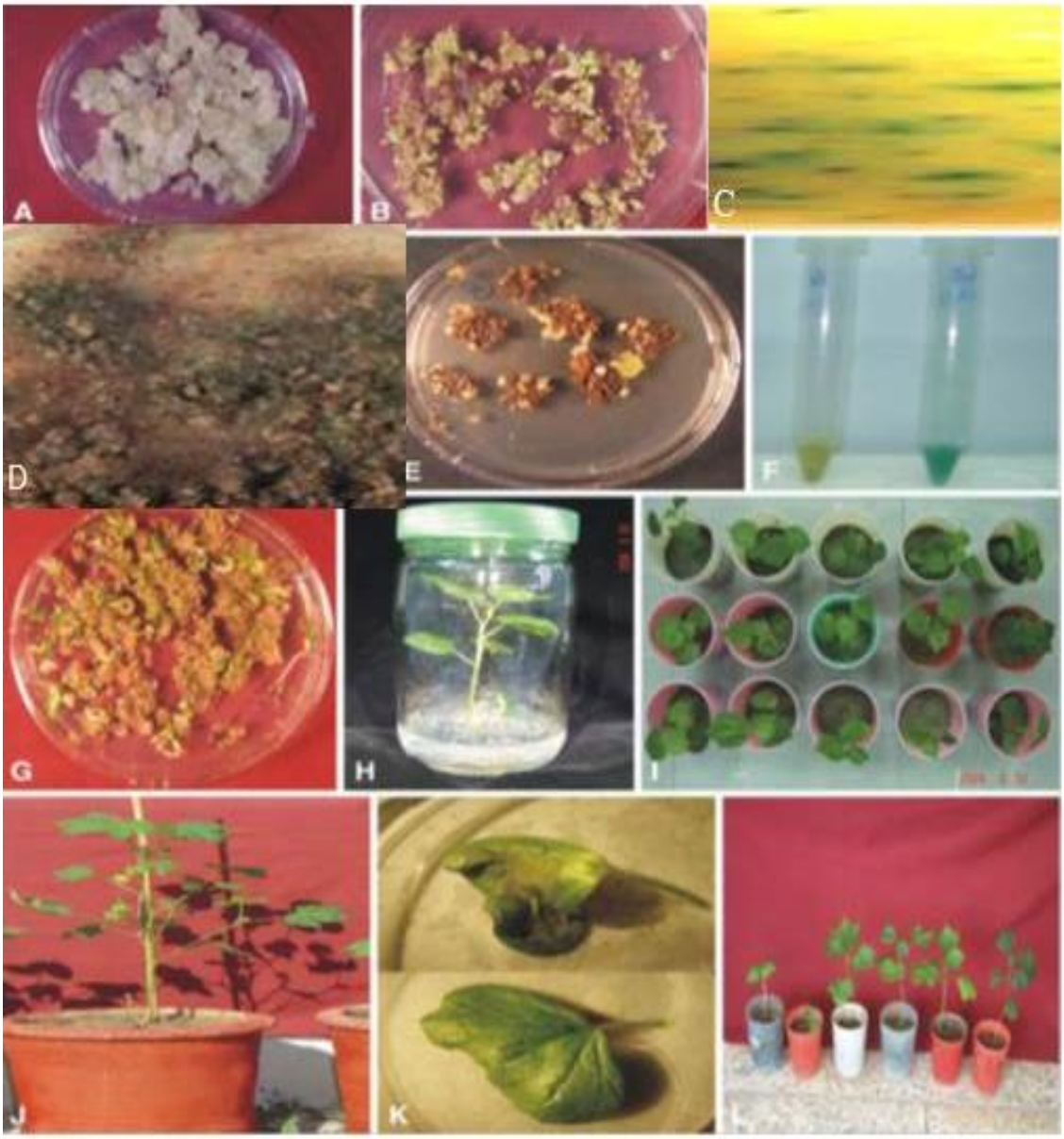


Figure 2.2 Tissue culture steps in whisker-mediated cotton callus transformation: (A) non embryogenic callus, (B) embryogenic callus, (C) transient GUS expression in whisker treated embryo after 72 h, (D) GUS expression in kanamycin resistant callus colonies after six weeks, (E) development of km resistant callus colonies for *AVP1* after six weeks, (F) GUS expression in stable colony; Right control, left transgenic callus, (G) transgenic embryogenic callus with regenerated embryos, (H) regenerated transgenic plant, (I) population of T_0 regenerated transgenic cotton plants, (J) T_0 regenerated plant in soil pot, (K) Km-swabbing test of transgenic (lower) and control (upper) plant cotyledonary leaves, (L) salt treatment of control (left 2) and *AVP1* transgenic T_1 cotton plants (Right).

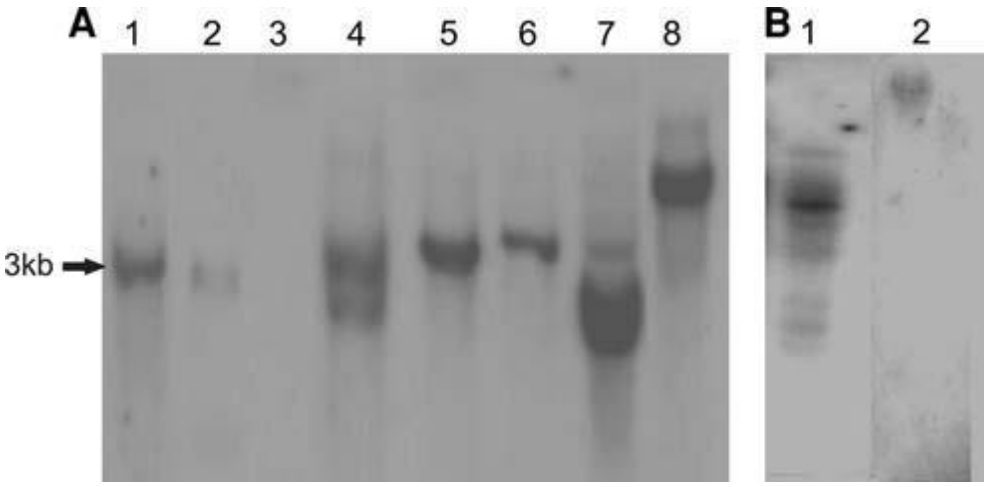
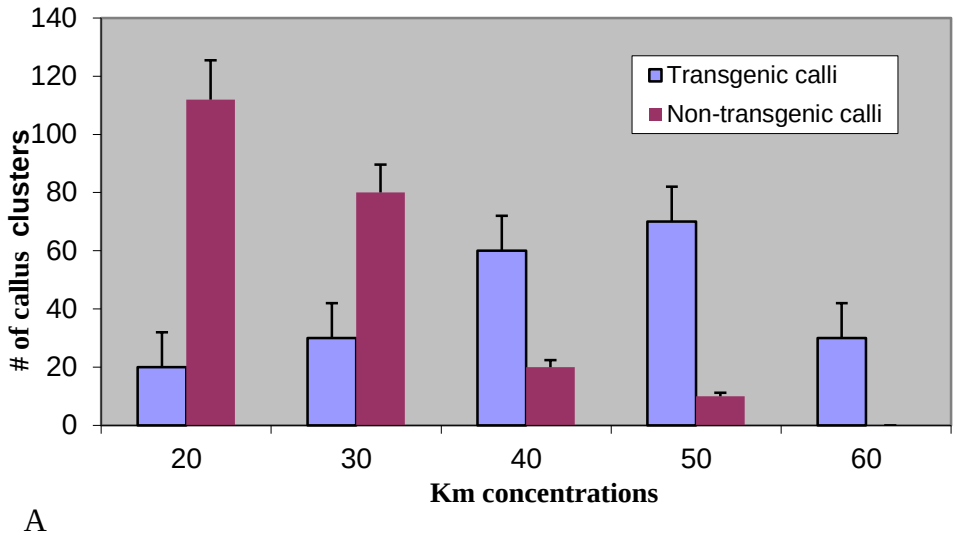


Figure 2.3 Southern analysis of nuclear DNA isolated from kanamycin resistant and non-treated control calli probed with the *AVP1* gene. (a) Total genomic DNA (approx. 15µg) was digested with *Hind*III and electrophoresed on 0.8 % agarose gel followed by transfer to nylon membrane. Lanes 1(1x-5pg) and 2 (5x-25pg) are one copy and five copies of 3.0 kbp *AVP1* containing fragment obtained by *Hind*III digestion of pRG229, lanes 3 (non-treated control), lanes 4, 5, 6, 7 and 8 represent integration of *AVP1* gene into nuclear DNA either in one or more copies. (b) shows transgene integration in uncut genomic DNA (lane 2) and in multiple copies in genomic DNA digested with *Pst*1(lane1). The blots were probed with a 32P-labelled 3.0 kbp *AVP1* gene containing expression cassette for *AVP1* samples.



A

B

Figure 2.4 Optimization of three transformation parameters. (a) Effect of callus type and days after subculture on the emergence of transgenic and non-transgenic cotton calli clusters. (b) Effect of kanamycin concentration on the emergence of transgenic and non-transgenic cotton calli clusters. (n=20).

2.3.5. Transformation efficiency

Genomic DNA was isolated from transgenic calli and subjected to PCR analysis. It was found that most of the PCR positives were transgenic. By this method, we were able to get 94 % transgenic plants which were screened by PCR analyses (Table 2.1).

Table 2.1 Summary of stable transformation efficiency from six whisker-mediated transformation experiments (4-6 g of embryogenic callus was used in each experiment).

Experiment No.	Number of Km-resistant clusters	PCR positive	Southern positive	Stable Transformation efficiency
1	40	38	36	94
2	60	56	50	89
3	45	40	34	85
4	45	38	35	92
5	39	38	36	94
6	60	50	44	88
7	64	34	30	88
8	58	54	51	94

Stable transformation efficiency (%)= Southern positive/PCR positive x 100

2.3.6. Inheritance of transgene

Progeny from several transgenic cotton plants obtained from independent events were analyzed to determine the inheritance of the *nptII* gene. Its expression was assessed by the applying kanamycin on cotyledonary leaves obtained from 7- to 9-day-old plantlets.

Figure 2.2 k shows that plants expressing the *nptII* as selectable marker gene developed no symptoms of yellowing, whereas the non-transformed plants (upper leaf) showed necrosis and damage after 72 h of treatment. The kanamycin swabbing made it feasible to monitor the segregation of *nptII* in large numbers of progeny plants from many events. The 3:1 segregation ratio was expected in T_2 generation. One population of clones S9 and S5 showed a 3:1 ratio, while the other population of these two clones was 100 % kanamycin-resistant, indicating they were homozygous for the *nptII* gene. These segregation results confirm that the *nptII* gene was stably inherited up to the T_3 generation in a Mendelian manner (Table 2.2).

Table 2.2 Segregation of *nptII* gene based on kanamycin swabbing assays of cotyledons in T_2 and T_3 generation transgenic cotton plants (n=20).

Generation	Cross	Kanamycin resistant	Kanamycin sensitive	X^{2c}	P^d
T_2[a]					
	Self	56	23	050	0.46
S31	Male	30	37	053	0.47
	Female	36	34	0.01	0.90
	Self	30	9	0.01	0.90
S9	Male	28	22	0.50	0.50
	Female	37	50	1.64	0.49
	Self	50	46	0.09	0.76
S2	Male	52	43	0.67	0.41
	Female	57	24	0.70	0.40
	Self	64	27	0.82	0.36
S5	Male	48	47	0.00	1.00
	Female	37	56	3.48	0.06
T_3[b]					
	Self1	61	14	1.28	0.20
S31	Self2	64	31	2.56	0.11
S9	Self1	66	27	0.61	0.44
	Self2	97	0	Homozygous	

S2	Self1	64	28	1.17	0.28
	Self2	56	27	2.12	0.14
S5	Self1	62	0	Homozygous	
	Self2	66	22	0.29	0.65

[a] kanamycin resistant one T_1 were either selfed or crossed as male and female parent in crosses with non-transformed plants.
[b] kanamycin resistant plants were selfed.
X^{2c} (Chi square) value with Yates Correction

2.4. Discussion

Molecular biology and DNA delivery methods are state-of-the-art technologies for fundamental studies and agronomic improvement of crop plants. The new DNA delivery methods endeavors the gene deliveries into chromosomes "stably" with efficient selection facilitating the regeneration of transgenic plants from several events within short time. With successful and commercial deployment of GM plants, efforts were geared up towards develop different transformation methods with unique characteristics like genotype flexible, throughput and rapid to accelerate the engineering of plants for novel traits (Chen *et al.,* 2014). In these efforts different transformation methods were developed like *Agrobacterium tumefaciens* mediated transformation, microparticle bombardment, electroporation, microinjection and silicon carbide whiskers (Hussaini *et al.,* 2010; Arshad *et al.,* 2013). Being a versatile and premium crop i.e. cotton, these developments led to focusing on the transformation of cotton via different transformation methods. But cotton remains non-accessible to these transformation methodologies by its tedious and recalcitrance for embryogenesis and regeneration potential. Up till now few cotton lines like Coker-312 or derived lines, have been transformed (Wilkins *et al.,* 2004; Singh and Pental, 2015).

In the present study, cotton embryogenic calli was transformed with reporter green *GUS* and agronomic gene *AVP1* to see the efficacy of the newly developed method. Silicon whiskers possess enough mechanical strength for effective penetration into cells including those with difficult- to-penetrate cell walls such as yeast and pollens (Mcknight *et al.,* 2006). Silicon carbide-mediated DNA delivery affected the transient as well as stable transformation of cotton callus cultures. The evidences for transformation included expression of three genes, the selectable marker *nptII,* the reporter *GUS* gene, and agronomic trait gene (*AVP1-*H^+ pump). For stable transformation, Southern blot of genomic DNA digested with restriction enzymes showed the transgenes integration in intact form into higher molecular weight genomic DNA. Ratios of the number of stable transformants to the average number of transient expression were 0.9 and 0.8 for GUS (data not shown) and *AVP1* genes. Finer and McMullen *et al.,* (1990) reported a stable-to-transient ratios of 0.7 in cotton by physical procedure of biolistic gun. Thus current

protocol resulted in efficiency higher than that reported for cotton transformation by biolistic gun and similar to those reported for *Agrobacterium*-mediated transformation.

The success in producing fertile transgenic cotton is due to use of reporter *GUS* gene delivery to assess the validity through optimization of three crucial factors like callus type, days after subculture and antibiotic concentration. It was observed that embryogenic callus was significantly better than non-embryogenic callus in the induction of km resistant callus colonies at all days after subculture (particularly at 18 days after subculture). Plant cells have negative water potential relative to their surrounding environment, which is balanced by an equal pressure provided by the cell wall. So plant tissues such as callus remained turgid. The pre-treatment of callus tissue via exposure to media of high osmotic potential like mannitol/sorbitol has been used to improve cell survival following microparticle bombardment (Klein et al.., 1989; Vain *et al.,* 1993 Southern *et al.,* 1973). In this case, callus at 12 days after subculture might be fully turgid, mainly responsible for low occurrence of callus colonies, and cell turgidity decreased with increased days after subculture, although physiological status of cotton calli at 12 days after subculture has been reported best for transformation via *Agrobacterium* (Jin *et al.,* 2005). Fully turgid callus is more sensitive to whisker damage and pre-plasmolysis provides some level of protection as described earlier (Petolino *et al.,* 2000).

Callus has been reported to possess cells with condensed cytoplasm and big karyon at 12–18 days after subculture possessing sufficient strength to face the physical piercing of silicon whisker fibers, thereby allowing the efficient transformation without affecting the survivability and regeneration potential. It is callus type and days after subculture that significantly determine the cotton transformation (Jin *et al.,* 2005). Another major critical factor for optimum transformation of the tissue from putatively transformed cotton calli is the kanamycin antibiotic used, which was found that at low concentration of kanamycin there was high emergence of callus colonies with low number of transgenic events at low selection pressure which decreased to minimum at 50 mg l^{-1} and the total number of transgenic calli was decreased when selection concentration was higher. Kanamycin concentration of 50 mg l^{-1} km was found optimum concentration to get maximum number of transformation events. It is the selection

schemes, type, and level of antibiotic used which markedly affect the nature of stable transformation in the sense of nature of integration and number of integrations (Wu *et al.*, 2005). Stable transformation was confirmed by Southern blot analysis of genomic DNA from km resistant cotton calli cultures. In addition to expected band hybridization, there was hybridization of bands of unexpectedly smaller or larger sizes than predicted (Figure 2.3a), which might have resulted from rearrangement and fragmentation of plasmid sequences during delivery and integration.

DNA delivery into plant cells varies considerably showing copy number integrated into the genome by the different methods, but it appears to be on the same order of magnitude (Toms *et al.*, 1990). This may indicate that, although DNA may enter the cells by different means, there is common mechanism of integration of the DNA once it enters a cell. Restriction fragments, both larger and smaller than the predicted sizes observed in present study, have also been noted in other studies (Finer and McMullen 1990) .polymorphisms observed in transgene sequences maybe originate by (Wilkins *et al.*, 2004)) concatamerization of the transforming DNA by homologous recombination, concomitant loss, and rearrangement of restriction sites prior to integration; (Gould *et al.*, 1990) shearing or partial digestion of the DNA prior to integration; and methylation of the restriction sites within the integrated transgene. These mechanisms may explain the appearance of fragments smaller or larger than the expected ones. Despite the possible occurrence of rearrangements, shearing and or methylation of transgenes integrated into the plant genome, many transgenic plants possessed intact expression cassette of *AVP1* and cell lines expressing high levels of various transgenes have been recovered by current protocol as reported for other plant species (Toms *et al.*,1990: Fomm *et al.*, 1990: Gordon-Kamm *et al.*, 1990).

