Novel Vacuolar Na^+/H^+ Antiporters Of Cowpea And Mungbean Confer Salt Tolerance In Transgenic Plants

SAGARIKA MISHRA

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PhD SUPERVISOR

Prof. Lingaraj Sahoo

PLANT GENETIC ENGINEERING AND FUNCTIONAL GENOMICS LABORATORY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
NORTH GUWAHATI, GUWAHATI – 781039, ASSAM, INDIA

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भारतीय प्रौद्योगिकी संस्थान गुवाहाटी
INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
North Guwahati, Guwahati – 781039, Assam, India

Dr. Lingaraj Sahoo
Professor
Department of Biotechnology

Tel: +91 361 258 2204
Fax: +91 361 258 2249
E-mail: ls@iitg.ernet.in

CERTIFICATE

It is certified that the work described in this thesis, entitled "**Novel Vacuolar Na^+/H^+ Antiporters Of Cowpea And Mungbean Confer Salt Tolerance In Transgenic Plants**", done by Ms. Sagarika Mishra for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

April 2014

Dr. Lingaraj Sahoo
Professor
(Thesis Supervisor)

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI
Department of Biotechnology
Guwahati – 781039

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, under the guidance of Prof. Lingaraj Sahoo.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

April, 2014 Sagarika Mishra

Dedicated to my beloved parents

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ABSTRACT

Cowpea (*Vigna unguiculata* L. Walp.) and mungbean (*Vigna radiata* L. Wilczek) are two important grain legumes that supply vegetable proteins in nutritionally deprived regions of the world. Cowpea and mungbean are cultivated mainly in the semi-arid and arid regions of Africa, India, Middle East, South America, Southern Europe and USA. However, their sustainable agricultural production worldwide is limited by salt salinity. Plant Na^+/H^+ antiporters play a major role in maintaining cellular homeostasis under salinity stress. Vacoular Na^+/H^+ antiporters compartmentalize excess cytosolic Na^+ into vacuole thereby reduce ionic toxicity in cytosol and assist in maintaining cellular homeostasis and ionic equilibrium. Isolation and functional characterization of vacuolar Na^+/H^+ antiporters from many glycophytes and halophytes have clearly indicated the role of vacuolar NHX in salt tolerance mechanisms. We report for the first time, the cloning and functional characterization of vacuolar Na^+/H^+ antiporters of cowpea (*VuNHX1*) and mungbean (*VrNHX1*). The *VuNHX1* (Genbank Acc. No. JN641304.2) cDNA is 1981 bp and *VrNHX1* (Genbank Acc. No. JN656211.1) cDNA is 2095 bp, both consist of an ORF of 1629 bp encoding a polypeptide of 542 amino acid residues, with a deduced molecular mass of 59.6 kDa. *VuNHX1* and *VrNHX1* displayed a conserved amiloride binding domain (^{84}LFFIYLLPPI^{93}) in their third transmembrane (TM3) region. Sequence alignment and phylogenetic analysis revealed *VuNHX1* and *VrNHX1* belong to Class-I clade of plant NHX exchangers and have high similarity with legume Na^+/H^+ antiporters. Southern hybridization indicated presence of single copy of *VuNHX1* and *VrNHX1* in respective genomes. Heterologous expression of *VuNHX1* and *VrNHX1* in salt sensitive AXT3 yeast mutant complemented for the loss of yeast

NHX1 under NaCl, KCl and LiCl stress. The expression of *VuNHX1* and *VrNHX1* is upregulated under salt, cold and dehydration stress. Expression analysis and ion estimation in plants exposed to salt stress indicated higher *VuNHX1* and *VrNHX1* transcript accumulation, preferentially in roots of both cowpea and mungbean. Ectopic expression of *VuNHX1* and *VrNHX1* under both constitutive and stress inducible promoter increased salt tolerance in transgenic *Arabidopsis* lines. The role of *VrNHX1,* under a constitutive promoter under salinity stress, was studied in transgenic cowpea. Polymerase chain reaction and southern blot assays confirmed the stable integration of *VrNHX1* in cowpea genome. Comparative expression anlsysis by semi-quantitative RT-PCR revealed higher expression of *VrNHX1* in transgenic cowpea plants. The salt stress assay revealed independent T_2 transgenic cowpea lines exhibiting higher tolerance to salt stress (200 mM NaCl) as compared to wild type plants. Under salt stress, the transgenic lines exhibited a higher K^+/Na^+ ratio in their aerial part and higher $[Na^+]$ accumulation in roots as compared to wild-type plants. The physiological analysis revealed lower level of lipid peroxidation, hydrogen peroxide and oxygen radical production and further, higher levels of proline, ascorbate, relative water and chorophyll content in transgenic cowpea lines indicating higher cellular tolerance to salnity stress.

Cisgenesis represents avenues for generation of crops with inserted gene(s) derived from organisms sexually compatible with the target crop and therefore, have less environmental concerns and increased consumer's acceptance. We generated cisgenic cowpea lines for the first time, by overexpressing mungbean *NHX1* and identified stable cisgenic T_1 plants or descendants thereof, in the early seedling stage, by implementing an efficient salt screening approach that

substantially reduces the labor and time involved in identification of stable cisgenic plants. The cisgenic cowpea lines were selected over four generations by employing salt screening approach and validated with PCR detection of introduced gene(s). No escapes were observed through salt screening approach. No penalty, either on growth or yield factors, was detected in cisgenic cowpea plants selected through the salt screening method. Soil salinity is a major threat to cowpea production and salt tolerant cowpea therefore, would facilitate sustainable production in salinity inflicted regions of the world.

ABBREVIATIONS

ABA	Abscisic acid
Acc	Accession
ANOVA	Analysis of variance
B5	Gamborg's medium
BAP	6-benzylaminopurine
BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
$CaCl_2$	Calcium chloride
CAMBIA	Centre for application of molecular biology to international agriculture
CaMV	Cauliflower mosaic virus
cDNA	Complementary deoxyribonucleic acid
CDS	Coding sequence
Cl^-	Chloride ion
CTAB	Cetyltrimethyl ammonium bromide
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
gus-A	*β-1-4 glucuronidase*
h	Hour
H^+	Hydrogen ion
HCl	Hydrochloric acid
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonic acid
Hgy	Hygromycin
HNO_3	Nitric acid
H_2SO_4	Sulfuric acid
IBA	Indole-3- butyric acid
IC-NHX	Intracellular sodium hydrogen exchanger
IPTG	Isopropyl β-d-1-thiogalactopyranoside
K^+	Potassium ion
KCl	Potassium chloride
LB	Luria-Bertani
LCM	Liquid co-cultivation media
Li^+	Lithium ion

LiCl	Lithium chloride
MES	2-(*n*-morpholino)ethanesulfonic acid
$MgCl_2$,	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MS	Murashige and Skoog's medium (1962)
MSB_5	MS medium supplemented with B5 vitamins
Na^+	Sodium ion
NaCl	Sodium chloride
Na_2HPO_4	Disodium hydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
NHX	Sodium hydrogen exchanger
No	Number
nptII	*Neomycin phosphotransferase II*
OD	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PM_NHE	Plasma membrane sodium hydrogen exchanger
RACE	Rapid amplification of cdna ends
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase- polymerase chain reaction
SDS	Sodium dodecyl sulfate
SE	Standard error
SSC	Sodium chloride and sodium citrate buffer
T-DNA	Transferred-DNA
TDZ	N-phenyl-1,2,3-thidiazol-5-yl-urea
TM	Transmembrane
ura	Uracil
UTR	Untranslated region
vir	Virulence gene
X-Gal	5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside

UNITS

^{0} C	Degree celsius
µg	Microgram
µg/ml	Microgram per millilitre
µl	Microlitre
µmol $m^{-2}s^{-1}$	Micromoles per square metre per second
µM	Micromolar
%	Percentage
bp	Basepair
cm	Centimetre
DW	Dry weight
dS m^{-1}	Decisiemens per metre
FW	Fresh weight
g	Gram
g/l	Gram per litre
g/min	Gravitational force per minute
kb	Kilo basepair
kDa	Kilodalton
kHz	Kilohertz
mF	Millifarad
mg/l	Milligram per litre
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
mm Hg	Millimetres of mercury
MW	Molecular weight
ng	Nanogram
ng/ml	Nanogram per millilitre
nm	Nanometre
ohm	SI derived unit of electrical resistance
pH	Negative log of H^+
psi	Pounds per square inch
rpm	Revolution per minute
sec	Second
U	Unit
w/v	Weight/volume (concentration)

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CHAPTER 1

Introduction

1.1. Introduction

Plants are constantly exposed to a range of environmental stresses such as salinity, drought, cold and high temperature, which exerts adverse effects on plant growth, metabolism and development, ultimately causing extensive crop losses. Among abiotic stresses, salinity causes massive damage to agricultural productivity worldwide (Tuteja 2007). Nearly 3.6 billion of world's 5.2 billion hectares of dryland used for agricultural purposes have already suffered erosion, soil degradation and salinization (Koyro et al. 2012).

Most of the crop plants including legumes are categorized as glycophytes, or salt sensitive species that are affected by 100 mM of salt stress (Tuteja 2007). Salinity stress brings in an array of morphological, physiological, biochemical and molecular changes which forces the plant to succumb to ionic imbalance, hyperosmotic stress, oxidative damage, impaired nutrient acquisition, improper mineral acquisition and partitioning within the plant, damage to photosynthetic machinery and programmed cell death (Bohnert et al. 1999; Gratan and Grieve 1999; Hasegawa et al. 2000; Tester and Davenport 2003; Botella et al. 2005; Tuteja 2007). Development of more resilient crops that can cope up better with extremes of salinity can assist in achieving sustainable and economic agricultural production (Yamaguchi and Blumwald 2005).

Salinity tolerance is essentially a multigenic trait in plants. Previous physiological, molecular genetics and functional genomics studies have provided some molecular and physiological knowledge of plant salt tolerance. Some important genes encoding proteins for osmolyte synthesis, ion channels, signaling factors and salt-responsive enzymes have been cloned and characterized, which revealed their fundamental functions in plant response and adaptation to salinity (Tuteja 2007).

One of the important mechanisms of salt tolerance in plants include sequestration of toxic Na^+ into vacuoles, mediated by membrane bound Na^+/H^+ antiporter for detoxification of Na^+ at cellular and whole plant level. Compartmentalization of toxic Na^+ in vacuoles helps in maintaining osmoticum and turgor of plant cells (Blumwald et al. 2000). Plants engineered to overexpress *AtNHX1*, first known member of the Arabidopsis tonoplast Na^+/H^+ antiporter family (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001) have shown

enhanced salinity tolerance. The fact that the Na^+ accumulation occurs mainly in the green parts of the plant, but not in fruits (Zhang and Blumwald 2001), has extended its application to other crops and has been found to be highly effective in improving salinity tolerance in majority of crop plants (Hasegawa 2013; Schroeder et al. 2013).

Mungbean (*Vigna radiata* L. Wilczek.) and cowpea (*Vigna unguiculata* L. Walp.) are important grain legumes, cultivated widely in Asia and Sub-Saharan Africa (Sahoo et al. 2003). They provide a rich and vital source of dietary protein, essential amino acids, fiber, complex carbohydrates, vitamins and minerals for both humans and grazing animals. However, their productivity is limited by severe loss owing to exposure to various biotic and abiotic stresses, of which, high salinity poses as a major constraint affecting mungbean (Singh and Singh 2011) and cowpea (Lawlor 2013) producion. However, no efforts have been made yet to find genes and their inherent mechanisms for salt tolerance in mungbean and cowpea, and development of genetically engineered legumes through cisgenic and transgenic approaches to enhance salt tolerance.

The present study is undertaken with the objectives to clone vacuolar NHX exchanger gene from commercial cultivars of mungbean and cowpea, and study their function in imparting salt tolerance through complementation assays in yeast mutant lacking genes required for efficient survival under salt stress. Further, transgenic approach has been employed to express vacuolar NHX exchangers in model and crop plant systems, and understand the resulting morphological, physiological and biochemical changes under salt stress, in these genetically engineered plants.

1.2. Objectives

The objectives are outlined as

- Isolation and functional characterization of a cowpea vacuolar Na^+/H^+ antiporter gene, *VuNHX1* and its ectopic expression in *Arabidopsis thaliana* for studying its role in salt tolerance
- Isolation and functional characterization of a mungbean vacuolar Na^+/H^+ antiporter gene, *VrNHX1* and its ectopic expression in *Arabidopsis thaliana* for studying its role in salt tolerance

- Development of salt tolerant transgenic cowpea plants by ectopic overexpression of *VrNHX1*
- Development of a salt screening method for identification of salt tolerant cisgenic cowpea

CHAPTER 2

Review of Literature

2.1. Soil salinity- a major abiotic stress

Climate change and population growth continue to impose enormous pressures on agricultural production worldwide (Schroeder et al. 2013). Global demand for food is predicted to increase by 40% by 2030 (Schroeder et al. 2013). Innovative solutions are required to increase production on the land currently used for agriculture, as we are already close to the sustainable limit of 15 % of earth's surface that can be exploited for crop production (Rockstrom et al. 2009). Today, salinity is perhaps the greatest threat to sustainable irrigated agriculture (Yadav et al. 2011). Increasing food production on limited land resources with impeding salinity threat will rely on innovative agronomic practices coupled to the genetic improvement of crops (Conway 2012).

2.1.1. Primary reasons of soil salinity

The global land salinization covers nearly 830-950 million hectares, and 50 % of which exhibit sodic nature wherein, accumulation of high concentration of Na^+ contributes to devastating effect on crop growth and production (Hasegawa 2013). Global soil salinization is largely influenced by natural, environmental and man-made factors. Climatic variation over specific geographic regions accounts for alteration in precipitation, evotranspiration and landscape hydrology, often resulting in salinity enhancement. Naturally, saline and sodic soil occur in seasonally water logged regions with poor drainage systems, deserts, grasslands, certain savanna ecosystems, and mainly arid along with semi-arid regions. Inland salinity is attributed due to seawater droplet drift, rising sea levels, tidal changes that cause intermixing of fresh and salt water. Receding oceans also resulted in lowland areas with large pools of concentrated seawater. Further, secondary salinization is accounted due to improper irrigational practice and deforestation. Soil salinity is characterized with the presence of harmful concentration of chlorides, sulfates, nitrates, bicarbonates of sodium, calcium, magnesium and potassium at alkaline pH conditions, affecting crop growth and sustainability. Saline soils exhibit an electrical conductivity >4 dS m^{-1} (~36 mM NaCl) which further increases with extent of salinity (Flowers and Flowers 2005; Brady and Weil 2008; Yadav et al. 2011; Hasegawa 2013; Bui 2013).

2.1.2. Detrimental effects of salinity on plant growth and development

Under typical physiological conditions, plants tend to retain higher K^+/Na^+ ratio in their cytosol by preferentially maintaining high K^+ (100-200 mM), an essential macronutrient owing to the involvement of K^+ in a wide array of functions like maintenance of electrical potential gradients across cell membranes, generation of turgor, activation of numerous enzymes, protein and starch synthesis, respiration and photosynthesis (Maathuis and Amtmann 1999; Blumwald et al. 2000, Britto and Kronzucker 2008; Hauser and Horie 2010).

Salt at higher concentration in the apoplast of cell generates primary and secondary effects that negatively affect survival, growth and development of plant. Both Na^+ and Cl^- affect cytosolic, organellar and metabolic processes (Niu et al. 1995; Tester and Davenport 2003). The primary effects are ionic toxicity and hyperosmolarity (Botella et al. 2005). This disruption of homeostasis occurs at both cellular and whole plant level (Zhu 2001). High concentration of salt impose hyperosmotic shock by lowering the water potential causing turgor reduction or loss that restricts cell expansion (Hasegawa et al. 2000). This generates dehydration of cell due to severe water loss, also known as "physiological drought" condition. Low water potential also induce ABA production and signaling, which cause guard cell depolarization and reduction in stomatal aperture and conductance resulting in biomass and yield reduction (Smith and Stitt 2007; Yoo et al. 2009; Kim et al. 2010). Principal secondary effects of NaCl stress includes disturbance of K^+ acquisition, membrane dysfunction, lower stomatal conductance, impairment of photosynthesis, carbon metabolism, lower transpiration rates, impaired nutrient uptake ability, generation of reactive oxygen species (ROS) and programmed cell death (Hasegawa et al. 2000; Wilkinson and Davies 2002; Zhu 2003; Yoo et al. 2009; Salekdeh et al. 2009; Hasegawa 2013).

2.2. Plant adaptive mechanisms to salinity stress

Plants respond and adapt to the environmental cues by activating appropriate physiological, molecular, developmental and biochemical changes to overcome the damage incurred during stress (Atkinson et al. 2013). However, abiotic stress tolerance mechanism is multigenic in nature and thus, a very complex phenomenon. The complexity of abiotic stress response is attributed to the variability in

occurrence of different stress exposures at multiple stages of plant development individually or concurrently as observed in field conditions (Yamaguchi-Shinozaki and Shinozaki 2006; Mittler and Blumwald 2010). Although, the constant re-adjustment of physiology and metabolism takes place throughout plant life cycle within the framework of their genetic background, however, the survival or death of plant is dependent on severity of the stress (Pastori and Foyer 2002). The stress adaptive mechanisms of plants are shown in Fig. 2.1.

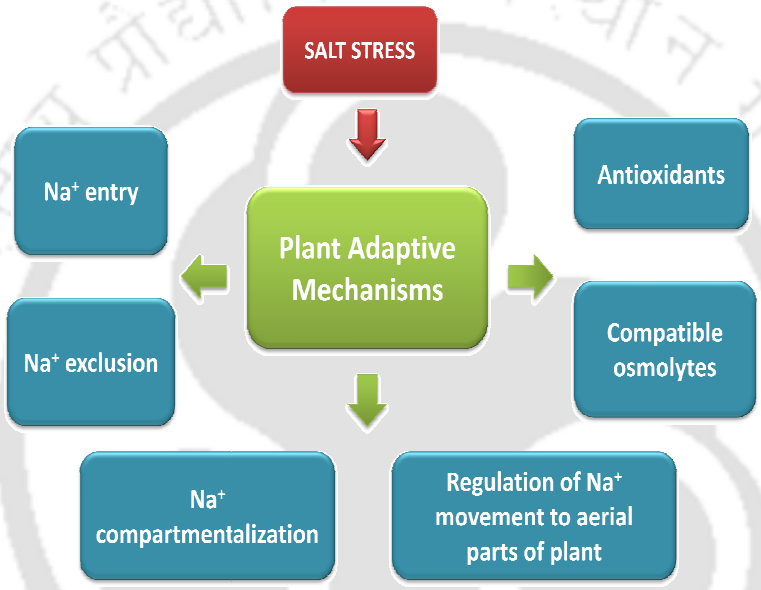


Fig. 2.1 Diagrammatic representation of adaptive response mechanisms to salinity stress in plants

2.2.1. Entry of Na^+ in plant cells

Presence of Na^+ have been suggested to be detected externally by plasma membrane receptor(s) or internally by sensor(s), membrane protein(s) and cytosolic enzymes (Kader and Lindberg 2010; Zhu 2003). The unidirectional passive influx of sodium ions to plant cells under saline conditions owing to the electrochemical potential gradient across plasma membrane is mediated mainly by apoplastic pathway components. Moreover, Na^+ is known to compete with K^+ for entry into plant cell owing to their similar ionic radii. Thereby, entry of Na^+ is mediated by membrane

transporters such as low affinity K^+ channel (AKT1), high affinity K^+/Na^+ symporter (HKT1), both voltage dependent non-selective cation channels, NSCC (cyclic nucleotide gated channels, CNGCs and glutamate activated channels, GLRs) and independent (VIC) cation channels (Blumwald et al. 2000; Apse and Blumwald 2007; Manchanda and Garg 2008). However, selective uptake of K^+ over Na^+ by membrane transporters also stands as a possible mechanism for regulation of salinity stress (Schachtman and Liu 1999). Interestingly, in halophytes, *Thellungiella salsuginea* and *Suede maritime*, stringent regulation of Na^+ uptake and K^+/Na^+ selectivity are exhibited indicating them as important attributes of salt tolerance mechanisms (Wang et al. 2007; Amtmann and Beilby 2010).

2.2.2. Na^+ exclusion from plant cells

The salt stress signaling and perception plays an evident role in triggering gene networks playing vital role in detoxification of toxic Na^+ at cellular as well as whole plant level. The detection of Na^+ inside the cytosol is mediated by sensor(s), membrane protein (s) and Na^+-sensitive cytosolic enzymes (Zhu 2003). Altered primary calcium signatures amidst salt and physiological drought stress, is witnessed with activation of IP_3-gated calcium channels and mechano-sensitive stretch-activated channels. The calcium sensor proteins are further activated to foreplay signal transduction cascades involved in up-regulation of stress dependent genes in response to salinity stress (Knight et al. 1997; Saijo et al. 2000). The salt overly sensitive (SOS) pathway has been a major breakthrough elucidating the interactive role of sensors and downstream genes in achieving cellular and ion homeostasis. The SOS pathway comprises of a calcium binding protein SOS3 which interacts with SOS2, a multifunctional serine/threonine protein kinase to form a SOS3-SOS2 kinase complex further, phosphorylating SOS1, a plasma membrane Na^+/H^+ antiporter, homologous to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi et al. 2000). The sodium extrusion from plant cell is an active process mediated by plasma membrane Na^+/H^+ antiporter (SOS1) and H^+-ATPase. The SOS1 extrudes excess Na^+ from cytosol to apoplast against its electrochemical gradient by coupling the downhill movement of H^+ into the cell along its electrochemical gradient (Blumwald et al. 2000; Zhu 2002, 2003; Apse and Blumwald 2007). Analyses of *sos1* mutant plants have revealed regulation of [Na^+] in

xylem sap by unloading Na^+ from or loading Na^+ into xylem vessels by SOS1, depending on the strength of salinity stress, thereby influencing long-distance Na^+ transport from roots to shoots (Shi et al. 2002).

2.2.3. Vacuolar compartmentation of Na^+ in plant cell

Despite the efflux of toxic Na^+ by the plasma membrane Na^+/H^+ antiporter, an internal adaptive measure is also followed by plants. The compartmentalization of excess Na^+ in vacuole is mediated by Na^+/H^+ antiporter (NHX exchanger) coupled with multisubunit V-type ATPases (H^+-ATPases) and Pyrophosphatases (H^+-PPases) present on the vacuolar membrane. The driving force for entry of Na^+ into vacuole by NHX1 antiporter is obtained from the electrochemical proton gradient generated by vacuolar H^+-translocating enzymes. The first reported plant vacuolar Na^+/H^+ antiporter gene, *AtNHX1* isolated from model plant *Arabidopsis thaliana*, was originally identified by sequence homology to *NHX1* gene in *Saccharomyces cerevisiae* and to members of *NHX* family in *Caenorhabditis elegans* and *Homo sapiens* (Apse et al. 1999; Gaxiola et al. 1999). Several orthologues of *NHX1* from glycophytes and halophytes share functional similarity with *AtNHX1*. Besides reducing cytosolic Na^+ for regaining ionic and cellular homeostasis, vacuoles use accumulated Na^+ as osmoticum to restore water potential of cells (Blumwald 2000). Further, NHX antiporters play important role in maintaining cellular and pH homeostasis (Blumwald et al. 2000; Flowers and Colmer 2008), transport of K^+ into vacuole for maintaining osmotic potential and intracellular K^+ homeostasis (Jiang et al. 2010; Barragán et al. 2012), vesicular trafficking and protein targeting (Bowers et al. 2000; Brett et al. 2005). The diagrammatic representation of Na^+/H^+ membrane transporters involved in entry, exclusion and restriction of Na^+ in plant cell is shown in Fig. 2.2.

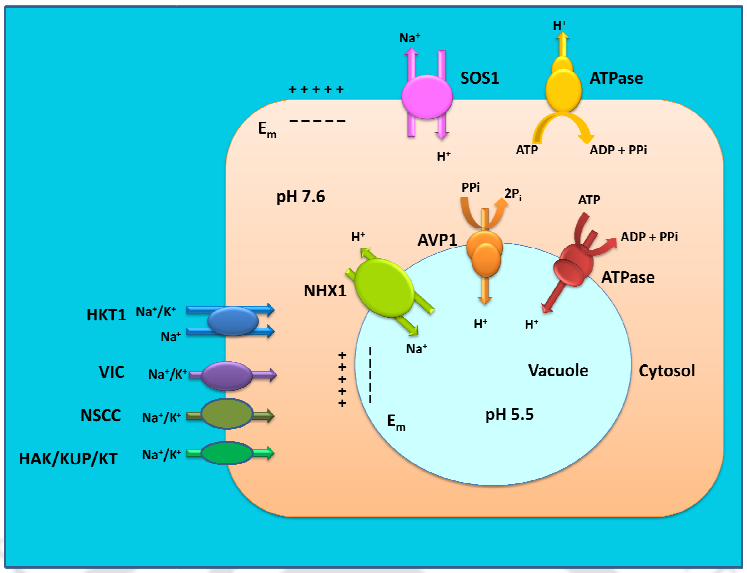


Fig. 2.2 Diagrammatic representation of role of membrane transporters in adaptive response mechanisms to salt stress in plants. Na^+ entry is mediated by membrane transporters: HKT1, VIC, NSCC and HAK/HUP/KT (High affinity potassium transporters/ high uptake potassium transporters/ potassium transporters), and tightly regulated in some cases. Plasma membrane Na^+/H^+ antiporter, SOS1 and vacuolar antiporter, NHX1 mediates extrusion and compartmentalization of Na^+ in plant cell, respectively. This is an energy driven process involving membrane bound ATPases and pyrophosphatases. The figure is generated by involving components and pathways explained by Serrano and Rodriguez-Navarro (2001); Pardo and Quintero (2002) and Mäser et al. (2002)

2.2.4. Regulation of movement of Na^+ to aerial part of plants

Na^+ entry into plant cell is mediated through both symplastic and apoplastic pathways. However, restriction of radial movement of Na^+ into transcriptional stream can avoid negative impact of toxic Na^+ in leaves of plants. It has been observed that vacuolar compartmentalization of Na^+ mediated by *NHX1* exchanger, in root epidermal and cortical cells, as well as pericycle and xylem parenchyma cells can regulate the apoplastic movement of Na^+ from entering into xylem vessels (Apse and Blumwald 2007; Munns and Tester 2008; Rodríguez-Rosales et al. 2009). The casparian strip in the endodermis poses as a hindrance in the radial movement of Na^+ to root stele and transcriptional pathway in certain crop species (Chen et al. 2011). Further, the long distance transport of Na^+ in plants has been found to be regulated by plasma membrane transporters, belonging to HKT (High affinity

potassium transporters) family. *AtHKT1;1* was originally identified as an *Arabidopsis* homologue of *T. aestivum TaHKT2;1* (Schachtman and Schroeder 1994; Uozumi et al. 2000) that showed strong preference for Na^+ selective transport when expressed in heterologous systems such as *S. cerevisiae* and *Xenopus laevis* oocytes (Uozumi et al. 2000). Furthermore, recent experimental evidences have proposed two important functions of *AtHKT1;1* regarding control of [Na^+] in aerial part of plants by its function in recirculation of Na^+ in phloem (Berthomieu et al. 2003) and exclusion of Na^+ from xylem vessels in roots by absorption of accumulated Na^+ in the vessels into xylem parenchyma cells, in order to reduce the amount of Na^+ transported to aerial parts by long-distance Na^+ transport (Sunarpi et al. 2005). Similarly, orthologues of *AtHKT1;1* in rice (*OsHKT1;5*) and durum wheat (*HKT1;4* and *HKT1;5*) also exhibit a similar functions in unloading of Na^+ from xylem vessels (Ren et al. 2005; Huang et al. 2006; Byrt et al. 2007). The diagrammatic representation for movement of Na^+ in whole plant level is explained in Fig. 2.3.