Development of salt tolerance in transgenic cotton generated via silicon carbide whisker mediated transformation was observed in T_1 transgenic cotton progenies of four events. Significant enhancement in salt tolerance was observed due to transgene over-expression of *AVP1* in cotton plants as compared to controls (Figure 2.2 l). Thirty days of salt treatment showed loss of growth and vigor on wild plants, whereas transgenic cotton lines remained tolerant and continued its growth and vigor by increase in height and leaves number as well as size (Figure 2.2 l). The transgenic status of the transformed

callus and plants was determined by a combination of PCR and Southern blot analysis, together with salt/GUS assays demonstrating functional gene products (kanamycin and GUS) activity). Transmission and expression of the introduced *nptII* and *GUS* reporter gene in the progeny of transgenic plants was as expected for a single dominant allele, providing further evidence of integrative transformation. To test further the inheritance of *nptII* activity in the following generations, 16 kanamycin resistant T_2 plants representing each of the four transformation events were self-pollinated. Plants from two T_3 populations of each transformant were germinated and swabbed for kanamycin resistance. As shown in Table 2.1 the populations of clones S-9, S-2, and S-5 showed the expected 3:1 segregation ratio. One population of clones S9 and S5 showed a 3:1 ratio, while the other population of these two clones was 100 % kanamycin resistant, indicating they were homozygous for the *nptII* gene. These segregation results confirm that the *nptII* gene was stably inherited to the T_3 generation in a Mendelian manner. The fate of genes introduced into cotton calli cells via whisker transformation does not appear to differ from other direct DNA delivery approaches.

Through the present protocol, it has been possible to get fertile plants of different independent transgenic events from small amount (~1g) of embryogenic cotton calli treated with whiskers within a period of 4–5 months that showed the potential of the protocol for scaling up to develop large number of transgenic plant To populations enhancing the probability of getting transgenic line with optimum transgene expression. Moreover, the present protocol is efficient, simple and inexpensive than either biological method, protoplast electroporation or biolistic gun method of cotton transformation and is equally applicable to transform any gene.

Chapter 3

Development of salt and drought tolerant cotton

3.1. Introduction

Understanding how plants respond to drought and salt stress at the cellular and molecular levels is pivotal for engineering future crops with improved stress tolerance (Wang *et al.,* 2002; Zhang *et al.,* 2004). Glycophytic plants usually rely on Na^+ exclusion and compartmentation strategies for the maintenance of a low cytosolic Na^+ concentration (Roy *et al.,* 2014).

Na^+ transport out of the cell is brought through operation of plasma membrane-bound H^+-pumps energizing Na^+/H^+ antiporters as confirmed by the characterization of SOS1, a putative plasma membrane Na^+/H^+ antiporters from *Arabidopsis thaliana* (Apse *et al;* 1999; Gaxiola, *et al.,* 2002; Shi *et al.,* 2000). These ions, in turn, act as an osmoticum within the vacuole to maintain water flow into the cell (Glenn *et al.,* 1999; Gaxiola *et al.,* 2002). Cotton is a versatile crop due to its contribution in foreign export becoming lifeline for Pakistan's textile sector. Its cultivation is under threat due to salinity and drought problems and threat of water scarcity is mounting pressure on cotton breeders and scientist to develop cotton varieties for efficient use of available water facilities (Deng *et al.,* 2004; Bhattarai *et al.,* 2005; Sahito *et al.,* 2015).

Abiotic factors are responsible for 30% losses in cotton production. Due to intensive cropping system, salinity and drought problems are being expected to be major threat in the way of sustained cotton production (Sahito *et al.,* 2015). After exploring natural genetic variation by conventional breeding for the improvement of abiotic stress tolerance, genetic engineering has emerged as an alternate option to meet the future challenges for abiotic stress tolerance. Therefore, main goal of present study was to develop salt and drought tolerance in cotton coker-312 by transferring *AVP1* and *AtNHX1* genes. *AVP1* encodes 770 amino acid proteins of molecular weight 80 KDa (Rea *et al*., 1992) while *AtNHX1* encodes 500 amino acids proteins and are well characterized for sequestration of Na^+ ions into the lumen of vacuole (Zhang *et al*., 2001). Both of these proteins are embedded in the tonoplast and catalyse the H^+-pumping and material

exchange into the vacuolar lumen to generate proton electrochemical gradient energizing the movement and exchange of radicals by transporters/exchangers.

Keeping in view the importance of major tonoplast genes (*AVP1/AtNHX1*), present study was carried out for the transformation of both genes into cotton (Coker-312) through *Agrobacterium tumefaciens*. Transgenic cotton plants were tested under salt and drought stresses.

3.2. Materials and Methods

3.2.1. Cloning of *AtNHX1* gene

3.2.1.1. PCR amplification of *AtNHX1*gene

Primers were designed to amplify the 1.643 kbp of *AtNHX1* gene from *Arabidopsis* cDNA clone (available at NIBGE). Restriction sites of *Eco*RI and *Hind*III were introduced in forward and reverse primers for directional cloning respectively. The primer sequences were *AtNHX1* forward (*Eco*R1) 5′GGAAGCTTATGTTGGATTTCTCTAGTGTC3′, *AtNHX1* reverse (*Hin*dIII) 5′AGGGAATTCCTAAGATCAGGAGGGTTTCTC3′.

PCR reaction was carried out in thermal cycler (Eppendorf, Model 22331, Hamburg Germany) by adding cDNA 2 µl (50 ng), dNTPs (0.25 mM each), Buffer (10X) 5 µl (1.0 X), MgCl2 1.5 µl (25mM), 3 µl (1.5 mM), forward primer 1µl (1µM), reverse 1µl (1µM), Taq polymerase 0.5 µl (2.5U), deionized water 36.5 µl of total volume of 50 µl in a PCR tube. PCR profile was as follows: denaturation 94 °C for 5 min followed by 40 cycles of denaturation at 94°C for 1min, annealing at 55°C for 1min, extension at 72°C for 1 minute. The final extension was carried out at 72°C for 10 minutes.

3.2.1.2. Purification and digestion of *AtNHX1* PCR product

The PCR amplified fragment was electrophoresed on a 1 % (w/v) agarose gel. The amplified DNA fragment (1.643kbp) was eluted by GeneJET^{TM} Gel Extraction Kit (Fermentas, Life Sciences USA). The area of the gel containing required DNA fragment was cut with a razor under UV and placed in an eppendorf containing 30 µl of gel (10 mg) solubilization buffer. The tube was incubated at 50°C for 15 min to dissolve the gel. The mixture was transferred to cartridge and centrifuged at 6000 x *g* for 1 minute. The flow through was discarded, 700 µl of wash buffer was loaded to the catridge, incubated for 5 min at room temperature and centrifuged at 6000 x *g* for 1 minutes. The flow-through was discarded and again centrifuged to remove the traces of wash buffer. The DNA was eluted with 50µl warm TE buffer (10 mM Tris and 1 mM EDTA) after centrifugation at 6000 x *g* for 1 minute.

The purified PCR products were digested with *Eco*R1 and *Hind*III enzymes. The reaction reagents were as followed: DNA (PCR product) 15µl (10µg), reaction buffer 2µl

(10X) 1µl *Eco*R1 (10U μl^{-1}), 1µl *Hin*dIII (10U μl^{-1}), making total volume 20µl. The digestion mixture was incubated at 37^0C for 1 hr. Digested fragments were precipitated by adding $1/10^{th}$ 3M sodium acetate and 2 volumes of absolute ethanol.

3.2.1.3. Digestion of plant expression vector pJIT60

Plant expression vector pJIT60 was digested with *Eco*RI and *Hind*III. The reagents for the reaction were as follows DNA fragment (2µg) 16µl, reaction buffer (10X) 2µl *Eco*R1 10U μl^{-1}) 1µl *Hind*III (10U μl^{-1}) 1µl to a total volume of 20µl. The digestion mixture was incubated at 37 °C for 1 hr. The digested plasmid was treated with phenol-chloroform to inactivate enzymes and precipitated by 2.5 volume of ethanol. The digested plasmid (pJIT60) and *AtNHX1* DNA fragments were electrophoresed on 1 % agarose gel along with 1 kbp DNA marker.

Restricted PCR fragments and pJIT60 plasmids were eluted by GeneJET^{TM} Gel Extraction Kit (Fermentas, Life Sciences USA; as described earlier. The digested eluted DNA fragment of *AtNHX1* gene and plasmid DNA were ligated to yield pSA1. The ligation reaction was carried out as follows DNA fragment (digested) 6µl (500ng), digested plasmid DNA 2µl (200 ng), ligase buffer (10X) 2µl, ligase (5u μl^{-1}) 2µl, deionized water 8µl to a total volume of 20µl. The ligation reaction mixture was incubated for 12-16 hour at 16°C.

3.2.1.4. Preparation of electrocompetent cells

Electrocompetent cells of *E. coli* (DH5α) and *Agrobacterium tumefaciens* were prepared (Sambrook *et al*., 1989), Single colony of *E. coli* strain DH5α and *A. tumefaciens* strain LBA 4404 were picked and grown in 10 ml Lauria Bertani (LB) medium (Tryptone 10 g l^{-1}, yeast extract 10 g l^{-1}, NaCl 5 g l^{-1}, and pH, 7.0), at 37°C and 28°C respectively, on a rotary shaker overnight. For *E. coli*, five ml of the overnight culture was inoculated into fresh LB medium and allowed to grow at 37°C and 28°C until $O.D^{600}$ reached between 0.5-1.0 (10^{10} cells ml^{-1}). The cells were transferred aseptically to an ice cold 50 ml propylene tube and kept cool on ice for 10 minutes. The cells were then centrifuged at 4000 × *g* for 10 minutes in a centrifuge (Sorvall RT6000) at 4^oC.

The cells were pelleted, and then resuspended in 50 ml of sterile cold deionized H_2O. The cells were again centrifuged at 4000 × *g* in the same centrifuge at 4°C for 10

mins. The supernatant was decanted and the cells were pelleted, and then resuspended in 25 ml of sterile cold deionized H_2O. After another wash, cells were resuspended in 10 ml of filter sterilized cold 10 % (v/v) glycerol. This wash was repeated twice. Finally the cells were resuspended in 1.5 ml filter sterilized cold 10 % (v/v) glycerol , aliquoted as 50 µl of cells in sterile 1.5ml eppendorf tubes and stored at -70°C.

3.2.1.5. Transformation of pSA1 in *E. coli* by electroporation

Transformation of pSA1 plasmid (pJIt60AtNHX1) in to the competent cells of *E. coli* DH5α was carried out by the electric shock method (Sambrook *et al*., 1989) using an Electro-Cell Manipulator 600^{TM} (BTX San Diego, California). Electroporation cuvettes of 1 mm gap were placed on ice. Vials of frozen electro-competent cells were allowed to thaw on ice, 1µg DNA of the plasmid was mixed with 50 µl of electro-competent cells in the electroporation cuvettes on ice. The conditions for electroporation were set as; choose mode T 1.2 KV *(E. coli) s*et resistance R5 (129 ohm) and set charging voltage 1.44 KV. A pulse was given and 1 ml of liquid LB medium was added immediately, mixed gently and transferred to a 1.5 ml eppendorf tube and incubated at 37°C for 1 hour with vigorous shaking. Just 400 µl of transformed culture were spread on petri plates containing solid LB medium supplemented with ampicillin (100mg l^{-1}) antibiotic, the plates were sealed with parafilm and kept at 37°C for 10 hour.

A single colony was picked with sterile toothpick and cultured in 5 ml liquid LB medium in 50 ml tube containing ampicillin (100 mg l^{-1}) antibiotic. Culture tubes were kept at 37°C for 12 hours with vigorous shaking. Plasmid was isolated from the cultures.

3.2.1.6. Plasmid isolation from *E. coli*

Mini plasmid isolation was carried out by following the method of (Sambrook *et al.*, 1989). Ten colonies of trans-conjugants were individually grown in 2 ml of LB liquid medium containing ampicillin (100µg ml^{-1}). In each 1.5 ml eppendorf tube, 200 µl of individually grown overnight culture was added and centrifuged at 4000 × *g* for 10 minutes. The pellet was resuspended in 200 µl of buffer (25 mM Tris, 10 m M EDTA, pH 8.0). The cells were lysed by adding 200 µl of lysis buffer (0.2 N NaOH, 1 % (w/v) SDS) and mixed the contents by inverting the tube. The reaction was neutralized by

adding 200 µl of 3M potassium acetate and mixed by inverting the tubes five times. The tubes were centrifuged at 4000 × *g* for 10 minutes. The supernatant was transferred to fresh eppendorf tube and plasmid was precipitated by adding 600 µl of ethanol. The tube was incubated at –20°C for 30 min and centrifuged at 4000 × *g* for 10 minutes. The supernatant was discarded and the plasmid pellet was washed with 70 % (v/v) ethanol and air dried for 10 min and dissolved in 20µl of deionized water. The isolated plasmid DNA was digested with *Eco*RI and *Hin*dIII enzymes and separated on 1 % (w/v) agarose gel.

3.2.1.7 Cloning of plant expression cassette into plant transformation vector pGA482 to yield plasmid pSA2

Plamids pSA1 were digested with *Kpn*I/*Eco*RV (to release the plant expression cassette size) and pGA482 with *Kpn*I/*Hpa*I respectively, separated on 1 % agarose gel. The restricted plasmid and expression cassette were ligated to produce pSA2, transformed in *E. coli* competent cells and screened to isolate the clone with pSA2 plasmid.

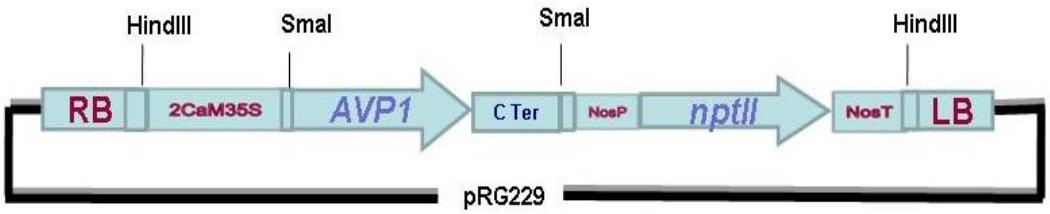
3.2.2. Transformation of pRG229 (*AVP1*) and pSA2 (*AtNHX1*) into *A. tumefaciens* strain LBA 4404

Plasmids pRG229 and pSA2 were electroporated into *A. tumefaciens* strain LBA 4404 competant cells as described in section 3.2.1.5. Figure 3.1 a, b shows the schematic representation of the plasmids maps.

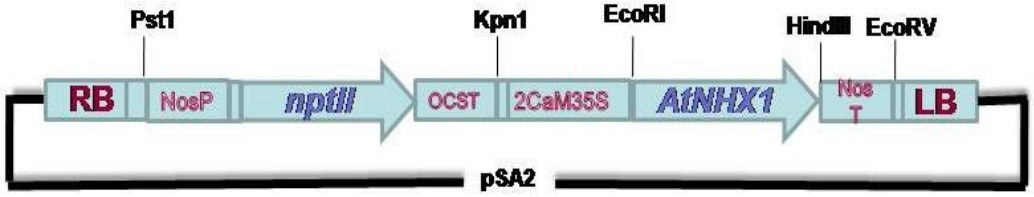
3.2.3. *Agrobacterium* mediated cotton transformation with pRG229 (*AVP1*) and pSA2 (*AtNHX1*) plasmids

Agrobacterium tumefaciens strain LBA 4404 was used to transform the *AVP1* and At*NHX1* genes (Figure 3.1) in cotton Coker-312 following the method as described in Hashmi *et al*. (2011). Seeds of Coker-312 (*Gossypium hirsutum* L.) were delinted in H_2SO_4 for 3-5 min, surface-sterilized with a solution of 1 % (w/v) SDS and 1 % (w/v) $HgCl_2$ for 20 min, and washed three times with sterile water. Seeds were allowed to germinate on MS0 medium (Murashige and Skoog 1962) at 30°C. Hypocotyls of 8 day old *in-vitro* grown plants were used as explants. An *Agrobacterium tumefaciens* single colony was inoculated in 10 ml LB medium containing the respective antibiotics

(rifampicin, tetracycline) and grown at 28 °C for 48 hours. The hypocotyl explants were inoculated



A



B

Figure 3.1 schematic representations of plasmids maps. A) pRG229 (*AVP1*) and B) pSA2 (*AtNHX1*). 2×35S, double 35S promoter of cauliflower mosaic virus (CaMV) with duplicated enhancer region, *AVP1*, *Arabidopsis vacuolar pyrophosphatase I*; *AtNHX1*, *Arabidopsis thaliana* Na^+/H^+ exchanger; OCS, octopine synthase terminator; C Ter, CaMV terminator; NOSP, nopaline synthase promoter, *nptII*, *neomycin phosphotransferase II* gene. Unique enzymes used were *Pst*I, *Kpn*I, *Eco*RI, *Hin*dIII, *Eco*RV and *Sma*I.

with *Agrobacterium* cultures for 30 min, blotted on sterile filter paper and co-cultivated for 48 hours at 28 °C on callus induction (CCiCK) medium plates (MS medium with B5 vitamins; 4.5g l^{-1}, 30 g l^{-1} glucose, 0.1 mg ml^{-1} 2, 4-D, 0.5 mg ml^{-1} kinetin, pH 5.8 and solidified with phytagel 2.9 g l^{-1} and autoclaved). After inoculation and co cultivation, explants were transferred to fresh CCiCK selection medium containing kanamycin 50 mg l^{-1} and cefotaxime 500 mg l^{-1}. Sub culturing was carried out after 3-4 weeks. Calli (4mm) were cut from the hypocotyls to encourage faster growth and after 3-5 subcuturing on

CCiCK selection medium, the calli were transferred to embryo maturation medium (MSK plates) (CCiCK + 1.9 g l^{-1} KNO3, kanamycin 25 mg l^{-1} and cefotaxime 500 mg l^{-1}).

After 6-8 weeks, matured embryos were transferred to selection germination medium (Stewart and Hsu 1977) containing 25 mg l^{-1} kanamycin. After 7-8 weeks, the plants were shifted to small pots and after hardening transferred to larger soil pots until maturity. T_0 seeds were selfed and subsequently the T_1 and T_2 populations were harvested.