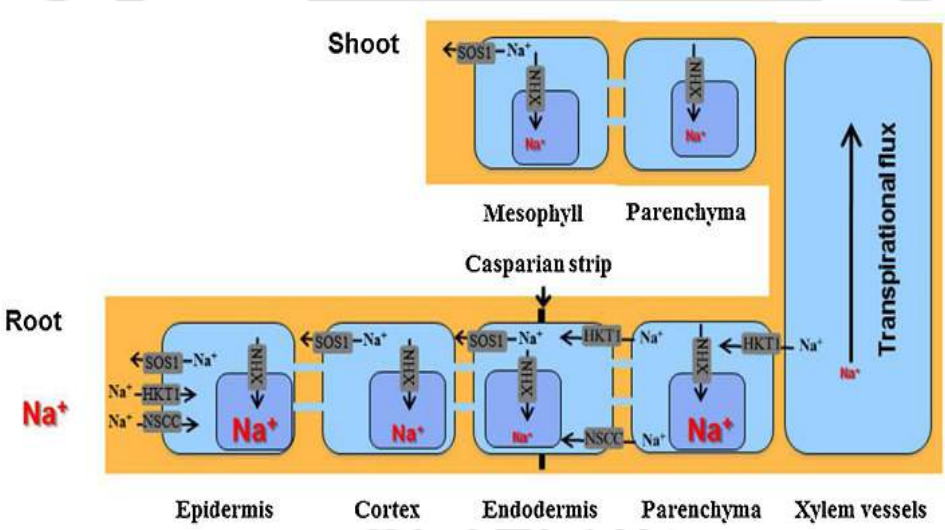


Fig. 2.3 Transport of Na^+ in whole plant. The diagrammatic representation of radial movement of Na^+ through epidermal cells to aerial part of plants and tight regulation is maintained as a defense mechanism against salinity stress in some plants (Hasegawa 2013)

2.2.5. Synthesis of compatible osmolytes

One of the mechanisms to combat water deficit condition arising due to salinity and drought stress is synthesis of osmoprotectants (osmolytes or compatible solutes). These are non-toxic metabolites which pose non-hinderance to normal metabolism in plants, accumulate in cytoplasm to restore osmotic potential in cytosol against Na^+ in apoplastic and vacuolar space (Valliyodan and Nguyen 2006; Negrao et al. 2011). The accumulation of osmoprotectants like non-structural carbohydrates (sucrose, trehalose, sugar alcohols like mannitol and glycerol), amino acids (mainly proline), quaternary amino compounds (glycine-betaine, proline-betaine, hydroxyproline betaine) varies from species to species (Flowers and Colmer 2008, Ashraf and Harris 2004; Serraj and Sinclair 2002). Sugars are believed to contribute towards protection of macromolecules, cellular membrane stabilization, turgor maintenance and signaling (Manchanda and Garg 2008; Mohammadkhani and Heidari 2008). Among amino acids, proline is synthesized at higher levels than other stress active amino acids, in a wide range of salt and drought stressed plants.

2.2.6. Antioxidant production

The production of reactive oxygen species (ROS) is observed under normal condition as a byproduct of cellular metabolism and under various abiotic stresses in plants. The excessive presence of ROS such as free radicals (superoxide anion, $0_2^{\bullet-}$ and hydroxyl radical, $^{\bullet}OH$) and non-radicals (hydrogen peroxide, H_2O_2 and singlet oxygen, O_2^*) affects the cellular machinery causing lipid peroxidation, membrane integrity, protein oxidation by damage to macromolecules, enzyme inhibition, damage to DNA and RNA, and ultimately programmed cell death. Apart from its destructive function, ROS are also considered as secondary messengers or signaling molecules transmitting vital signals for activating stress defense mechanisms. The ROS-scavenging mechanisms are employed by plants to maintain optimum concentration of ROS required for proper functioning of cell. The ROS-scavengers are mainly enzymes (superoxide dismutase, ascorbate peroxidase, monodehydroascorbate reductase, dehydroascorbate reductase, catalase, glutathione peroxidase), ascorbic acid, glutathione, α —tocopherol, caretenoids and phenolics (Mittler 2002). The oxidative damage by ROS production is mitigated in legume nodules which pose several ROS scavengers (Becana et al. 2000).

2.3. Mungbean and cowpea- two important grain legumes

Mungbean and cowpea are two important grain legumes belonging to Fabaceae or Leguminosae family, considered nutritionally and economically significant food crops. Grain legumes, considered second next to cereals in terms of agricultural importance are rich source of dietary protein (17-40%), essential amino acids, fiber, complex carbohydrates, vitamins and minerals, and are often quoted as "poor man's meat". The nitrogen fixation ability of legumes is exploited in field crop rotation with cereal crops as a cost-effective strategy for improving soil fertility and crop yield (Tharanathan and Mahadevamma 2003; de Almeida Costa et al. 2006).

2.3.1. Mungbean

Mungbean (*Vigna radiata* L. Wilczek) is a self-pollinated diploid (2n = 2x=22), short duration warm seasonal crop with a genome size of 579 Mb categorized under *Vigna* genus and sub-genus *Ceratotropis* (Arumuganathan and Earle 1991*;* Lambrides and Godwin 2007*).* Mungbean is an important Asiatic grain legume, mainly cultivated in India, southeast and east Asia. It is grown on more than 6 million hectares worldwide (about 8.5 % of the global pulse area) with annual production around 3 million ton of grain (about 50 % of global mungbean production and 5% of global pulse production). India is the largest producer of mungbean where it stands as the third most important pulse crop with an area of approximately 3.5 million hectares (about 15 % of the national pulse crop area) producing 1.2 million ton of grain (Nair et al. 2013).

Mungbean primarily serves as nutritionally rich source for human food consumption besides being used as animal fodder, in crop rotation on account of its nitrogen fixing ability. On dry weight basis mungbean seeds constitute (25-28 %) protein, (1-5 %) fat, (3.5-4.5 %) fiber, (4.5-5.5 %) ash and (60-65 %) carbohydrate content. Presence of high lysine, ascorbic acid, potassium, iron, phosphorus and calcium content, easy digestibility, low flatulence on consumption and low phytic acid content together constitute to serve as an excellent complement for cereal based diets. Further, mungbean is popularly consumed in form of sprouts in both developing and developed countries. It also exhibits hepatoprotective characteristics and antioxidant effect against lipid peroxidation and non-lipids

oxidative damage (Ali et al. 1997; Duh et al. 1999; Wu et al. 2001; Weinberger 2005; Lambrides and Godwin 2007; Nair et al. 2013).

2.3.2. Cowpea

Cowpea (*Vigna unguiculata* L. Walp.), a diploid species (2n=2x=22) with a genome size of 620 Mbp is categorized under genus *Vigna* Savi. (subgenus *Vigna* sect. Catiang) in the Phaseoleae (Das et al. 2008). It is divided into 11 subspecies that includes ssp. *unguiculata* having the cultivated forms (var. *unguiculata*) and wild forms (var. *spontanea*) and 10 wild perennial subspecies (Pasquet 2000; Maxted et al. 2004). Based on pod and seed characteristics, cultivated forms of cowpea have been distributed to five groups: *Unguiculata*, *Melanophthalmus, Sesquipedialis, Biflora* and *Textilis* (Fang et al. 2007). It is highly self pollinating herbaceous warm seasonal legume crop. It is an important food and forage legume cultivated throughout the tropics and subtropics including Asia, Africa, South America, Southern Europe and USA (Singh 2005). As per reports, 80 % of cowpea production takes place in the dry savannah of tropical West and Central Africa from nearly 21 million acres grown worldwide (Timko et al. 2008). Cowpea annual worldwide production estimated around 4.5 million metric tons on 12 to 14 million ha (Diouf 2011). It is cultivated by economically poor farmers of developing countries primarily for rich dietary protein .

Cowpea seeds possess nutritional and therapeutic attributes such as rich source of lysine, methionine and tryptophan as compared to other legumes (Lambot 2002), substantial amount of mineral and vitamins (folic acid and vitamin B) (Hall et al. 2003), low amount of fat and high level of fiber helpful in preventing heart disease by reducing the low-density lipoprotein. Further, the slow digestibility of its starch content promotes slow accumulation of glucose in blood level and therefore cowpea based diet is advisable for diabetics (Phillips et al. 2003). It is a pantropical herbaceous nitrogen fixing plant also well known for its drought tolerance trait and survival ability on poor quality soil (Timko et al. 2008; Diouf 2011).

2.3.3. Production constraints

The genetic potential of both mungbean and cowpea is still not realized owing to several production constraints. These include both biotic and abiotic constraints

that impose severe threat to their production. Legumes, generally considered salt sensitive species and soil salinity imposes adverse effects on their growth, symbiotic association with *Rhizobium*, development of root nodules and nitrogen fixing ability (Fougere et al. 1991; van Hoorn et al. 2001; Rao et al. 2002).

Mungbean is considered as salt susceptible crop with ≥70 % yield reduction observed at 50 mM NaCl stress (Singh and Singh 2011; Sehrawat et al. 2013). Reports on reduction in germination percentage, shoot and root length, fresh mass, leaf area, carbohydrate content nitrogen fixation ability, nodule formation, mineral uptake, cellular metabolism, chlorophyll content in mungbean plants upon salt stress elucidate the negative impact of salinity on its development (Ashraf and Rasul 1988; Hafeez et al. 1988; Misra et al. 1996; Zayed and Zeid 1997).

Cowpea is known to exhibit good tolerance against drought and heat stress (Rachie and Roberts 1974). However, several experimental evidences have demonstrated retardation of its growth and development upon salinity stress. Extensive work has been performed in measuring parameters like reduction in germination rate, shoot and root length, leaf area, dry weight, leaf conductance, seed yield, and inhibition of photosynthesis which indicated negative impact of high salt stress on growth and yield of different cowpea cultivars (Maas and Poss 1989; Plaut et al. 1989, 1990; Sousa et al. 2003; Patel et al. 2010).

2.4. Transgenic approaches for generation of salt tolerant legumes

Salinity tolerance being a multigenic trait complicates the various efforts made towards generation of salt tolerant crops (Flowers 2004). However, the fact that ectopic expression of a single gene (or more than one related genes) have contributed in enhancing salt tolerance in various crops, probably by activating related stress responsive genes including membrane transporters, transcription factors and other important regulatory genes exhibiting crosstalk and networking processes, is likely to be the important strategy for developing salt tolerance in legumes.

2.4.1. Role of ion transporters in salt tolerance in legumes

Membrane transport in plants is associated with influx and efflux of ions, solute molecules including organic nutrients, sugars, osmolytes, and metabolic wastes.

Moreover, membrane transporters are also associated with signal perception and transduction to various environmental stress.

Plant Na^+/H^+ antiporters play a vital role in maintaining ion homeostasis by detoxification of toxic Na^+ from cellular space. The exclusion of excess toxic Na^+ from plant cells is mediated by plasma membrane Na^+/H^+ antiporter, *SOS1* as reported firstly in model plant *A.thaliana* (Shi et al. 2000). Moreover, SOS1 has also been reported in various legumes like *G. max* (GenBank Acc. No. JQ287499.1) and *V. radiata* (GenBank Acc. No. KC855193.1). Overexpression of *AtSOS1*, *TdSOS1* in *A. thaliana* (Shi et al. 2003; Feki et al. 2014) and *AtSOS1* in *N. tobacum* (Yue et al. 2012) have increased salt tolerance in transgenic plants. Therefore, overexpression of *SOS1* in legumes from glycophytes and salt-tolerant halophytes can be exploited for development of improved salt tolerant crops.

Another adaptive mechanism of plants in response to salt stress involves sequestration of sodium ions in vacuoles to reduce ionic toxicity. There have been several reports of isolation and characterization of vacuolar Na^+/H^+ antiporters in legumes under salinity stress such as *G. max* (GenBank AY972078.1 , Li et al. 2006), *V. radiata* (GenBank JN656211.1) , *V. unguiculata* (GenBank JN641304.2), *T. repens* (GenBank EU109427.1, Tang et al. 2010), *M. sativa* (GenBank AY456096.1, Yang et al. 2005), *Galega orientalis* (Xin et al. 2009) and *A. hypogaea* (GenBank HM590627.1, Xing et al. 2011).

The regulation of Na^+ movement in plant from root-soil interface to leaves is of prime importance for salt tolerance. Members of the HKT (High affinity potassium transporters) family are attributed with functions involving unloading of Na^+ from xylem vessels into stele, thereby reducing Na^+ concentrations in transpirational stream and also plausibly phloem recirculation (Hasegawa 2013). A HKT1 has been isolated from *G. max* and functionally validated in tobacco confirming the involvement in imparting salt tolerance by regulating K^+/Na^+ homeostasis (Chen et al. 2011).

2.4.2. Transgenic Asiatic grain legumes for salt tolerance

We focus on the advancement in legume stress biology by development of transgenic salt-tolerant legumes achieved through overexpression of vacuolar

membrane transporter, mainly NHX1 involved in adaptive mechanism of plants in response to salt stress by vacuolar sequestration of Na^+ to reduce ionic toxicity. Further, generation of transgenic legumes have been achieved by overexpression *SsNHX1* and *TaNHX2* in *M. sativa* (Li et al. 2011; Zhang et al. 2012), *AtNHX1* in *A. hypogaea* (Banjara et al. 2012), *GmNHX1* in *Lotus corniculatus* (Sun et al. 2006), *GmNHX2* in *A. thaliana* (Zhou et al. 2009) and *TaNHX2* in *G. max* (Cao et al. 2011). Vacuolar membrane H^+-pyrophosphatases and H^+-ATPases are involved in ion regulation along with NHX1 in vacuole. Further, overexpression of *AtH⁺-PPase* also enhanced the salt and drought tolerance ability of transgenic *M. sativa* (Bao et al. 2009). Similar results were also observed in case of transgenic *M. sativa* co-expressing *ScNHX1* and *ScVP* (Liu et al. 2013) as well as in *L. corniculatus* co-expressing *ZxNHX* and *ZxVP1* (Bao et al. 2014).

2.5. Concluding remarks and future perspectives

Regulation of the ever increasing menace of salinity has become a prime concern to overcome the devastating effect exerted on agricultural production which in turn, poses as a major challenge in fulfilling human consumption rates across global population. Legumes are economically important crops, generally categorized as salt sensitive and the gross production suffers a major setback owing to salt stress in cultivable lands.

Traditional breeding techniques have been employed for decades for development of wide range of crop species. Legumes mostly have a limited genetic variability to explore for abiotic stresses like salinity, drought and low- and high-temperature conditions. Another technique employed for generation of salt-tolerant crops is the transgenic approach, which further requires improvisation of transgene delivery strategies in recalcitrant legume crops. The transgenic approach emphasizes on altering expression levels of known “stress responsive genes” and also exploits the level of salt tolerance upon introduction of novel genes (Yamaguchi and Blumwald 2005). Successful attempts have been made till date, in improving salt tolerance trait(s) in transgenic legumes by exploiting role of membrane transporters, osmoprotectants, antioxidants, regulatory elements and transcription factors. Further detailed understanding of stress responses at the molecular front, can be beneficial in deciphering pathways and regulatory networks involved in

salinity tolerance mechanisms in plant. Thereby, generation of improved cultivars can be achieved by detailed study of stress responses and their cross-talks and conjoint efforts in molecular breeding programs, transgenic approaches, and newly emerging biotechnological tools and high-throughput technologies.

CHAPTER 3

Isolation and functional characterization of a cowpea vacuolar Na^+/H^+ antiporter gene, *VuNHX1* and its ectopic expression in *Arabidopsis thaliana* for studying its role in salt tolerance

3.1. Introduction

Soil salinity is a major abiotic stress that limits crop growth and productivity worldwide (Kronzucker and Britto 2011). Advances in molecular genetics and understanding of complex physiological mechanisms involved in plant salt tolerance in genetically tractable glycophyte model, *Arabidopsis thaliana*, has lead to identification of a number of genes involved in salt acclimation or adaptation (Flowers et al. 1977, Flowers and Yeo 1986; Hasegawa et al. 2000; Zhu 2002, 2003; Flowers and Colmer 2008; Munns and Tester 2008). The salt tolerance genes identified through genetic loss- and gain-of-function approaches are those encoding transport determinants that mediate Na^+ homeostasis (Zhu 2002, 2003; Apse and Blumwald 2007; Horie et al. 2009; Plett and Møller 2010). Some of these determinants identified in glycophyte genetic screens are responsible for enhancing salt tolerance of halophytes and crops (Ren et al. 2005; Oh et al. 2010; Dassanayake et al. 2011a, b; Munns et al. 2012). The genes encoding transport determinants responsible for Na^+ homeostasis are $Na^+(K^+)/ H^+$ exchangers (also known as NHX-type cation/proton antiporters) that function cooperatively to prevent Na^+ uptake, increase efflux of Na^+ from cell and compartmentalize Na^+ into vacuole (Reguera et al. 2014). Sequestration of Na^+ into vacuole through the action of tonoplast Na^+/H^+ antiporter plays a key role in adjusting ionic balance (Pardo et al. 2006). Na^+/H^+ antiporters localized in vacuolar membrane, pump Na^+ in exchange of H^+ using the proton motive force generated by H^+-ATPase and pyrophosphatase to reduce Na^+ toxicity and maintain a high K^+/Na^+ ratio in cytosol leading to alleviation of salt stress (Blumwald 2000). Compartmentalization of Na^+ minimizes the exposure of cytoplasmic enzymes to toxic Na^+ (Blumwald et al. 2000). Cloning and characterization of vacuolar Na^+/H^+ antiporters have been reported in a few legumes including, *Medicago sativa* (Bao-Yan et al. 2008), *Glycine max* (Li et al. 2006), *Trifolium repens* (Tang et al. 2010), *Lotus tenuis* (Teakle et al. 2010), and *Caragana korshinskii* (Yang et al. 2012).

Cowpea (*Vigna unguiculata* L. Walp.) is one of the most important legumes that serve both as food and fodder for resource poor populace in semi-arid tropics (Singh 2005; Timko et al. 2007). High nitrogen fixing ability favoring easy adaptation to nutrionally poor soil, capacity to tolerate a wide range of soil pH (Fery

1990) and high temperatures, and ability to tolerate drought and moderate tolerance towards salinity (Murillo-Amador et al. 2006; Duzdemir et al. 2009) are the salient features of cowpea (Hall 2004). However, limited water availability is still the major environmental constraint that threatens its growth and yield. Consequently, development of cowpea cultivars endowed with enhanced tolerance to drought and salinity is expected to enhance its productivity in hostile soil. Further, cowpea serves as an ideal model legume for mining various stress-responsive genes, including *NHX1*. However, till date, NHX1 gene of cowpea has not been cloned and characterized.

In this study, we report isolation of a vacuolar Na^+/H^+ antiporter gene of cowpea (*VuNHX1*), is reported and functional characterization of cowpea *NHX1* by complementation studies in AXT3 yeast mutant. Additionally, we present the transcriptional regulation of *VuNHX1* and concomitant Na^+/K^+ content in leaves and roots of cowpea under salt stress is reported. Further, we demonstrate the functional validation of *VuNHX1* in model plant *Arabidopsis thaliana* indicating the potential role of *VuNHX1* in salt tolerance. The *VuNHX1* could be used for improvement of salt tolerance through its overexpression by cis- or trans-genic approaches in crop legumes.

3.2. Materials and methods

3.2.1. Plant material and stress treatment

Cowpea (cv. Pusa Komal) plants were grown hydroponically in Hoagland's solution in a growth chamber at 25 °C and 70 % relative humidity with a 16 hour/8 hour photoperiod and photosynthetic flux intensity of 300 µmol $m^{-2}s^{-1}$. Two weeks old seedlings were treated with 200 mM NaCl (Himedia, Mumbai, India) for 12 hours and roots were harvested, frozen immediately, and stored at -80 °C until further use.

3.2.2. Molecular cloning of *VuNHX1* by RACE approach

3.2.2.1. PCR amplification of partial fragment of *VuNHX1* using degenerate primers

Total RNA was isolated from salt-treated roots of cowpea using AMBION RNAqueous® Kit (Ambion Inc., Foster City, California, USA), according to the manufacturer's instructions. One microgram RNA was used for cDNA preparation

using Revert Aid ™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA), as per manufacturer's protocol. The partial fragment of *VuNHX1* was amplified using a pair of degenerate primers (FP: 5'-GGKTTTCARG TRAAAAAGAAGCA-3' and RP: 5'-GTRCTSGTGATCATRAYTGCATT-3') designed from the conserved region of transmembrane domains of plant NHX antiporters submitted in NCBI database. The PCR reaction consisted of 1X Taq Buffer, 200 µM dNTP mix, 20 µM forward and reverse primers, 1 unit of Ex-Taq™ ploymerase (Takara Inc., Otsu, Shiga, Japan), sterile water and 1 µl cDNA as template, in a reaction volume of 25 µl. The PCR was performed in Thermal cycler (BioRad, Hercules, California, USA) with the following condition: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 50 ^{0}C for 30 sec, 72 ^{0}C for 1 min with 35 cycles, and a final extension at 72 ^{0}C for 10 min. Based on the resulting partial fragment, gene specific primers were designed for amplification of 5'- and 3'- untranslated regions of *VuNHX1*.

3.2.2.2. 5'-RACE for amplification of 5'-UTR fragment of *VuNHX1*

The 5'-untranslated sequence was obtained by using "The 5'RACE System for Rapid Amplification of cDNA Ends Kit" (Version 2.0, Life Technologies/ Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. The cDNA synthesized using gene specific primer (GSP: 5'- GCACCCAAAGTTATGACAGCAC -3') and superscript ™ II reverse transcriptase enzyme (Invitrogen, Carlsbad, CA, USA), was purified using a SNAP column for efficient removal of unincorporated dNTPs and remaining GSP. Terminal transferase (TdT) enzyme generated a homopolymeric tail by incorporating dCTPs to the 3'end of the purified cDNA. The dC-tailed cDNA was amplified using abridged anchor primer (AAP: 5'- GGCCACGCGTCGACTAGTACG GGIIGGGIIGGGIIG-3') and gene specific primer (GSP1: 5'- TGTACCAATAGCACCAA ACAACATGATG -3'). The PCR condition was: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 60 ^{0}C for 30 sec, 72 ^{0}C for 1 min with 30 cycles, and a final extension of 72 ^{0}C for 10 min. A nested PCR was performed with abridged universal anchor primer (AUAP: 5'- GGCCACGCGTCGACTAGTAC -3') and gene specific primer (GSP2: 5'- GTCATGAAGT TAACAAAAAACTGC -3') using the first pcr product as template. The PCR condition was: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, 72 ^{0}C for 30 sec with 35 cycles, and a final extension of 72 ^{0}C for 10 min.

3.2.2.3. 3'-RACE for amplification of 3'-UTR fragment of *VuNHX1*

The 3'-untranslated sequence was obtained by using "Rapid Amplification of cDNA Ends Kit" (Version E, Life Technologies/ Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. cDNA was synthesized using a dT Adapter primer (AP: 5'- GGCCACGCGTCGACTAGTAC$(T)_{17}$-3'). The 3'-RACE PCR was performed with gene specific primer (GSP3:5'- GCTGTATATTGGAAGGCACTCT -3') and abridged universal anchor primer (AUAP: 5'- GGCCACGCGTCGACTAGTAC -3'). The PCR condition was: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 1 min and 30 sec with 30 cycles, and a final extension of 72 ^{0}C for 10 min.

3.2.2.4. Cloning of PCR fragments of *VuNHX1* into TA-cloning vector

The PCR products obtained from the above conditions were cloned into TA cloning vector pTZR/T (Ins TAclone™ PCR Cloning Kit, Thermo Fisher Scientific, Waltham, MA, USA) and transformed into *E. coli* DH5α competent cells. The blue-white screening was employed for selection of recombinant bacterial colonies on solid Luria Broth (LB) (Himedia, Mumbai, India) plates supplemented with 100 mg/l ampicillin (Duchefa, Haarlem, Netherlands) , 40 µl of 20 mg/l X-Gal (Thermo Fisher Scientific, USA) and 40 µl of 20 mg/l IPTG (Thermo Fisher Scientific, USA), incubated at 37°C in static incubator chamber. The white clones were selected, recombinant plasmid was isolated and confirmed by digestion with restriction digestion enzymes XbaI and BamHI and sequenced. The contiguous sequences were aligned to obtain full length of *VuNHX1* cDNA, and the sequence was submitted in NCBI database.

3.2.2.5. PCR amplification of CDS of *VuNHX1*

The full length *VuNHX1* was amplified using forward (FLP: 5'- ATGGTCTTTGAAA TCAGTTCTGTTGTTTC -3') and reverse (RLP: 5'- TCAACGCCATTGATGACC ATTACGTTC -3') primers, cloned into TA cloning vector pTZR/T (2.8 kb) and sequenced (GenBank Accession No. JN641304.2). The PCR reaction consisted of 1X Taq Buffer, 200 µM dNTP mix, 10 µM forward and reverse primers, sterile water, 1 unit of Ex-Taq ploymerase and 1 µl cDNA as template, in a final reaction volume of 25 µl. The PCR conditions were 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 58 ^{0}C for 30 sec, 72 ^{0}C for 1 min and 30s with 35 cycles, and 72 ^{0}C for 10 min. The PCR amplicon was

cloned into a TA cloning vector pTZR/T (2.8 kb) and the recombinant clone pTZR/T*VuNHX1* (4.4 kb) was sequenced.

3.2.2.6. Agarose gel electrophoresis

The PCR products were mixed with 6x loading dye and electrophoresed on 1% agarose gel containing ethidium bromide (10 mg/ml). Electrophoresis was carried out at 90 V for 1-2 h. An aliquot (500 ng) of Lambda HindIII/EcoRI DNA ladder (Thermo Fisher Scientific, USA), and middle range DNA ruler (Thermo Fisher Scientific, USA; Bangalore Genei, India) were used as molecular weight markers. Standard molecular biology techniques were used for all DNA manipulations (Sambrook 1989).

3.2.3. In-silico analysis

Multiple sequence alignment and phylogenetic analysis were performed using Clustal W (Thompson et al. 1997). Based on the neighbor-joining (NJ) algorithm, an unrooted phylogenetic tree was constructed using MEGA4: Tree Explorer software (Tamura et al. 2007). Hydrophobicity plot and transmembrane domain prediction was performed using TMPRED (Hofmann and Stoffel 1993). Secondary structure prediction was carried out using SOPMA (Geourjon and Deleage 1995). Post-translational modification of VuNHX1 protein was predicted by searching for conserved motifs of N- and O- glucosylation and N-myristoylation sites using ScanProsite (Gattiker et al. 2002).

3.2.4. Southern hybridization for *VuNHX1* copy number analysis

For *VuNHX1* copy number analysis, 20 µg of cowpea genomic DNA was digested with restriction endonucleases, EcoRI, HindIII and PstI. Digested genomic DNA was electrophoretically fractionated on 1% agarose gel and blotted onto Zeta-Probe membrane (BioRad, Hercules, California, USA). The blot was hybridized with DIG-labeled PCR product (0.9 kb), corresponding to the coding region of *VuNHX1* using the primers, *VuNHX1*-SHFP: 5'- GCTGTATATTGGAAGGCACTCT- 3' and *VuNHX1*-SHRP: 5'- TCAACGCCATTGATGACCAT TACGTTCAG - 3'. Southern hybridization was carried out using hybridization solution containing 50% formamide, 5X SSC, 5X Denhardt's solution, 0.05 M sodium phosphate pH 6.5, 0.1% SDS, 10% Dextran sulfate, 0.1 mg/ml sheared denatured salmon-sperm DNA and 20 ng/ml probe at

42°C for 18 hours. Washing and detection was performed according to instructions of the DIG Labeling and Detection system (Roche Diagnostics, Mannheim, Germany).

3.2.5. Complementation studies of *VuNHX1* in yeast mutant

3.2.5.1. Cloning of *VuNHX1* in yeast expression vector

The ORF of *VuNHX1* cloned in TA cloning vector pTZR/T was digested and subcloned in the yeast expression vector pYES2.0 (Invitrogen, USA). The ligation reaction was carried out using 1U of DNA ligase enzyme (NEB, UK) at 16°C overnight and transformed into *E.coli* DH5α competent cells. The recombinant colonies were screened and confirmed by restriction digestion of the recombinant plasmid pYES*VuNHX1* (7.5 kb). The digested fragments were resolved in 1% agarose gel, stained with 10 mg/ml ethidium bromide and visualized under UV transilluminator.

3.2.5.2. Yeast complementation assay

The functional characterization of *VuNHX1* was investigated by yeast complementation assays. The *VuNHX1* ORF cloned to yeast expression vector pYES2.0 was transformed into the mutant yeast strains, W303-1B (*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and AXT3 *(Δ ena1- 4::HIS3 Δnha1::LEU2 Δnhx1::TRP1, ura3-1)*. Yeast strains were grown in YPD (1% yeast extract, 2% peptone and 2% glucose), YPGal (1% Yeast extract, 2% peptone and 2% galactose), SC and APGal synthetic minimal media (10 mM arginine, 8 mM phosphoric acid, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, 2% Galactose, trace vitamin, and minerals; pH-4.0) supplemented with appropriate amino acids as indicated. AXT3 was transformed with empty pYES2.0 and recombinant pYES2.0*VuNHX1* vector and thereby, labeled as AXTYES2.0 and AXTYESVuNHX1, respectively, by Lithium acetate method (Gietz et al. 1992) and selected on SC ura^- medium.

3.2.5.3. Growth assay under stress treatment

For growth assay, seed cultures were diluted to an OD_{600} of 0.006, inoculated to liquid APGal media (Rodriguez-Navarro and Ramos 1984) supplemented with different concentrations of NaCl, KCl, LiCl and grown at 30 °C for 48 hours. For complementation assay, saturated liquid cultures (OD_{600}- 0.8) of each strain were serially diluted to 10, 100 and 1000 fold and spotted on APGal and YPGal solid media

supplemented with or without 75 mM NaCl, 0.5 M KCl, 50 mM LiCl and 50 µg/ml Hygromycin-B. Growth was monitored after 3 days.

3.2.5.4. Intracellular measurement of Na^+ and K^+ content

Yeast strains were grown in liquid APGal media, pH 4.0 supplemented with or without 70 mM NaCl, and harvested at an OD_{600} of 0.3-0.4. Cells were centrifuged at 3000 g/ 3 min, washed twice in ice-cold 10 mM $MgCl_2$, 10 mM $CaCl_2$ and 1mM HEPES buffer and resuspended in the same buffer. The relationship between cell density (Absorbance at OD_{600}) and yeast dry weight was determined. Total intracellular ion was determined by addition of HCl to a final concentration of 0.4 % and incubated at 95 °C for 20min. After removal of cell debris, the supernatant was measured for presence of total Na^+ and K^+ content. Similarly, cells were grown and washed as above and resuspended in 2 % cytochrome c, 18 µg/ml antimycin, 1 mM HEPES, 10 mM $MgSO_4$, 10 mM $CaCl_2$, and 5 mM 2-Deoxy D-Glucose solution. Cytochrome c selectively permeabilizes the plasma membrane. After 20 min incubation at room temperature, cells were washed thrice with the same solution without cytochrome c. Cytoplasmic ion content was determined by pooling the supernatants. The remaining vacuolar ions were extracted with addition of HCl to a final concentration of 0.4 % and incubated at 95 °C for 20 min (Venema et al. 2003). The Na^+ and K^+ distribution in the cytoplasmic and vacuolar fractions were measured in Flame Photometer (Systronics, MP, India). The formula used for calculation of Na^+ and K^+ :-

$$\text{Ion content}\left[\frac{\mu\text{mol}}{\text{g DW}}\right] = \left[\frac{(\text{Conc. in } \mu\text{g/ml}) \times \text{Dilution factor}}{\text{DW of tissue used in extraction} \times \text{MW of ion 39.098 for } K^+ \text{ and 22.99 for } Na^+}\right]$$

3.2.6. Expression analysis of *VuNHX1* using Semi-quantitative RT-PCR

Ten days old cowpea seedlings were subjected to various abiotic stress conditions such as salt, dehydration and cold. The seedlings were transferred to 200 mM NaCl solution and incubated at 4 °C in growth chamber for salt and cold stress, respectively. For dehydration stress, the seedlings were removed from the nutrient solution (Hoagland's medium) and dehydrated on filter paper at room temperature

and 60% humidity under dim light. The stressed tissues were sampled at different time intervals, frozen immediately and stored at -80 °C until further use.

Expression pattern for *VuNHX1* in response to different abiotic stresses such as salt (200 mM NaCl), dehydration and cold (4 ^{0}C) was studied at different time intervals (0, 6, 12, and 24 hours). Tissue specific response to salt stress was investigated at different time intervals (0, 6, 12, and 24 hours). Total RNA was isolated using RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands) and quantified in Nanovue™ Plus Spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Semi-quantitative RT-PCR was performed using gene specific primers (RFP: 5'- GCTGTATATTGGAAGGCACTCT- 3' and RRP: 5'-CAATGTCCAAGGCATCCATACC -3'). The PCR reaction consisted of 1X Taq Buffer, 200 µM dNTP mix, 10 µM forward and reverse primers, 1 unit of standard Taq ploymerase (Sigma-Aldrich, St. Louis, MO, USA), sterile water and 1 µl cDNA as template, in a reaction volume of 25 µl. The PCR was performed in Thermal cycler with the following conditions: 95 °C for 10 min; 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec and 72 °C for 10 min for 28 cycles. Housekeeping *VuNSR9* primers (FNP: 5'- GCACAGTTTGGGTATATTG-3' and RNP: 5'- GAGTAAAACTGGCAAAAATTAG-3') were used as an internal control. The PCR conditions were as follows: 95 °C for 10 min; 95 °C for 30 sec, 49 °C for 30 sec, 72 °C for 30 sec and 72 °C for 10 min for 28 cycles. Semi-quantitatative RT-PCR was repeated three times. The PCR products were analyzed in 2 % agarose gel stained with 10 mg/ml ethidium bromide and visualized under UV-transilluminator. The intensity of the DNA bands was analyzed using ImageJ software.

3.2.7. Measurement of total ion content in salt stressed cowpea seedlings

Leaves and roots of untreated and salt-treated cowpea seedlings were harvested at different time intervals (0, 6, 12, 18, 24, and 48 hours). The samples were dried, digested with concentrated HNO_3 at 90°C for 30 min and centrifuged at 12,000 rpm for 10 min (Munns et al. 2010). The suspension was diluted with milliQ water and analyzed for Na^+ and K^+ content in flame photometer, as described by the manufacturer's instructions. The formula used for calculation of Na^+ and K^+ :-

$$\text{Ion content}\left[\frac{\mu\text{mol}}{\text{g DW}}\right] = \left[\frac{(\text{Conc. in } \mu\text{g/ml}) \times \text{Dilution factor}}{\text{DW of tissue used in extraction} \times \text{MW of ion 39.098 for } K^{+} \text{ and 22.99 for } Na^{+}}\right]$$

3.2.8. Plant binary constructs preparation for plant transformation

3.2.8.1. Preparation of pCAMBIA230135S::*VuNHX1*

The binary vector pCAMBIA2301 (Cambia, Canberra and Brisbane, Australia) used for the plant transformation harbored *nptII* as antibiotic selection marker and *gus-A* as reporter gene in the T-DNA region. The *VuNHX1* CDS (1.6 kb) was subcloned into plant binary vector pCAMBIA 2301 (11.6 kb) flanked by cauliflower mosaic virus, CaMV 35S promoter and terminator at *Pst*I restriction site from intermediate vector pRT101*VuNHX1* (4.9 kb). The resulting recombinant plant binary vector was labeled as pCAMBIA230135S::*VuNHX1* (13.9 kb) and transformed into *E.coli* competent cells. The recombinant colonies were screened using blue-white selection of bacterial colonies and confirmed by restriction digestion.

3.2.8.2. Preparation of pCAMBIA2301RD29A::*VuNHX1*

Further, a 0.898 kb promoter fragment of AtRD29A (DQ071887.1) was amplified from *A. thaliana* genomic DNA using primers RD29F: 5'- CTGAAATTTCTGCAAGA ATC -3' and RD29R: 5'- TCCAATAGAAGTAATCAAACC -3', and cloned into TA cloning vector pGEMT easy (3.015 kb) (Promega, Madison, WI, USA). The promoter fragment *Eco*RI- RD29A- *Eco*RI obtained from recombinant vector pGEMTRD29A (4.2 kb) was cloned into EcoRI digested recombinant binary vector pCAMBIA230135S::*VuNHX1* (13.9 kb) by replacing the 0.4 kb 35S promoter fragment from 35SP::*VuNHX1*::35STer cassette. The resulting binary vector was named pCAMBIA2301RD29A::*VuNHX1* (14.4 kb) and transformed into *E.coli* competent cells. The recombinant colonies were screened using blue-white selection of bacterial colonies and confirmed by restriction digestion. All molecular biology techniques were employed for all DNA manipulations (Sambrook et al. 1989).

3.2.8.3. Mobilization of plant binary constructs to *Agrobacterium tumefaciens*

The recombinant plant binary vectors, pCAMBIA230135S::*VuNHX1* (13.9 kb) and pCAMBIA2301RD29A::*VuNHX1* (14.4 kb) were transferred into *A. tumefaciens* GV3101 strain via electroporation at 1250 V with capacitance of 25 mF and resistance of 400 ohm. The constructs were used for transformation of *Arabidopsis thaliana* (ecotype Columbia) plants via floral dipping method (Clough and Bent 1998). The T_1 transgenic plants were screened on ½ MS medium (Duchefa, Haarlem, Netherlands) supplemented with 50 mg/l kanamycin (Duchefa, Haarlem, Netherlands). The transgenic selections were continued until T_4 generation to obtain homozygous 35S::*VuNHX1* and RD29A::*VuNHX1* transgenic lines.

3.2.9. RNA extraction and quantitative expression analysis in transgenic *Arabidopsis* by Real Time PCR

Total RNA was extracted from wild-type and T_4 transgenic independent lines for pCAMBIA230135S::*VuNHX1* (13.9 kb), and pCAMBIA2301RD29A::*VuNHX1* (14.4 kb) binary constructs using RNeasy Plant Mini Kit quantified in Nanovue™ Plus Spectrophotometer and cDNA was prepared using Revert Aid ™ First Strand cDNA Synthesis Kit. The gene specific forward primer (VrRTF: 5'- TGATCCAATCCATCG TCCAA -3') and 35S poly-A reverse primer (TerparR: 5'-GCGAAACCCTATAAGAACC CTAATTCC-3') were used for amplification of a 0.28 kb fragment of *VuNHX1*::35S poly-A cassette in transgenic *A. thaliana* lines. Housekeeping (UBQ1FP: 5'-AGAGCTGTCAACTGC AGGAAGAA-3' and UBQ1RP- 5'-ACAAGAAAAACAAACCCTATCA AAGG) primers were used to amplify a 150 bp fragment of *AtUbiquitin* to be used as an internal control. Real time PCR was performed using USB VeriQuest™ SYBR Green qPCR Master Mix (2X) (Affymetrix, Santa Clara, CA, USA) and primers at a final concentration of 200nM for both genes in 7500 Real-Time PCR System (Applied Biosystem, Foster City, California, USA), as per manufacturer's protocol. The experiment was repeated twice independently with three replicates. The expression values relative to the standard curve was calculated for each sample. The relative expression level of transgene *VuNHX1* in wild-type (WT) and transgenic *Arabidopsis* lines was estimated by normalizing *VuNHX1* expression values with respect to housekeeping *AtUBQ1* expression values. The real-time PCR products were run on 2 % agarose gel for analying the expected product size.

3.2.10. Salt tolerance assays of transgenic 35S::*VuNHX1* and RD29A::*VuNHX1* *Arabidopsis* lines

3.2.10.1. Effect of salt stress on seed germination

Wild-type (WT) and T_4 homozygous transgenic *Arabidopsis* seeds were vernalized at 4 °C on ½ MS medium (Murashige and Skoog 1962) supplemented with or without 150 mM NaCl for 3 days and transferred to growth chamber maintained at 22 °C and 60 % relative humidity with a 16 hour /8 hour photoperiod for 7 days.

3.2.10.2. Measurement of growth parameters

Wild-type (WT) and T_4 homozygous transgenic *Arabidopsis* seeds were grown on ½ MS medium (Murashige and Skoog 1962) for 5 days and thereafter transferred to ½ MS medium supplemented with 150 mM and 200 mM NaCl. The difference in root length of wild-type and transgenic lines (35S::*VuNHX1* and RD29A::*VuNHX1*) was measured after 7 days of NaCl treatment. The emergence of lateral roots in each case was also studied. Mean data was collected from ten replicates (n=10) for wild-type (WT) and T_4 kanamycin selected transgenic *Arabidopsis* lines.

3.2.10.2.1. Measurement of physiological parameters under salt stress

To test the effect of salt stress on mature plants, germinated seedlings were initially grown on ½ MS medium for 5 days and then subsequently transferred to soilrite and grown for 2 weeks. The WT and transgenic lines (35S::*VuNHX1* and RD29A::*VuNHX1)* were subjected to salt stress for a period of 2 weeks by watering them with ½ MS nutrient liquid media supplemented with 200 mM NaCl. Mean data was collected from three replicates (n=3) for wild-type (WT) and T_4 kanamycin selected transgenic *Arabidopsis* lines.

3.2.10.2.1.1. Measurement of Na^+ and K^+ in transgenic plants

The leaves were harvested for Na^+ and K^+ estimation using method described in section 3.2.7.

3.2.10.2.1.2. Chlorophyll content

The chlorophyll content was measured according to Lichtenthaler (1987). Briefly, shoot samples were homogenized in 95 % ethanol and the lysate was centrifuged at 3,000 rpm for 10 min. The absorbance was recorded for the extract at wavelength of

648 and 664 nm in Beckman UV/Vis Scanning Spectrophotometer (Beckman Coulter, USA). The total chlorophyll content was calculated using the formulae:-

$$Chl_{(a+b)}\,(mg/ml) = ((5.248 \times A_{664}) + (22.4 \times A_{648}))$$

3.2.10.2.1.3. Lipid peroxidation

Lipid peroxidation was measured according to Heath and Packer (1968) as the amount of malondialdehyde (MDA) determined by the thiobarbituric acid (TBA) reaction. 0.2 g of fresh leaf samples were homogenized with 5 ml of 0.25 % TBA containing 10 % TCA (tricloroacetic acid). The homogenate was boiled for 30 min at 95 °C and centrifuged at 10,000 g for 10 min Absorbance values were recorded at 532 nm and values corresponding to non-specific absorption at 600 nm were substracted. The MDA content was calculated according to the molar extinction coefficient of MDA (155 $mM^{-1}cm^{-1}$) using the formulae:-

$$\text{MDA content}\left[\frac{\text{nmol}}{\text{g FW}}\right] = \frac{\left[\frac{1000 \times (Abs_{532}-Abs_{600})}{155}\right]}{\text{g tissue sample}} \times \text{Dilution factor}$$

3.2.10.2.1.4. Proline content

Proline colorimetric estimation was performed following method by Bates et al. 1973. Leaf samples (0.5 g) were homogenized with 5.0 ml of sulfosalicylic acid (3%). 2 ml of homogenate was filtered through Whatman filter paper (No. 2) and incubated with 2 ml glacial acetic acid and 2 ml ninhydrin reagent at a ratio of 1:1:1 in boiling water bath at 100°C for 30 min. After cooling, 4 ml toluene was added to the reaction mixture, mixed vigorously and absorbance measured at 520 nm. The standard graph for proline was made and the formulae used for calculation of proline was:-

$$\text{Proline}\left[\frac{\mu\text{moles}}{\text{g tissue}}\right] = \frac{\left[\frac{\mu\text{g proline/ml} \times \text{ml toluene}}{115.5}\right] \times 5}{\text{g tissue sample}}$$

3.2.11. Statistical analysis

Statistical comparison between the variances was determined by ANOVA and significant differences between mean values were determined by Bonferroni analysis. Statistically significant mean values were denoted as different letters ($P \leq 0.05$).

3.3. Results and discussion

3.3.1. Isolation and in-silico analysis of *VuNHX1*

The isolation of full length *VuNHX1* cDNA was performed from salt stressed cowpea roots. Using degenerate primers designed from conserved domains of reported plant legume NHX proteins in NCBI (Fig. 3.1), a 0.977 kb PCR amplicon was obtained (Fig. 3.2) and gene specific primers were designed specific to partial *VuNHX1* fragment. The 0.335 kb (Fig. 3.3) and 0.904 kb (Fig. 3.4) fragments corresponding to the 5'- and 3'-untranslated regions of *VuNHX1* cDNA, respectively were obtained by 5'- and 3'-RACE approach. The *VuNHX1* cDNA was found to be 1981 bp (Genbank Accession No. JN641304.2). The open reading frame of *VuNHX1* consisted of 1,629 bp (Fig. 3.5), encoding a polypeptide of 542 amino acid residues with an estimated molecular mass 59.60 kDa and isoelectric point 6.76 as predicted using ExPaSy bioinformatic tools for protein structure analysis (http://www.expasy.org/tools/). The PCR fragments obtained in the process were cloned into TA cloning vector pTZR/T (2.8 kb) (Fig. 3.2b-3.5b) and confirmed by restriction digestion (Fig. 3.2-3.5). The multiple sequence alignment of VuNHX1 with other plant Na^+/H^+ exchangers showed higher identity with *Vigna radiata* VrNHX1 (97.42 %), *Glycine max* GmNHX1 (92.44 %), *Caragana korshinskii* CkNHX1 (88.1 %), *Lotus tenuis* LtNHX1 (87.45 %), *Medicago sativa* MsNHX1 (87.25 %), *Cicer arietinum* CaNHX1 (86.9 %), *Trifolium repens* TrNHX1 (86.51 %), and *Galega orientalis* GoNHX1 (84.84 %) (Fig. 3.6). Phylogenetic analysis indicated that *VuNHX1* clustered into Class-I type IC-NHX exchangers and was more closely related to legume NHX homologs, thus distinct

from Na^+/H^+ antiporters belonging to Class-II type IC-NHX and PM-NHE clade (Fig. 3.7). The hydropathy plot of VuNHX1 protein sequence indicated highly hydrophobic N-terminal end with 11 putative transmembrane domains and a longer hydrophilic C-terminal end (Fig. 3.8). The amiloride binding motif, ^{84}-LFFIYLLPPI-^{93} in VuNHX1 is highly conserved among eukaryotic Na^+/H^+ exchangers (Fig. 3.8). Secondary structure prediction by SOPMA indicated occurrence of alpha helix (49.45%), extended strand (14.21%), beta turn (3.69%), and random coil (32.66%) as shown in Fig. 3.9. The putative post-translational modification sites predicted by ScanProsite software are as follows: 2 *N*-glycosylation sites, 14 phosphorylation sites, 9 *N*-myristoylation sites, and 1 Leucine Zipper site (Table 3.1).

3.3.2. Copy number analysis of *VuNHX1*

Southern blot of digested cowpea genomic DNA hybridized with the probe specific to *VuNHX1* CDS showed two, one and three hybridization signals for EcoRI, HindIII, and PstI digested genomic DNA, respectively (Fig. 3.10). It was presumed that a single copy of *VuNHX1* existed in cowpea genome, as a single band was detected for HindIII digested genomic DNA. The detection of more than one signal for *Eco*RI and *Pst*I digested genomic DNA might be due to occurrence of these restriction sites in *VuNHX1* genomic region. However, previous reports indicated occurrence of *NHX1* in multiple copies in *B. napus* and *M.zumi* (Wang et al. 2003; Qingxia et al. 2009).

3.3.3. *VuNHX1* complements salt sensitive yeast mutant

3.3.3.1. Yeast complementation assay

Substantial evidence affirm functional similarity between plant and yeast endosomal Na^+/H^+ exchanger, thereby providing an excellent platform to elucidate structural and regulatory attributes of novel plant Na^+/H^+ exchangers in relation to salt tolerance mechanisms (Darley et al. 2000; Quintero et al. 2000). It has been observed that plasma membrane and endosomal Na^+/H^+ antiporters in yeast are involved in ion homeostasis, and cellular pH maintenance. The yeast mutant AXT3 lacks the potential plasma membrane antiporters, ENA 1-4 and NHA1 required for Na^+ efflux, along with vacuolar antiporter *NHX1* for efficient compartmentation of toxic Na^+ in vacuole. Therefore, *Δena Δnha1 Δnhx1* mutant shows high sensitivity to increased level of Na^+ and K^+ at low pH condition.

The ORF of *VuNHX1* was cloned into yeast expression vector pYES2.0 and used for validation of role of *VuNHX1* in yeast mutant AXT3 (Fig. 3.11). Thus, AXT3 cells show increased sensitivity to Na^+ in deficit of effective protective machinery. In liquid medium, growth condition was analyzed under different concentrations of NaCl (0, 50, 75, and 100 mM), LiCl (0, 15, 20 and 25 mM) and KCl (0, 0.5, 1.0, and 1.5 M) as shown in Fig. 3.12. A statistically significant difference ($P \leq 0.05$) was observed in growth pattern of W303-1B wild-type and AXTYES2.0 cells under stress conditions. Under salt stress, no significant difference in growth was observed between AXTYES2.0 and AXTVuNHX1 cells at 50 mM NaCl, however, a marked difference was observed with increase in NaCl concentration (75 and 100 mM NaCl) (Fig. 3.12). Similarly, pronounced difference under increase in external [K^+] between AXTYES2.0 and AXTVuNHX1 cells was observed at 1 M KCl unlike, 0.5 and 0.75 mM KCl stress. AXTYES2.0 cells were able to survive at a similar rate as that of AXTVuNHX1 cells under 15 and 20 mM LiCl stress (Fig. 3.12). But, significant growth difference was observed at 25 mM LiCl suggesting the efficient survival of AXTVuNHX1 than AXTYES2.0 cells owing to the cation/ proton antiporter activity of *VuNHX1*. The yeast complementation assay was performed on solid APGal minimal medium and similar results were obtained as observed in liquid media supplemented with different concentrations of Na^+, K^+ and Li^+. The survival efficiency of AXT3 strains expressing *VuNHX1* was greater than AXT3 cells transformed with empty pYES2.0 vector (Fig. 3.13a). Wild-type strain W303-1B was taken as control in each case. Similarly, heterologous expression of plant endosomal Na^+/H^+ antiporters, *AtNHX1* (Hernández et al. 2009), *MzNHX1* (Qingxia et al. 2009), *MsNHX1* (Bao-Yan et al. 2008), *TNHXS1* (Gouiaa et al. 2012), *AeNHX1* (Qiao et al. 2007), *CmNHX1* (Wang et al. 2011), *ThNHX1* (Wu et al. 2009), *PgNHX1* (Rajagopal et al. 2007) in AXT3 have been able to ameliorate its salt sensitive phenotype.

Hygromycin-B, a cationic protein synthesis inhibitor is known to confer hypersensitivity in *Δnhx1 and Δnhx1 Δnha1* mutants, owing to its increased uptake by cells under changes in plasma membrane electrical potential but, no effect on wild-type and Δnha1 mutants with intact *ScNHX1* (Ali et al. 2004; Brett et al. 2005). Heterologous expression of *VuNHX1* under GAL1-inducible promoter restored salt tolerance in AXTVuNHX1 cells at 50 µg/l Hygromycin-B (Fig. 3.13b). The growth sensitivity of AXTVuNHX1 cells under a higher concentration of Hygromycin-B was

least affected indicating the functional complementation of *ScNHX1* by heterologous expression of *VuNHX1* as compared to AXTYES2.0 cells lacking effective salt tolerance machinery.

3.3.3.2. Measurement of Na^+ and K^+ accumulation

The total intracellular ion distribution in the yeast cells (W303-1B, AXTTES2.0, AXTVuNHX1) was determined using flame photometer. Under normal physiological and salt stress condition, no significant difference was observed in total [Na^+] in yeast cells, moreover, AXTYES2.0 cells showed relatively higher value than W303-1B and AXTVuNHX1 cells. However, statistically significant difference ($P \leq 0.05$) was observed in total [K^+] in yeast cells (Fig. 3.14). This can be assumed as the activity of other antiporters in vacuole involved in K^+ transport other than ($Na^+/K^+/H^+$) antiporter, VuNHX1. Further, ion analysis in vacuolar and cytoplasmic region was studied for better understanding of distribution of ions in wild-type and mutants harboring null pYES2.0 vector and pYES*VuNHX1*. Under normal physiological condition, no significant difference was observed in vacuolar Na^+ accumulation between yeast strains, unlike, K^+ accumulation which was significantly higher in W303-1B and AXTYESVuNHX1 as compared to AXTYES2.0 (Fig 3.14). Under salt stress, Na^+ accumulation in W303-1B and AXTYESVuNHX1 was comparable, however, AXTYES2.0 cells exhibited 2.1 and 1.9 times lower vacuolar Na^+ than wild-type and *Δena Δnha1 Δnhx1* mutant expressing *VuNHX1* (Fig. 3.14). This can be attributed to the fact that AXTYYES2.0 cells lack vacuolar antiporter *ScNHX1* involved in sequestration of cations. Further, vacuolar K^+ accumulation under salt stress was significantly different in each strain with W303-1B and AXTVuNHX1 sequestering 2.4 and 1.94 times higher amount of K^+ as compared to AXTYES2.0 cells, respectively. The comparatively higher K^+ in AXTVuNHX1 than AXTYES2.0 cells indicated the possible role of *VuNHX1* in compartmentalizing K^+ along with Na^+ in vacuole. Higher [Na^+] was observed in the cytoplasmic region of AXTYES2.0 cells as compared to W303-1B and AXTVuNHX1 cells owing to lack of plasma membrane and vacuolar antiporters involved in exclusion and compartmentalization of excess toxic ions. Though W303-1B and AXTVuNHX1 cells were statistically non-significant ($P \leq 0.05$), but, AXTVuNHX1 cells exhibited 1.25 times higher cytoplsamic Na^+. This can be explained due to the loss of NHA exchanger activity in AXT3 cells which

cannot be solely compensated by *VuNHX1* complementation of Sc*NHX1* (Fig. 3.14). Similar findings were cited for *OsNHX1* expressed in *Δena Δnha1 Δnhx1* mutant (Kinclová-Zimmermannová et al. 2004). Moreover, *VuNHX1* expression in AXT3 showed enhanced K^+ distribution within both cytoplasmic and vacuolar fractions in coherence with the suggested vacuolar cation/proton activity of *VuNHX1*. This data was in accordance with expression of *AtNHX1* (Quintero et al. 2000) and *TNHXS1* (Gouiaa et al. 2012) in AXT3 mutant.

3.3.4. Expression pattern of *VuNHX1* under abiotic stress by semi-quantitative RT-PCR

The expression of *VuNHX1* was studied in roots and leaves of cowpea plants under various abiotic stresses by semi-quantitative RT-PCR. The expression of *VuNHX1* varied with salt, cold and dehydration stress at whole plant level. In particular, treatment with salt stress showed marked increase in transcript accumulation after 12 hours in both leaves and roots of cowpea seedlings (Fig. 3.15). The increased expression of *VuNHX1* under salt stress clearly indicated its role in restoring tolerance in plants under salinity condition. Similar expression pattern was also observed for *ZmNHX* (Zorb et al. 2005), *ThNHX1* (Wu et al. 2009) and *AlNHX1* (Zhang et al. 2008) but, contradictory expression pattern was observed for *TrNHX1* (Tang et al. 2010), *MsNHX1* (Bao-Yan et al. 2008) and *DmNHX1* (Zhang et al. 2012). Intriguingly, sharp increase in expression of *VuNHX1* at 6 and 12 hours of dehydration stress followed by decrease at 24 hours. This experimental evidence was in contradiction with the results shown for *AtNHX1* (Shi and Zhu 2002). However, in case of *PeNHX1,* an initial up-regulation in transcript level was observed at 2 hours of dehydration treatment followed by a gradual decrease (Rajagopal et al. 2007). Interestingly, an up-regulation of *NHX1* transcript level was observed for *E. globulus* (Baltierra et al. 2012), *G. max* (Li et al. 2006), and *O. sativa* (Fukuda et al. 2004) under dehydration stress. A 3.75 fold increase in expression was recorded at 6 hrs of cold stress and decrease to 2.2 fold with further increase in cold stress duration (Fig. 3.16). However, down-regulation of *NHX* transcripts under cold stress has also been observed in *P. glaucum* (Rajagopal et al. 2007), *T.halophila* (Wu et al. 2009) with no expression in *A. thaliana* (Shi and Zhu 2002). The increased

expression level under cold stress might be accounted due to cross-talk between *NHX1* and other stress components involved during low temperature stress.