3.2.4. Molecular analysis of transgenic cotton plants

3.2.4.1. Isolation of genomic DNA

Genomic DNA was isolated from putative T_0 and T_1 transgenic cotton plant leaves using the CTAB method as mentioned in section 2.2.7.1

3.2.4.2. PCR and Southern analysis

Genomic DNA was subjected to PCR analyses by *nptII, 35S, AVP1* and *AtNHX1* primers (Table 3.1). After confirmation of trasngene in transgenic plants through PCR, Southern analysis was performed on genomic DNA as described in section 2.2.7. Ten µg genomic DNA was restricted with specific unique enzymes and resolved on 1 % (w/v) agarose gel and transferred to nylon membrane by capillary action following protocols as described by Sambrook *et al*., (1989) crosslinked and hybridized to gene specific DNA fragments labeled with αP^{32} radioisotopes. Non-transgenic plant DNA template and positive control plasmid were included along with *AVP1* and *AtNHX1* plants.

Table 3.1 List of primers used in PCR for molecular analyses of transgenic cotton plants

Sr. No.	Gene Name	Orientation	DNA sequence	Number of nucleotides	Amplicon size (bps)
1	AtNHX1	F	AGGAAGCTTATGTTGGATTCTCTAGTGTC	29	1617
		R	AGGGAATTCCTAAGATCAGGAGGGTTTCTC	30	
2	*AVP1*	F	ATGGTGGCGCCTGCTTTGTTACCGGAGCTC	30	2300
		R	TTAGAAGTACTTGAAAAGGATACCACCGTG	30	
3	nptII	F	GATAGCGGTCCGCCACAC	18	700
		R	CGAGGATCGTCGTGACAC	18	
4	35S	F	GAAACCTCCTCGGATTCCAT	20	1000

		R	CGTGGTTGGAACGTCTTCTT	20	
5	Actin	F	AAGGAGCTAGCTGGGCTTTC	20	286
		R	TCAAATGCATTGCAGGAGTC	20	
7	AtNHX1	F	ACCACGAAGCTGCTTTTCAT	20	200
		R	AGTCGAAAAGCTCAGCAAGC	20	

3.2.4.3. Expression analyses

The T_2 cotton seeds were sterilized as described earlier in section 2.3. Seeds were germinated on MS0 germination medium containing kanamycin at the concentration of 100 μg ml^{-1} to screen transgenic plants. Resistant plants with a complete root system including branch roots were selected as transgenic plants. The sensitive plants without branch roots were segregating non-transgenic plants. Transformants T_2 were transplanted into soil, allowed to self pollinate and T_2 (second generation of seeds and plants after self-cross on T_1 plants) seeds were individually harvested. The T_2 seeds that showed at 3:1 ratio of kanamycin-resistant to kanamycin-sensitive plants were sterilized and germinated on kanamycin-containing MS0 medium.

Seeds that were all resistant to kanamycin when grown on kanamycin-MS_0 medium were considered homozygous for single insertion of the target gene. An alternative approach is to apply kanamycin solution 50 mg ml^{-1} on the one week-old cotyledonary leaves on $1cm^2$. as described by Yan *et al*., (2004); Asad *et al.*, (2008). After a week, if the cotyledon turns yellow or bleaching, it would be considered as a segregating non-transgenic plant. Otherwise, the plant in which the cotyledonary leaves remained green were considered as transgenic plants. These seeds from parent plants were bulked for further analyses.

After PCR analysis and confirmation of transgene integration through Southern hybridization, transgenic cotton plants were selected for transgenes (*AVP1* and AtNHX1) expression studies.

3.2.4.4. Isolation of total RNA

Solutions, plastic/glassware's and water were DEPC treated for at least 2 hours, autoclaved and oven dried over-night. Total RNA was extracted from leaves of the *AVP1* and *AtNHX1* transgenic cotton by using Concert Plant RNA Reagent (Invitrogen

Waltham, Massachusetts, USA) according to the manufacturer's instructions. Before starting the procedure the centrifuge was set at 4 ^{0}C. Young and fresh leaves weighing ~200 mg were cut from each plant and ground into a very fine powder in pestle and mortar in the presence of liquid nitrogen and transferred to 1.5 ml RNase free tubes. Exactly 0.5 ml cold Concert Plant RNA Reagent was added to each tube of tubes and mixed by flicking. The tubes were laid down horizontally and incubated for 5 minutes at room temperature. Solutions were clarified by centrifugation at room temperature for 2 minutes at 10,000 × *g* in microcentrifuge (Eppendorf Minispin 22331, Hamburg, Germany). Supernatant was collected in fresh tubes and 100 µl of 5 M NaCl and 300 µl chloroform was added to each tube. The tubes mixed by inversion and centrifuged at 4 ^{0}C for 10 minutes at 10000×g. The top aqueous phase of each solution was transferred to a fresh tube and an equal volume of isopropanol was added, mixed thoroughly and allowed to stand at room temperature for 10 minutes. The tubes were again centrifuged at 4 ^{0}C for 10 minutes at 6000 × *g*.

The supernatant was discarded and the pellet was washed by resuspension in 80 % (v/v) ethanol and centrifuged at room temperature for two minutes at 8,000 × *g*. After a brief interval the air dried pellet was dissolved in 30 µl RNase free water. The integrity/purity of RNA was checked on 1 % (w/v) agarose gel and stored at -80 ^{0}C.

3.2.4.5. RNA quantification

The concentration of each RNA sample was checked using a SmartSpec Plus (Bio-Rad, Hercules, USA) flourimeter. Dilutions of each RNA sample were made to uniform concentration by adding DEPC treated double distilled deionized H_2O and loaded in flourimeter. The final concentrations were found by multiplying the samples reading with dilution factor and absorption constant.

3.2.4.6. Synthesis of first cDNA strand

DNase-treated RNA samples were reverse-transcribed using a M-MuLV reverse transcription kit. First strand cDNAs were synthesized from 4 µg total RNA using 1 ml M-MuLV reverse transcriptase, 1 ml Oligo (dT)18 primer and 2 ml dNTP mix at 37 °C for 1 h, then the reaction was terminated at 70 °C for 10 minutes. The total volume of the reverse-transcription reaction system was 20 ml. Four microlitres (100 ng) of the cDNA

solution was used as template for PCR amplification with a pair of gene-specific primers of the *AtNHX1* (Table 3.1) 5TGTTATCTTCGGCCTTGCTC3 and 3´ CAGCAATACCACCAGCATTG5´ which amplify a 195 bp fragment. A 286 bp fragment of the Actin gene was used as an internal control amplified with the specific primers: 5´AAGGAGCTAGCTGGGCTTTC3´ and 5´CAAATGCATTGCAGGAGTC3´. Amplification conditions for the *AtNHX1* fragment were an initial denaturation at 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 60 s, 72 °C for 60 s, then a final extension at 72 °C for 10 minutes. The PCR products were electrophoresed on a 1.2 % (w/v) agarose gel containing ethidium bromide.

3.2.4.7. Northern analysis

Just 20µg of total RNA isolated from selected *AVP1* T_2 transgenic cotton along with control plants was incubated with 10 µl gel loading buffer in a 0.250 ml microcentrifuge tubes for 10 minutes at 65 °C and chilled on ice. One gram agarose was melted in 72 ml DEPC water. Ten ml of 10X formaldehyde gel buffer and 18 ml of formaldehyde was added to it and pour it into gel tray. The agarose gel was used for electrophoresis of RNA samples. The gel was run in 1X MOPS buffer (10X MOPS buffer: 200 mM NaMOPS, 50 mM Na-acetate, 1 mM EDTA) and incubated for 4 hours. After electrophoresis, the resolved RNA from the gel was transferred to a nylon membrane using 10X SSC as described by Sambrook (1989). Conditions for preparations of probe, carrying out of prehybridization, hybridization, washing, cross-linking and exposing X-ray films were same as described in section 2.2.7.

3.2.5. Salt stress analysis

Transgenic cotton containing AVP1 and AtNHX1 genes were analysed for salt stress. T_2 generation of transgenic lines and non-transformed control plants were raised in hydroponic and earthen pots containing soil (16 Kg) under a photoperiod of 16/8 h (light/dark, and 65 % relative humidity. The pots were watered (2L) every 2 days with 1/8 Hoagland nutrient solution (KNO_3 33.3 g l^{-1}, $Ca(NO_3).4H_2O$ 9.4 g l^{-1}, $MgSO_4.7H_2O$ 2.2 g l^{-1}, $NH4NO_3$ 2.4 g l^{-1}, Fe.EDTA 0.6 g l^{-1}) for 4 weeks. At the 8^{th} week old stage, then the nutrient solution was supplemented with NaCl. Concentrations of NaCl were incrementally applied after each successive watering from 100 mM through 150 and 200

mM. Leaves from transgenic plants and non-transformed controls were taken for estimation of Na^+ and K^+ content.

3.2.6. Measurement of biomass and Na^+, K^+ contents

Shoot dry weight of the transgenic and non-transformed control plants from hydroponic experiment was determined after drying samples at 70 °C±2 for 48 h. Na^+ and K^+ were measured according to the method described by Flowers and Hajibagheri (2001) with slight modifications. Leaves were rinsed with deionized water for 10 s, and then washed with cold $LiNO_3$ isotonic solution. The leaves from the transgenic and non-transformed control plants were dried at 80°C for 48 h, the dry weight was measured. Na^+ and K^+ were extracted from dried plant tissue with 100 mM acetic acid at 90°C for at least 2 h, and then the cation contents were determined using a Flame Photometer (Janway Co., USA).

3.2.7. Measurement of free amino acids

Transgenic cotton plants were analysed for total free amino acids as described by Ashraf *et al.* (2005). A non-transformed control Coker-312 plant was also included in the experiment. One ml of each sample extract (as extracted for the soluble proteins) was treated with 1 ml of 10 % (w/v) pyridine and 1 ml of 2 % (w/v) ninhydrin solution. The optical densities of these colored solutions were then read at 570 nm on a spectrophotometer.

3.2.8. Determination of leaf proline and total soluble sugar contents

Mature leaves were taken from transgenic and non-transformed control cotton plants separately and processed for the measurement of free proline and total soluble sugar accumulation. About 0.5 g of frozen plant material was homogenized in 1 ml of sterilized ion-free water; the remnant was removed by centrifugation at 3,800 × *g*. Proline was measured as described by Bates *et al*. (1973) with minor modifications. One hundred micro liters of the extract was allowed to react with 1 ml glacial acetic acid and 1 ml acid ninhydrin reagent (1.25 g ninhydrin warmed and dissolved in 30 ml glacial acetic acid, after cooling at room temperature, and 20 ml of 6M phosphoric acid was added) for 1 hour at 100 °C, in 2 ml test tube. Reaction was then terminated by placing the tubes in an ice bath. The reaction mixture was mixed with 4 ml toluene. The chromophore-

containing toluene was separated from the aqueous phase and warmed to room temperature, and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve.

Total soluble sugar content was determined using the method described by Dubois *et al*. (1956) with few modifications. Twenty five micro liters of the leaf cellular extract was pipetted into a colorimetric tube with 50 µl of 80 % (w/v) phenol. Five milli liters of concentrated sulfuric acid was then added with rapid mixing. The tubes were allowed to stand still for 10 min, and then shaken and placed in a water bath at 28 °C for 15 minutes. The absorbance of the yellow-orange solution was measured at 490 nm for total soluble sugars. The amount of sugar was determined by reference to a standard curve developed using AR grade glucose.

3.2.9. Measurement of nitrate reductase activity

Nitrate reductase activity was determined by following the method of Giordano *et al*. (2000). Fresh leaf materials of about 400 mg was ground in liquid nitrogen and dissolved in 0.8 ml NR extraction buffer [50 mM HEPES (pH7.8), 10 mM $MgSO_4$, 1 mM EDTA, 5 mM DTT, 0.1 % Triton X100, 0.4 µg ml^{-1} leupepsin 1 µg ml^{-1} pepstatin, and 1 % (w/v) PVP (MW 40,000]. The extracts were vortexed for 5 min and centrifuged at 13,000×*g* at 4 ºC for 10 minutes. The supernatant was desalted using a Sephadex G25 column pre-equilibrated with NR extraction buffer, and centrifuged at 2000 × *g* for 3 minutes. Fifty micro liters of extract was mixed with 940 µl reaction buffer containing 30 mM KPO_3 buffer (pH 7.5), KNO_3 10 mM, and EDTA 0.1mM) and 10 µl 20 mM NADH; incubated at 30 ºC in a water bath for 10 minutes. Then 50 µl 1 M zinc acetate was added to stop the reaction, and the reaction mixture was centrifuged at 12,000 × *g* for 2 minutes. Supernatant (0.9 ml) was mixed with 0.5 ml color reagent (1 % (w/v) sulfanilamide in 3N HCl) and 0.5 ml color reagent-2 (0.02 % N-1-Naphtylethylendiamine dihydrochloride). Incubated at room temperature for 15 minutes to develop the color and absorbance at 540 nm was obtained. Protein content in the extract was determined using the method of Bradford (1976) with BSA as standard. NR activity was determined from a standard curve of NO_2 and expressed in nM min^{-1} mg^{-1} weight. For each transgenic line, four to five plants were analyzed. The experiments were repeated three times and expressed values are the means of these values.

3.2.10. Drought stress treatment

For drought stress treatments, plants from AVP1 transgenic cotton lines and the non-transformed control were transplanted to earthen pots containing soil (16 kg) under a photoperiod of 16/8 h and 65 % relative humidity. The pots were irrigated on alternate day with 1/8 Hoagland nutrient solution to maintain the soil field capacity for 6 weeks, then the water was withheld until all plants showed severe drought stress symptoms (i.e., visible wilting). During drought-stress treatments, the physiological parameters of transgenic and non-transformed plants were measured on second, 4th days intervals up to extreme stress (10 days).

3.2.11. Quantification of solutes

Quantification of solutes in leaf tissue was detected as described by Gaxiola *et al*. (2001) Fully expanded leaves were excised from the transgenic and control cotton plants of each treatment and rinsed in deionized water and blotted immediately on sterile filter paper. The leaves were then frozen in liquid nitrogen and thawed to extrude sap by a syringe. The resulting sap was determined with a cryoscopic osmometer (OSMOMAT-030, GONOTEC GmbH, CLG Slamed, Germany). The readings (mmol kg^{-1}) were used to calculate the solute potential (Cs) in MPa (mega Pascals) with the formula Cs = moles of solute - RK, where R = 0.008314 and K = 295.

3.2.12. Measurement of the relative water content

During drought stress, the relative water contents (RWC) of transgenic and non-transformed control cotton plants were measured according to the method described by Gaxiola *et al*. (2001). Leaves from cotton plants were excised, and immediately their fresh weights were measured. After floating them in deionized water at 4-8 °C overnight, their rehydrated weight was determined. Finally, they were dried in an oven at 70 °C for 48 h and weighed. The RWC was calculated as RWC = [(fresh weight-dry weight)/ (rehydrated weight-dry weight)] × 100.

3.2.13. Measurement of the net photosynthetic rate (*Pn*)

The Pn was measured using an Infrared Gas Analyzer (LI-6400, LI-COR Biosciences, USA) as described by Qiu *et.al.* (2002).

3.2.14. Measurements of plant agronomic parameters

The plant agronomic parameters were recorded at maturity. Cotton fiber yield (with seeds) was estimated when all bolls had opened and matured.

3.2.15. Fiber quality analysis

Harvested fibers were ginned using a small 10-saw experimental gin. Three lint samples from each transgenic line and non-transformed control cotton plants were analyzed on Uster model 900-A High Volume Instrument (HVI). The fiber quality parameters, i.e., micronaire, fiber length, uniformity, and strength were determined for transgenic and control plants.

3.2.16. Scanning electron microscopy of cotton fibers

Cotton fiber was soaked first in 70 % (v/v) ethanol for 5 minutes and then washed thoroughly with deionized water to get rid of contaminants. Samples were dried by placing them in between filter paper in an oven set at 60 °C. After drying, 1-2 cotton fibers were isolated with the help of tweezers under optical stereo microscope. Fibers were pasted on the copper stub with the help of double end sticky carbon tape. Then using vacuum evaporating unit (JEE 420), carbon coating was done by graphite plasma. A carbon plasma was generated under vacuum 2×10^{-4} bar and sample was coated for 60 sec. sample was analyzed in SEM mode at accelerated voltage.

3.2.17. Statistical analyses

ANOVA (Analysis of variance) with a complete randomized block design and Chi square (X^2) tests were used to determine the significance effects regarding the influence of transgenes *AVP1* and *AtNHX1* in all studied traits. The Type I error for testing the hypothesis of no significant difference between transgenic lines and control plants was set at 5 % level of probability All experiments were conducted in triplicates and statistical analysis was performed by using Microsoft® Office Excel 2003.

3.3. Results

3.3.1. PCR amplification of *AtNHX1*gene

PCR amplification of *AtNHX1* gene, separation on 1 % agarose gel was carried out (section 3.2.1.1). Figure 3.2 shows the PCR amplified product of exact size of *AtNHX1* gene (1.617 kbp).

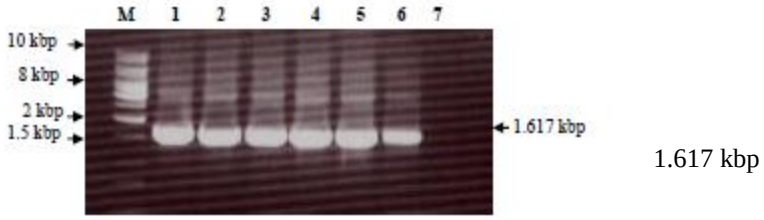


Figure 3.2 PCR amplified *AtNHX1* gene. Lane M, 1 kbp DNA marker; lanes 1-6 amplified fragment of *AtNHX1* gene (1.617 kbp); lane 7, negative control.