3.3.5. Na^+ and K^+ measurement in salt stressed cowpea seedlings

The Na^+ and K^+ content in leaves and roots of untreated and salt-treated cowpea seedlings at different time intervals (0, 6, 12 18, 24, and 48 hours) was measured by flame photometer (Systronics, India). Under normal physiological (control) condition, [Na^+] was almost similar in leaves and roots of cowpea seedlings. Na^+ accumulation was significantly greater in roots than leaves under salt stress and increased concomitantly with exposure time. The statistically significant difference ($P \leq 0.05$) in [Na^+] was observed after 6 and 18 hours of salt stress in roots and leaves, respectively. Roots and leaves showed 9.3- and 6.1-fold increase in [Na^+], respectively, after 48hrs of salt treatment (Fig. 3.17). However, no significant difference was observed in [K^+] in leaves under salt stress, unlike in roots, after 6 hours of salt stress. As compared to control condition, 2.9 and 33.7 times higher Na^+/K^+ ratio values were observed in case of leaves and roots, respectively, at 48 hours of salt stress (Fig. 3.17). The restriction of movement of Na^+ to leaves might serve as a salt tolerance mechanism in cowpea as observed at 48 hours of salt stress, a K^+/Na^+ ratio of 2.1 was maintained in leaves as compared to roots with a K^+/Na^+ ratio of 0.12. Maintenance of higher K^+ in leaves is essential for cellular and ionic homeostasis to attain unhindered growth under hypersalinity and hyperosmotic condition. Therefore, it can be concluded that higher Na^+ are sequestered in root vacuoles thus, restricting their movement to the aerial part of plant, wherein, a higher K^+/Na^+ ratio is maintained. The increased *VuNHX1* transcript level coupled with higher sequestration of Na^+ in roots can be attributed as the tolerance mechanism of cowpea against salinity stress condition.

3.3.6. Salt tolerance assay in transgenic *Arabidopsis* lines

In order to functionally validate *VuNHX1* in plant system, kanamycin selected T_4 homozygous *Arabidopsis* lines expressing *VuNHX1* constitutively, under the control of cauliflower mosaic virus (CaMV) 35S promoter (Fig. 3.18) and inducibly, under a stress-responsive AtRD29A promoter (Fig. 3.19), were studied under salt stress. The difference in the growth and survival efficiency of transgenic *Arabidopsis* lines

expressing transgene *VuNHX1* under constitutive and inducible promoters was performed.

3.3.6.1. Effect of salt stress on seed germination

Inhibition of seed germination, plant growth and induced senescence are accelerated by salt stress (Lee and Zhu 2010). Therefore the effect of salt stress on germination of *Arabidopsis* lines (Wild-type WT, Col-0; 35S::*VuNHX1*, Line #4 and RD29A::*VuNHX1*, Line #13) after one week exposure to 150 mM NaCl treatment was studied. Wild-type and transgenic lines exhibited undifferentiated growth under normal physiological condition (Fig. 3.20). However, reduced growth and germination rate was observed in WT than transgenic lines #4 and # 13, which exhibited better survival efficiency under salt stress (Fig. 3.20).

3.3.6.2. Effect of salt stress on root growth

The physiological growth parameter (root length and lateral root development) was monitored in wild-type (WT, Col-0) and independent transgenic *Arabidopsis* lines expressing *VuNHX1* constitutively (Line #4, 35S::*VuNHX1*) and inducibly (Line #13, RD29A::*VuNHX1*). WT and transgenic lines were initially grown on ½ MS medium for 5 days prior to transfer onto ½ MS medium supplemented with or without150 mM and 200 mM NaCl. The difference in root length was measured after 7 days of salt stress. Under control unstressed condition, no significant difference was observed between WT and transgenic *Arabidopsis* lines (Fig. 3.21a). However, a statistically significant difference ($P \leq 0.05$) in root length was observed in transgenic *Arabidopsis* lines under 150 and 200 mM NaCl stress as compared to WT plants. Root growth inhibition was lower in transgenic 35S::*VuNHX1* and RD29A::*VuNHX1 Arabidopsis* lines than WT, under salt stress (Fig. 3.21b). Lateral root development is affected by salinity stress. Under 200 mM salt stress, a statistically significant ($P \leq 0.05$) higher lateral root number was observed in transgenic lines #4 and #13 as compared to WT (Col-0) *Arabidopsis* plants (Fig. 3.21b).

3.3.6.3. Expression of *VuNHX1* in transgenic *Arabidopsis*

The effect of salt stress (200 mM NaCl) on mature *Arabidopsis* plants was studied by growing WT and transgenic lines (Line #4, 35S::*VuNHX1* and Line #13,

RD29A::*VuNHX1*) on ½ MS medium for 5 days prior to growth in soilrite for 2 weeks. The WT and transgenic lines expressing transgene *VuNHX1* were watered with ½ MS liquid medium supplemented with 200 mM NaCl. In response to salt stress, WT exhibited growth inhibition with induced leaf senescence whereas, 35S::*VuNHX1* and RD29A::*VuNHX1* exhibited better survival response and comparatively lesser salinity induced leaf senescence (Fig. 3.22a). Further, quantitative measurement of *VuNHX1* expression in transgenic lines by real-time PCR analysis indicated a 1.34-fold higher expression in line #13, RD29A::*VuNHX1* as compared to line #4, 35S::*VuNHX1* (Fig. 3.22b).

3.3.6.4. Effect of salt stress on physiological parameters

The leaves of salt-stressed WT and transgenic lines were analyzed for Na^+ and K^+ accumulation, chlorophyll, malondialdehyde (MDA) and proline content, after exposure to salt stress (200 mM NaCl) for 2 weeks. Under normal physiological condition, no statistical difference ($P \leq 0.05$) was observed between WT and transgenic lines #4 and #13 (Fig. 3.23). However, post salt stress, transgenic line #4 and #13 exhibited 1.06 and 1.34 times higher Na^+ in leaves than WT, respectively (Fig. 3.23). The maintenance of K^+ homeostasis under hypersalinity is essential for tight regulation of plant growth and survival. Transgenic line #4 overexpressing *VuNHX1* exhibited 1.93 times, and line #13 expressing *VuNHX1* under stress indicble promoter had 1.66 times higher accumulation of K^+ in leaves, than WT. Consequently, transgenic lines #4 and #13 maintained a 1.8 and 1.2 times higher K^+/Na^+ ratio, respectively than WT (Fig. 3.23). Higher compartmentalization of Na^+ in leaves provides a better strategy towards protecting the photosynthetic machinery, active metabolic and enzymatic activities, and protein synthesis from damage incurred due to hyperionic imbalance and oxidative stress. Effect of *VuNHX1* expression in transgenic *Arabidopsis* protected the component of photosynthesis, chlorophyll as wild-type plants displayed a 1.9 times lower chlorophyll content (Fig. 3.24). Similarly, malondialdehyde, a measuring factor for lipid peroxidation was estimated to be 1.2 times higher in WT as compared to transgenic lines (Fig. 3.24). The detection of osmoprotectant, proline has been observed in transgenic line #4 overexpressing *VuNHX1* at 1.53 times higher than WT. Further, transgenic line #4,

35::*VuNHX1* exhibited higher proline accumulation than line #13, RD29A::*VuNHX1* (Fig. 3.24).

3.4. Conclusion

In conclusion, *VuNHX1*, a vacuolar Na^+/H^+ antiporter has an important role in salt tolerance mechanisms of cowpea. Phylogenetic analysis revealed that *VuNHX1* shared highest homology with reported legume Na^+/H^+ antiporters. It was evenly categorized under Class-I category of IC-NHE/NHX group distinct from Class-II and PM-NHE category. *VuNHX1* could functionally complement yeast vacuolar *NHX* exchanger, Sc*NHX1.* Under abiotic stress conditions, like salt, cold and dehydration stress, the induction of *VuNHX1* suggested for its potent role in stress tolerance mechanisms inherent to cowpea. Further, constitutive and inducible expression of *VuNHX1* in model plant *Arabidopsis thaliana*, conferred increased salt tolerance. Therefore, its potential against salinity stress can be exploited through genetic engineering approach to generate salt tolerant crop plants.

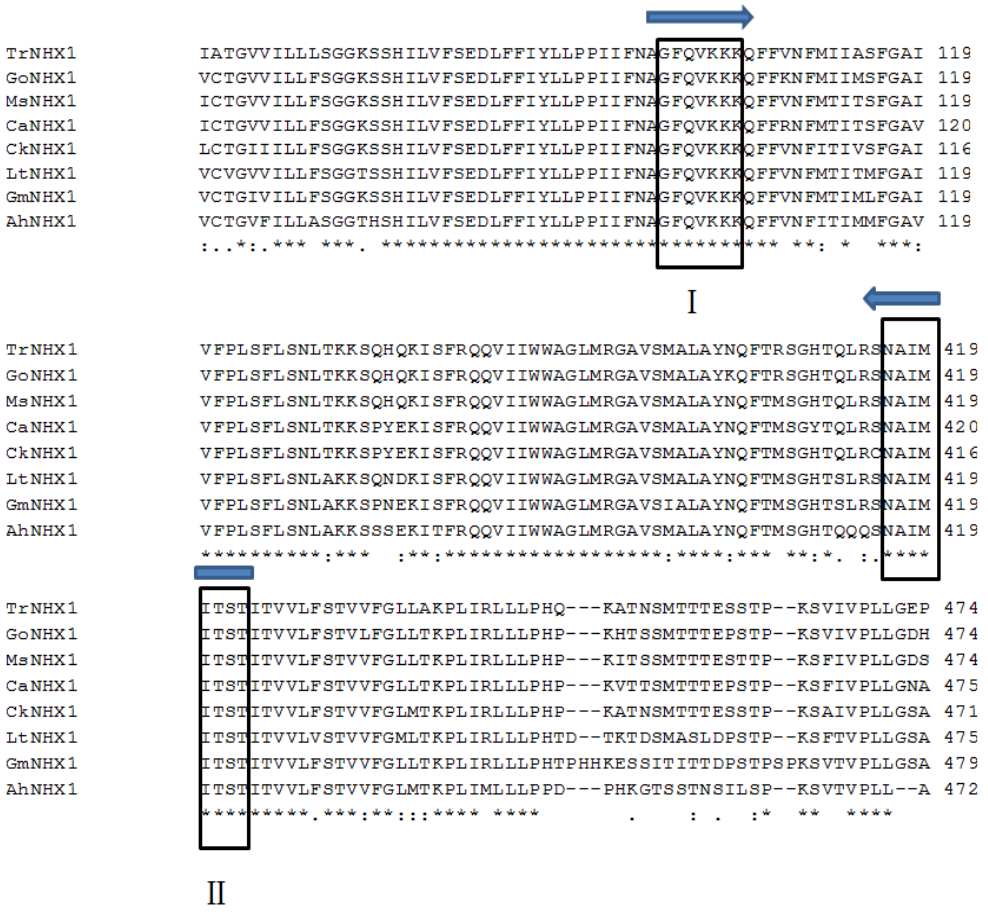


Figure 3.1 Designing of degenerate primers for partial amplification of *VuNHX1* gene. Multiple sequence alignment of amino acid sequences of NHX1 from various legume species submitted in NCBI database was performed using Clustal W. The forward and reverse degenerate primers (position is indicated by arrows) were designed from conserved regions designated as box I and II, respectively. The "*" sign indicates the highly identical residues constituting the conserved donains in NHX1 protein. The GenBank Accession numbers for NHX proteins are: GmNHX1 (AAY430061.1); CkNHX1 (ABG89337.1), MsNHX1 (AAS84487.1), CaNHX1 (ADL28385.1), TrNHX1 (ABV00895.1); LtNHX1 (ACE78322.1), AhNHX1 (ADK74832.1), GoNHX1 (ABY59540.1)

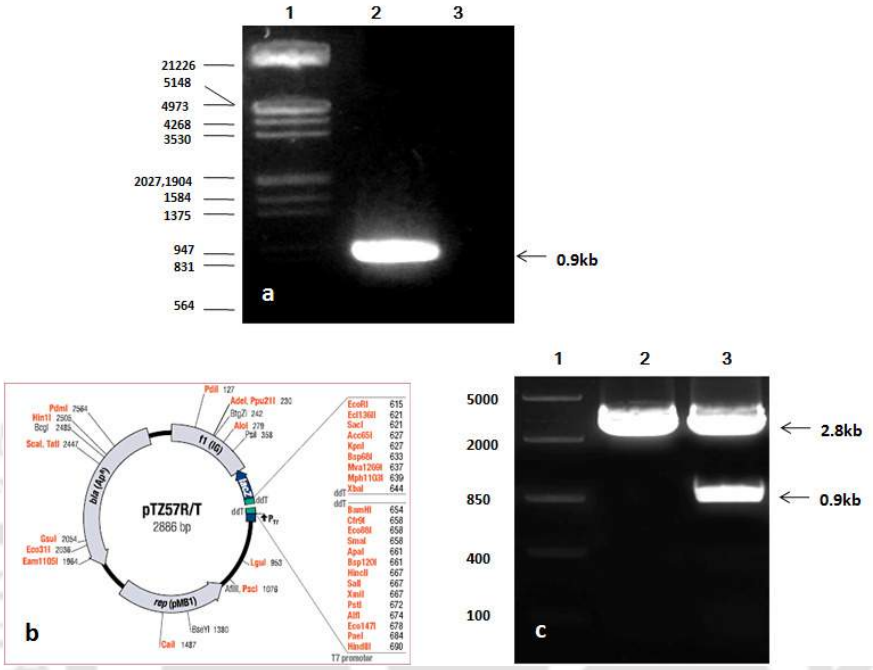


Figure 3.2 Cloning of partial fragment of *VuNHX1.* (a) Isolation of partial fragment of *VuNHX1*. L1: Lambda HindIII/EcoRI DNA Ladder; L2: 0.977 kb PCR amplicon of *VuNHX1* was obtained using degenerate primers; L3: Blank. (b) Vector map of TA cloning vector pTZR/T (2.8 kb). (c) Cloning of 0.977 kb partial fragment of *VuNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Middle range DNA ruler; L2: Uncut plasmid; L3: Release of partial fragment from pTZR/T-Deg*VuNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI

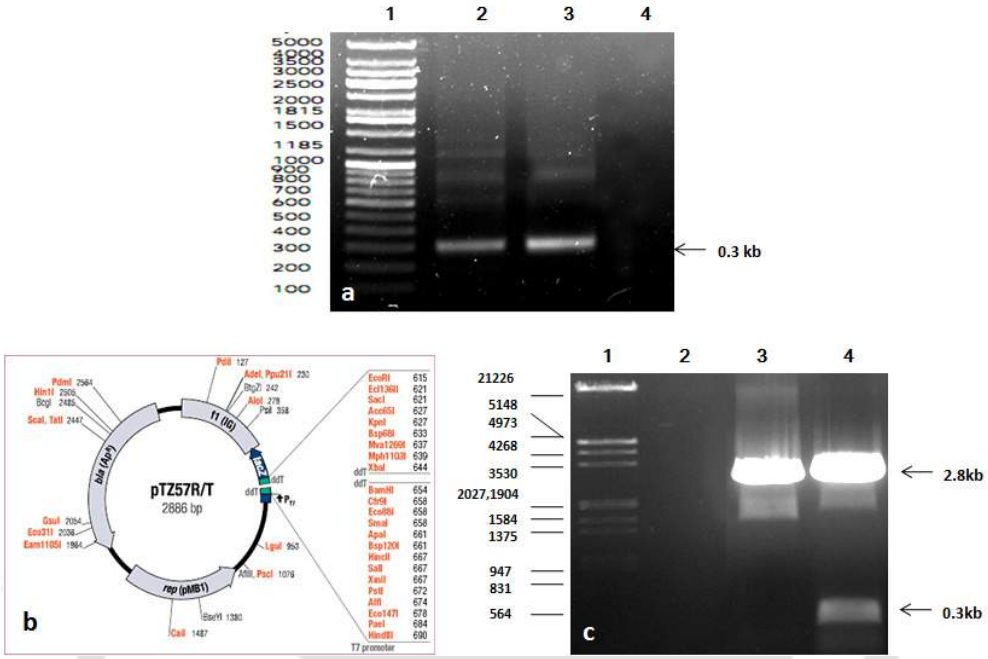


Figure 3.3 Cloning of 5'- untranslated region of *VuNHX1*. (a) Isolation of 5'- UTR fragment of *VuNHX1*. L1: Medium range DNA ruler; L2: PCR amplicons of 1st PCR using GSP1 and AAP primers; L3: Isolation of 5'- UTR fragment of *VuNHX1* gene using nested GSP2 and AUAP primers. b) Vector map of TA cloning vector pTZR/T (2.8 kb). c) Cloning of 0.335 kb partial fragment of *VuNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder; L2: Uncut plasmid (pTZR/T-5'*VuNHX1*); L3: Release of partial fragment from vector backbone upon disgestion with restriction enzymes XbaI and BamHI

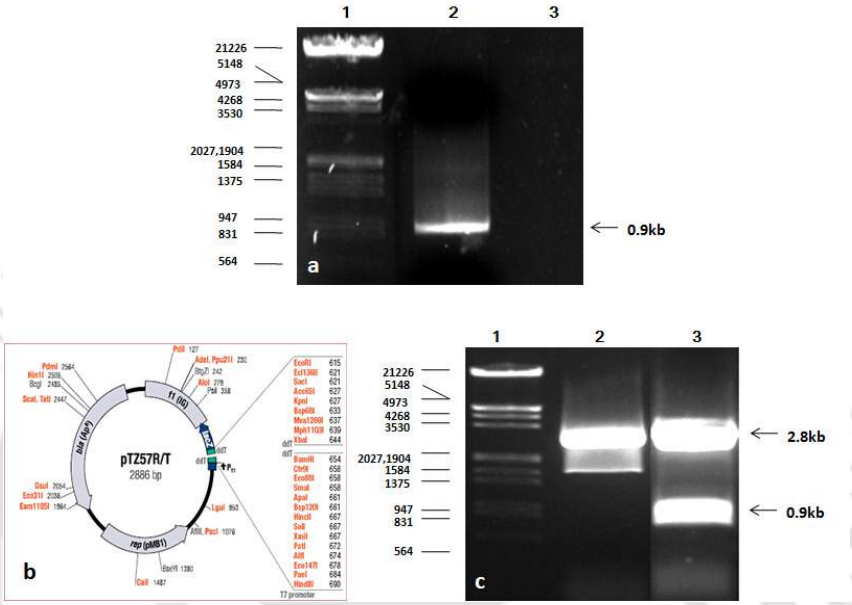


Figure 3.4 Cloning of 3'- untranslated region of *VuNHX1*. (a) Isolation of 3'- UTR fragment of *VuNHX1* using GSP3 and AUAP primers. L1: Lambda HindIII/EcoRI DNA Ladder; L2: Blank. (b) Vector map of TA cloning vector pTZR/T (2.8 kb). (c) Cloning of 0.904 kb fragment of *VuNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder; L2: Uncut plasmid (pTZR/T-3'*VuNHX1*); L3: Release of partial fragment from pTZR/T plasmid upon disgestion with restriction enzymes XbaI and BamHI

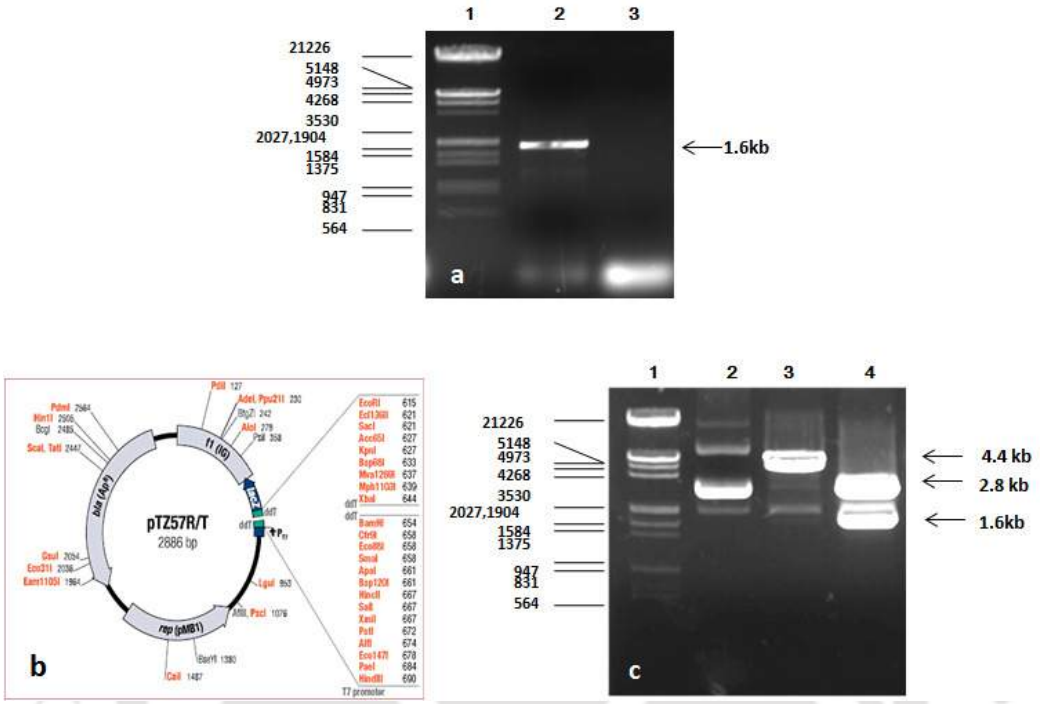
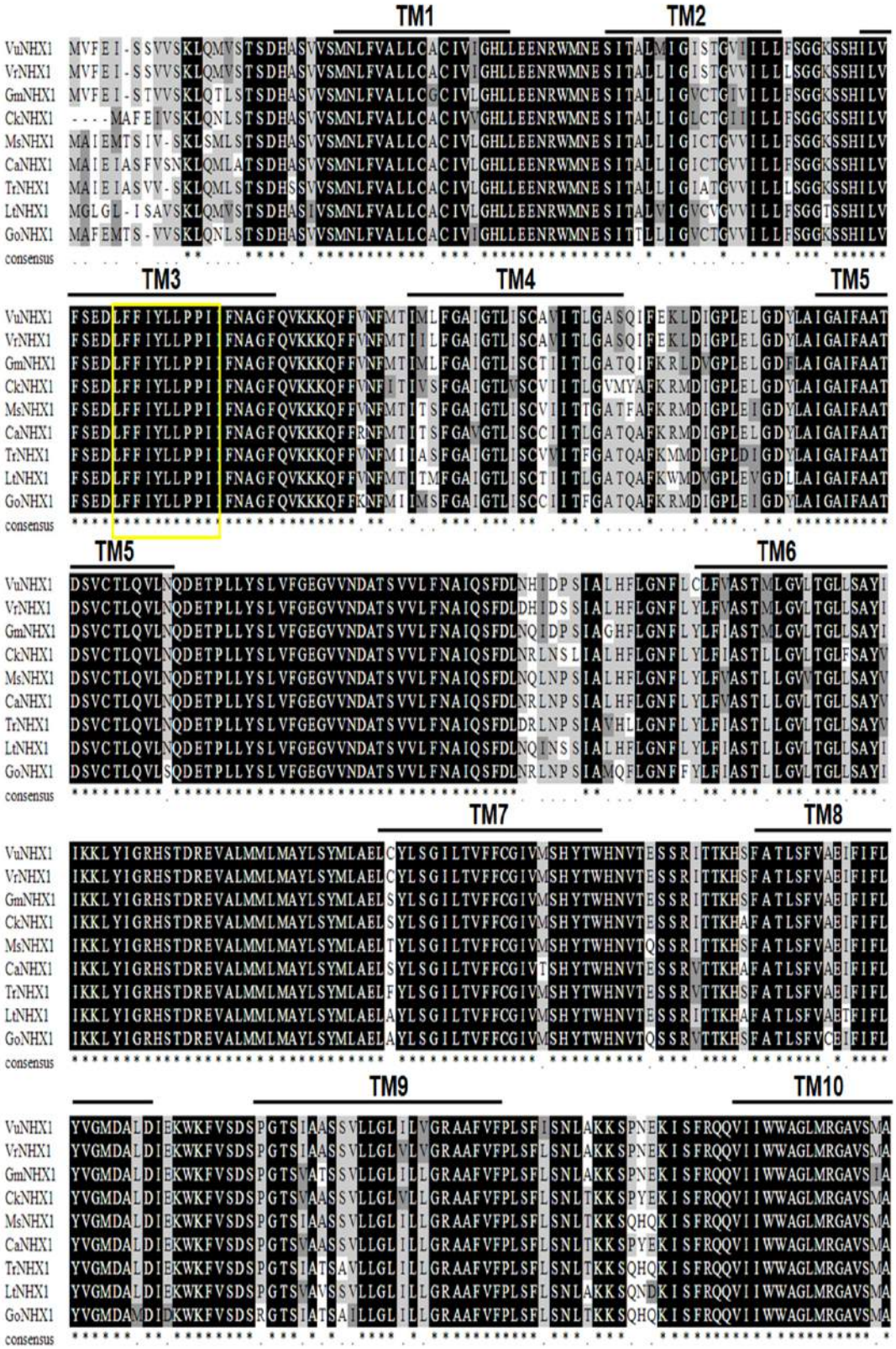


Figure 3.5 Cloning of CDS of *VuNHX1*. (a) PCR amplification of CDS of *VuNHX1* using gene specific primers. L1: Lambda HindIII/EcoRI DNA Ladder; L2: 1.6 kb PCR amplicon corresponding to CDS of *VuNHX1*; L3: Blank. (b) Vector map of TA cloning vector pTZR/T (2.8 kb). (c) Cloning of 1.6 kb CDS fragment of *VuNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder (Thermo Fisher Scientific); L2: Uncut plasmid pTZR/T-*VuNHX1* (4.4 kb); L3: Release of partial fragment from pTZR/T-*VuNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI


TM1
TM2
TM3
TM4
TM5
TM5
TM6
TM7
TM8
TM9
TM10
VuNHX1
VrNHX1
GmNHX1
CkNHX1
MsNHX1
CaNHX1
TrNHX1
LtNHX1
GoNHX1
consensus

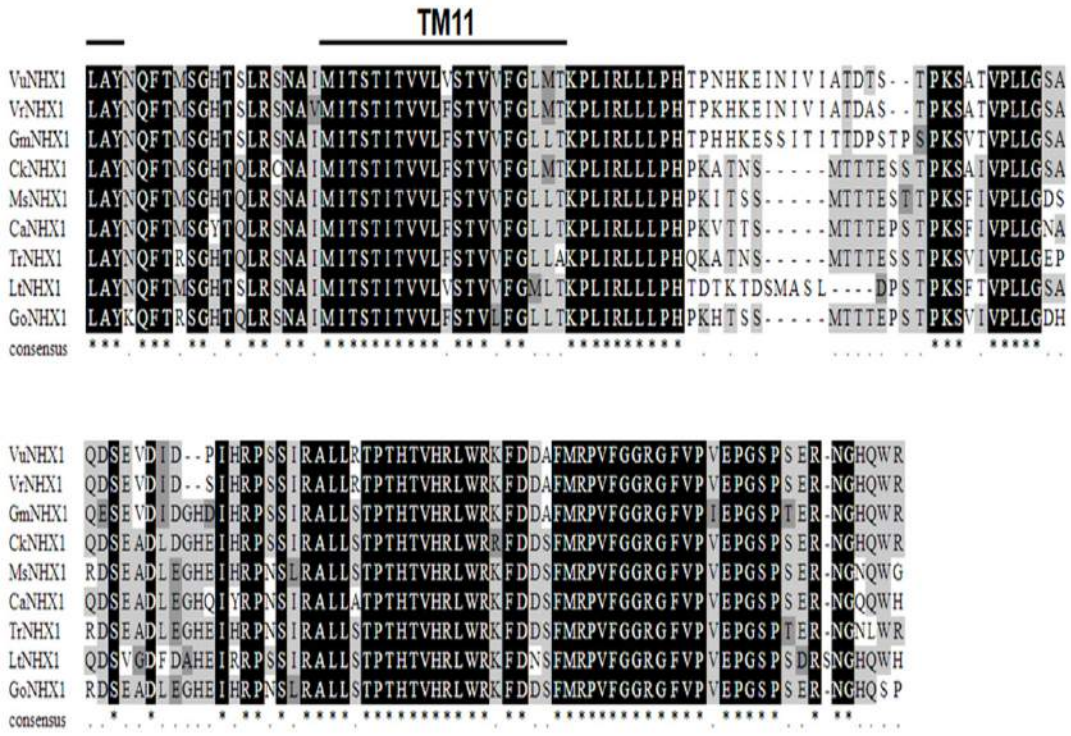


Figure 3.6 Amino acid sequence analysis of Na^+/H^+ antiporters from several plant species. Multiple sequence alignment was performed using CLUSTAL W. Boxshade was used to highlight identical (dark shading with white letters) and similar (grey shading with black letters) residues. The transmembrane domain of VuNHX1 is shown as TM 1-11. The amiloride conserved motif is shown TM3 in box. The GenBank Accession numbers for NHX proteins are: VuNHX1 (AEO72079.2), VrNHX1 (AEO50758.1), GmNHX1 (AAY430061.1), TrNHX1 (ABV00895.1), GoNHX1 (ABY59540.1), CkNHX1 (ABG89337.1), LtNHX1 (ACE78322.1), CaNHX1 (ADL28385.1), MsNHX1 (AAS84487.1)