3.3.2. Cloning of *AtNHX1* in plant expression vector pJIT60 to yield pSA1

Cloning of *AtNHX1* gene was confirmed by cutting plasmid pSA1 with *Eco*RI/*Hin*dIII restriction enzymes, separated on 1 % agarose gel as described in section 3.2.1.6. Figure 3.3 shows the release of DNA fragment of exact size of *AtNHX1* gene (1.617 kbp).

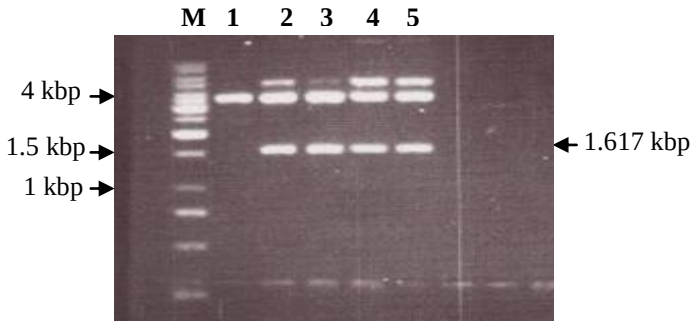


Figure 3.3 Verification of the *AtNHX1* gene in plant expression vector pSA1. Lane M, 1 kbp DNA marker, lane 1 –ve plasmid control; lanes 2-6 *Eco*R1/*Hin*dIII restriction released fragment of 1.617 kbp of *AtNHX1* gene from pSA1

3.3.3. Cloning of plant expression cassette in binary vector pGA482 to yield pSA2

Cloning of *AtNHX1* expression cassette was carried out (section 3.2.1.7).Verification of cloned plant expression cassette in pSA2 was confirmed (section 3.2.1.8). Figure 3.4 A, B shows the release of DNA fragment of exact size of expression cassette (2.6 kbp) and *AtNHX1* gene (1.617 kbp).

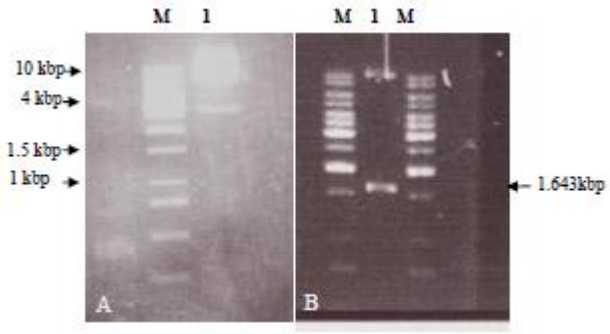


Figure 3.4 Verification of *AtNHX1* cloning in pSA2. A) Lane M 1kbp DNA marker, lane 1, *Kpn*1/*Eco*RV restricted recombinant pSA2 showing release of 2x35S:1.643kbp *AtNHX1* expression cassette (2.6 kbp). B) Lane M 1kbp DNA marker, lane 1 *Eco*R1/*Hin*dIII restricted pSA2 showing the release of 1.617 kbp *AtNHX1* gene.

3.3.4. Transformation and verification of pRG0029 and pSA2 into *Agrobacterium tumefaciens* strain LBA 4404

The pRG229 (*AVP*1*)* and pSA2 (*AtNHX*1) were transformed into *A. tumefaciens* strain LBA 4404 and verified by PCR (section 3.2.1.8). Figure 3.5 shows the amplified products of *AVP1* and *AtNHX1* genes from *Agrobacterium.*

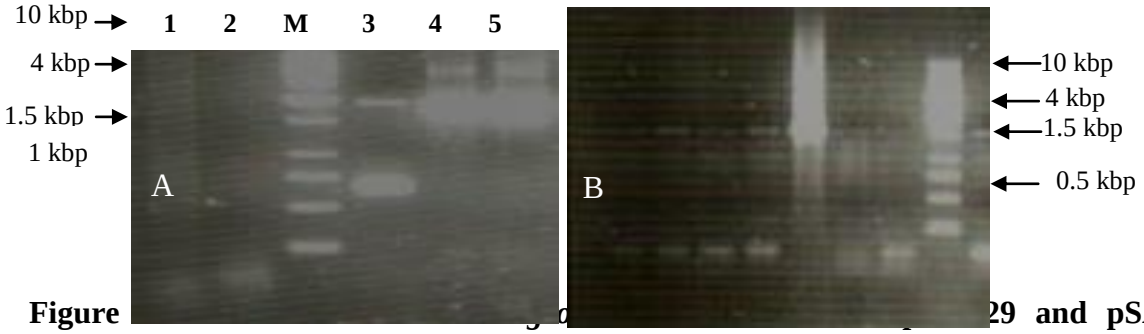


Figure [illegible] 29 and pSA2 containing *AVP1*/*AtNHX1*genes. A) Lanes 1, 2 water and culture control, M 1Kbp DNA marker, lane 3 +ve positive plasmid control, lanes 4,5 *A. AVP1* amplified from *Agrobacterium* culture **B**) M 1Kbp DNA marker, lanes 1, 2 water and culture –ve control,

lane 3 +ve positive plasmid control, lanes 4, 5, 6 and 7 *AtNHX1* from *Agrobacterium* culture

3.3.5. Transformation of cotton Coker-312 through *Agrobacterium*

A. tumefaciens harboring plasmid pRG229 and pSA2 were grown separately (section 3.2.2). Cotton Coker-312 transformation was carried as described (section 3.2.3; Figure 3.2). Overall cotton transformation efficiency with *AVP1 and AtNX1* ranged between 18-28 % (Table 3.2, 3.3). Out of 19 and 15 events of *APV1* and *AtNHX1* transgenic cotton respectively, four events were randomly selected for further studies. Figure 3.6 shows the steps of cotton Coker-312 transformation and regeneration.

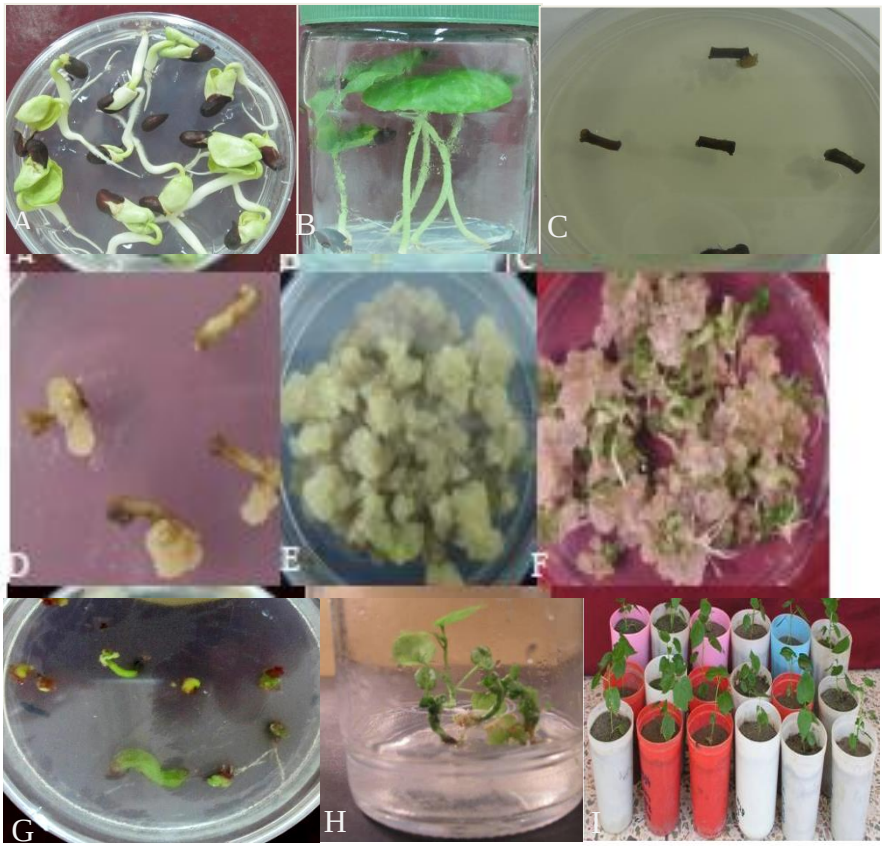


Figure 3.6 Steps in the development of transgenic cotton.
a) *In-vitro* seed germination, b) cotton seedlings (7-9 days), c) negative control explants on selection medium without agro-inoculation, d) putatively transformed hypocotyls explants on selection medium and induction of calli, e) multiplication of calli, f) embryogenic calli showing embryos and plantlet development, g) recovery of embryos,

h) embryo germination for root/shoot development, I) regenerated cotton plantlets

Sr. No	No. of explants placed on callus induction medium (X)	No. of explants producing callus (Y)	Callusing efficiency (%)=Y/X×100	Embryogenic callus (Z)	Embryogenic efficiency (%)=Z/Yx100	Number of events/plant regeneration
1	23	7	32	2	25	2/4
2	35	14	40	4	23	4/8
3	25	12	48	3	25	3/8
4	40	16	40	3	15	3/6
Total	123	49	39	12	24	12/26

transferred to glass pots.

Table 3.2 Transformation of *AVP1* into cotton Coker-312.

Table 3.3: Transformation of *AtNHX1* into cotton Coker-312

Sr. No	Explants placed on callus induction medium (X)	Explants producing callus (Y)	Callusing efficiency (%)=Y/X×100	Embryogenic callus (Z)	Embryogenic efficiency (%)=Z/yx100	Number of events/plant regeneration
1	40	10	25	4	10	3/8
2	45	20	42	9	20	5/12

3	**45**	**22**	**48**	**11**	**25**	**7/20**
Total	**135**	**52**	**38**	**23**	**28**	**15/40**

3.3.6. Molecular analyses of transgenic plants

3.3.6.1. Polymerase Chain Reaction (PCR) analyses

Genomic DNA was isolated from the putative transgenic plants (T_0). PCR analysis was performed for the detection *nptII* gene, CaMV35S promoter, *AVP1* and *AtNHX1* transgenes (Section 3.2.4). The Figure 3.7 (A-C) indicates the presence CaMV35S promoter (750bp), *nptII* (750bp), *AVP1* (2.3 kbp) while Figure 3.8 (A and B) shows the amplified fragment of *AtNHX1* (1.643 kbp) and *nptII* (750bp) transgenes in representative T_0 putative transgenic cotton lines as well as in the positive control (plasmid). No band was amplified from the DNA of the negative control (untransformed plant).

3.3.6.2. Southern Analyses

Southern hybridization showed the varied (1-5) transgene copy number of *AVP1* and *AtNHX1* in the genome of selected transgenic cotton lines. Figures 3.7 D and 3.8 C show the presence of hybridized signals which indicated the integration of *AVP*1 and *AtNHX1* genes respectively into the cotton genome whereas no signals were observed in genomic DNA of non-transgenic control plants. Lines APP-4 and APP-8 showed the insertion of *AVP1* gene at two and three loci while in lines APP2 and APP- 6 it was integrated at one locus (Figure 3.7 D).

In transgenic AtNHX1 cotton, integrated copies ranged from 1-5 (Figure 3.8 C). Transgenic lines AX1, AX2, AX3, AX4 and AX5 contains three, two, 7, two and five copies. (Figure 3.9, lanes 1, 3, 5, 6 and 7). Non-transgenic cotton plants did not show hybridization signals (Figure 3.8 C, lanes -ve, 2, 4).

3.3.6.3. Northern Analyses

Northern blots were used to study the transgene expression in selected T2 AVP1 transgenic lines at transcriptional level under stress condition. The hybridization signals

were observed (Figure 3.9 B) in transgenic lines indicating the variable expression of transgenes. No expression was observed in non-transgenic control plant.

3.3.6.4. RT-PCR analyses

RT-PCR was used to detect the expression of AtNHX1 transgene in cotton. Expression was observed in the transgenic lines while no expression was found in non-transgenic control plants (Figure 3.10 C)

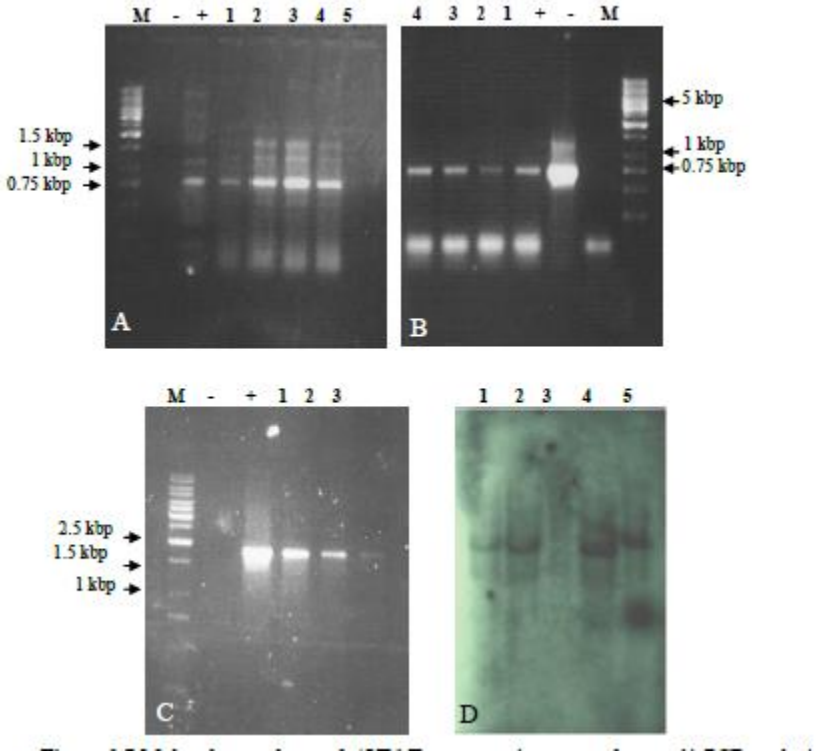


Figure 3.7 Molecular analyses of *AVP1* T_0 transgenic cotton plants. A) PCR analysis for CaMV 35S promoter. Lane M 1 kbp DNA marker, lane – negative control, + control plasmid, lanes 1-5 transgenic lines. B) PCR analysis for *nptII* gene. Lane M 1kbp DNA marker, lane – negative control, lane + control plasmid, lanes 1-4 transgenic lines C) PCR analysis for *AVP1*gene . Lane M 1kbp DNA marker, lane– negative control, + control plasmid, lanes 1-4 transgenic lines D) Southern analysis of *AVP1* transgenic T_0 cotton plants; lane 3 control plant , lanes 1,2,4,5 transgenic plants of APP2, APP-3, APP4- and APP-5 lines.

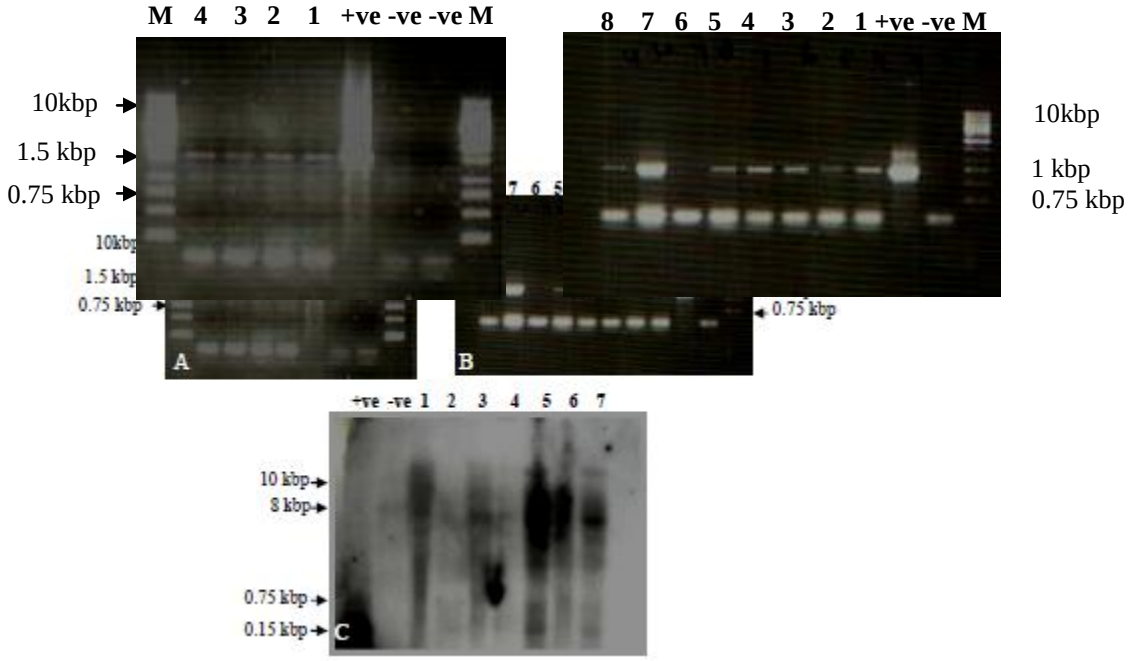


Figure 3.8 Molecular analyses of T_0 *AtNHX1* transgenic cotton plants. A) PCR analysis for *nptII* gene, lane M 1Kbp DNA marker, lane –ve, negative control, lane +ve, positive plasmid control, lanes 1-8 transgenic lines. B) PCR analysis for *AtNHX1* gene, lane M 1Kbp DNA marker, lane –ve negative control (no DNA), -ve negative control (control plant DNA), lane +ve positive plasmid control, lanes 1-4 transgenic lines. C) Southern analysis of putative transgenic *AtNHX1* cotton T_0 plants. Lane +ve, positive control, lane–ve, negative control (control plant DNA), lanes 1, 3-7 transgenic lines.

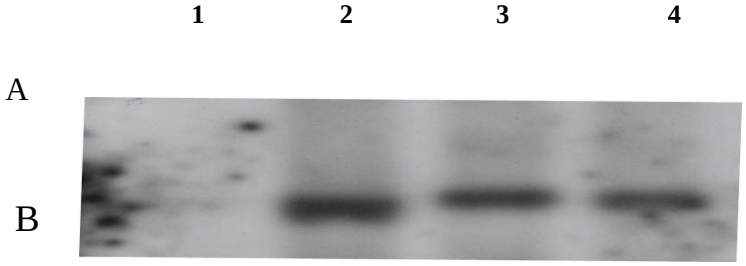


Figure 3.9 Northern analysis of *AVP1* T_2 transgenic cotton plants under stress conditions. A) Total RNA isolation, lanes 1, non-transformed control plant , lanes 2-4 transgenic lines. B) Hybridization of RNA blot to *AVP1* specific probe. Lane 1, non-transformed control plant, lanes 2-4, transgenic lines

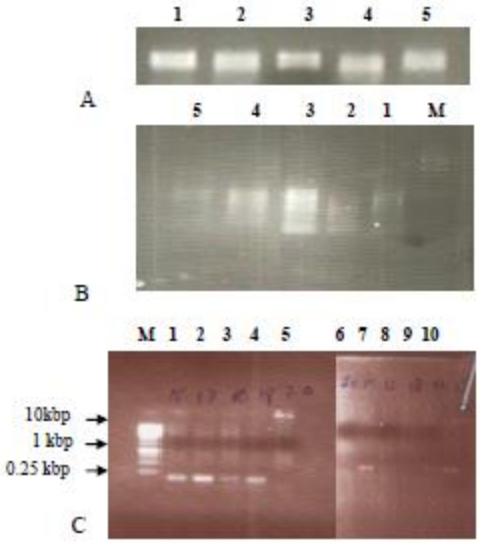


Figure 3.10 Expression of *AtNHX1* in T_2 transgenic cotton plants. A) Total RNA isolation from transgenic and control plants. B) cDNA synthesis from total RNA. C) PCR analysis of cDNA, lane M 1Kbp DNA marker, lane 1 endogenous actin control, lane 2 +ve positive control, lane 5 –ve negative control, lanes 3, 4, 6, 8, 9, 10 transgenic lines

3.3.7. Salt tolerance analyses

Transgenic cotton lines (T_2) overexpressing *AVP1* and *AtNHX1* were exposed to salt stress (section 3.2.5-9). Transgenic cotton lines grown either hydroponically or in pots having soil showed significantly high accumulation of Na^+, K^+ ions, greater root biomass and length as compared to non-transformed control (Figure 3.11---3.17). Transgenic cotton lines SV2 and APP-4 (AVP1) cotton showed high accumulation of Na^+, K^+ and followed by SV5 and APP-3 whereas least accumulation was observed in non-transformed control plants. Among the transgenic lines, SV2 and APP-4 produced maximum root biomass while SV5 and APP-2 were able to produce intermediate root system (Table 3.4).

Figure 3.11 Hydroponic set-up for the salt tolerance study of *AVP1* transgenic plants. Four litres Hoagland solution was taken in each pot and uniform air supply was provided to all pots for 4 hours daily, pH was maintained at 5-8 and weekly fresh Hoagland's solution was replaced. After six weeks of NaCl (200mM) treatment, leaf samples were harvested for Na^+/K^+ determination.

Plants of AVP1 transgenic lines were able to grow at 200 mM NaCl and set healthy root system in saline Hoagland solutions whereas roots of non-transformed control plants were found retarded, weak and poor of fade color (Figure 3.12 a, b). Under hydroponic conditions, maximum Na^+ accumulation was observed in SV2 and AAP-4. SV5 showed 0.9 % (w/w) Na^+ DW followed by APP-2 which was able to accumulate Na^+ to a level 1.0 % DW. Other transgenic lines showed intermediate Na^+/K^+ accumulation. Non-transformed control showed 0.8 % of DW of Na^+ ions which is less than exhibited by other transgenic genotypes (Figure 3.13 a, b).

Fresh root biomass produced by transgenic *AVP1* cotton lines was more than non-transformed control plants in saline hydroponic media. Transgenic *AVP1* plants grown under hydroponic conditions displayed remarkably higher root biomass and root length. Transgenic *AVP1* cotton lines SV2 and APP-4 produced 53 and 55 cm length respectively while other transgenic cotton lines i.e. SV5 and APP-3 set healthy root

length. Transgenic lines produced roots of greater length and weight than non-transformed control plants (Table 3.4).

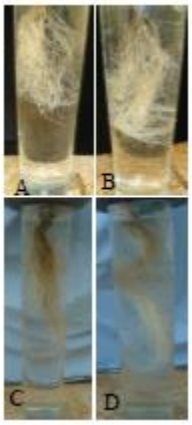


Figure 3.12 Root growth of hydroponically grown *AVP1* T_2 cotton plants (10 week) Rooting system of (a) non-transformed control plant (b) Transgenic plants without salt treatment. After salt treatment for 4 weeks, root growth of (c) non-transformed control/standard and (d) transgenic plants.

Table 3.4 Root growth and biomass of *AVP1* T_2 transgenic cotton under salt stress.

Plant line #	Root fresh weight (g)	Root dry weight (g)	Root length (cm)
(ctr)	5.6 ±2.54c	0.7 ±1.52d	35.75 ±2.52d
SV2	15.1 ±0.74a	1.5 ±1.32a	55.25±0.42a
SV5	11.43 0.52b	1.11 ±0.62b	48.5 ±0.60b
APP-3	5.65 ±0.52c	0.69 ±.56c	40.25 ±2.72c
APP-4	13.95 ±1.62a	1.21 ±0.72a	53.25 ±1.08a

Root biomass was harvested from transgenic and non-transformed control plants after salt treatment under hydroponic conditions. Five plants were taken from each transgenic event with same number of non-transformed control plants.. Values are the means of three replications ± standard errors and variants possessing same letters in one column are not statistically significant at P=0.05 according to Fisher's protected LSD.

Fresh root biomass was also significantly higher in transgenic *AVP1* cotton lines i.e. SV2 and APP-4 producing 15.1 and 13.95 g followed by other transgenic lines SV5 and APP-3 producing 11.3 and 5.75 g of fresh roots. Least root fresh biomass was produced by non-transformed control plants. Transgenic *AVP1* cotton lines also performed best in root dry biomass by producing 1.11 and 1.21 g of dry roots followed by other two transgenic linesSV5 and APP-3 producing 1.5 and 0.88 g respectively whereas non-transformed counterpart were able to produce only 0.7 g (Table 3.4).

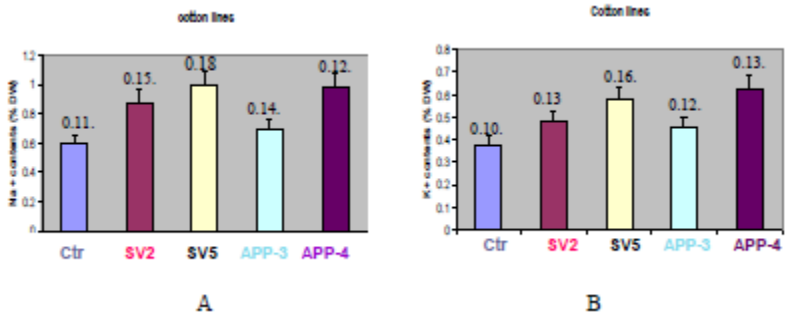
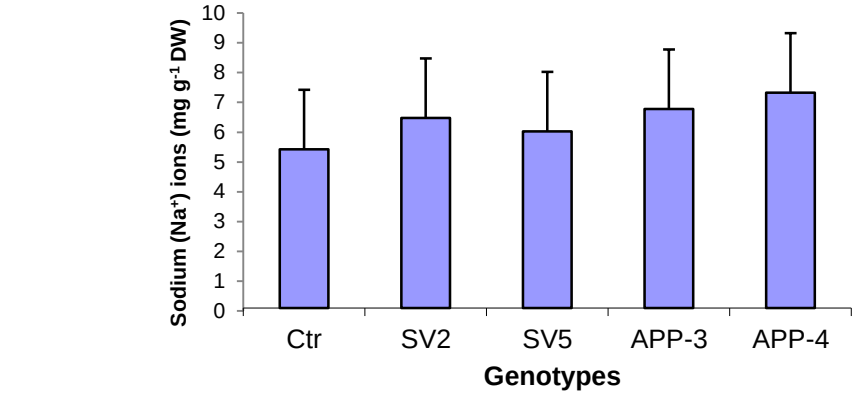


Figure 3.13 Determination of A) Na^+ and B) K^+ contents from leaves of hydroponically grown non-transformed control (ctr) and *AVP1* T_2 transgenic cotton plants. Values are means of 5 values. Non-transformed control (ctr) and four transgenic plants line (*AVP1*) (five plants from each) were grown hydroponically for 9 week in a 16-h light 8-h dark photoperiod at 32-35°C with 65 % RH. Values on the bar peak are standard errors (±).

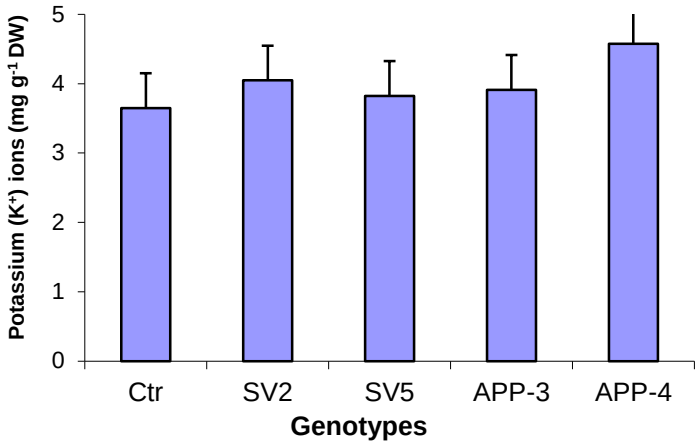
Figure 3.14 Salt treatment of *AVP1* T2 transgenic cotton: A) Transgenic cotton, B) non-transformed control plants, phenotypic condition of transgenic plant and non-transformed control plant for 20 days.

A B

Figure 3.15 Phenotypic conditions of non-transformed control (A) and *AtNHX1* T_2 transgenic cotton (B) after salt treatment for 20 days.



A

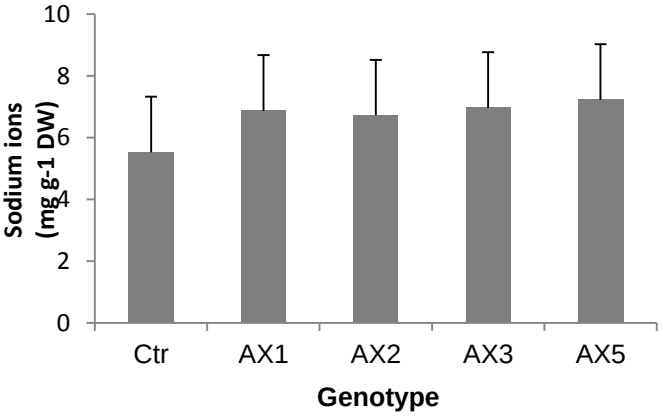


B

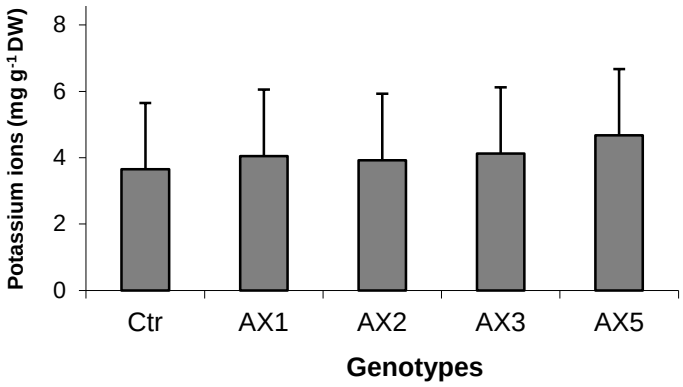
Figure 3.16 Sodium (A) and potassium (B) contents after salt stress in T_2 transgenic *AVP1* cotton plants. Values are the means (n=5. ctr- non-transformed control and SV2, SV5, APP-3, APP-4 *AVP1* T_2 transgenic cotton lines

AVP1 T_2 transgenic cotton plants showed high salt tolerance than non-transformed control plants under soil condition. After three week of slat treatment, prominent symptoms exhibited by cotton plants were losing of color freshness, leaf yellowing, wilting, growth stunting rapid shedding of old leaves, square shedding and boll abortion (Figure 3.15). Transgenic lines SV2 and APP-4 showed better salt tolerance with less leaf shedding, leaf yellowing and boll abortion (Figure 3.15 a,) than non-transformed control plants (Figure 3.15 b) while SV5 and APP-3 exhibited mild severity in symptoms of salt stress. It was also observed that extension in salt treatment beyond 24 days leads to plant death in non-transformed control plants whereas mortality was delayed in transgenic cotton plant progenies. Transgenic lines SV2 and APP-4 exhibited

high accumulation of Na^+ and K^+ contents in leaves followed by SV5 and APP-3 lines whereas less accumulation was observed in non-transformed control plants after salt stress. . These results indicated that transgenic cotton lines over expressing *AVP1* were more tolerant to salt stresses when compared to their respective non-transformed counterparts (Figures 3.16)



A



B

Figure 3.17 Sodium (A) and potassium (B) contents after salt stress in T_2 transgenic *AtNHX1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and AX1, AX2, AX3 and AX5 T_2 transgenic lines.

AtNHX1 transgenic cotton lines, AX2 and AX3 showed lesser severity of salt stress symptoms. It was also observed that extension in salt treatment beyond three week leads to plant death in non-transformed control plants whereas mortality was delayed in transgenic cotton plants. After third week of salt treatment, plant leaves were harvested and analyzed. It showed higher contents of Na^+ and K^+ than that measured from control

plants. Highest Na^+/K^+ accumulation was observed in AX1 and AX5 followed by AX3 and AX2 transgenic lines. The least Na^+/K^+ accumulation was observed in non-transformed control plants (Figure 3.17 a, b).

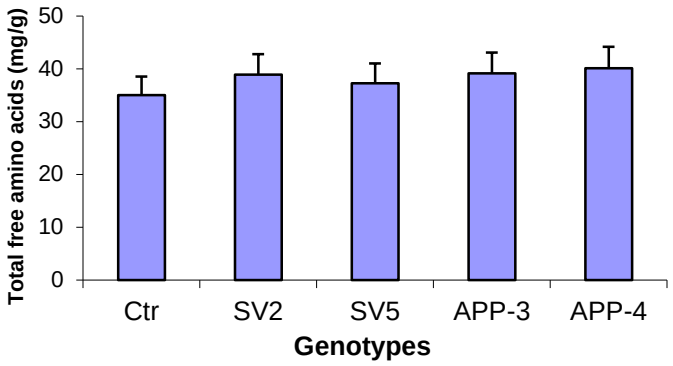


Figure 3.18 Total free amino acids contents after salt stress in T_2 transgenic *AVP1* cotton plants. Values are the means (n=5) . ctr- non-transformed control and SV2, SV5, AAp-3 and APP-4 are transgenic *AVP1* plants from independent events.

Analyses of leaf samples after salt stress showed high accumulation of free amino acid contents by transgenic lines than non-transformed control plants. Maximum accumulation was found in SV2 and APP-4 followed by SV5 and APP-3 transgenic lines than non-transformed control plants. These results indicated that transgenic cotton lines over expressing *AVP1* are tolerant to salt stress as compared to non-transformed plants (Figure 3.18).

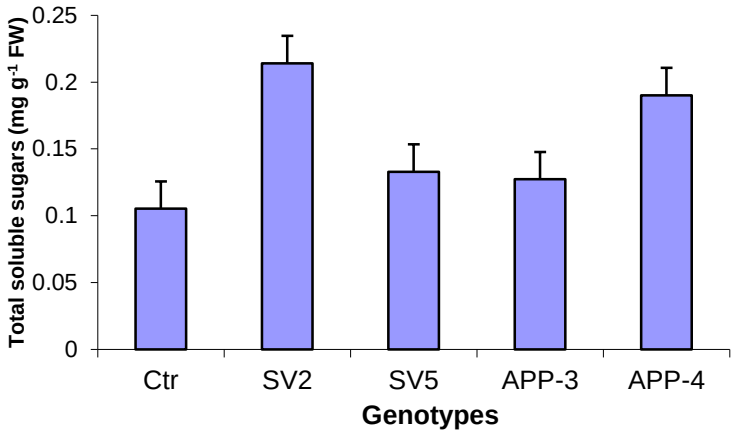


Figure 3.19 Total soluble sugar contents after salt stress in T_2 transgenic *AVP1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and SV2, SV5, APP-3 and APP-4 are transgenic *AVP1* plants

Accumulation of soluble sugars in transgenic lines was higher than non-transformed control plants under salt stress. Maximum accumulation was exhibited by transgenic lines SV2 and APP-4 followed by SV5 and APP-3 than non-transformed control plants. These results indicated that transgenic cotton lines over expressing *AVP1* are highly salt tolerant due to high accumulation of total soluble sugars compared to non-transformed plants (Figure 3.19)

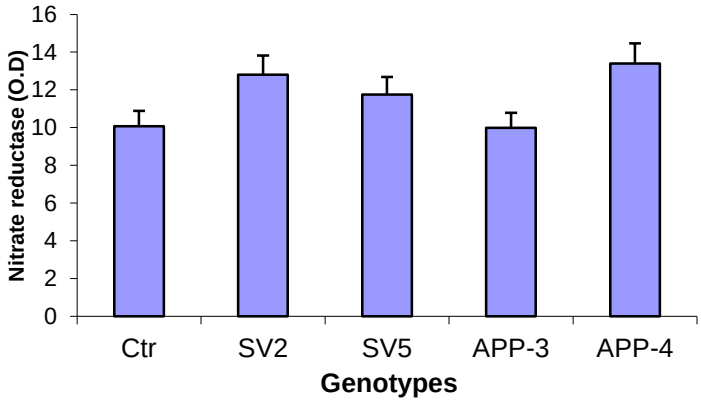


Figure 3.20 Nitrate reductase activity (NRA) after salt stress in T_2 transgenic *AVP1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and SV2, SV5, APP-3 and APP-4 are transgenic *AVP1* plants from independent events.