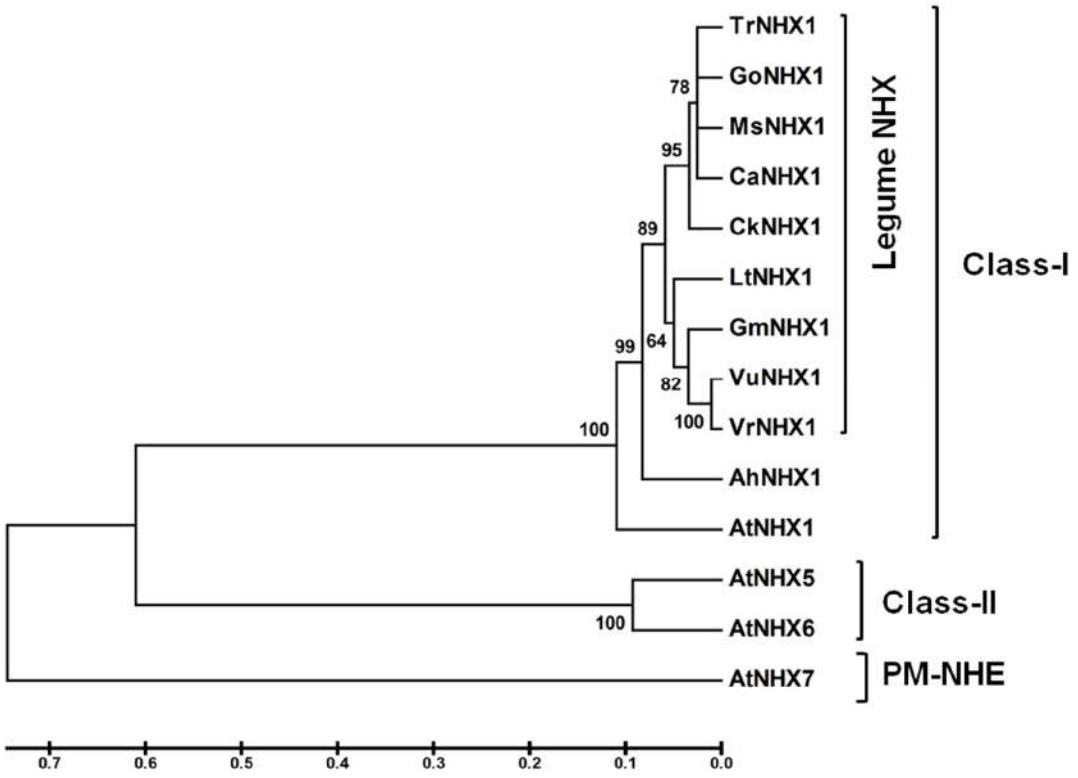


Figure 3.7 Phylogenetic relationship of plant Na^+/H^+ antiporters. The phylogenetic tree was generated using MEGA4: Tree Explorer software. The evolutionary history was inferred using the Neighbour-Joining method. The scale bar indicates branch length as calculated from multiple sequence alignment. The *VuNHX1* shows high homology with legume NHX1 proteins and clusters in Class-I NHX clade. The GenBank Accession numbers for NHX proteins are: VuNHX1 (AEO72079.2), VrNHX1 (AEO50758.1), GmNHX1 (AAY430061.1), TrNHX1 (ABV00895.1), GoNHX1 (ABY59540.1), CkNHX1 (ABG89337.1), LtNHX1 (ACE78322.1), CaNHX1 (ADL28385.1), MsNHX1 (AAS84487.1), AhNHX1 (ADK74832.1), AtNHX1 (NM_122597.2), AtNHX5 (AEE33089.1), AtNHX6(AEE36272.1), AtNHX7 (AEC05529.1)

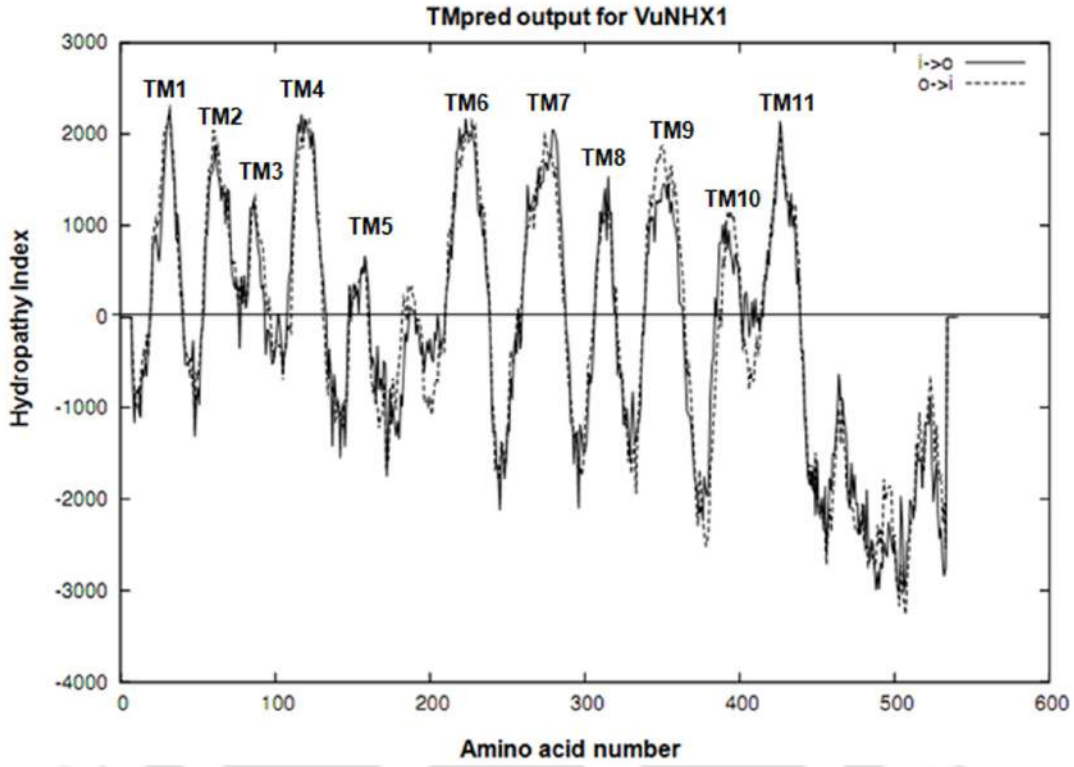


Figure 3.8 Prediction of transmembrane helices of VuNHX1. The hydropathy plot was generated using TMPred online software. The positive values indicate putative transmembrane domains as indicated as TM 1-11

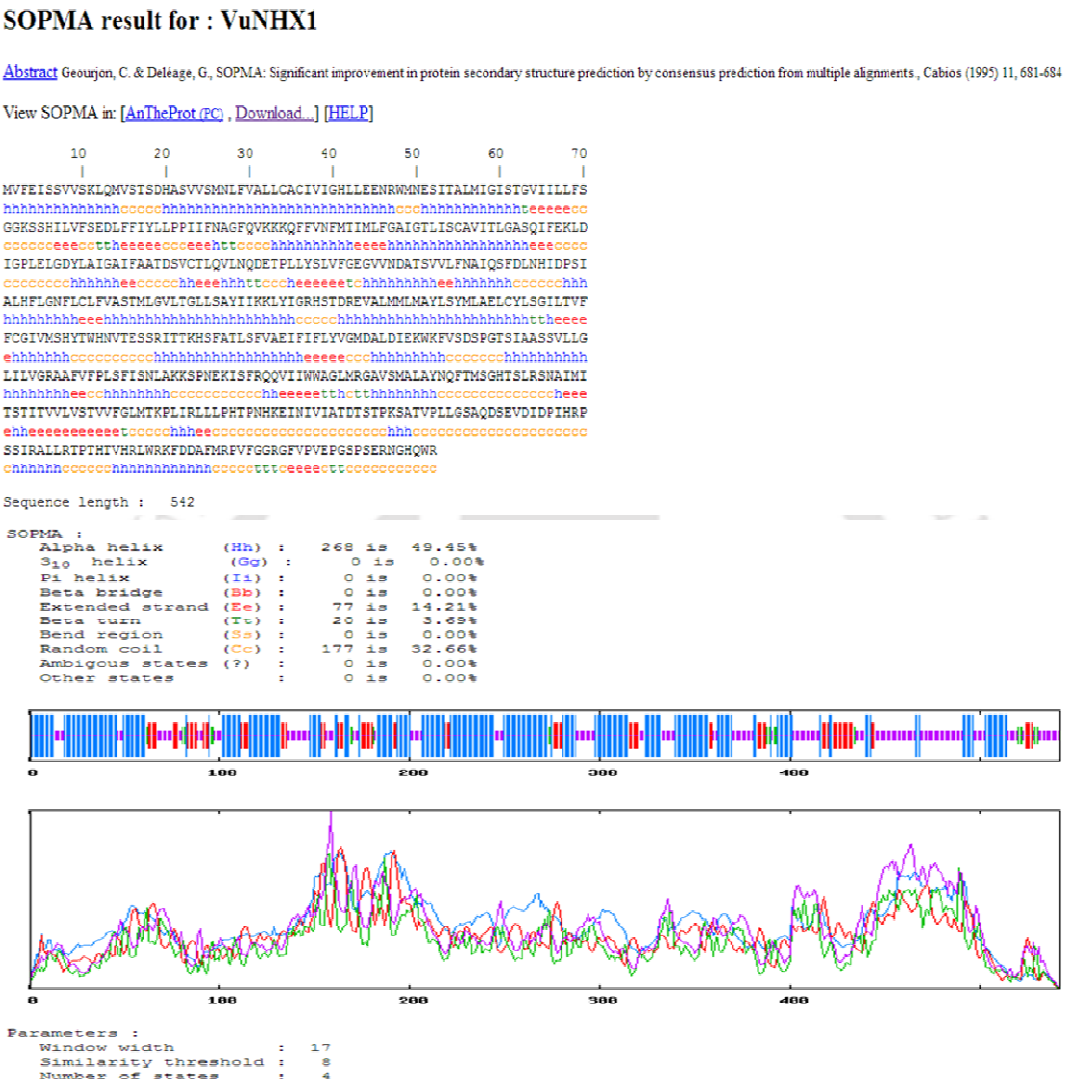


Figure 3.9 Secondary structure prediction of VuNHX1. Secondary structure using SOPMA indicated occurrence of alpha helix (49.45%), extended strand (14.21%), beta turn (3.69%), and random coil (32.66%) in VuNHX1

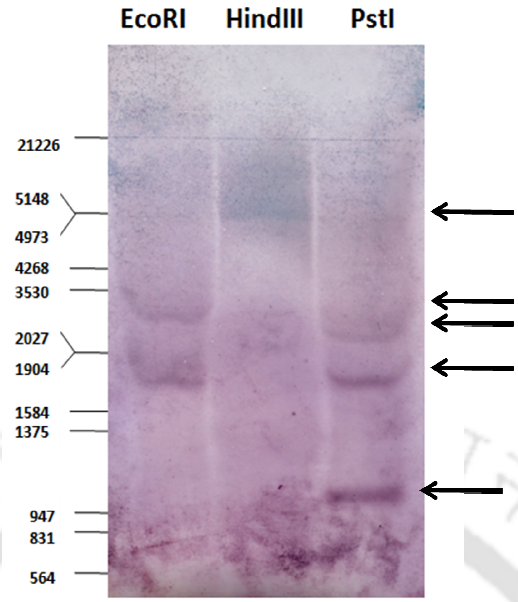


Figure 3.10 Copy number analysis of *VuNHX1* in cowpea genome. Cowpea genomic DNA (20µg) was digested with EcoRI, HindIII and PstI and hybridized with DIG-labeled probe (0.9 kb) corresponding to the CDS of *VuNHX1*. The arrows are shown against the hybridization signals

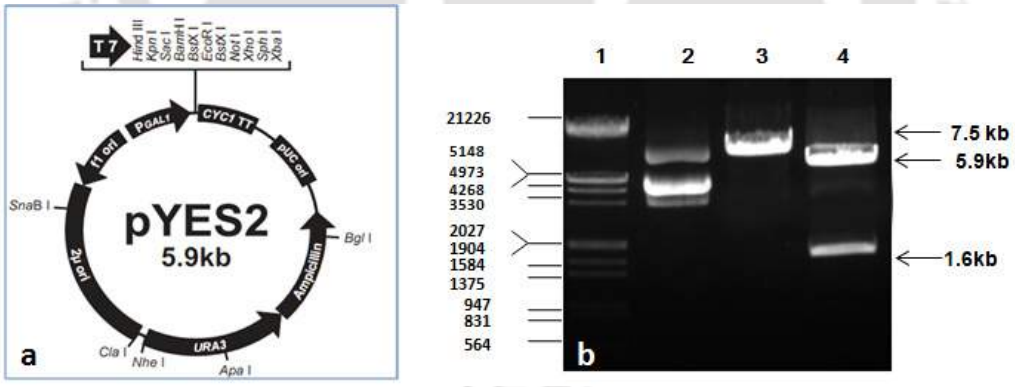


Figure 3.11 Cloning of *VuNHX1* CDS into yeast expression vector pYES2.0. (a) Vector map of yeast expression vector pYES2.0. (b) Cloning of *VuNHX1* CDS (1.6 kb) into yeast expression vector pYES 2.0 (5.9 kb). L1: Lambda HindIII and EcoRI digested DNA ladder; L2: Uncut pYES*VuNHX1* plasmid (7.5 kb); L3: pYES*VuNHX1* plasmid was linearized with EcoRI; L4: Release of the cloned *VuNHX1* CDS from the vector backbone

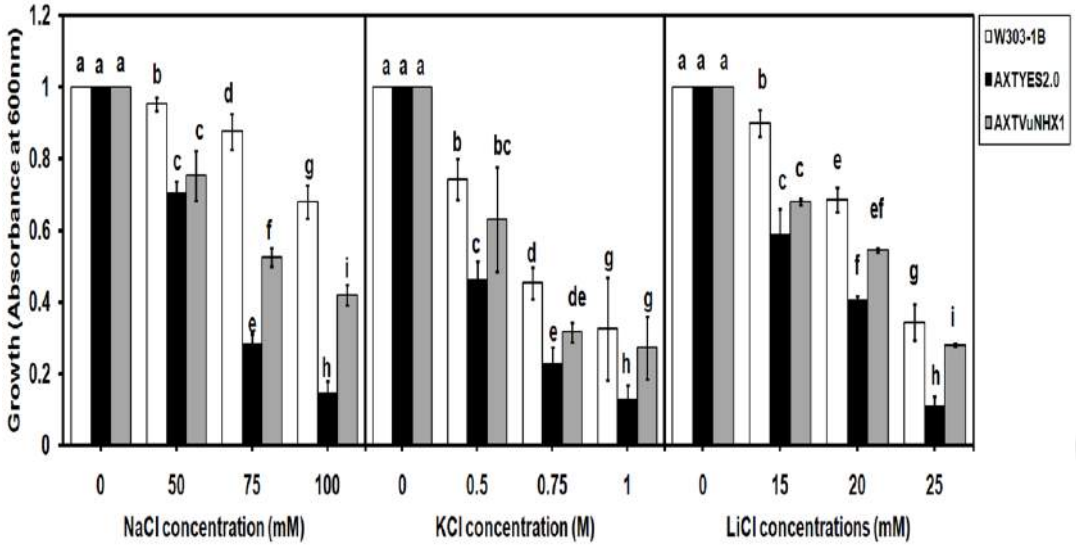


Figure 3.12 Cation sensitivity assay of transformed yeast strains under various concentrations of NaCl (0, 50, 75, 100 mM), KCl (0, 0.5, 0.75, 1.0 M), and LiCl (0, 15, 20, 25 mM). Saturated seed cultures for each strain was diluted to an OD_{600} of 0.006 and inoculated to liquid APGal medium (pH 5.5) supplemented with or without above mentioned concentrations of NaCl, KCl, and LiCl. Growth was observed at 30°C after 3 days and absorbance recorded at 600nm. Data are means of 3 independent events (n=3) and standard errors are plotted in the graph. Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

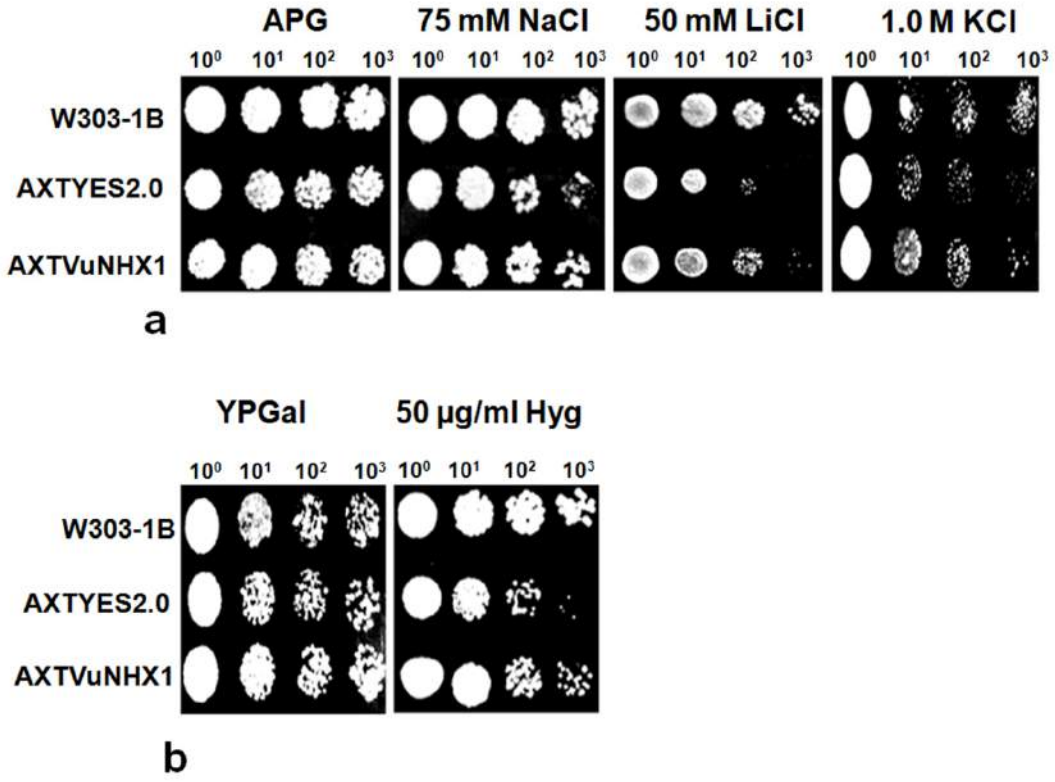


Figure 3.13 Heterologous expression of *VuNHX1* in yeast mutant. Wild type (W303-1B) and *Δ ena1- 4 Δnha1 Δnhx1* mutant (AXT3) strains used for the complementation assay were transformed with pYES2.0 vector only and pYES*VuNHX1* recombinant vector and labeled as AXTYES2.0 and AXTVuNHX1, respectively. 10^1, 10^2, and 10^3 fold serial dilutions of saturated seed cultures of each strain were spotted onto (a) APGal media (pH-5.5) supplemented with or without 75 mM NaCl, 50 mM LiCl, 1.0 M KCl and (b) YPGal media (pH-5.5) supplemented with or without 50µg/ml Hyg. The plates were incubated at 30°C for 3 days

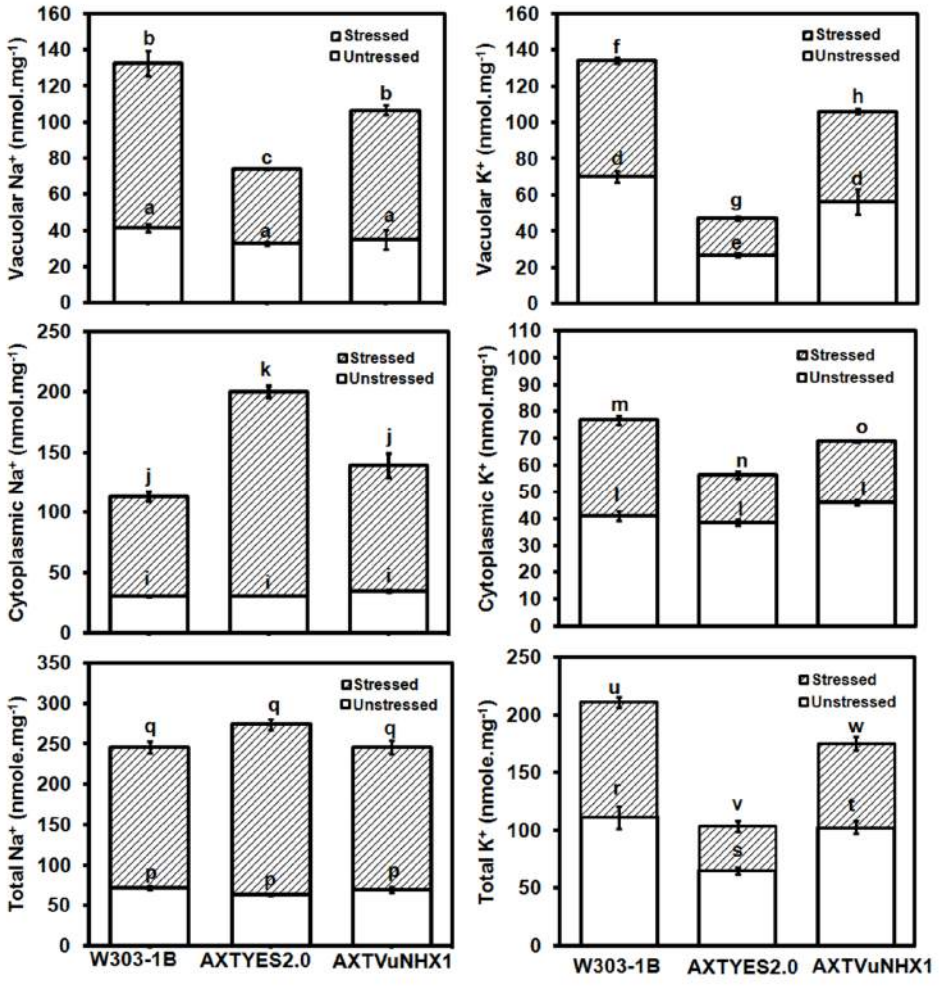


Figure 3.14 Total intracellular ion estimation in yeast strains. Wild type (W303-1B) and *Δena1- 4 Δnha1 Δnhx1* mutant (AXT3) strains used for the complementation assay were transformed with pYES2.0 vector only and pYES*VuNHX1* recombinant vector and labeled as AXTYES2.0 and AXTVuNHX1, respectively. Yeast cells were grown in APG medium (pH 4.0) with 1mM KCl supplemented in presence (stressed) or absence of 70mM NaCl (unstressed) and harvested at a cell density of 0.3. Total intracellular, vacuolar and cytoplasmic Na^+ and K^+ content was determined as described in the materials and methods section. Data are means of 3 independent events (n=3) and standard errors are plotted in the graph. Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

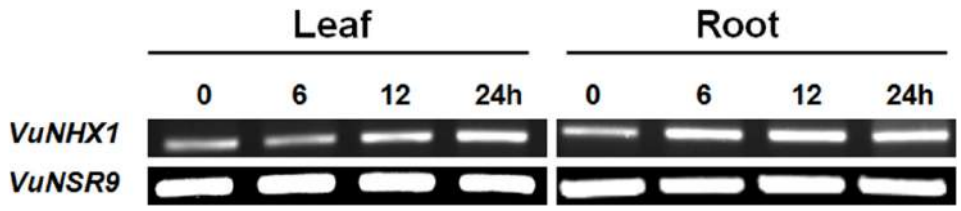


Figure 3.15 Semi-quantitative RT-PCR for studying expression patterns of *VuNHX1* under salt stress (200 mM NaCl). Total RNA was isolated from leaves and roots of 10 days old cowpea seedling under 200mM NaCl treatment at time intervals of 0, 6, 12, and 24 hours. PCR fragments of 260bp and 292bp size corresponding to *VuNHX1* and *NSR9* gene were fractionated electrophoretically on 2% agarose gel stained with 10mg/ml ethidium bromide

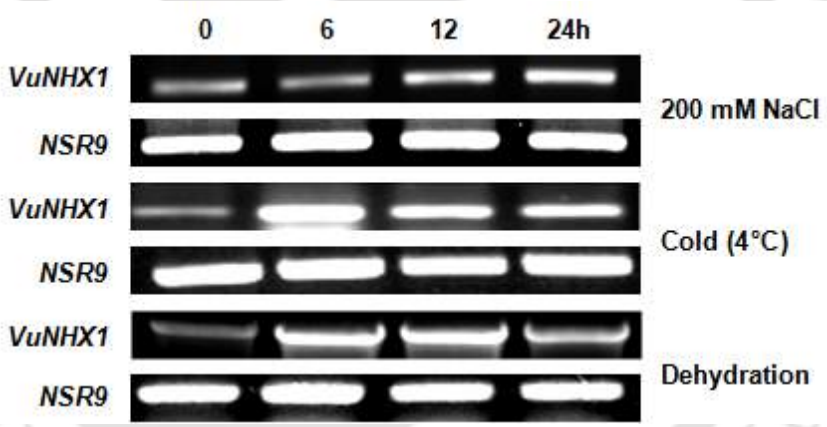


Figure 3.16 Semi-quantitative RT-PCR for studying expression patterns of *VuNHX1* under different abiotic stress conditions such as salt, cold and drought stress. Total RNA was isolated from 10 days old cowpea seedling under 200mM NaCl, Cold (4°C), and dehydration treatment at time intervals of 0, 6, 12, and 24 hours. PCR fragments of 260 bp and 292 bp size corresponding to *VuNHX1* and *NSR9* gene were fractionated electrophoretically on 2% agarose gel stained with 10mg/ml ethidium bromide