Nitrate reductase contents in transgenic lines were higher than non-transformed control plants after salt stress. Maximum accumulation was exhibited by transgenic lines SV2 and APP-4 followed by SV5 and APP-3 than non-transformed control plants. These results indicated that transgenic cotton lines containing *AVP1* are highly efficient for NRA along with tolerance to salt stresses when compared to their non-transformed plants (Figure 3.20).

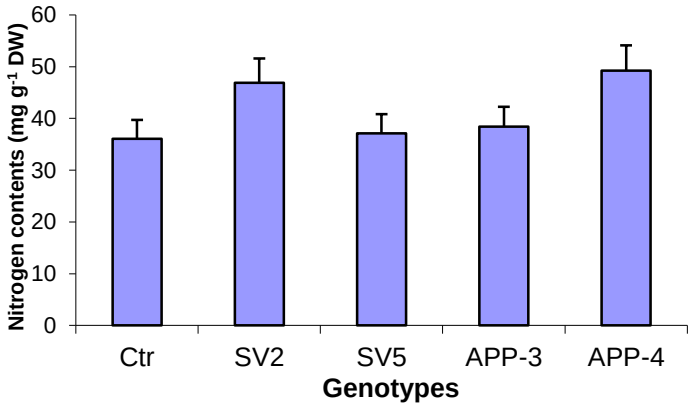


Figure 3.21 Total nitrogen contents after after salt stress in T_2 transgenic *AVP1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and SV2, SV5, AAp-3 and APP-4 are transgenic *AVP1* lines.

Analysis of leaf samples after salt stress for total nitrogen (N) indicated the higher nitrogen contents in *AVP1* transgenic lines than non-transformed control plants. The maximum accumulation was exhibited by transgenic lines SV2 and APP-4 followed by SV5 and APP-3 while minimum was in non-transformed control plants. These results clearly indicated that transgenic cotton lines containing *AVP1* possessed the higher nitrogen contents necessary for the synthesis of protein plant hormones and enzymes which contribute in tolerance to salt stress compared to their non-transformed plants (Figure 3.21).

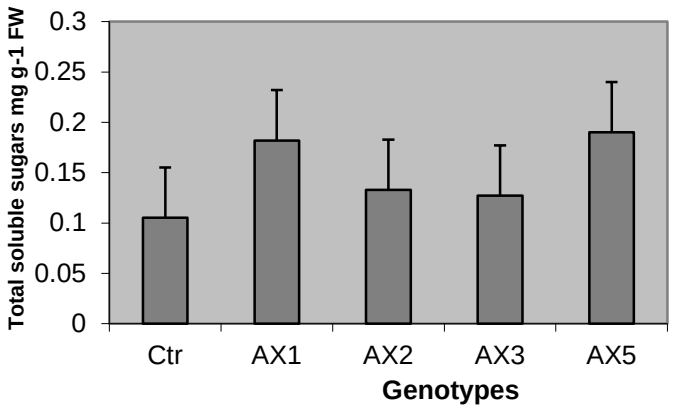


Figure 3.22 Total soluble sugars contents after salt stress in T_2 transgenic *AtNHX1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and AX1, AX2, AX3 and AX5 are transgenic *AtNHX1* plants from independent events.

After salt treatment, analysis of leaf samples showed high accumulation of total soluble sugar contents by transgenic lines than non-transformed control plants. Highest accumulation was found in AX1 and AX5 lines of AtNHX1 transgenic as compared to non-transformed control plants. High contents of total soluble sugars indicated that transgenic cotton lines over-expressing *AtNHX1* are highly tolerant to salt stresses as compared to non-transformed plants (Figure 3.22).

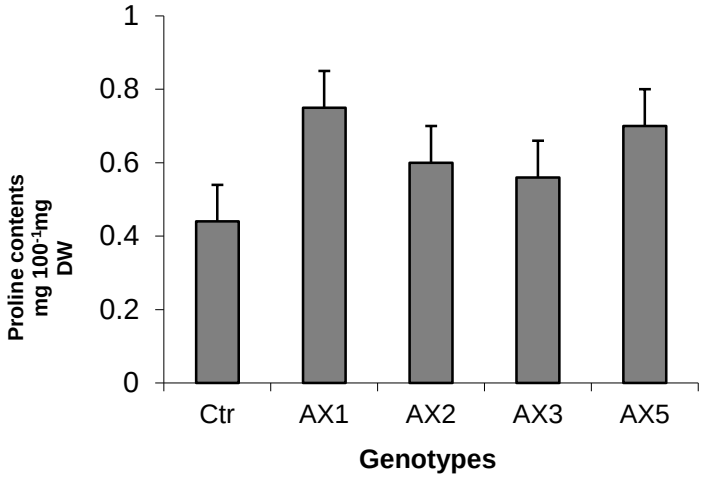


Figure 3.23 Measurement of proline contents after salt stress in T_2 transgenic *AtNHX1* cotton plants. Values are the means (n=5). Ctr, non-transformed control and AX1, AX2, AX3 and AX5 are transgenic *AtNHX1* plants from independent events.

Proline contents measured from salt stressed plants showed that transgenic plants possessed high proline contents than non-transformed control plants. Transgenic AX1 and AX5 lines over-expressing *AtNHX1* contained highest proline contents followed by AX2 and AX3 whereas lower proline contents were found in non-transformed control plants (Figure 3.23).

3.3.8. Drought analysis

Drought analyses showed that transgenic plants possessed higher water contents than control plants under water deficit conditions. Transgenic lines expressing *AVP1* (SV2 and APP-4) maintained highest water contents as compared to non-transformed control plants. Other two transgenic lines SV5 and APP-3 remained intermediate in relative water contents (Figure 3.24 a).

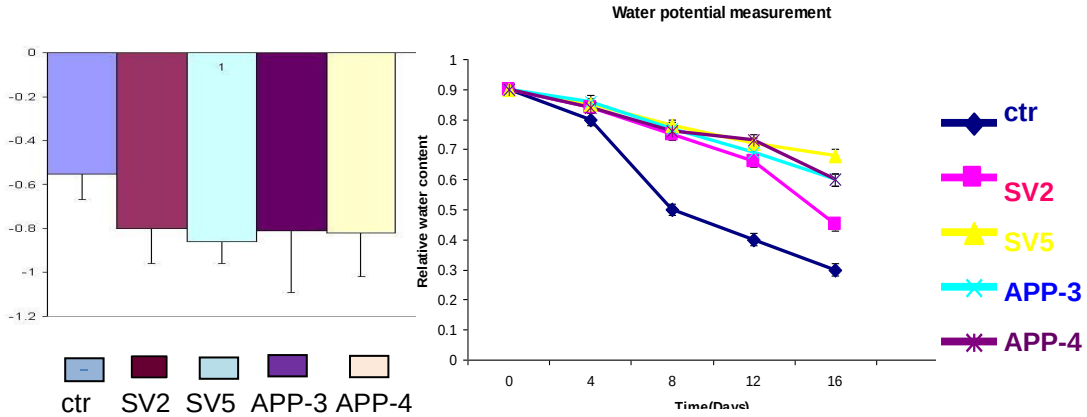


Figure 3.24 Solute potential and water retention in non-transformed and transgenic *AVP1* cotton plants. (A) Solute potential of fully hydrated leaves from fully watered non-transformed control and four independent *AVP1* over-expressing lines SV2, SV5, APP-2 and APP-4. Values are means ± SD (n=10). Light blue bars depict non-transformed control solute potential values, and blue, Red, green, pink and yellow bars represent SV2, SV5, APP-3 and APP-4 transgenic cotton lines (B) Relative water content of non-transformed control and *AVP1* transgenic cotton plants under a water-deficit stress. Ten non-transformed control plants and ten of each *AVP1* transgenic line plants were grown and stressed. The RWC non-transformed control (blue lines circles) and transgenic plants was determined every 4 days. One fully expanded leaf per plant (four plants of each group every 4 days) was removed from the growing upper part. Values are means ± SD (n=10 for each).

Water potential was found lowest in transgenic lines SV5 and APP-4 than other two transgenic lines (SV9 and APP-2) and non-transformed cotton plants. Higher RWC and highly negative potential exhibited by transgenic *AVP1* lines indicates that under drought conditions, transgenic plants significantly had drought tolerance potential than the non-transformed control plants as displayed by growth and physiological performance. Transgenic cotton over-expressing *AVP1* was found to manifest high growth rate, more number of leaves, fruiting bodies/squares and boll setting as compared to control plants (Table 3.5). Drought experiments showed that transgenic *AVP1* plants are highly drought tolerant at seedling and reproductive stages as compared to non-transformed control plants (Figure 3.25, 3.26). Two transgenic lines were significantly fast in growth performance and set large, healthy bolls during pot study for 6 months (Table 3.5).

Table 3.5 Agronomic parameters of transgenic cotton (*AVP1)* after drought stress.

Genotypes	**Plant Height (cm)**	**# of leaves**	**# of squares**	**# of bolls/plant**	**Seed + lint yield (g)**
Ctr	64 ±2.12c	45 ±1.52c	16 ±2.52c	6 ±1.62c	15±2.33d
SV2	74 ±1.92b	48 ±1.52b	22 ±1.52b	10 ±0.32a	23 ±0.62a
SV5	76 ±0.22a	49 ±0.75b	20 ±0.57b	9 ±0.56a	20 ±1.59b
APP-3	74 ±1.62b	50 ±0.82b	18 ±2.5c	8 ±2.53b	18 ±2.54c
APP-4	79 ±0.52a	70 ±0.33a	26 ±2.52a	11 ±0.99a	24±0.52a

ctr- non transformed control and SV2, SV5, AAp-3 and APP-4 are transgenic *AVP1* plants from independent eventsn=10 average plants were grown for six months. Values are the means of three replications ± standard errors and variants possessing same letters in one column are not statistically significant at P=0.05 according to Fisher's protected LSD. Ten T_2 plants of each transgenic event with same number of non-transformed control plants were grown in containment under 16/8 photoperiod 37 ±2°C, RH 65 % (n-20) for six months

Figure 3.25 Drought tolerances in cotton under water deficit conditions after 12 week of sowing (reproductive stage) in glass house. Water was withdrawn for 8-10 days and phenotypic performance was observed in control and transgenic plants. Non-transformed control plants (a, c) and transgenic cotton plant T_2 populations (b, d).

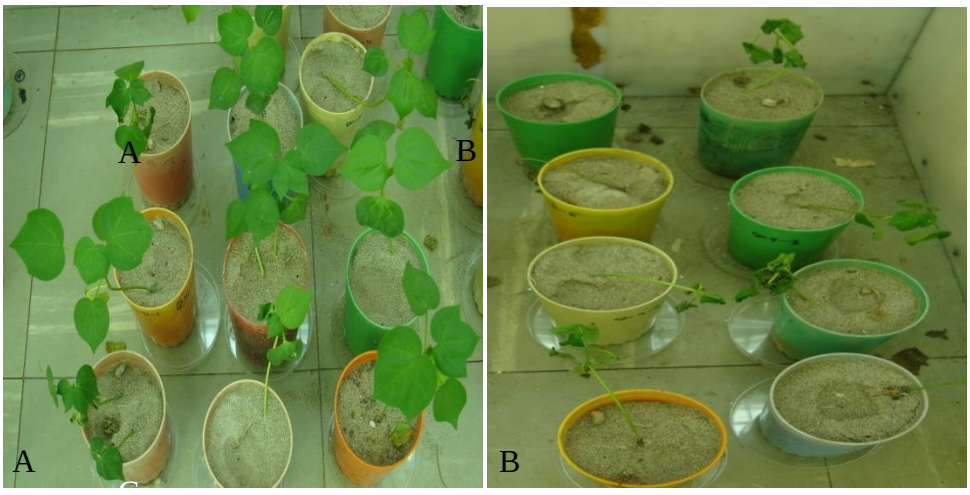


Figure 3.26 Drought tolerance in transgenic *AVP1* cotton at seedling stage. Water was withdrawn for 7 days and phenotypic performance was observed in control and transgenic plants. Phenotypic condition of transgenic *AVP1* cotton lines (A) and non-transformed control plants. Growth conditions were kept as 28 °C, RH 65 % and 16/8 h. photoperiod.

A

1 2 3 4 5

B

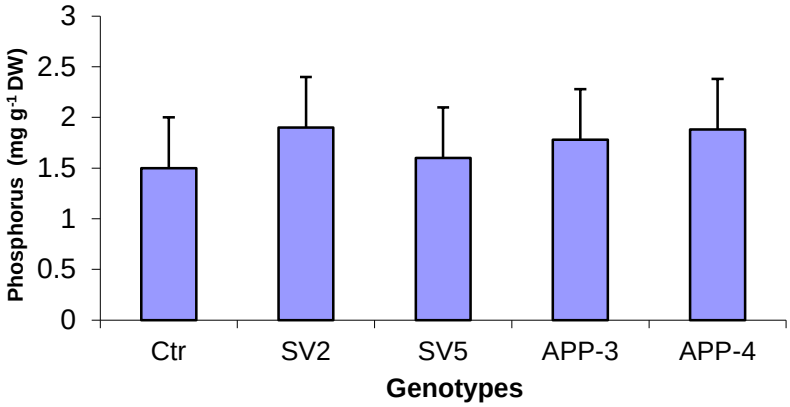
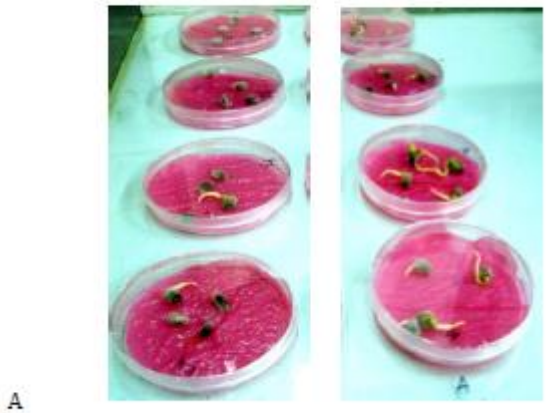
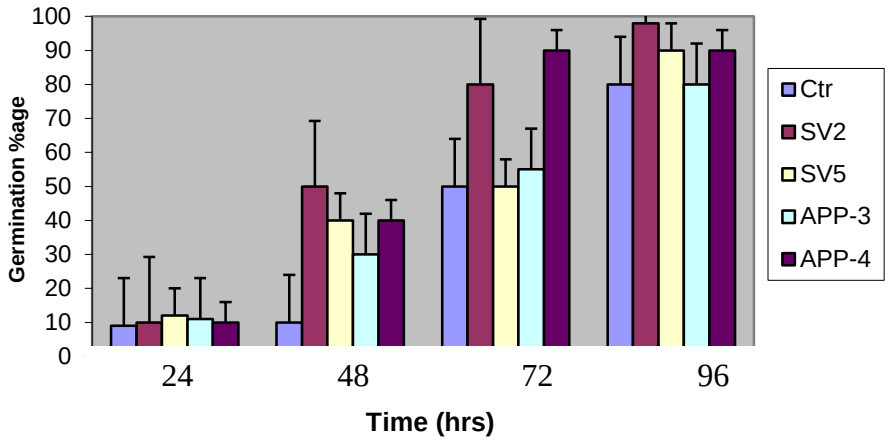


Figure 3.27 A) Transgenic T_2 *AVP1* cotton plants growing under phosphorous limited conditions. A) 1 Non- transformed control and 2-5 plants of transgenic cotton. **B)** Total Phosphorus contents after growing under phosphorus-limited conditions by non-transformed control and transgenic *AVP1* cotton plants. Values are the means (n=5). Ctr non-transformed control; SV2, SV5, AAP-3 and APP-4 are *AVP1* transgenic lines.

Under phosphorus limited conditions, transgenic *AVP1* T_2 population performed significantly better than non-transformed plants (Figure 3.27a). Transgenic *AVP1* cotton showed high growth rate and number of bolls. Plant height remained less than those plants grown under normal fertilized conditions but boll setting by transgenic plants were higher than non-transformed control plants (Figure 3.27 a). Phosphorus contents were higher in tissues of transgenic plants than non-transformed control plants. Transgenic AVP1 cotton lines SV2 and APP-4 showed highest phosphorus accumulation followed by SV5 and APP-3 transgenic lines whereas least phosphorus contents were observed in non-transformed control plants (Figure 3.27 b).

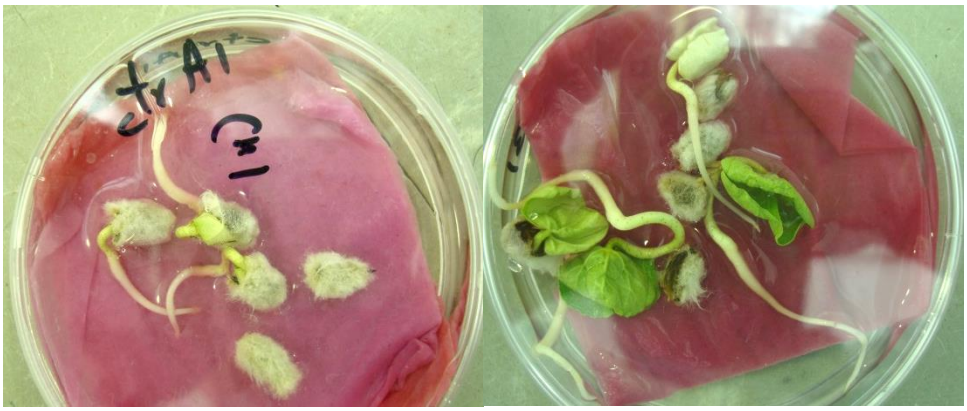


A



B

Figure 3.28 Seed germination of transgenic *AVP1* cotton (T_2). A) Seeds were incubated on moistened tissues in petri-plates. Germination percentage was evaluated in transgenic seeds (right) and non-transformed control plants (left). Four to five seeds were incubated in one petri-plate with four replications. B) Germination percentage over time after 24, 48, 72, 96 hr.



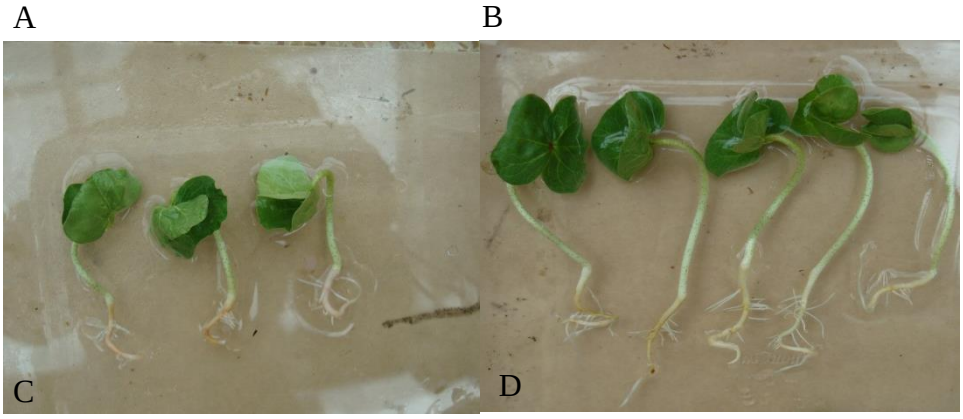


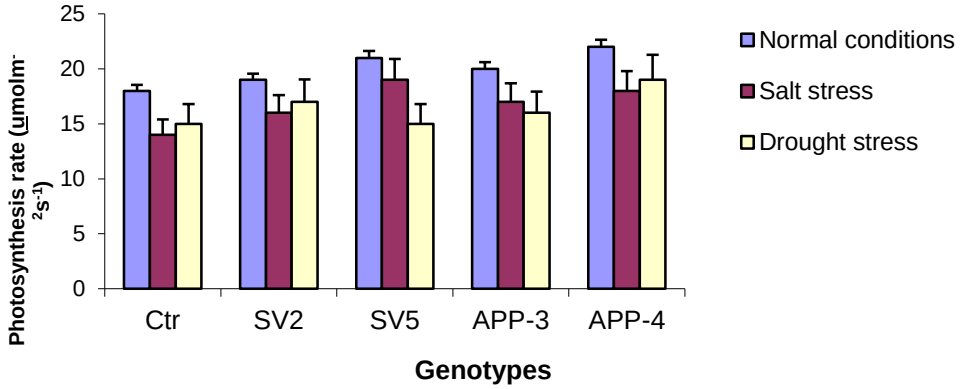
Figure 3.29 Growth and roots of germinated seedlings of *AVP1* transgenic cotton. Germination of T_2 transgenic plant seeds after 48 and 96 hours in control (a, c) and transgenic seeds (b, d).

3.3.9. Germination analyses of *AVP1* transgenic plants

T_2 seeds from transgenic lines exhibited early and high germination rate as compared to non-transformed control plants. After 24 h, 10-12 % germination was observed in transgenic plants of events SV2 and APP-4 against the 9 % germination in non-transformed control plants. Germination rate increased with time and after 48 hours seed germination was recorded as 84 % in transgenic whereas only 40 % seeds were able to germinate in non-transformed control plants. After 96 h, seed germination rate of SV2 and APP-4 transgenic lines were 96-100 % while 80 % germination was found in non-transformed control plants. SV5 and APP-3 lines showed intermediate germination rate (Figure 3.28 a, b). Transgenic seedlings possessed larger cotyledonary leaves and hypocotyls growth was greater among transgenic seeds (Figure 3.29 b, d). Similarly high vigor and rapidly developing lateral roots from main root system was exhibited by transgenic seedlings as compared to that of non-transformed control seedlings (Figure 3.29 a, c and 3.29 b, d).

A

B



C

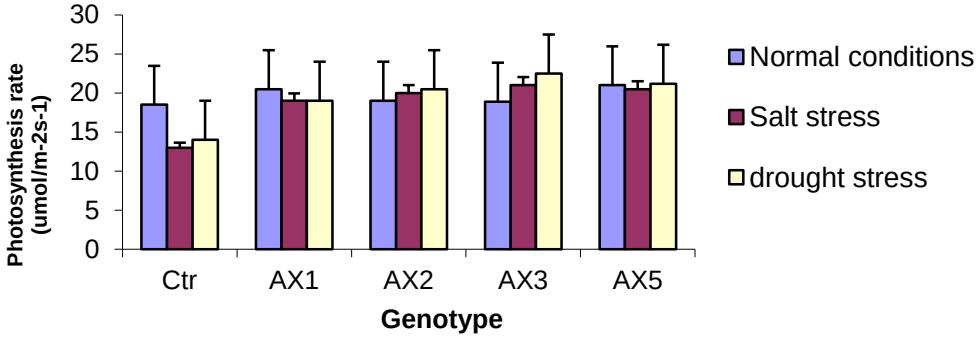


Figure 3.30 Measurement of Photosynthesis. A) IRGA analysis of transgenic cotton plants. B) Transgenic AVP1 plants photosynthesis rate (µmol m^{-2} S^{-1}) and AtNHX1 C) under normal, alt and drought stresses. Values are the means (n=5). ctr, on-transformed control and SV2, SV5, AAp-3 and APP-4 are transgenic *AVP1* and plants from independent events and AX1, AX2, AX, AX5 transgenic lines.

3.3.10. Photosynthetic performance

Maximum photosynthetic performance was observed in SV2 and APP-4 transgenic AVP1 lines with less decline in efficiency during salt and drought stresses when compared normal and stressed condition photosynthesis (Figure 3.30 a & b).

Photosynthesis rate was also higher in AtNHX*1* transgenic T_2 cotton populations, maximum photosynthetic performance was observed in AX1 and AX3 plants with less

decline in efficiency during salt stress when compared to normal and stressed condition photosynthesis. Photosynthesis rate was found to be affected by salt stresses in non-transformed control plants while transgenic *AtNHX1* plants showed lesser decline in photosynthetic rates under salt stress (Figure 3.30 c)

3.3.11. Plant growth and yield

Transgenic *AVP1* T_2 cotton plants showed high growth rate, high leaf bearing and plant height under normal conditions. Transgenic cotton also showed better growth and agronomic performance under normal conditions in soil pots in glasshouse (Table 3.6).

Table 3.6 Plant agronomic parameters under normal growth conditions in non-transformed control and transgenic plants.

Genotypes[n]	Plant height (cm)	# of leaves	# of squares	# of bolls /plant	Seed + lint yield (g)
Ctr	80 ± 2.11c	45 ± 1.99c	26 ± 2.31c	10 ± 1.81c	30 ± 2.71d
SV2	92 ± 1.47b	53 ± 1.44c	33 ± 1.45a	13 ± 1.53b	40 ± 1.27b
SV5	85 ± 1.37c	50 ± 1.57b	30 ± 1.07b	12 ± 1.27b	37 ± 1.11c
APP-3	95 ± 2.2a	60 ± 1.25a	32 ± 1.26b	14 ± 1.27a	42 ± 1.59a
APP-4	94 ± 1.33a	58 ± 2.34b	35 ± 1.83a	15 ± 1.43a	44 ± 1.63a

ctr- non transformed control and SV2, SV5, AAp-3 and APP-4 are transgenic *AVP1* plants from independent events. Values are the means of three replications ± standard errors and variants possessing same letters in one column are not statistically significant at P=0.05 according to Fisher's protected LSD. Twenty T_2 plants of each transgenic event with same number of non -transformed control plants were grown in containment under 16/8 photoperiod 37 ±2°C, RH 65 % (n-20) for six months.

After six months, maximum plant height was observed in SV2 and APP-4 transgenic lines which were 92 and 94 cm respectively whereas SV5 and APP-3 showed intermediate growth performance. Squaring and boll setting was maximum in transgenic lines SV2. Non-transformed control plants showed low growth rate and boll setting

Table 3.7 Plant agronomic parameters of AtNHX1 cotton (pot study) after salt stress. n=10 (plants were grown for six months

ctr- non transformed control and AX1, AX2, AX3 and AX5 are transgenic *AVP1* plants from independent events. Values are the means of three replications ± standard errors and variants possessing same letters in one column are not statistically significant at P=0.05 according to Fisher's protected LSD. Ten T_2 plants of each transgenic event with same number of non -transformed control plants were grown in containment under 16/8 photoperiod 37 ±2°C, RH 65 % (n-10) for six months.

Genotypes[n]	**Plant height (cm)**	**# of leaves**	**# of squares**	**# of bolls/plant**	**Seed + lint yield (g)**
Ctr	64 ±1.12c	45 ±1.12c	16 ±2.52c	7 ±1.62c	17±1.33d
AX1	68 ±0.92b	47 ±0.66b	23 ±1.52b	9 ±1.32a	22 ±0.652a
AX2	67 ±1.22a	49 ±0.65a	24 ±0.57b	7 ±1.56c	20 ±1.569b
AX3	66 ±1.00b	46 ±0.62b	20 ±2.5c	8 ±1.53b	19 ±2.50c
AX5	70 ±0.72a	49±053a	25 ±2.52a	10 ±1.99a	23±1.52a

(Figure 3.32) than their transgenic counterparts i.e. SV2 and APP-4 bearing 14 and 15 bolls respectively while SV5 and APP-3 showed significant squaring and boll setting whereas non-transformed control showed less squaring and boll setting giving fiber yield i.e. 30 than the transgenic plant populations. Results in table 3.6 showed that transgenic lines SV2 (40g) and APP-4 (44g) performed very well by giving high fiber yield (Table 3.6).

Similarly, AtNHX1 transgenic cotton plants showed better yield than non-transformed control plants showed low growth rate and boll setting (Figure 3.32).Genotypes AX1 and AX5 performed significantly high by setting more number of bolls and plant yield (Table 3.7).

Figure 3.32 Growth under normal condition. A) left row *AVP1* transgenic cotton T_2 plants, right row non-transformed control plants. B) an *AVP1* transgenic plants at the reproductive stage (left) and a non-transformed control plant (left row).

3.12. Scanning electron microscopic analysis of fibers

Fibers analysis was carried out by scanning electron microscopy. Fibers from non-transformed plant were found shriveled, weak and uneven twisting (Figure 3.33 a-c) Fibers from transgenic plants were found healthy, strong and uniformly twisted (Figure 3.33 d-h) when examined under different resolutions. Under closer view, cotton fibers from transgenic plants were found to be well developed (Figure 3.34 d-f) as compared to fibers obtained from non-transformed control plants (Figure 3.34 a-c).

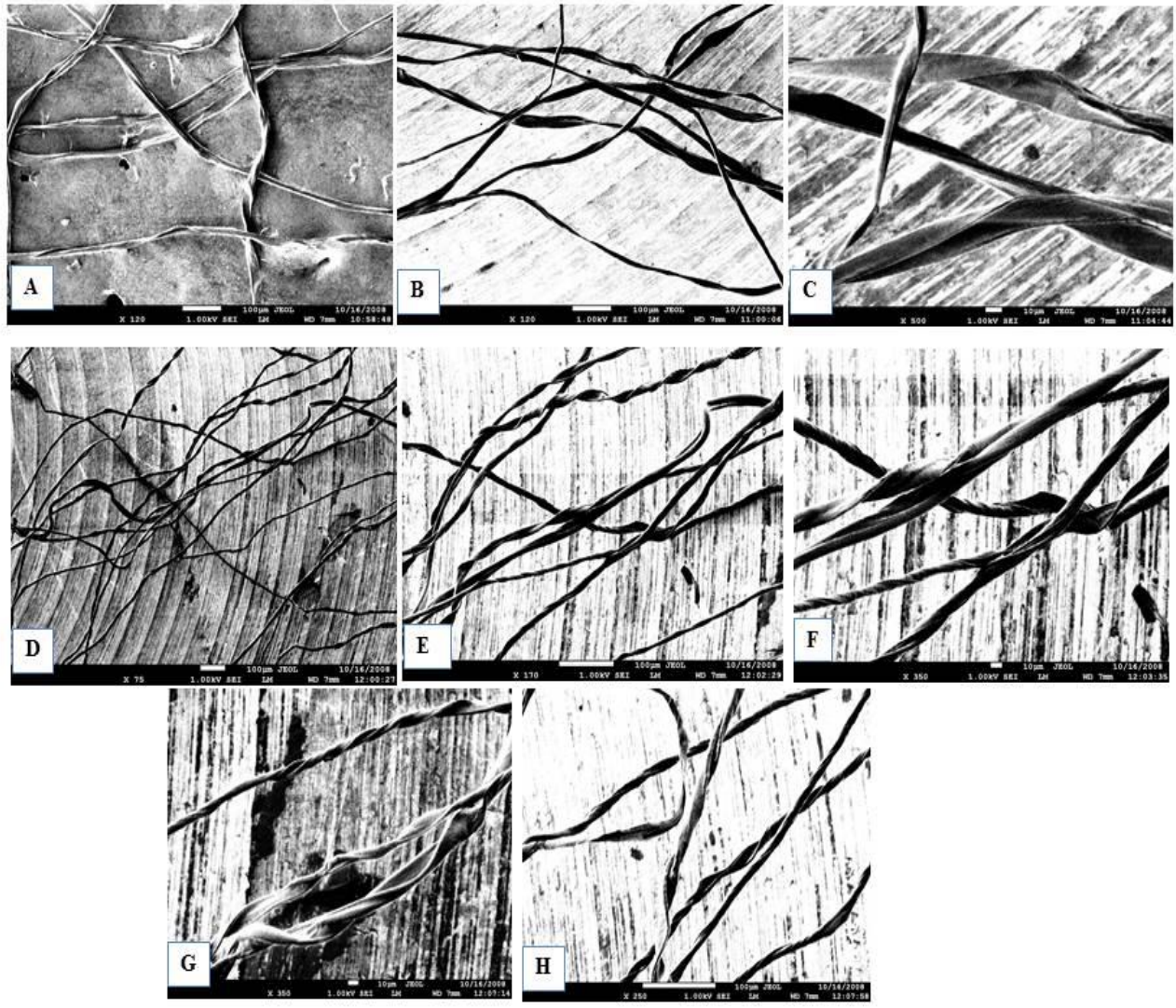
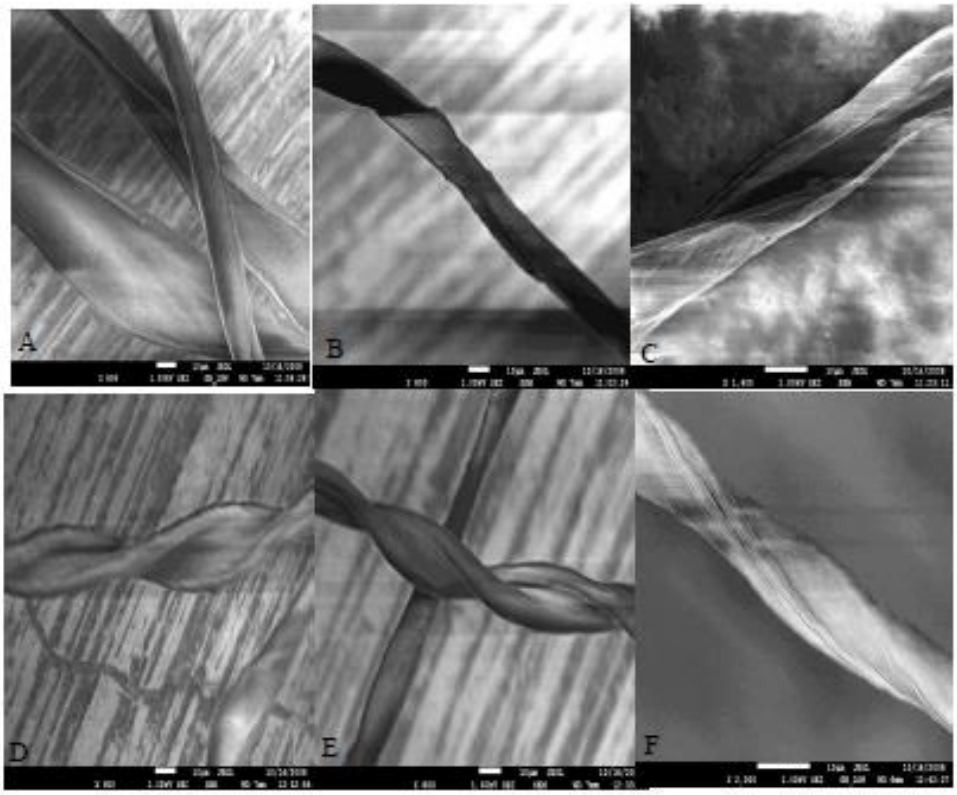


Figure 3.33 Scanning electron microscopy of cotton fibers. a-c) cotton fibers from non-transformed control plants shriveled shriveled, low coiled and twisted. (d-h) Cotton fiber from transgenic *AVP1* cotton plants showing uniform twisting and shape.



.34 High resolution and closer view under scanning electron microscopy of cotton fibers. a-c) cotton fibers from non-transformed control plants showing shriveled and low twisted fibers, (d-f) cotton fibers from transgenic *AVP1* cotton plants showing well developed, healthy and uniform twisting.

3.3.13. Study of fiber characteristics

In addition to high fiber yield, HVI analyses of fibers harvested from *AVP1* transgenic cotton lines showed improved fiber characteristics particularly micronair value, fiber length, strength and elongation % age. The lowest mic value was observed in fibers of APP-3 and APP-4 transgenic cotton lines. Similarly greater fiber length was shown in SV2 and APP-4 at 29.74 and 29.88mm respectively. Moreover, fiber strength was 31.6 and 30.5 g/tex observed in SV2 and APP-3 *AVP1* overexpressing cotton plants. Whereas non-transformed control plants possessed short fiber length, high mic value, low fiber strength and less elongation percentage (Table 3.8).