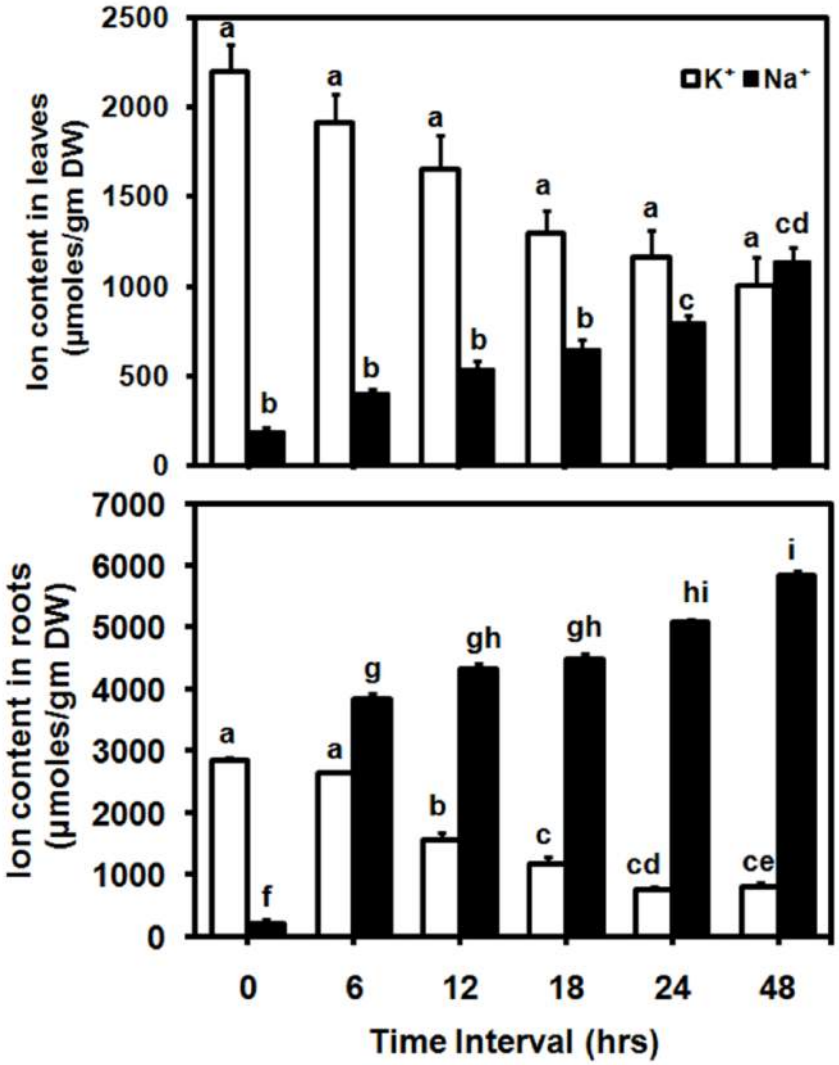


Figure 3.17 Total intracellular ion measurement in leaves and roots of cowpea plants. Na^+ and K^+ content in (a) leaves and (b) roots of unstressed and salt stressed 10 days old cowpea seedlings harvested at time intervals of 0, 6, 12, 24, 48, and 72 hrs was measured using Flame Photometer. Values indicate means ± SE (n=3)

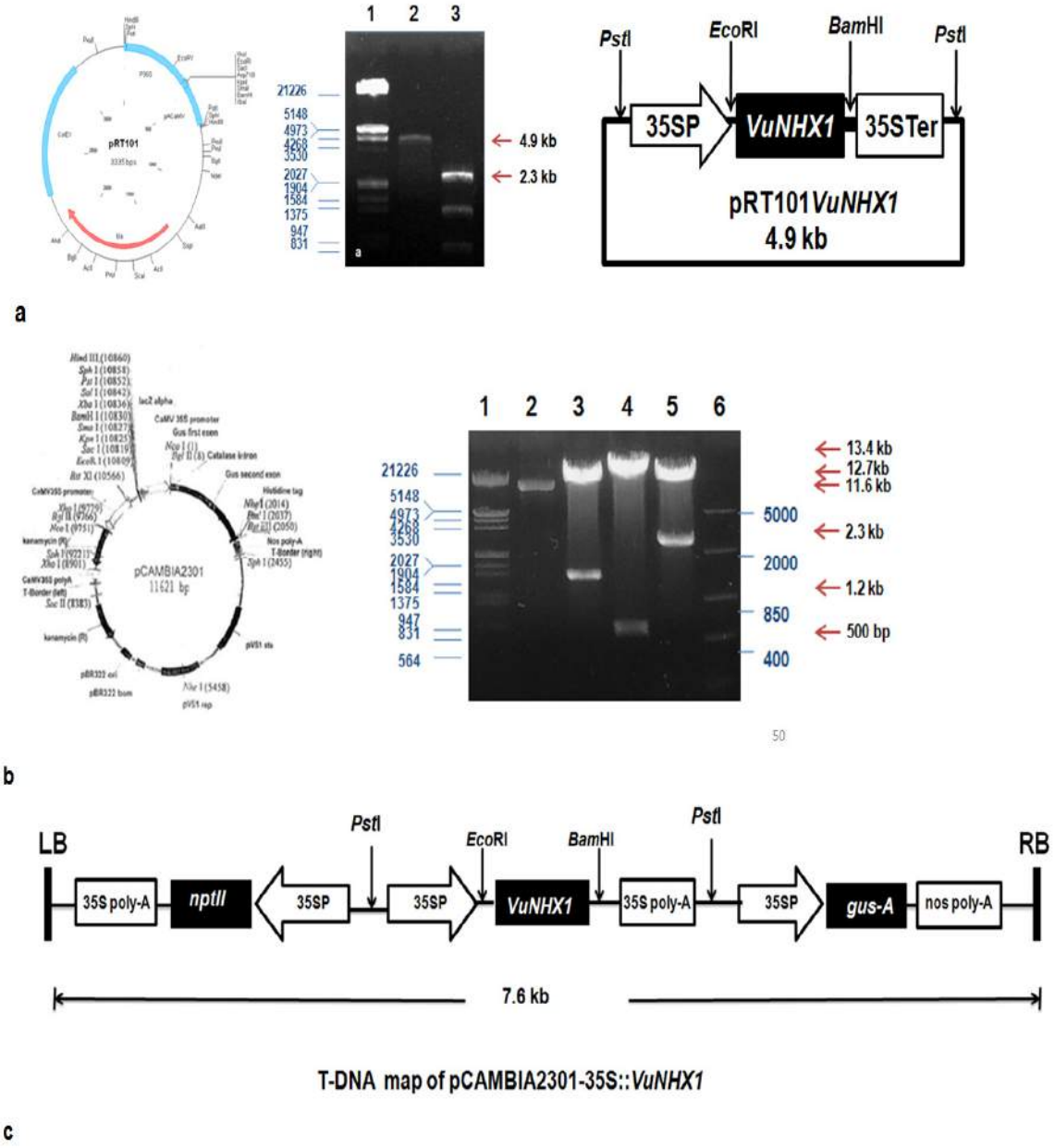


Figure 3.18 Cloning of CDS of *VuNHX1* into plant binary vector pCAMBIA2301. (a) The *VuNHX1* CDS was cloned into intermediate pRT101 (3.3 kb) plasmid at *Eco*RI and *Bam*HI sites and the recombinant plasmid pRT101*VuNHX1* (4.9 kb) was generated with the *VuNHX1* CDS flanked by CaMV 35S promoter and 35S poly-A. L1: Lamda HindIII/EcoRI DNA ladder; L2: Linearized pRT101VuNHX1 plasmid digested with BamHI; L3: The release of *Pst*I-35SP::*VuNHX1*::35STer-*Pst*I fragment upon digestion with PstI and BglI. (b) The *Pst*I-

35SP::*VuNHX1*::35STer-*Pst*I digested DNA fragment was cloned into plant binary vector pCAMBIA2301 (11.6 kb) and confirmed with restriction digestion. L1: Lamda HindIII/EcoRI DNA ladder; L2: Uncut pCAMBIA230135S::*VuNHX1* recombinant plasmid (13.9 kb); L3, L4: Confirmation of orientation of the 35SP::*VuNHX1*::35STer cassette (2.3 kb) in pCAMBIA2301 upon digestion with HindIII and EcoRI; L5: Release of 35SP::*VuNHX1*::35Ter cassette (2.3 kb) from vector backbone (11.6 kb) upon digestion with PstI; L6: Middle Range DNA Ladder. (c) T-DNA region (7.6 kb) of pCAMBIA230135S::VuNHX1 recombinant plasmid (13.9 kb) Restrcition enzyme PstI used for cloning 35SP::*VuNHX1*::35STer cassette (2.3kb) into plant binary vector pCAMBIA 2301 is also highlighted. Abbreviations: LB, left border; RB, right border; 35SPromoter, cauliflower mosaic virus 35S promoter; RD29A promoter, stress indicible AtRD29A promoter; CaMV 35S poly-A, cauliflower mosaic virus 35S terminator; nos poly-A, nopaline transferase terminator; *nptII*, neomycin phosphotransferase; *intron-gus-A*, intron interrupted β-glucuronidase; *VuNHX1*, *Vigna unguiculata NHX1*

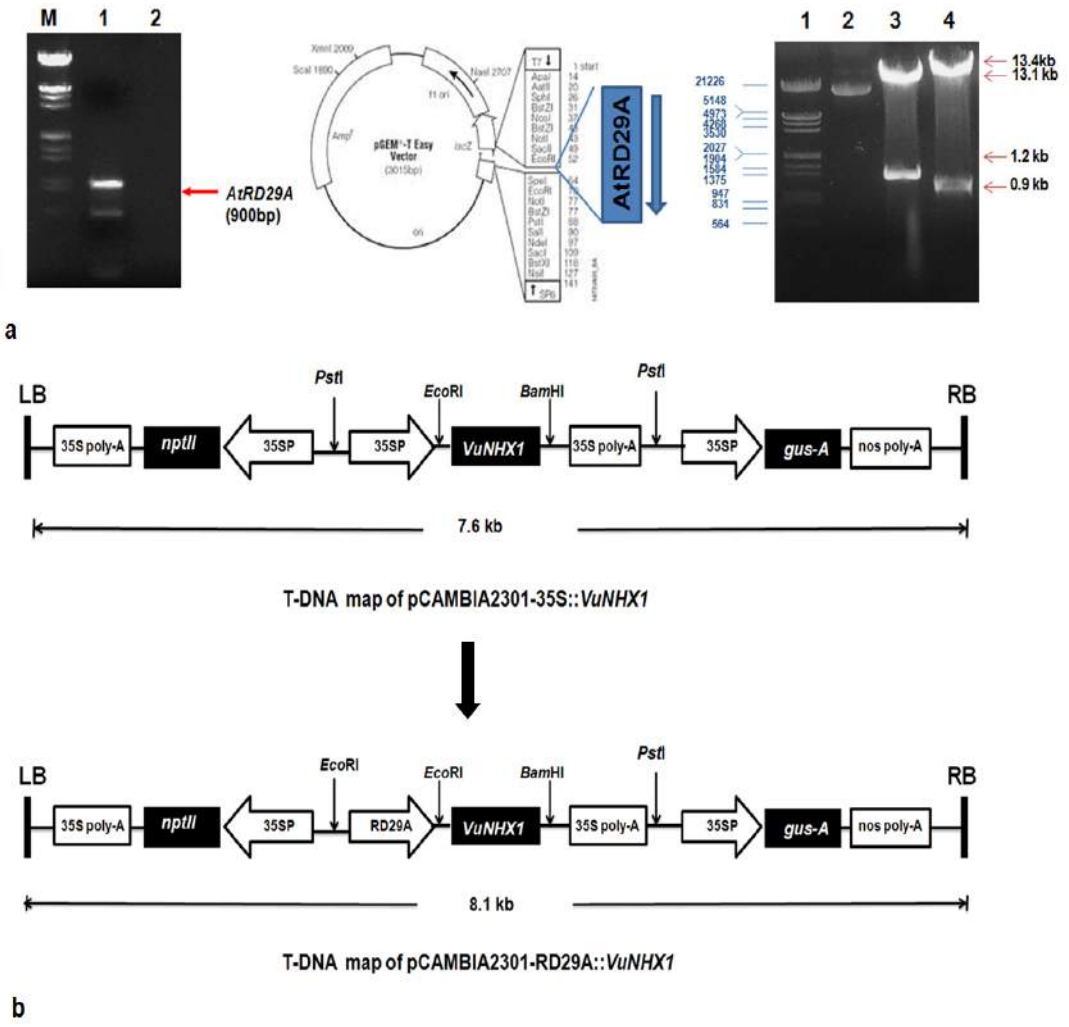


Figure 3.19 Preparation of recombinant pCAMBIA2301RD29A::*VuNHX1* plasmid (14.4 kb). (a) The first figure depicts the isolation of stress inducible promoter fragment RD29A (0.9 kb) from *Arabidopsis thaliana*. M: Lambda HindIII and EcoRI digested DNA ladder; 1: PCR amplicon for AtRD29A (0.9 kb) promoter fragment; 2: Blank. The second figure depicts the vector map of recombinant pGEMTRD29A plasmid (3.9 kb). The AtRD29A (0.9 kb) promoter fragment was cloned into TA cloning vector pGEMT-easy (3 kb). The third figure depicts the restriction digestion for confirmation of generation of recombinant plant binary vector pCAMBIA2301RD29A::*VuNHX1* (14.4kb). L1: Lambda HindIII and EcoRI digested DNA ladder; L2: Uncut pCAMBIA2301RD29A::*VuNHX1* (14.4kb); L3: Checking of orientation of RD29A::*VuNHX1*::35STer cassette in pCAMBIA2301; L4: Release of 0.9kb RD29A promoter from vector backbone. (b) The T-DNA map (8.1 kb) of pCAMBIA2301RD29A::*VuNHX1* is shown. The AtRD29A promoter fragment has been replaced with 35SP in T-DNA of pCAMBIA2301 35S::VuNHX1 plasmid

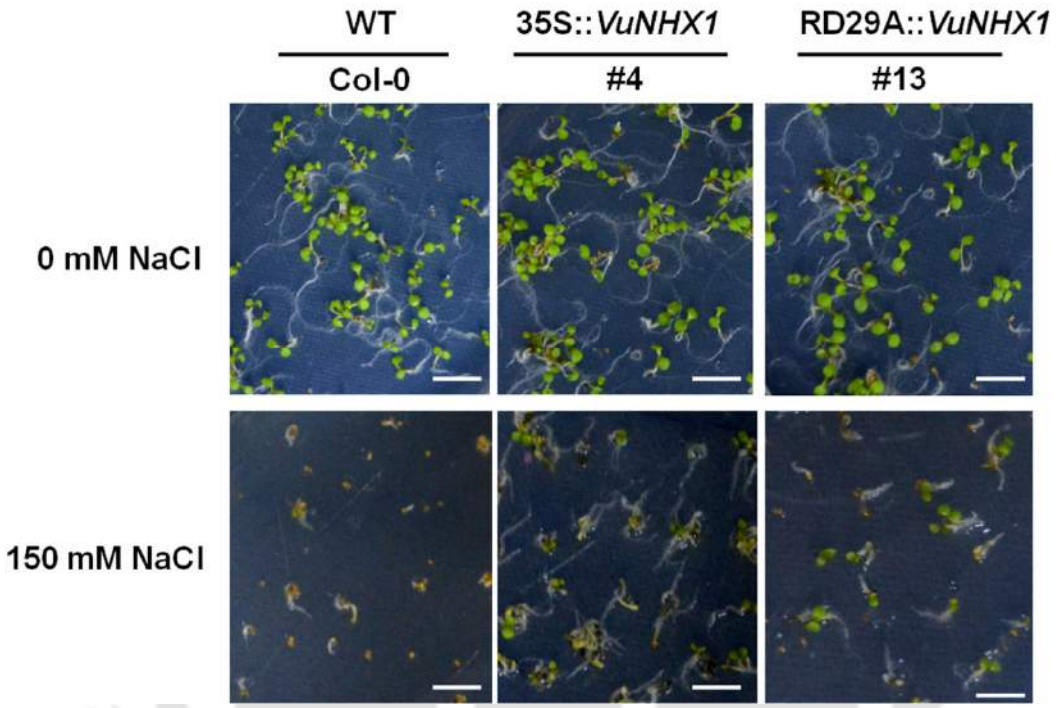


Figure 3.20 Seed germination efficiency in wild-type and transgenic *Arabidopsis* lines under salt stress. Study on effect of salt stress (150 mM NaCl) on germination efficiency of wild-type WT (col-0) and transgenic *Arabidopsis* 35S:*VuNHX1* (#4) and RD29A::*VuNHX1* (#13) lines, monitored after 7 days of salt treatment

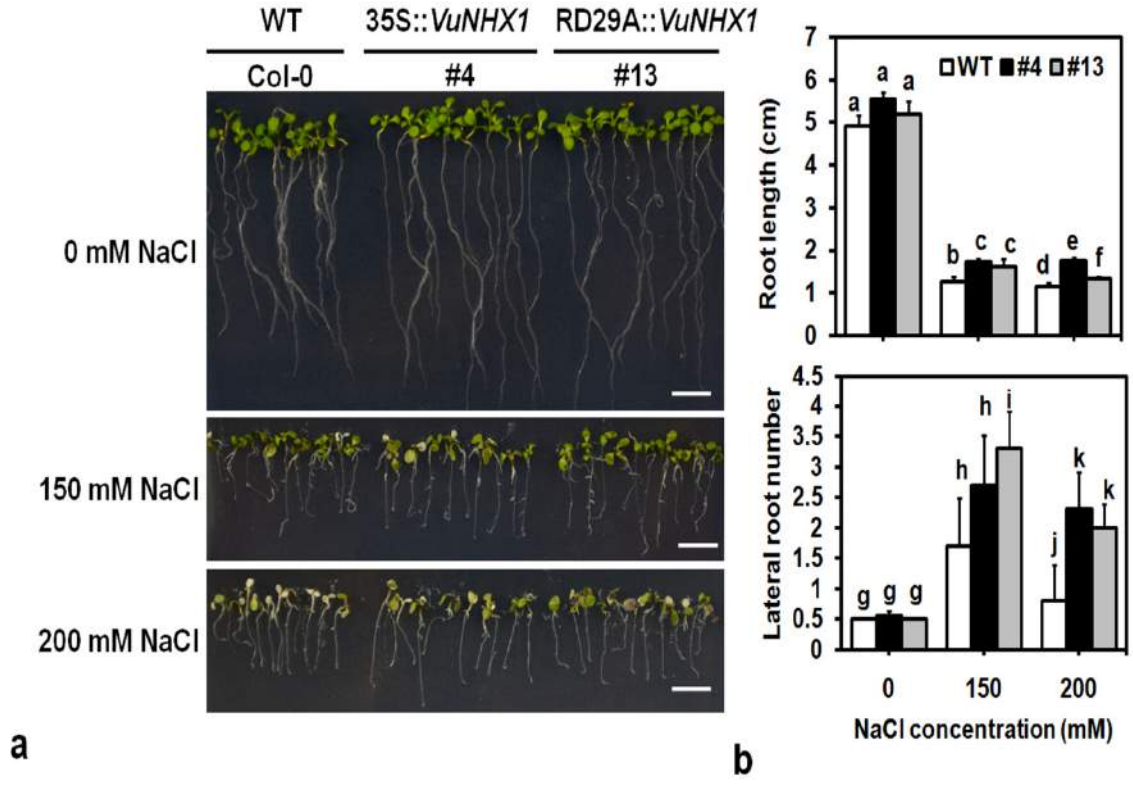


Figure 3.21 Root growth inhibition study under salt stress in wild-type and transgenic *Arabidopsis* lines (a) Study of root growth inhibition in wild type (WT, Col-0) and transgenic *Arabidopsis* lines(Line#4, 35S::*VuNHX1* and Line #13, RD29A::*VuNHX1*) upon salt stress. Excessive NaCl-induced root growth inhibition in Col-0 wild-type (WT) plants was observed as compared to transgenic lines. (b) Root length (cm) and lateral root number was measured after exposure of WT and transgenic lines to 150 and 200 mM NaCl stress for one week. Values indicate means ± SE (n=10). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

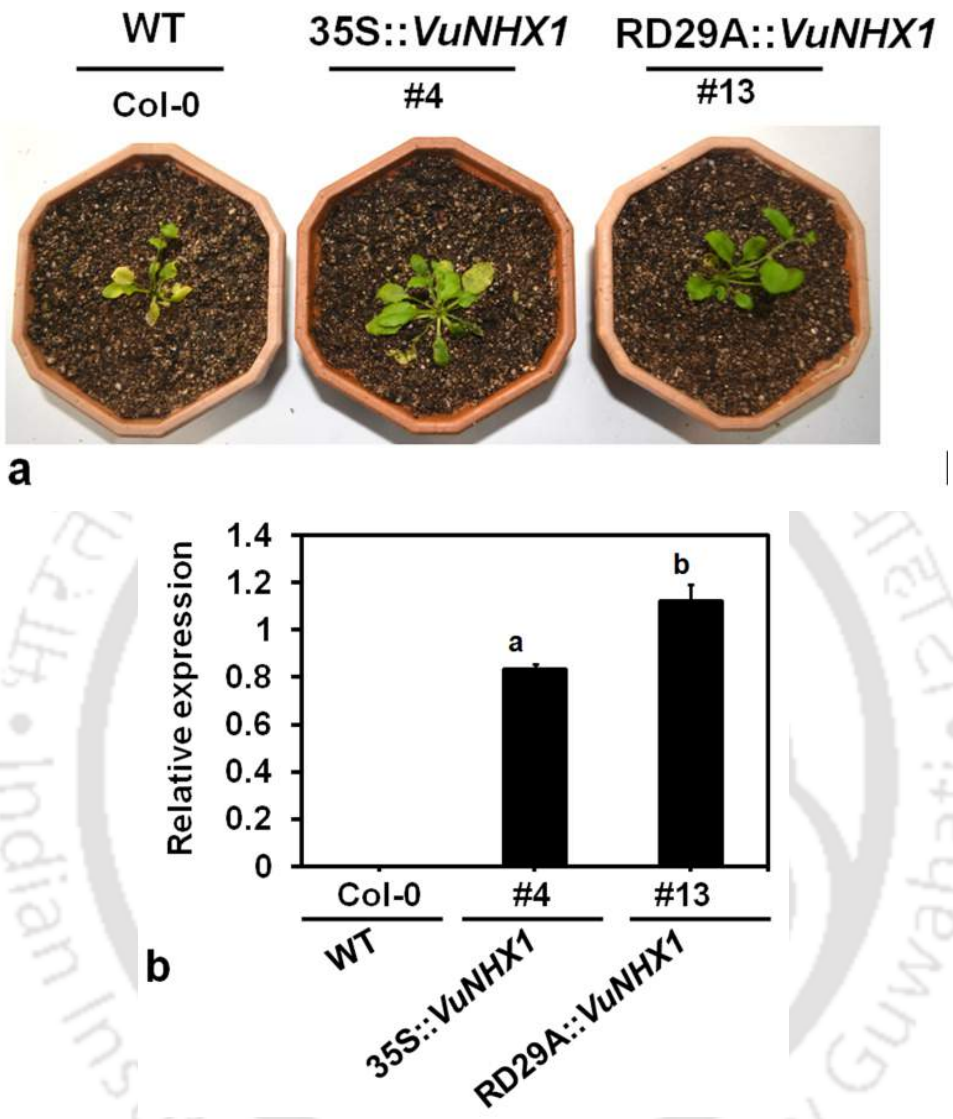


Figure 3.22 Salt stress effect on mature wild-type and transgenic *Arabidopsis* and measurement of relative transgene expression. (a) Salt tolerance assay in wild type (WT, Col-0) and transgenic *Arabidopsis* plants expressing *VuNHX1* constitutively (Line #4, 35S::*VuNHX1*) and inducibly (Line #13, RD29A::*VuNHX1*) subjected to 200 mM NaCl treatment for 2 weeks. (b) Relative transgene expression level of *VuNHX1* in transgenic *Arabidopsis* lines. No transgene expression was observed in WT. A 0.28 kb fragment of *VuNHX1*::35SployA and 0.150 kb fragment of *AtUBQ1* was amplified in case of transgenic *Arabidopsis* lines

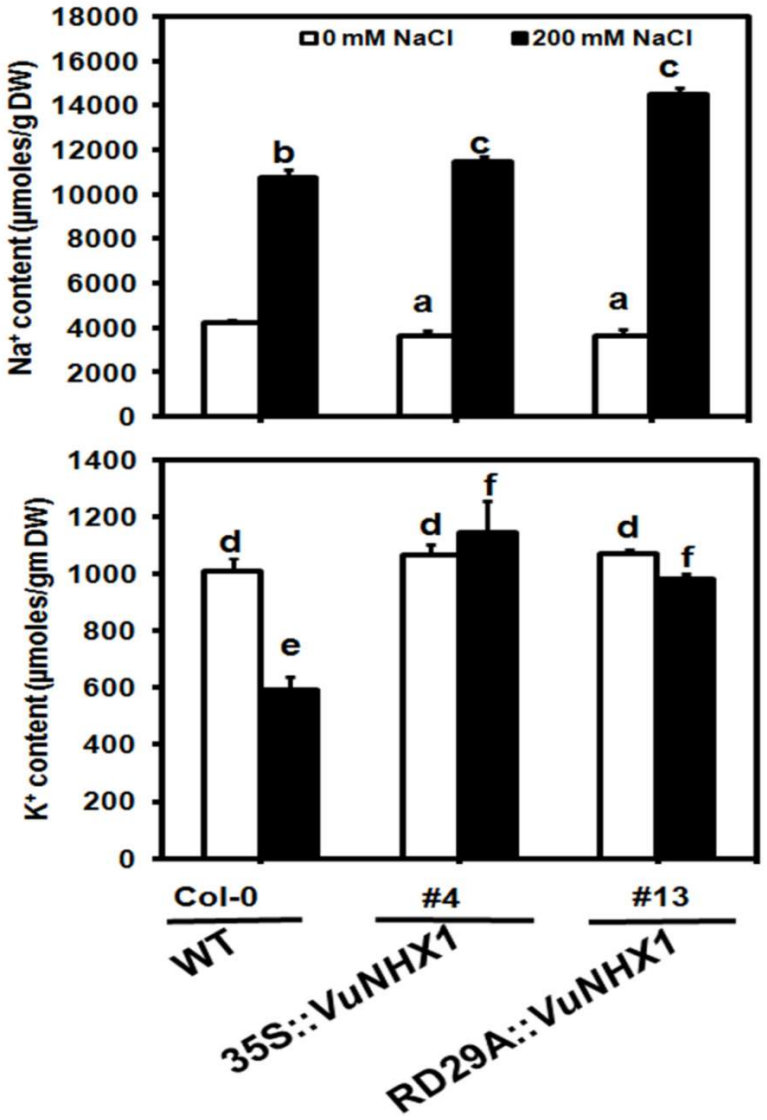


Figure 3.23 Sodium and potassium ion measurement in wild-type and transgenic *Arabidopsis* lines under control and salt stress condition. Na^+ and K^+ content (µmoles/ g DW) was estimated in leaves of salt stressed (200 mM NaCl) and unstressed (0 mM NaCl) WT and transgenic lines (Line #4, 35S::*VuNHX1* and Line #13, RD29A::*VuNHX1*) , as described in materials and methods. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

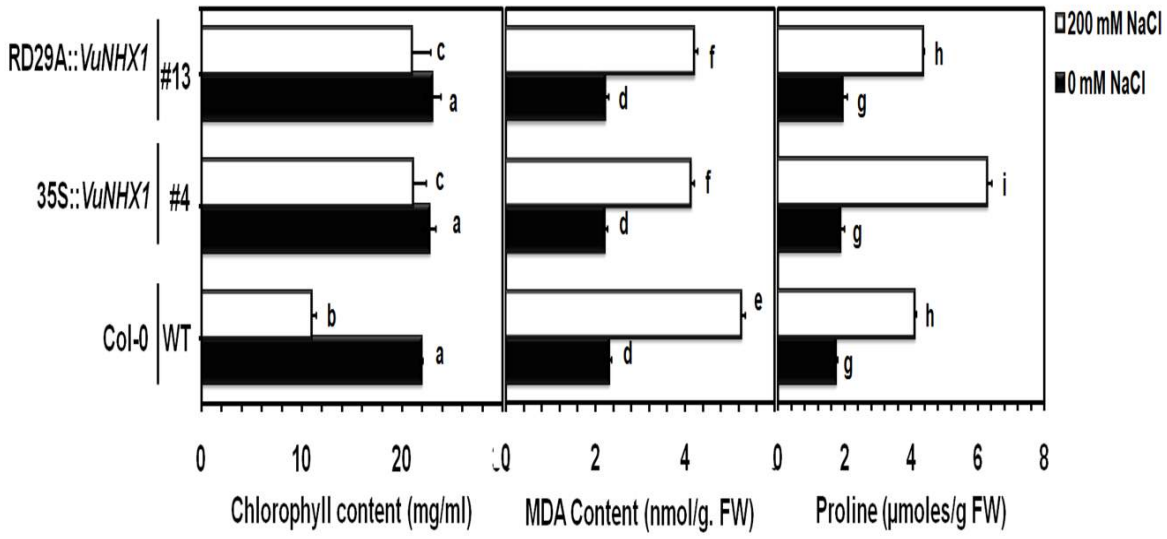


Figure 3.24 Measurement of chlorophyll, malondialdehyde and proline content in wild-type and transgenic *Arabidopsis* lines under salt stress. Physiological analysis of WT (Col-0) and transgenic *Arabidopsis* plants expressing *VuNHX1* constitutively (Line #4, 35S::*VuNHX1*) and inducibly (Line #13, RD29A::*VuNHX1*) upon salt stress. Changes in chlorophyll, MDA and proline content were estimated and analysed as explained in materials and methods section. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

Table 3.1 The putative post-translational modification sites predicted by ScanProsite software for *VuNHX1*

Predicted sites/patterns	Amino acid positions
ASN_GLYCOSYLATION	50-53 293-296
CK2_PHOSPHO_SITE	16-19 250-253 373-376 476-479 480-483 532-535
PKC_PHOSPHO_SITE	250-252 297-299 301-303 379-381 412-414 465-467 492-494 534-536
MYRISTYL	59-64 71-76 117-122 153-158 216-221 229-234 283-288 339-344 390-395
LEUCINE_ZIPPER	256-277

ASN_GLYCOSYLATION- N-Glycosylation site, CK2_PHOSPHO_SITE- Casein kinase II phosphorylation site, PKC_PHOSPHO_SITE- Protein kinase C phosphorylation site, MYRISTYL- N-Myristylation site, LEUCINE_ZIPPER- Leucine zipper pattern

CHAPTER 4

Isolation and functional characterization of a mungbean vacuolar Na^+/H^+ antiporter gene, *VrNHX1* and its ectopic expression in *Arabidopsis thaliana* for studying its role in salt tolerance

4.1. Introduction

More than 20 % of the cultivated area and nearly half of the world's irrigated lands are adversely affected by salinity (Mahajan and Tuteja 2005). Enhanced crop production on salinity inflicted areas will rely on innovative agronomic practices coupled with use of genetically improved crop varieties (Schroeder et al. 2011). In addition to the known role of Na^+/H^+ antiporter gene family in ion homeostasis, recent development indicated its involvement in other cellular processes. Vacuolar compartmentalization of Na^+ is a critical process in the salt adaptation, which is conserved in both halophytes and glycophytes. The genes encoding for NHX proteins have been discovered in more than 60 plant species, including gymnosperms and dicotyledonous and monocotyledonous angiosperms (Xu et al. 2013). The expression of most of these NHX genes was induced by NaCl treatment (Pardo et al. 2006). Overexpression of vacuolar Na^+/H^+antiporter NHX genes suppressed the salt sensitive phenotype of a yeast mutant defective for endosomal/vacuolar Na^+/H^+ antiporters and conferred salt tolerance to transgenic plants (Yokoi et al. 2002; Zhang and Blumwald 2001). Several reports on improvement of salt tolerance through overexpression of vacuolar NHX genes in agriculturally important but, glycophtic crops implicate a pivotal function of the NHX genes in intracellular compartmentalization of Na^+ and salt tolerance (Rodriguez-Rosales et al. 2009; Schroeder et al. 2013). However, no salt-tolerant related genes including *NHX* has yet been reported from mungbean.

Mungbean (*Vigna radiata* L. Wilczek) is an important grain legume widely cultivated in south, east and south-east Asian countries for its protein rich grains. Salinity is recognized as major constraint in the production of mungbean (Hasanuzzaman et al. 2013; Jacoby 1999). Mungbean is moderately drought tolerant (Nair et al. 2013) and therefore, this distinctive character makes it a valuable tropical crop legume for studying the molecular tolerance mechanisms for various abiotic stresses including salinity.

In this chapter, we report the cloning and molecular characterization of NHX1 antiporter from *V. radiata*, (*VrNHX1*), expression pattern of *VrNHX1* under various abiotic stresses like salt, dehydration and cold stress, and functional complementation of *VrNHX1* in salt sensitive mutant (AXT3). We demonstrate the

role of *VrNHX1* in enhancing salt tolerance by constitutive and inducible expression of *VrNHX1* in transgenic *Arabidopsis thaliana*.

4.2. Materials and methods

4.2.1. Plant material and stress treatment

Mungbean (cv. K-851) seeds were surface sterilized with 0.2% mercuric chloride and rinsed three times with distilled water. The seeds were germinated in dark chamber for 2 days, transferred to Hoagland's solution, grown in a controlled growth chamber at 25^{0}C, 80% relative humidity with a 16hour/8hour photoperiod and photosynthetic flux intensity of 300 µmol $m^{-2}s^{-1}$. Two weeks old seedlings were treated with 200 mM NaCl for 12 hours and roots were harvested, frozen immediately, and stored at -80°C until further use.

4.2.2. Molecular cloning of *VrNHX1* cDNA by RACE approach

4.2.2.1. PCR amplification of partial fragment of *VrNHX1* using degenerate primers

Total RNA was isolated from salt-treated roots of mungbean using AMBION RNAqueous® Kit, according to the manufacturer's instructions and quantified in Nanovue™ Plus Spectrophotometer. One microgram of RNA was used for cDNA synthesis using Revert Aid ™ First Strand cDNA Synthesis Kit, as per manufacturer's instructions. The cDNA was amplified with a pair of degenerate primers (Deg FP: 5'-TAT(A/T)ATATTCAATGC(C/A)GGGTTTCA(G/A)GT(A/G) -3' and Deg RP: 5'-GCATT(A/G)TGCCA(A/G)GT(A/G)TAATG(A/T)GACAT(A/G/C)AC -3') designed from the conserved region of transmembrane domains of plant NHX antiporters submitted in NCBI database. The PCR reaction consisted of 1X Taq Buffer, 200 µM dNTP mix, 20 µM forward and reverse primer, 1 unit of Ex-Taq™ ploymerase, sterile water and 1 µl cDNA as template, in a reaction volume of 25 µl. The PCR was performed in Thermal cycler with the following condition: 94^{0}C for 3 min; 94^{0}C for 30 sec, 52 ^{0}C for 30 sec, 72 ^{0}C for 30 sec with 35 cycles, and a final extension at 72 ^{0}C for 10 min. Based on the resulting partial PCR fragment, gene specific primers were designed for amplification of 5'- and 3'- untranslated regions of *VrNHX1*.

4.2.2.2. 5'-RACE for amplification of 5'-UTR fragment of *VrNHX1*

The 5' RACE was performed using the 5' RACE System for Rapid Amplification of cDNA Ends Kit, according to the manufacturer's instructions. Five micrograms of RNA was used for the first strand cDNA synthesis using a gene specific primer (VrGSP1: 5'- CTGCTTCTTTTTCACCTGAAACCCAGC -3') and superscript™ II reverse transcriptase. The cDNA was purified using SNAP column to remove unincorporated dNTPs and primer that might interfere in the homopolymeric tailing of cDNA. Terminal transferase enzyme was used to add dCTPs to 3' end of cDNA. The dc-tailed cDNA was amplified using abridged anchor Primer (AAP: 5'-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG -3') and gene specific primer (VrGSP2: 5'- ACCTGAAACCCAGCATTGAATAT-3'). The PCR condition was: 94^{0}C for 3 min; 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 30 sec with 30 cycles, and a final extension of 72 ^{0}C for 10 min. Further, nested PCR was performed using abridged universal anchor primer (AUAP: 5'- GGCCACGCGTCGACTAGTAC -3') and nested gene specific primer (VrGSP3: 5'- GGTATATGAAGAAAAGATCTTC -3') using the first PCR product as template to obtain 5' end product. The PCR conditions were 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, 72 ^{0}C for 30 sec with 35 cycles, and a final extension of 72 ^{0}C for 10 min.

4.2.2.3. 3'-RACE for amplification of 3'-UTR fragment of *VrNHX1*

The 3' RACE was performed using 3' RACE System for Rapid Amplification of cDNA Ends Kit, according to the manufacturer's instructions. Five micrograms of RNA was used to synthesize cDNA using a dT-Adapter primer (AP: 5'- GGCCACGCGTCGACTA GTAC$(T)_{17}$ -3'). The first 3'-RACE-PCR was carried out using gene specific primer (VrGSP4: 5'- AGTGGCATCCTCACTGTATTCTTTTGTG -3') and abridged universal anchor primer (AUAP: 5'- GGCCACGCGTCGACTAGTAC -3'). The PCR condition was: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 60 ^{0}C for 30 sec, 72 ^{0}C for 1min and 30sec with 30 cycles, and a final extension of 72 ^{0}C for 10 min. The PCR product was diluted 10 times (1:10) and used as template for nested 3' RACE-PCR. The nested 3'-RACE-PCR was carried out using gene specific primer (VrGSP5: 5'-GCTGTATATTG GAAGGCACTCT-3') and abridged universal anchor primer (AUAP: 5'-GGCCACGCG TCGACTAGTAC -3'). The PCR condition was: 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 55 ^{0}C for 30 sec, 72 ^{0}C for 1 min and 30 sec with 30 cycles, and a final extension of 72 ^{0}C

for 10 min. The above PCR products were sequenced and contiguous sequences aligned to obtain full length of *VrNHX1* cDNA.

4.2.2.4. Cloning of PCR fragments of *VrNHX1* into TA-cloning vector

The PCR products (*VrNHX1* cDNA) obtained from the above conditions were cloned into TA cloning vector pTZR/T (2.8 kb), as per the manufacturer's instructions and transformed into *E. coli* DH5α competent cells. The blue-white screening was performed for selection of recombinant bacterial colonies on solid Luria Broth (LB) plates supplemented with 100 mg/l ampicillin antibiotic, 40 µl of 20 mg/l X-Gal substrate and 40 µl of 20 mg/l IPTG inducer, grown at 37 °C in static incubator chamber. The white clones were selected, confirmed by digestion and sequenced. The contiguous sequences aligned to obtain full length of *VrNHX1* cDNA and submitted in NCBI database.

4.2.2.5. PCR amplification of *VrNHX1* CDS

The forward (VrFGP: 5'- ATGGTCTTTGAAATCAGTTCTGTTGTTTC- 3') and reverse (VrRGP: 5'-TCAACGCCATTGATGACCATTACGTTC- 3') primers were used for the amplification of the CDS region of *VrNHX1*. The PCR conditions were 94 ^{0}C for 3 min; 94 ^{0}C for 30 sec, 58 ^{0}C for 30 sec, 72 ^{0}C for 1 min and 30 sec with 35 cycles, and 72 ^{0}C for 10 min. The 1.6 kb amplicon obtained was cloned into TA cloning vector pTZR/T (2.8 kb), the resulting recombinant plasmid pTZR/T*VrNHX1* (4.4 kb) was sequenced.

4.2.2.6. Agarose gel electrophoresis

The PCR products obtained from the above conditions were mixed with 6x loading dye and electrophoresed on 1% agarose gel containing ethidium bromide (10 mg/ml). Electrophoresis was carried out at 90 V for 1-2 h. An aliquot (500 ng) of Lambda HindIII/EcoRI DNA ladder were used as molecular weight markers. Standard molecular biology techniques were used for all DNA manipulations (Sambrook 1989).

4.2.3. In-silico analysis of *VrNHX1*

Multiple sequence alignment and phylogenetic analysis were performed using ClustalW (Thompson et al. 1997). A phylogenetic tree was constructed using neighbor joining method and reliability of the tree was analyzed with bootstrap

analysis with 500 replicates using MEGA4 (Molecular Evolutionary Genetics Analysis): Tree Explorer software (Tamura et al. 2007). Hydrophobicity plot and transmembrane domain prediction was performed using TMpred software (Hofmann and Stoffel 1993). Secondary structure prediction was done using SOPMA (Geourjon and Deleage 1995). Post-translational modification of *VrNHX1* was predicted by searching for conserved motifs of N- and O- glucosylation and N-myristoylation sites using ScanProsite (Gattiker et al. 2002).

4.2.4. Southern hybridization for *VrNHX1* copy number analysis

Twenty µg of genomic DNA was used for gene copy analysis of *VrNHX1* and digested with restriction endonucleases EcoRI and HindIII. Digested genomic DNA was electrophoretically fractionated on 1% agarose gel and blotted onto Zeta-Probe membrane. The blot was hybridized with DIG-labeled 1.6 kb PCR product, corresponding to the coding region of *VrNHX1*. Southern hybridization was carried out using solution containing 50% formamide, 5X SSC, 5X Denhardt's solution, 0.05 M sodium phosphate pH 6.5, 0.1% SDS, 10% Dextran sulfate, 0.1 mg/ml sheared denatured salmon-sperm DNA and 20 ng/ml probe at 42°C for 18 hours. Washing and detection was performed according to instructions of the DIG Labeling and Detection system.

4.2.5. Complementation studies of *VrNHX1* in yeast mutant

4.2.5.1. Cloning of *VrNHX1* in yeast expression vector

The CDS of *VrNHX1* was cloned into yeast expression vector pYES2.0 (5.9 kb) with restriction sites of *Kpn*I and *Bam*HI. The ligation reaction was carried out using 1U of ligase enzyme (NEB, UK) at 16°C overnight and transformed into *E. coli* competent cells. The recombinant colonies were screened and confirmed by restriction digestion of the recombinant plasmid pYES2.0*VrNHX1* (7.5 kb). The digested fragments were resolved in 1% agarose gel and stained with 10 mg/ml ethidium bromide for visualization under UV transilluminator.

4.2.5.2. Yeast complementation assay

Functional complementation assay was performed in yeast strains, W303-1B (*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) and AXT3 *(Δ ena1- 4::HIS3 Δnha1::LEU2 Δnhx1::TRP1, ura3-1)*. Yeast strains were grown as described in section

3.2.5.2. AXT3 was transformed with empty pYES2.0 and recombinant pYES2.0*VrNHX1* vector and thereby, labeled as AXTYES2.0 and AXTYESVrNHX1, respectively, by Lithium acetate method (Gietz et al. 1992) and selected on SC ura$^-$ medium.

4.2.5.3. Growth assay under stress treatment

For growth assay, precultured cells were grown till OD_{600} of 1.0, diluted to an OD_{600} of 0.006, and inoculated to liquid APGal ura$^-$ synthetic minimal media supplemented with different concentrations of NaCl, KCl, and LiCl and grown at 30 °C for 48 hours. For complementation assay, saturated liquid cultures (OD_{600}- 0.8) of each strain were serially diluted to 10, 100 and 1000 fold and spotted on APGal and YPGal solid media supplemented with or without 50, 75 and 100 mM NaCl, 0.5 M KCl, 25 mM LiCl and 50 µg/ml Hygromycin-B. Plates were maintained at 30 °C and the growth was monitored after 3 days.

4.2.5.4. Intracellular measurement of Na^+ and K^+ content

Intracellular ion was extracted from yeast strains grown in liquid APGal media, pH 4.0 supplemented with or without 75 mM NaCl, following method described by Venema et al. (2003) and further, described in section 3.2.5.4.

4.2.5.5. Vacuolar pH estimation and fluorescence imaging

Yeast cells were grown in APGal medium (pH 5.0) supplemented with 75 mM NaCl to an OD_{600}: 0.25-0.3, pelleted, and washed with deionized distilled water. Further, the yeast cells were incubated with 50µM 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM) (Molecular Probes, Eugene, Oregon) for 30 minutes, centrifuged, washed thrice and resuspended in APGal medium (pH 5.0) and immediately used for fluorescence measurement. Single emission fluorescence measurement at 490 nm excitation wavelength and absorbance at 600 nm were measured using LS 55 Fluorescence Spectrophotometer (Perkin Elmer, Waltham, MA, USA). The calibration curve for fluorescence intensities at different pH was obtained for each strain following method as described by Brett et al (2005). Briefly, the yeast strains were incubated in experimental medium containing 50 mM MES, 50 mM HEPES, 50 mM KCl, 50 mM NaCl, 0.2 M ammonium acetate, 10 mM NaN_3, 10 mM 2- deoxy glucose, 50 µM carbonyl cyanide m-chlorophenylhydrazone, titrated to

five different pH values within the range of 4.0 to 8.0. Background substracted I_{490} values were normalized to cell density, NI_{490} and plotted against pH values. For vacuolar pH estimation, experimental NI_{490} values corresponding to each strain was analyzed with the calibration curve specific for each strain.

For vacuolar pH imaging the yeast cells were grown, pelleted to be suspended in the same medium with 50 µM BCECF-AM pH specific dye as above. For fluorescence imaging, 100 µl of BCECF-loaded yeast suspension was plated onto glass coverslips precoated with concavalin-A (Sigma-Aldrich, St. Louis, MO, USA) and placed on glass slides. Fluorescence images were captured in Nikon eclipse Ti-U Fluorescence microscope (Nikon, Chiyoda,Tokyo, Japan).

4.2.6. Expression analysis of *VrNHX1* using Semi-quantitative RT-PCR

Two different stages of growth in mungbean seedlings i.e. early and mid stage, were considered for expression analysis under salt stress (200 mM NaCl). Mungbean seedlings were germinated, grown in Hoagland's nutrient medium and transferred to 200 mM NaCl solution for salt stress assay. Leaves and roots of salt treated mungbean seedlings, five and ten days old, in case of early and mid stage respectively, were harvested at time intervals 0, 6, 12, 18, 24, and 48 hours. Similarly, expression pattern for *VrNHX1* in response to different forms of abiotic stress such as salt (200 mM NaCl), dehydration (200 mM mannitol) and cold stress (4 ^{0}C) was also studied at different time intervals (0, 6, 12, and 24 hours) for mid-stage (10 days old) mungbean seedlings. Total RNA was extracted using RNeasy Plant Mini Kit (Qiagen, Venlo, Limburg, Netherlands) and reverse transcribed using Revert Aid™ First Strand cDNA Synthesis Kit. Semi-quantitative RT-PCR was performed using gene specific primers (VrRFP: 5'- GTATTTCCACTGGCGTAGTCATT TTGC -3' and VrRRP: 5'- GCATCATTCACAGCACCCTCTCGG -3'). The PCR condition was: 94 °C for 3 min; 94 °C for 30 sec, 62 °C for 30 sec, 72 °C for 30 sec for 28 cycles, and a final extension at 72 °C for 10 min. Housekeeping *VrTubulin-β* primers (VrFNP: 5'- CTTGACTGCATCTGCTATGTTCAG-3' and VrRNP: 5'-CCAGCTAATGCTCGGCATACT G -3') were used as an internal control. The PCR condition was: 94 °C for 3 min; 94 °C for 30sec, 58 °C for 30 sec, 72 °C for 30 sec for 28 cycles, and a final extension at 72 °C for 10 min. Semi-quantitative RT-PCR was repeated three times. The PCR

products were analyzed in 2 % agarose gel stained with 10 mg/ml ethidium bromide.

4.2.7. Measurement of total ion content in salt stressed mungbean seedlings

Leaves and roots of untreated and salt-treated early and mid stage mungbean seedlings were harvested at different time intervals (0, 6, 12, 18, 24, 48 and 72 hours). The total ion analysis was performed as described in section 3.2.7.

4.2.8. Binary vector preparation and plant transformation

4.2.8.1. Preparation of pCAMBIA230135S::*VrNHX1*

The 1.6 kb CDS of *VrNHX1* was cloned into standard plant binary vector pCAMBIA2301 (11.6 kb) flanked by cauliflower mosaic virus CaMV 35S promoter and terminator at *Pst*I restriction site. The resulting recombinant plant binary vector was labeled as pCAMBIA230135S::*VrNHX1* (13.9 kb) and transformed into *E.coli* competent cells. The recombinant colonies were screened using blue-white selection of bacterial colonies and confirmed by restriction digestion.

4.2.8.2. Preparation of pCAMBIA2301RD29A::*VrNHX1*

Further, a 0.898 kb promoter fragment of *AtRD29A* (DQ071887.1) was amplified from *A. thaliana* genomic DNA and cloned into *Eco*RI digested recombinant binary vector pCAMBIA230135S::*VrNHX1* (13.9 kb) by replacing the 0.4 kb 35S promoter fragment from 35SP::*VrNHX1*::35STer cassette. The resulting binary vector was named pCAMBIA2301RD29A::*VrNHX1* (14.4 kb) and transformed into *E.coli* competent cells. The recombinant colonies were screened using blue-white selection of bacterial colonies and confirmed by restriction digestion. All molecular biology techniques were employed for all DNA manipulations (Sambrook et al. 1989).

4.2.8.3. Mobilization of plant binary constructs to *Agrobacterium tumefaciens*

The recombinant plant binary vectors, pCAMBIA230135S::*VrNHX1* (13.9 kb) and pCAMBIA2301RD29A::*VrNHX1* (14.4 kb) were transferred into *A. tumefaciens* GV3101 strain via electroporation at 1250 V with capacitance of 25 mF and resistance of 400 ohm. The constructs were used for transformation of *Arabidopsis thaliana* (ecotype Columbia) via floral dipping method (Clough and Bent 1998). The T_1 transgenic lines were screened on ½ MS medium supplemented with 50 mg/l

kanamycin. The transgenic selections were continued until T_3 generation to obtain homozygote transgenic lines with a single T-DNA locus (35S::*VrNHX1* or RD29A::*VrNHX1*).

4.2.9. RNA extraction and quantitative expression analysis in transgenic *Arabidopsis* by Real Time PCR

Total RNA was extracted from wild-type and T_3 independent 35S::*VrNHX1* and RD29A::*VrNHX1* transgenic lines using RNeasy Plant Mini Kit quantified in Nanovue™ Plus Spectrophotometer and cDNA was prepared using Revert Aid ™ First Strand cDNA Synthesis Kit. The gene specific forward primer (VrRTF: 5'-TGATTCAATCCATCGACCAA-3') and 35S poly-A reverse primer (TerparR: 5'-GCGAAACCCTATAAGAACCCTAATTCC-3') were used for amplification of a 0.283 kb fragment of *VrNHX1*::35S poly-A in transgenic *A. thaliana* plants. Housekeeping (UBQ1FP: 5'- AGAGCTGTCAACTGCAGGAAGAA-3' and UBQ1RP- 5'-ACAAGAAAAAC AAACCCTATCAAA GG) primers were used to amplify a 150 bp fragment of *AtUbiquitin* to be used as an internal control. Real time PCR was performed using USB VeriQuest™ SYBR Green qPCR Master Mix (2X) and primers at a final concentration of 200nM in 7500 Real-Time PCR System, following the manufacturer's protocol. The experiment was repeated twice independently with three replicates. The expression values relative to the standard curve was calculated for each sample. The relative expression level of transgene *VrNHX1* in wild-type (WT) and transgenic *Arabidopsis* lines was estimated by normalizing *VrNHX1* expression values with respect to housekeeping *AtUBQ1* expression values in each case.

4.2.10. Salt tolerance assays of transgenic *Arabidopsis* lines

Wild-type and T_3 transgenic *Arabidopsis* seeds were germinated on ½ MS medium (Murashige and Skoog 1962) in growth chamber maintained at 22 °C and 60% relative humidity with a 16h/8h photoperiod under controlled conditions.

4.2.10.1. Effect of salt stress on seed germination

The WT and T_3 transgenic 35S::*VrNHX1* and RD29A::*VrNHX1* lines were germinated on ½ MS medium supplemented with or without 150 mM NaCl and kept at 4 °C for 3

days, prior to, transfer to growth chamber. The germination efficiency was studied after 10 days of salt stress.

4.2.10.2. Measurement of growth parameters under salt stress

The 4 days old germinated seedlings were transferred to ½ MS medium supplemented with or without 150 mM NaCl for 1 week and the difference in root length of wild-type WT and T_3 independent transgenic lines of *Arabidopsis* seedlings expressing *VrNHX1* was measured. Mean data was collected from ten replicates (n=10) for wild-type (WT) and T_3 kanamycin selected transgenic *Arabidopsis* lines.

4.2.10.3. Measurement of physiological parameters under salt stress

The 10 days old germinated seedlings were transferred to ½ MS liquid medium supplemented with or without 200 mM NaCl for 5 days. For measurement of chlorophyll, malondialdehyde (MDA), and proline content, similar method was followed as described in section 3.2.10.2.1. Mean data was collected from three replicates (n=3) for wild-type (WT) and T_3 kanamycin selected transgenic *Arabidopsis* lines.

4.2.10.4. Measurement of Na^+ and K^+ in transgenic *Arabidopsis* lines

The germinated seedlings were initially grown in ½ MS medium (0.5% agar) for 5 days and then subsequently transferred to soilrite and grown for 2 weeks. The WT and transgenic lines were subjected to salt stress for a period of 2 weeks by watering them with ½ MS nutrient liquid media supplemented with 250 mM NaCl. The whole plant was harvested for Na^+ and K^+ estimation using method described in section 3.2.7. Mean data was collected from three replicates (n=3) for wild-type (WT) and T_3 kanamycin selected transgenic *Arabidopsis* lines.

4.2.11. Statistical analysis

Statistical comparison between the variances was determined by ANOVA (Analysis of variance) and significant differences between mean values were determined by Bonferroni analysis. Statistically significant mean values were denoted as " * " ($P \leq 0.05$).

4.3. Results and discussion

4.3.1. Isolation and in-silico analysis of *VrNHX1*

The degenerate primers designed from highly conserved regions in NHX1 proteins (Fig. 4.1) amplified a 600 bp partial fragment of *VrNHX1* (Fig. 4.2). Further, the 5'- (Fig. 4.3) and 3'- UTR of VrNHX1 (Fig. 4.4) were obtained by RACE-PCR approach. The *VrNHX1* cDNA was found to be 2095 bp (Genbank Accession No. JN641304.2). The open reading frame of *VrNHX1* consisted of 1,629 bp (Fig. 4.5), encoding 542 amino acid residues with an estimated molecular mass 59.60 kDa and isoelectric point 6.76 as predicted using ExPaSy bioinformatic tools for protein structure analysis (http://www.expasy.org/tools/). Multiple sequence alignment of deduced amino acid sequences of *VrNHX1* revealed that it has 97.42% sequence identity with *Vigna unguiculata*, 92.25% with *Glycine max*, 88.48% with *Caragana korshinskii*, 87.27% with *Lotus tenuis*, 87.25% with *Trifolium repens*, 87.06% with *Medicago sativa*, and 86.72% with *Cicer arietinum* (Fig. 4.6). Phylogenetic relationship analysis performed using MEGA4 software indicated that *VrNHX1* clustered into Class-I type IC-NHX legume *NHX* homologs, more closely to *VuNHX1* and *GmMHX1* (Fig. 4.7). The hydropathy plot of VrNHX1 protein predicted by TMpred software indicated highly hydrophobic N-terminal end with 11 putative transmembrane domains and a longer hydrophilic C-terminal end inside the vacuolar lumen (Fig. 4.8). The amiloride binding motif, ^{84}-LFFIYLLPPI-^{93}, a classic inhibitor of Na^+/H^+ antiporters (Harris and Fliegel 1999) and also highly conserved among eukaryotic Na^+/H^+ exchangers, was detected in TM3 region (Fig. 4.6). Secondary structure prediction by SOPMA (Self-Optimized Prediction Method with Alignment) indicated occurrence of alpha helix (48.34%), extended strand (14.76%), beta turn (3.32%), and random coil (33.58%) (Fig. 4.9). The prediction of putative post-translational modification sites by ScanProsite software indicated presence of two potential *N*-glycosylation (ASN_glycosylation) sites, fifteen phosphorylation sites for protein kinase CK2 and protein kinase C, ten *N*-myristoylation sites, and one Leucine Zipper site (Table 4.1).

4.3.2. Copy number analysis of *VrNHX1*

The Southern hybridization analysis revealed presence of single copy of *VrNHX1* in mungbean genome (Fig. 4.10). Two hybridization signals one each for *Hind*III and *Eco*RI digested mungbean genome were detected, possibly due to the occurrence of

a single *Hind*III site in *VrNHX1* (1.6 kb). Occurrence of a single *Eco*RI site in genome fragment of *VrNHX1* was accounted for getting two signals as probe lacked any *Eco*RI site. However, previous reports indicated occurrence of *NHX1* in multiple copies in *B. napus* and *M.zumi* (Wang et al. 2003; Qingxia et al. 2009).