Table 3.8 Characteristics of cotton fiber

Genotype	Micronaire	Length (mm)	Uniformity index	Strength (g/tex)	Elongation (%)
Ctr	4.67±2.44c	28.74±1.98c	82.4±2.13c	28.1± 1.87c	6.3± 2.54c
SV2	4.60±1.76c	29.736±b	83.4±b	31.6±1.27a	6.8±1.32a
SV5	4.67±2.43c	30.44±1.87a	85.4±1.96a	29.1±2.11b	6.7±1.84b
APP-3	4.44±1.99a	29.05±2.15b	83.6±1.19b	30.5±1.87a	6.5±0.67c
APP-4	4.52±0.35b	29.88±0.96a	84.7±1.56a	29.8±0.98b	6.6±0.77a

Ctr- non-transformed control and SV2, SV5, APP-3, APP-4 are four independent transgenic lines expressing *AVP1*. n=10. Values are the means of three replications ± standard errors and variants possessing same letters in one column are not statistically significant at P=0.05 according to Fisher's protected LSD. Ten T_2 plants of each transgenic event with same number of non -transformed control plants were grown in containment under 16/8 photoperiod 37 ±2°C, RH 65 % (n-10) for six months.

3.4. Discussion

Agricultural crop production suffers huge losses from salt and drought stresses. Modern systems of crop production have aggravated the salt and drought problems owing to climate change, commercial crop production and intensive crop input applications (Dai *et al.,* 2014; Roy *et al.,* 2015). Plant breeders require the development of plant species with enhanced genetic capability of performance under salinized land/water and drought stresses to meet the challenges posed by increased food demand, changes in life style and food preferences (Grover and Pental, 2003).

Transgenic plant cultivation has revolutionized agricultural system particularly in resource poor countries which have limited resources by better management of crop protection and input applications. In farmer's field, GM crops like cotton, soya bean, canola and maize superceded their non-GM counterparts in their performance allowing proper insect/weed pest management and other input applications (Carpenter 2010; Ezezika *et al.,* 2012; Gruskin *et al.,* 2012). These successes stress the need also to address non-biotic stresses by GM crop adoption developed through transgenic approaches because success is hampered in conventional breeding programs due to quantitative, complex, multi-genic and stage specific nature of salt/drought tolerance phenomenon in plants controlled by different overlapping and finely tuned pathways helping plants marginally to cope up the abiotic stresses (Munns and Tester 2008). Under salt and drought stresses, plants are armed with several strategies like synthesis of osmoprotectants (prolines, glycine betains, polyamines, mannitol, trehalose, galactinol), detoxifying enzymes, LEA proteins, lipid biosynthesis genes, heat shock proteins, regulatory genes, transcription factor genes, signal transduction genes and tonoplast protein genes (Golldack *et al.,* 2011; Sohito *et al.,* 2015; Roy *et al.,* 2015).

Due to the empirical nature of transformation techniques and available tissue culture systems, important genes seem attractive targets to explore their role in salt and drought tolerance. Among these genes, protein genes of vacuolar membranes i.e. *AVP1*, *AtNHX1* and transcription factors have huge potential of sequestering extra and toxic sodium into vacuole due to enhanced H^+-pumping and Na^+ exchanging properties engineered in transgenic plants leading to improved salt and drought tolerance and other

improved plant characteristics as well (Gaxiola *et al.,* 2011; Yarra *et al.,* 2012; Yu *et al.,* 2015).

The present study deals with the development of transgenic cotton coker-312 against abiotic stress tolerance (salt and drought). Transformation of cotton coker-312 was carried out with *AVP1* and *AtNHX1* genes through *Agrobacterium.* Cotton hypocotyls were used as explants and putative transgenic plants of 12 independent events for each gene were obtained (Figure 3.8; Tables 3.2 and 3.3). Transformation and regeneration efficiency (18-26%) in this study correlated with the cotton reports (Mishra *et al.,* 2003 Wilkins *et al.,* 2004 ; Hashmi *et al.,* 2011). Molecular analyses were performed to detect the integration, copy number and expression of transgenes in transgenic cotton. PCR results indicated that representatives lines of transgenic cotton contained *neomycin transferase II gene*, CaMV promoter, *AVP1* (Figure 3.8 a, b, c) and *AtNHX1* genes (Figure. 3.9 ab, b, c). PCR analyses of the transgenic plants for the detection of transgenes have been reported (Yara *et al., 2012*; Liu *et al*., 2012; Wu *et al.,* 2011; Zhang *et al.,* 2008).

In the present study, southern hybridization showed the integration of 1-5 copies of transgenes (*AVP1 and AtNHX1*) in the cotton genome (Figure. 3.8 d and 3.9c) which correlates with the finding of others (Hashmi *et al.,* 2011; Sunilkumar and Rathore, 2001; Zhu *et al*., 2006) who showed integration of 1-5 or multiple copies of transgenes in the plant genomes. Results of expression analyses by northern (*AVP1*) and RT-PCR (*AtNHX1*) of the selected lines of transgenic cotton under stress condition showed variable expression between transgenic lines (Figure 3.10; 3.11 C). The difference in expression among transgenic lines might be attributed to the gene copy number or site of insertion in the genome (Gaxiola *et al*., 2001; Pasapula *et al*., 2010; He *et al*., 2005).

In the present study, salt tolerance analysis under hydroponic and pot conditions, transgenic cotton *AVP1* lines i.e., SV2, APP-4 and AtNHX1 lines (AX1, AX2, AX3, AX5) showed significantly high Na^+/K^+ accumulation (Figures 3.14; 3.15; 3.17) which supports the findings of Gaxiola et al., (2001) and Pasapula *et al.,* (2010) who indicated the accumulation of high Na^+ and K^+ contents in the *AVP1* transgenic plants. Over-expression of *AVP1* in transgenic cotton results in enhancement in salt and drought tolerance implicating improvement in abiotic stress tolerance by increased H^+-pumping

thereby energizing the transport of materials by transporters/exchangers like Na^+/H^+, K^+/H^+ and Ca^{2+}/H^+ exchangers. There are 500 transporters found in *Arabidopsis* genome and found in the vacuolar membranes deriving energy for transport of ions from PEG generated by tonoplast H^+-pumps (Gaxiola *et al.,* 2002). High accumulation of Na^+ and K^+ contents is ascribed to PEG generated by transgenic expression of *AVP1* in transgenic cotton plants (Gaxiola *et al.,* 2011). Plant behavior was better in the sense of growth rate, health and leaf size in transgenic *AVP1* cotton (Figure 3.15). Transgenic SV2 and APP-4 lines showed high accumulation of proline contents, total soluble sugars, free amino acids, nitrate reductase activities nitrogen and phosphorus contents (Figures. 3.19-21). These findings are in consistent with the results of Gaxiola *et al.,* (2001) and Zhang and Blumwald, (2001) who reported that over-expressing *AVP1 and AtNHX1* transgenic tomato and rice accumulated high soluble sugars and proline contents.

In addition to salt tolerance, *AVP1* transgenic cotton plants also showed drought tolerance and gave good growth performance under water deficient conditions. This phenomenon is supported by the high occurrence of total free amino acids, total soluble sugars and nitrate reducatse activities. Transgenic lines SV2 and APP-4 showed significant drought tolerance by showing high total soluble sugars, free amino acids and nitrate reductase activities. The improvement in tolerance to drought condition in transgenic plants with high levels of *AVP1* is attributed to increased H^+-pumping enhancing ions transport across the tonoplast into the lumen alleviating toxic effects of excessive Na^+ (Gaxiola 2001). Most probably, the high *AVP1* activity leads to enhanced H^+ pumping generating PEG energizing secondary active uptake/exchange of cations across the tonoplast into the vacuole (Figure 3.14, 3.16). The resulting high lumen solute contents would make possible greater water retention, making plants able to thrive under conditions of low soil water potentials.

Furthermore, at high NaCl concentrations, the increased H^+-gradient across the tonoplast and other biological membranes could also strengthen the driving force for *AtNHX1*-mediated Na^+/H^+ exchange, thereby enhancing Na^+ sequestration into the vacuole of *AVP1* transgenic plants. Presumably, any toxic effects associated to Na^+ are alleviated by confining of extra and toxic Na^+ into the vacuole. These findings makes it clear that a transgenic plant engineered to over-expression simultaneously both the major

single action genes i.e., *AVP1* H^+- pump and *AtNHX1* Na^+/H^+ antiporters (Gaxiola *et al.*, 1999 and Apse *et al.*, 1999) would be able tolerate higher NaCl stresses than *AVP1* and *AtNHX1* alone in separate transgenic plants. Co-expression by pyramiding of tonoplast determinants (*AVP1* and AtNHX1) enabled transgenic cotton plants to survive under high salt and water deficient conditions than transgenic plants possessed either of the *AVP1* or AtNHX1 genes (Shen *et al.*, 2014).

Transgenic *AVP1* cotton plants showed accumulation of total soluble sugars. Increase in sugar contents under salt stress is well documented as it is conserved among prokaryotes and eukaryotes. High accumulation of sugar contents in transgenic plants than non-transformed control plants suggest that transgenic *AVP1* cotton possessed efficient mechanism to protect cells and cellular organelles with potentially improved growth under salt stress. Accumulation of sugar molecules under salt stress maintains osmotic adjustment protecting intracellular organelles under salt/drought stressed conditions (Meloni *et al.*, 2001). Sugar molecules also act as hydroxyl radical scavengers and osmoticum (Smirnoff *et al.*, 1989) and similar results were obtained in transgenic tobacco accumulating high proline contents under salt and drought conditions (Kishore *et al.*, 1995).

Under salt stress proline role is well documented in tomato and canola to alleviated the toxic effects of salinity (Zhang *et al.*, 2001; Zhang and Blumwald 2001).

Under salt stresses, photosynthesis and nitrate reductase activities are adversely affected and transgenic *AVP1* cotton lines maintained high photosynthesis performance and nitrate reductase activities than the non-transgenic plants (Figures 3.19; 3.24). High photosynthetic and nitrate reductase assays clearly demonstrates that transgenic *AVP1* plants are more salt tolerant with capability of high carbon and nitrogen assimilation. These findings are in consistent with those reported in other plant species such as *Arabidopsis*, rapeseed and canola (Apse *et al.*, 1999; Zhang *et al.*, 2001). Moreover, other studies have reported NO_3 uptake and NR activity decrease in plants under salt stress (Aslam *et al.*, 1984; Rao and Gnanam, 1990; Gouia *et al.*, 1994).

Drought analyses showed that transgenic cotton *AVP1* SV-2 and APP-4 lines possessed high water contents and low water potential indicating that under drought conditions, transgenic plants are significantly tolerant also manifested by growth and

physiological performance (Figure. 3.20). These findings are in agreement with others who reported drought tolerance in over-expressing *AVP1* transgenic plants (Gaxiola et al. 2001; Pasapula *et al*., 2010). Moreover high solute contents also must lead to plant capability to maintain higher water contents as compared to control plants and this inference is in compatible with other studies (Bray *et al.,* 2000).Transgenic lines SV2 and APP-4 also showed drought tolerance by showing high total soluble sugars, free amino acids and nitrate reductase activities.

The improvement in tolerance to drought condition in transgenic plants with high levels of *AVP1* is attributed to increased H^+-pumping enhancing ions transport across the tonoplast into the lumen alleviating toxic effects of excessive Na^+ (Gaxiola 2001). Most probably, the high *AVP1* activity leads to enhanced H^+ pumping generating PEG energizing secondary active uptake/exchange of cations across the tonoplast into the vacuole (Figure 3.14, 3.16). The resulting high lumen solute contents would make possible greater water retention, making plants able to thrive under drought conditions.

Furthermore, at high NaCl concentrations, the increased H^+-gradient across the tonoplast and other biological membranes could also strengthen the driving force for *AtNHX1*-mediated Na^+/H^+ exchange, thereby enhancing Na^+ sequestration into the vacuole of *AVP1* transgenic plants. Presumably, any toxic effects associated to Na^+ are alleviated by extra and toxic Na^+ confining in the vacuole. These findings makes it clear that a transgenic plant engineered to over-expression simultaneously both the major single action genes i.e., *AVP1* H^+- pump and *AtNHX1* Na^+/H^+ antiporters (Gaxiola *et al.,* 1999 and Apse *et al.,* 1999) would be able tolerate higher NaCl stresses than either *AVP1* or *AtNHX1* in separate transgenic plants. Transgenic *AVP1* cotton plants showed high accumulation of total soluble sugars. Increase in sugar contents under salt stress is well documented as it is conserved among prokaryotes and eukaryotes. High accumulation of sugar contents in transgenic plants than non-transformed control plants suggest that transgenic *AVP1* cotton possessed efficient mechanism to protect cells and cellular organelles with potentially improved growth under salt stress. Accumulation of sugar molecules under salt stress maintains osmotic adjustment protecting intracellular organelles under salt/drought stressed conditions (Meloni *et al.,* 2001). Sugar molecules also act as hydroxyl radical scavengers and osmoticum (Smirnoff *et al.,* 1989) and similar

results were obtained in transgenic tobacco accumulating high proline contents under salt and drought conditions (Kishore *et al.,* 1995). Under salt stress proline role is well documented in tomato and canola to alleviate the toxic effects of salinity (Zhang *et al.,* 2001; Zhang and Blumwald 2001).

Nitrate reductase activity was found high under salt stress in transgenic *AVP1* plants than non-transformed standards. Under salt stresses, photosynthesis and nitrate reductase activities are adversely affected and transgenic *AVP1* cotton T_2 plants maintained high photosynthesis performance and nitrate reductase activities than the non-transgenic plants (Figures 3.19; 3.24). High photosynthetic and nitrate reductase assays clearly demonstrates that transgenic *AVP1* plants are more salt tolerant with capability of high carbon and nitrogen assimilation. This finding is in consistent with those reported in other plant species such as *Arabidopsis*, rapeseed and canola (Apse *et al.,* 1999; Zhang *et al.,* 2001). Moreover, other studies have reported NO_3 uptake and NR activity decrease in plants under salt stress (Aslam *et al.,* 1984; Rao and Gnanam, 1990; Gouia *et al.,* 1994).

Drought analyses showed that transgenic plants possessed high water contents than control plants under water deprivation. Transgenic lines over-expressing *AVP1* i.e., SV-2 and APP-4 contained highest water contents followed by SV9, APP-2 and least by non-transformed control plants. Similarly solute potential was found lowest in transgenic lines SV-2 and APP-4 than other two transgenic lines i.e. SV-9 and APP-2 followed by non-transformed control indicating that under drought condition transgenic plants are significantly tolerant displayed by growth and physiological performance (Figure 3.20). Transgenic expression of *AVP1* have resulted in drought tolerance in plant species like Arabidopsis (Gaxiola *et al.,* 2001), tomato, rice, rapeseed and cotton. Moreover high solute contents also must lead to plant capability to maintain higher water contents as compared to control plants and this inference is in compatible with other studies (Bray *et al.,* 2000).

Transgenic cotton containing *AVP1* also possessed high growth rate, more number of leaves, fruiting bodies/squares and boll setting as compared to control plants in pot experiments. Two transgenic lines showed significantly fast in growth performance and sett large, healthy bolls during pots study for 6 months (Table 3.5). Fiber yield from transgenic plants was also higher than non-transgenic lines. Scanning electron

microscopy analyses showed that cotton fibers from transgenic plants were robust, healthy and uniformly twisted where as fibers of control plants was shriveled and less uniformly twisted (Figures. 3.26, 27). It is documented that *AVP1* requiring PPase a substrate is involved in secondary wall synthesis during fiber developmental stages. Biological membrane energizing by over-expression of *AVP1* contributes to activity of sucrose transporters thereby leading to uniform and strong fiber formation (Wilkins and Arpat, 2005).

AVP1 transgenic SV2 and APP-4 lines displayed significantly high and fast germination (Figure 3.12). These results correlated with the findings of other reports (Pasapula *et al*., 2010; He *et al*., 2005). Root development is a controversial agronomic character that determines plant adaptability under salt and drought conditions affecting crop productivity (Seo and Park, 2009; Aglawe *et al.,* 2012). Plant root formation is direct control of auxin ABA which is also responsible for regulating growth and developmental processes in plants. In additions to maintaining lumen pH, *AVP1* H^+-PPPase also affects the abundance/transport of auxin and thereby auxin-mediated organ development. *AVP1* over-expression in transgenic *Arabidopsis* has led to increased cell elongation at the time of organ formation and enhanced auxin transport. Transgenic expression of *AVP1* affects the abundance and distribution of two protein i.e. P-adenosine Triphosphatase and Pinformed-1 auxin-efflux facilitator that regulate auxin transport during organ formation and development events (Yan *et al.,* 2002; Li *et al.,* 2005).

Transgenic cotton containing *AVP1* also possessed high growth rate, more number of leaves, fruiting bodies/squares and boll setting as compared to control plants in pot experiments. Two transgenic lines showed significantly fast in growth performance and sett large, healthy bolls during pots study for 6 months (Table 3.5). Fiber yield from transgenic plants was also higher than non-transgenic lines. Scanning electron microscopy analyses showed that cotton fibers from transgenic plants were robust, healthy and uniformly twisted where as fibers of control plants was shriveled and less uniformly twisted (Figures. 3.26, 27). It is documented that *AVP1* requiring PPase a substrate is involved in secondary wall synthesis during fiber developmental stages. Biological membrane energizing by over-expression of *AVP1* contributes to activity of

sucrose transporters thereby leading to uniform and strong fiber formation (Wilkins and Arpat, 2005).

Transgenic *AtNHX1* cotton lines AX1, AX2, AX3 and AX5 showed salt tolerance with high accumulation of Na^+ , $K^{+,}$ proline contents and total soluble sugar (Figures 3.23; 3.24). Similar findings were also documented by over-expression of the *AtNHX1* gene and its orthologs (*OsNHX1, TaNHX2, MdNHX1, AmNHX2*) into different transgenic cotton and other plants ((He *et al*. 2005; Leidi *et al*. 2010; Li *et al*. 2010a, Fukuda *et al.,* 2011; Yarra *et al.,* 2012). So, transgenic cotton coker-312 over-expressing *AVP1* and *AtNHX1 gene* exhibiting salt and drought tolerance were developed and these characters could be introgressed into local elite cultivars for marginal lands and drought prone areas.

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