4.3.3. Yeast complementation studies

Previous work showed that heterologous expression of Na^+/H^+ antiporter genes in yeast mutant AXT3 could partly suppress its hypersensitivity to hygromycin-B and restore salt tolerance. The similar method was exploited to initially characterize the function of *VrNHX1*. The ORF of *VrNHX1* was cloned into yeast expression vector pYES2.0 and used for validation of role of *VrNHX1* in yeast mutant AXT3 (Fig. 4.11). The AXTVrNHX1 cells displayed enhanced Na^+, K^+ and Li^+ tolerance with statistically significant improvement in their survival at NaCl (75 and 100 mM) and 0.5 M KCl in contrast to AXTYES2.0 cells (Fig. 4.12). Expression of *VrNHX1* in AXT3 cells under GAL1-inducible promoter restored salt tolerance upto 100 times dilution in 75 and 100 mM NaCl, and better survival at 1000 times dilution range in 25 mM LiCl and 0.5 M KCl in AXTVrNHX1 cells on solid media (Fig.4.13). *ScNHX1* has been suggested to ameliorate sensitivity of yeast cells by sequestering hygromycin-B, a cationic aminoglycoside antibiotic in vacuole. Therefore, yeast mutant lacking *NHX1* is more susceptible to hygromycin treatment (Brett et al. 2005). *VrNHX1* expression showed suppression of hygromycin (50 µg/ml) sensitivity in AXTVrNHX1 cells (Fig. 4.13).

4.3.4. Na^+ and K^+ distribution in yeast mutants

The AXTYES2.0 cells displayed 2.3 times lower K^+ content than AXTVrNHX1 cells under normal condition owing to lack of yeast $Na^+/K^+/H^+$ antiporter activity (Fig. 4.14). Under salt stress, AXTVrNHX1 cells accumulated 2 times higher and 4.8 times lower vacuolar Na^+ content compared to AXTYES2.0 and W303-1B cells, respectively (Fig. 4.14). Similar findings were reported in functional complementation of rice NHX1 gene in *Δena Δnha1 Δnhx1* mutant (Kinclova-Zimmermannova et al. 2004). Similarly, vacuolar K^+ content observed for AXTVrNHX1 cells was 2.36 times higher than AXTYES2.0 cells (Fig. 4.14). The cytoplsamic Na^+ content was higher in both the cell types as compared to W303-1B, due to the loss of NHA exchanger activity which cannot be solely compensated by *VrNHX1* complementation. However, cytoplasmic K^+ fractions measured were not statistically significant, though AXTVrNHX1 cells

exhibited higher K^+ values as compared to AXTYES2.0 indicating the improved ability of AXTVrNHX1 cells in maintaining a higher intracellular K^+/Na^+ ratio for ionic homeostasis. This data was in accordance with previous reports of functional complementation of At*NHX1* (Yokoi et al. 2002) and *TNHXS1* (Gouiaa et al. 2012) in AXT3 mutant. The total ion content in yeast cells was in accordance with distribution of Na^+ and K^+ in cytoplasm and vacuole (Fig. 4.14).

4.3.5. Vacuolar pH estimation and imaging

2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM), a widely used fluorescent indicator was used to measure the change in vacuolar pH of yeast mutant expressing *VrNHX1* in low pH condition. The growth of AXT3 mutant was found highly affected as compared to wild-type strain W303 while, AXT3 mutant expressing *VrNHX1* showed improved growth under acidic condition (Fig. 4.15).

Vacuolar pH estimated following calibration curve plotted for each strain (Fig. 4.16a) showed an acidic vacuolar pH of 5.4 in AXT3 and 5.9 in AXT3 expressing *VrNHX1.* W303 wild type strain maintained a pH value 6.2 in response to low pH stress condition (Fig. 4.16b). Similarly, fluorescence images provided acidic vacuolar pH values for AXT3 mutant, and the cells expressing *VrNHX1* alkalinized the vacuolar compartment (Fig. 4.17).

4.3.6. Expression of *VrNHX1* in mungbean seedlings under abiotic stresses

The expression of *VrNHX1* was studied by semi-quantitative RT-PCR, in roots and leaves of mungbean seedlings at early (five days old) and mid (ten days old) growth stages exposed to salt stress (200 mM NaCl) for different time interval (0, 6, 12, 18, 24, and 48 hours). The results indicated that transcript levels of *VrNHX1* were induced by NaCl in both roots and shoots of early and mid stage mungbean seedlings indicating the potent role of *VrNHX1* in salt tolerance mechanisms in mungbean. In case of early seedling stage, higher expression level of *VrNHX1* was observed in leaves at 12, 24, and 48 hours and after 6 hours in roots (Fig. 4.18). The differential expression of *VrNHX1* in roots and leaves was also observed in mid stage seedlings, with a significant accumulation observed at 48 hours in leaves whereas, some basal level of *VrNHX1* transcript was observed in roots under normal condition which further, increased steadily with salt stress treatment period (Fig. 4.18). This result

was in accordance with expression pattern of *ZmNHX1*, *AeNHX1, AlNHX1,* and *ThNHX1* under salt stress (Zorb et al. 2005; Zhang et al. 2008; Qiao et al. 2007; Wu et al. 2009). However, some earlier studies have reported higher expression of *NHX1* in leaves/ shoots of rice*, AgNHX1, SsNHX1, PeNHX1, MsNHX1, TrNHX1, ZjNHX1, ZxNHX,* and *DmNHX1* (Fukuda et al. 1999; Hamada et al. 2001; Ma et al. 2004; Rajagopal et al. 2007; An et al. 2008; Tang et al. 2010; Du et al. 2010; Wu et al. 2011; Zhang et al. 2012).

To determine whether the expression of *VrNHX1* was also induced by dehydration (200 mM Mannitol) and cold (4 ^{0}C), mid-stage (10 days old) seedlings were given the respective stress treatments for different time intervals (0, 6, 12, and 24 hours). The *VrNHX1* expression varied with salt, cold and drought stress. The accumulation of *VrNHX1* transcript under salt, cold and dehydration stress reached its peak at 24 hours (Fig. 4.19). The results indicated that osmotic and low temperature stress is involved in the up-regulation of *VrNHX1* in addition to an ion-specific signaling component in mungbean. The *VrNHX1* expression analysis revealed involvement of cross talk between salinity, low temperature and osmotic stress in mungbean. The result was contrary to the previous reports on expression pattern of *PeNHX1* and *ThNHX1* (Wu et al. 2009; Rajagopal et al. 2007) under cold stress that showed decrease in the transcript accumulation and no change in expression pattern of *AtNHX1* (Shi and Zhu 2002). Up-regulation of *VrNHX1* under cold stress can be attributed to the other unknown functional mechanisms that still remain to be deciphered. However, involvement of *NHX1* in conferring freezing tolerance has been reported in transgenic *A. thaliana* overexpressing *SsNHX1*, although the exact mechanism has not been explained (Li et al. 2007). Water deficit and altered water potential along with ionic imbalance are known to be primary effects of salt stress (Blumwld 2000; Hasegawa et al. 2000). The result for dehydration stress was similar to previous reports on expression of *GmNHX1*, *ThNHX1* and *EgNHX1* which displayed up-regulation under dehydration stress (Li et al. 2006; Wu et al. 2009; Baltierra et al. 2012). However, contrasting results have been reported for expression of *PeNHX1* and *AtNHX1* (Shi and Zhu 2002; Rajagopal et al. 2007).

4.3.7. Na^+ and K^+ measurement in salt stressed mungbean seedlings

The measurement of Na^+ and K^+ content in leaves and roots of untreated and salt-treated mungbean seedlings at different time intervals (0, 6, 12 18, 24, 48, and 72 hours) showed that under salt stress, Na^+ accumulation increased in leaves/roots by 1.28/2.1, 1.1/2.3, 2.1/4.36, 4.8/4.3, 4.1/4.54 times whereas, K^+ accumulation decreased by 3.4/4.5, 1.6/1.78, 1.59/2.43, 2.2/3 and 2.1/3.5 times as compared to control condition at 6, 12, 18, 24 and 48 hours, respectively in early stage mungbean seedlings (Fig. 4.20a). Similarly, in mid stage seedlings, Na^+ accumulation in leaves/roots also increased by 1.1/1.4, 1.4/2.4, 4/3.3, 4.5/3.5, 9.8/4.2 and 7.1/4.7 times whereas, K^+ accumulation decreased by 1.05/1.1, 1.03/1.66, 1.1/3.57, 1.34/3.2, 1.36/4.07, 1.77/4.03 times as compared to control condition at 6, 12, 18, 24, 48 and 72 hours, respectively (Fig. 4.20b). However, higher accumulation of Na^+ ions under salt stress in leaves/ shoots than roots have been reported in *T. repens, Z. japonica, H. caspica, Z. xanthoxylum,* and *D. morifolium* (Du et al. 2010; Guan et al. 2010; Tang et al. 2010; Wu et al. 2011; Zhang et al. 2012). The overall higher accumulation of Na^+ (µmoles/g dry weight) in roots as opposed to leaves indicated the restriction of movement of toxic Na^+ to the aerial part of the plant as a plausible mechanism to confer salinity tolerance in mungbean.

4.3.8. Ectopic expression of *VrNHX1* resulted in enhanced salt tolerance in transgenic *Arabidopsis*

In order to characterize *VrNHX1* functionally *in planta*, T_3 homozygous *Arabidopsis* lines expressing *VrNHX1* under the control of constitutive CaMV35S promoter or a stress-responsive RD29A promoter were generated using the binary constructs pCAMBIA2301-35S::*VrNHX1* (Fig. 4.21) and pCAMBIA2301-RD29A::*VrNHX1* (Fig. 4.22), respectively, to study their performance under salt stress.

4.3.8.1. Effect of salt stress on seed germination

The germination efficiency was studied in transgenic lines 1 (35S::*VrNHX1*) and 4 (RD29A::*VrNHX1*) after exposure to 150 mM NaCl stress for 10 days. Under normal condition, no difference was observed in WT and transgenic lines (Fig. 4.23). However, the transgenic lines exhibited better survival and germination efficiency than WT under salt stress (Fig. 4.23).

4.3.8.2. Effect of salt stress on root growth

Further, inhibition of root growth in four days old WT and transgenic seedlings subjected to salt stress (150 mM NaCl) for one week was monitored (Fig. 4.24a). Transgenic lines 1 and 4 exhibited 2.65 and 3 times higher root length respectively, than WT (Fig. 4.24b).

4.3.8.3. Effect of salt stress on physiological parameters

The effect on physiological parameters was monitored in 10 days old wild-type (WT) and independent transgenic *Arabidopsis* lines expressing *VrNHX1* constitutively (Lines 1-3, 35S::*VrNHX1*) and inducibly (Lines 4-6, RD29A::*VrNHX1*) under 200 mM NaCl stress for 5 days, by analyzing the total chlorophyll, malondialdehyde (MDA) for lipid peroxidation and proline content. Under normal physiological condition, no qualitative and statistical difference was observed between wild-type and transgenic *Arabidopsis* lines (Fig. 4.25). However, under salt stress (200 mM NaCl), WT showed leaf senescence while transgenic *Arabidopsis* lines (Lines 1-3, 35S::*VrNHX1* and Lines 4-6, RD29A::*VrNHX1*) showed better growth and survival (Fig.4.25). The transgenic lines showed higher chlorophyll (18-20 mg/ml) (Fig. 4.26) and proline (4.8-6 µmoles/g FW) (Fig. 4.26) content than WT (Fig. 4.26). The 35S::*VrNHX1* lines showed 1.35 times higher proline than RD29A::*VrNHX1* lines. A lower lipid peroxidation was detected in transgenic lines as WT showed 1.33 times higher malondialdehyde (MDA) content (Fig. 4.26).

4.3.8.4. Effect of salt stress on mature *Arabidopsis* plants

Effect of salt stress was studied in mature WT and transgenic lines (Line 1, 35S::*VrNHX1* and Line 4, RD29A::*VrNHX1*). The transgenic lines displayed better survival efficiency while, WT exhibited leaf senescence and growth inhibition upon salt stress (200 mM NaCl) (Fig. 4.27a). The relative expression level of transgene *VrNHX1* normalized with the housekeeping *AtUBQ1* revealed that *Arabidopsis* transgenic line RD29A::*VrNHX1* displayed a 1.4 times higher expression value as compared to 35S::*VrNHX1* (Fig. 4.27b). The total Na^+ and K^+ accumulated in transgenic lines was higher than WT. Further, transgenic 35S::*VrNHX1* and RD29A::*VrNHX1* lines exhibited 1.3 and 1.14 times higher Na^+/K^+ ratio, respectively, as compared to WT (Fig. 4.27c).

4.4. Conclusion

This is the first report on isolation of a vacuolar Na^+/H^+ antiporter from mungbean and its functional characterization . Phylogenetic analysis, evolutionary relationship, and secondary structure prediction revealed that *VrNHX1* shared highest homology with reported legume Na^+/H^+ antiporters and categorized under Class-I group of Intracellular Sodium/Hydrogen Exchangers (IC-NHE/NHX). Restored growth of AXT3 mutant in presence of high concentrations of Na^+, K^+, and Li^+ and suppression of hygromycin sensitivity indicated the functional complementation of in yeast mutant due to the heterologous expression of *VrNHX1* . The measurement of higher vacuolar Na^+ and K^+ in yeast mutants transformed with pYES*VrNHX1* provided further evidence to functional role of *VrNHX1* as a $Na^+/K^+/H^+$ antiporter involved in conferring salt tolerance. Alkalinization of endolytic compartments has been reported to be mediated by *ScNHX1* which serves as a leak pathway for H^+, thus, regulating the pH level for efficient survival against external acid stress (Ali et al. 2004; Brett et al. 2005).

The growth sensitivity of AXTVrNHX1 cells was lower than AXTYES2.0 cells under external acidic pH environment. Vacuolar acidification was reduced in AXTVrNHX1 cells under low pH indicating the role of *VrNHX1* in extrusion of excess H^+ by its ion specificity.

Differential regulation of Na^+ uptake, extrusion, compartmentalization, radial transport to stele, loading and unloading into xylem is responsible for the varied response of plants against salinity stress. Under salt stress, *VrNHX1* expression was induced in both leaves and roots of mungbean seedlings with concomitant higher expression in roots than leaves in both early and mid stage seedlings. The expression pattern of *VrNHX1* was also induced under various abiotic stress conditions in mungbean.

Physiological response under salt stress, indicated higher Na^+ accumulation in roots than shoots in early and mid stage mungbean seedlings This indicated that higher K^+/Na^+ ratio is maintained in leaves owing to sequestration of higher Na^+ in root vacuoles thus, restricting their movement to the aerial part of plant. Combined together, increased *VrNHX1* transcript level coupled with higher sequestration of

Na^+ in roots can be attributed as the tolerance mechanism of mungbean under salt stress.

Ectopic expression of *VrNHX1* conferred salt tolerance in transgenic *Arabidopsis* lines. Both, 35S::*VrNHX1* and RD29A::*VrNHX1* homozygous T_3 lines displayed better growth response in comparison to WT. Salt stress affects the photosynthetic system components including chlorophyll contents (Demetriou et al. 2007). The reduction in chlorophyll content was less in transgenic lines (35S::*VrNHX1* and RD29::*VrNHX1*) as compared to WT. Lipid peroxidation is mediated by increase in accumulation of reactive oxygen species (ROS) under salinity stress (Bor et al. 2003). Therefore, the extent of lipid peroxidation was measured using malionaldehyde (MDA), a by-product of lipid peroxidation. Transgenic lines showed lower extent of MDA generation as compared to WT indicating protection against membrane damage process. Metabolic response against salt stress, generally includes generation of proline, an osmoprotectant and compatible osmolyte, as a protective measure in plants (Hasegawa et al. 2000). Transgenic lines expressed higher proline content in response to salt stress. Proline is also known as a potent ROS scavenger (Szabados and Savouré 2010) which might also be correlated with the lower levels of generation of ROS, thus rendering reduced lipid peroxidation in transgenic plants as compared to WT. Similar result was also reported for proline content in transgenic *Arabidopsis* lines overexpressing *DmNHX1* (Zhang et al. 2012).

The regulation of K^+/Na^+ ratio to maintain K^+ homeostasis for proper cellular and enzymatic functioning is an essential mechanism against salinity stress in plants (Maathuis and Amtmann 1999). The results demonstrated that the transgenic lines (35S::*VrNHX1* and RD29::*VrNHX1*) maintained a higher K^+/Na^+ ratio than WT plants under salt stress indicating effective tolerance in transgenic lines under salt stress. The phenotypical, physiological and expressional analysis using quantitative real-time PCR concluded that the transgenic RD29::*VrNHX1* line displayed comparable higher survival and growth than 35S::*VrNHX1* lines under salt stress and can be further exploited in crop plants.

The expression of *VrNHX1* under constitutive and inducible promoter enhanced salt tolerance in transgenic *Arabidopsis*. *AtNHX1* is one of the most effective genes in

improving plant salt tolerance, however, it played a dominant role mainly in leaf. The results suggested that *VrNHX1* might play an important role in the root resistance to Na^+ toxicity. Therefore, it can be assumed that overexpression of *VrNHX1* in crop plants might generate enhanced salt tolerance.

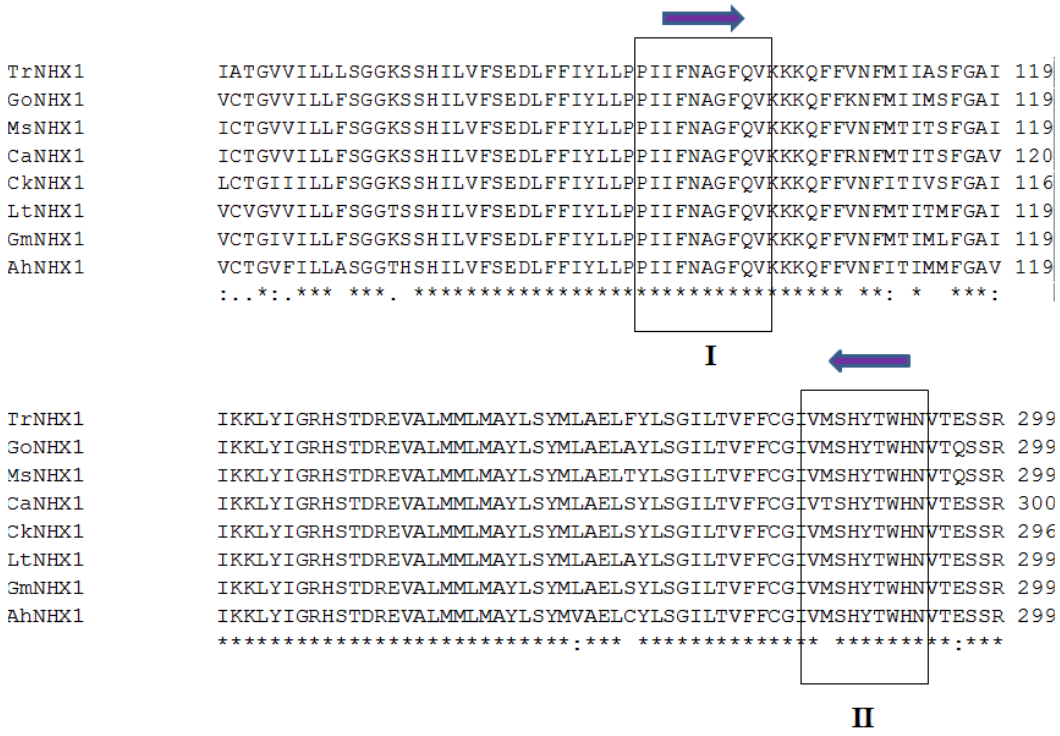


Figure 4.1 Designing of degenerate primers for partial amplification of *VrNHX1* gene. Multiple sequence alignment of amino acid sequences of NHX1 from various legume species submitted in NCBI database was performed using Clustal W. The forward and reverse degenerate primers (position is indicated by arrows) were designed from conserved regions designated as box I and II, respectively. The "*" sign indicates the highly identical residues constituting the conserved donains in NHX1 protein. The GenBank Accession numbers for NHX proteins are: *GmNHX1* (AAY430061.1); *CkNHX1* (ABG89337.1), *MsNHX1* (AAS84487.1), *CaNHX1* (ADL28385.1), *TrNHX1* (ABV00895.1); *LtNHX1* (ACE78322.1), *AhNHX1* (ADK74832.1), *GoNHX1* (ABY59540.1)

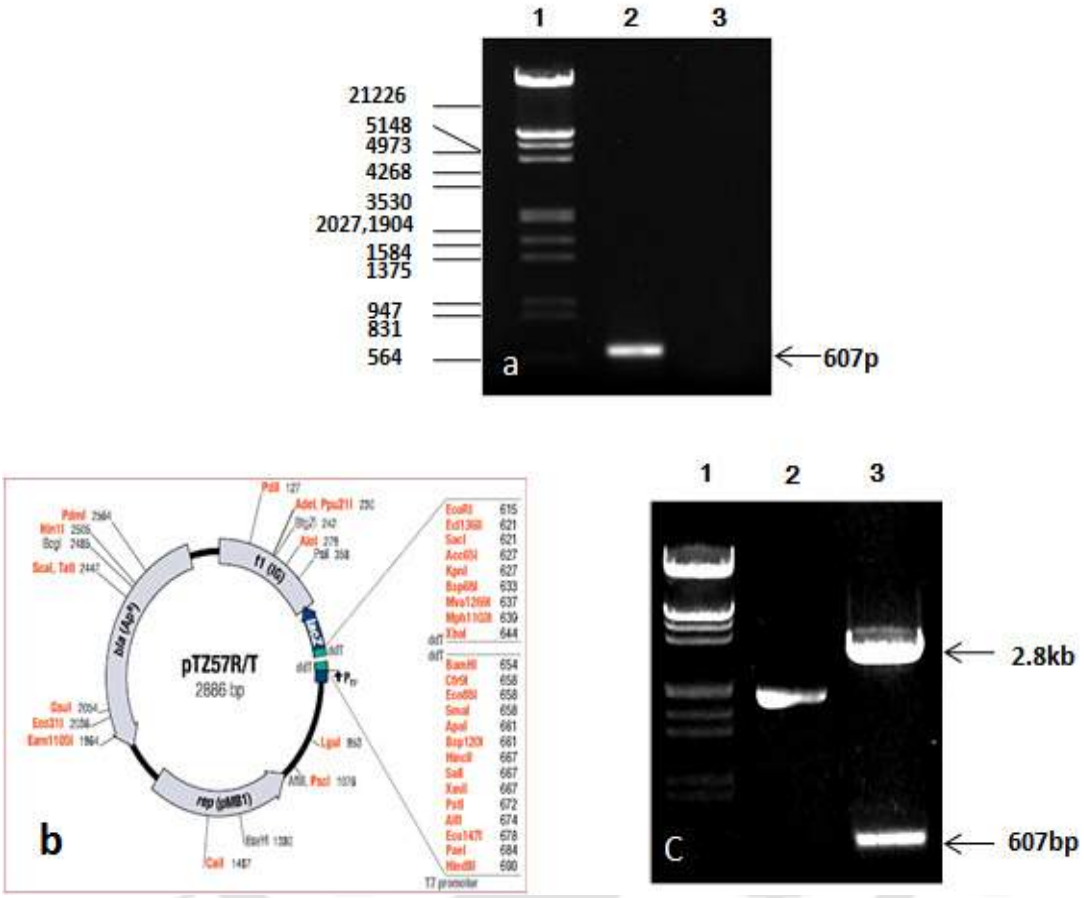


Figure 4.2 Cloning of partial fragment of *VrNHX1*. (a) Isolation of partial fragment of *VrNHX1* gene. L1: Lambda HindIII/EcoRI DNA Ladder; L2: 0.6 kb PCR amplicon of *VrNHX1* was obtained using degenerate primers; L3: Blank. (b) Vector map of TA cloning vector pTZR/T (2.8 kb). (c) Cloning of 0.6 kb partial fragment of *VrNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder; L2: Uncut plasmid (pTZR/T-Deg*VrNHX1*); L3: Release of partial fragment from pTZR/T-Deg*VrNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI

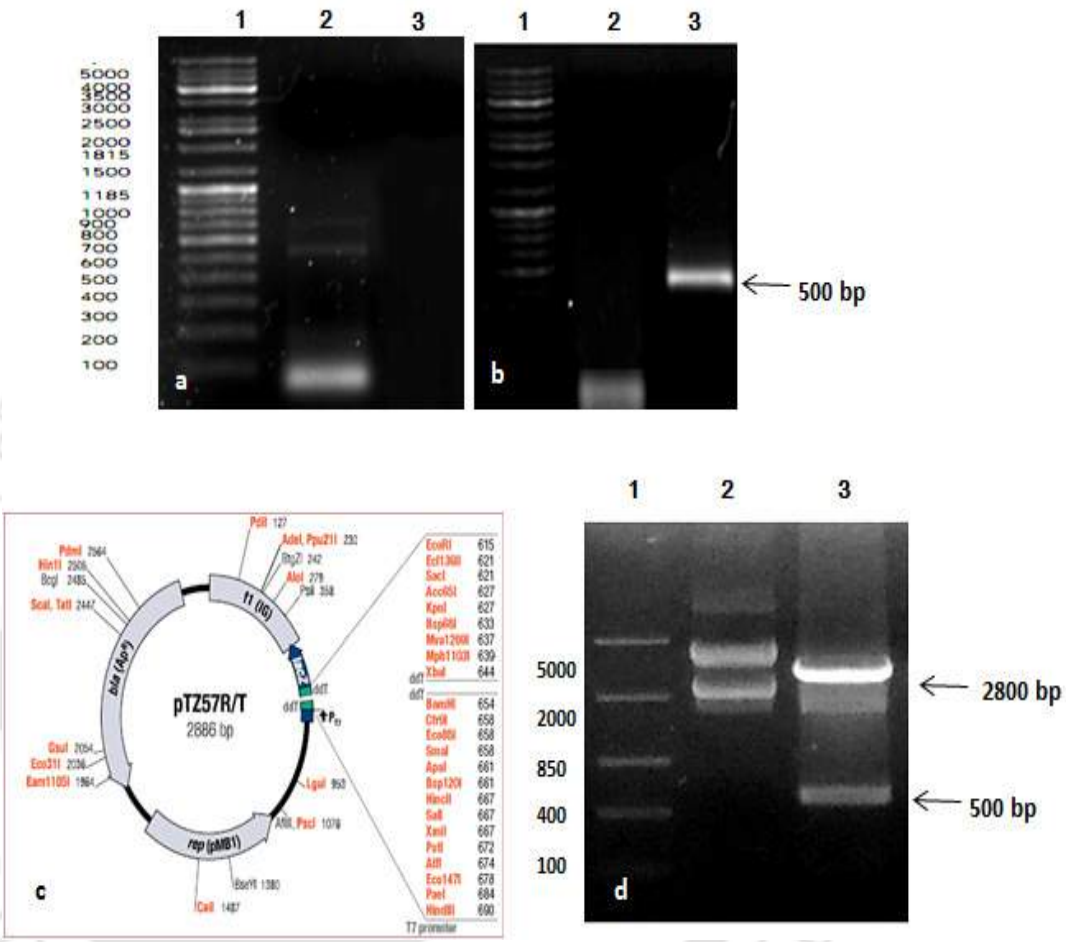


Figure 4.3 Cloning of 5'- untranslated region of *VrNHX1* (a) Isolation of 5'- UTR fragment of *VrNHX1* gene using GSP2 and AAP primers. L1: Middle range DNA ruler; L2: PCR amplicons of 1st PCR. L3: Blank. (b) Isolation of 5'- UTR fragment of *VrNHX1* gene using nested GSP3 and AUAP primers. L1: Medium range DNA ruler; L2: Blank; L3: 0.5 kb PCR amplicon of *VrNHX1.* (c) Vector map of TA cloning vector pTZR/T (2.8 kb). d) Cloning of 0.5 kb partial fragment of *VrNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Medium range DNA ruler; L2: Uncut plasmid pTZR/T-5'*VrNHX1*; L3: Release of partial fragment from pTZR/T-5'*VrNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI

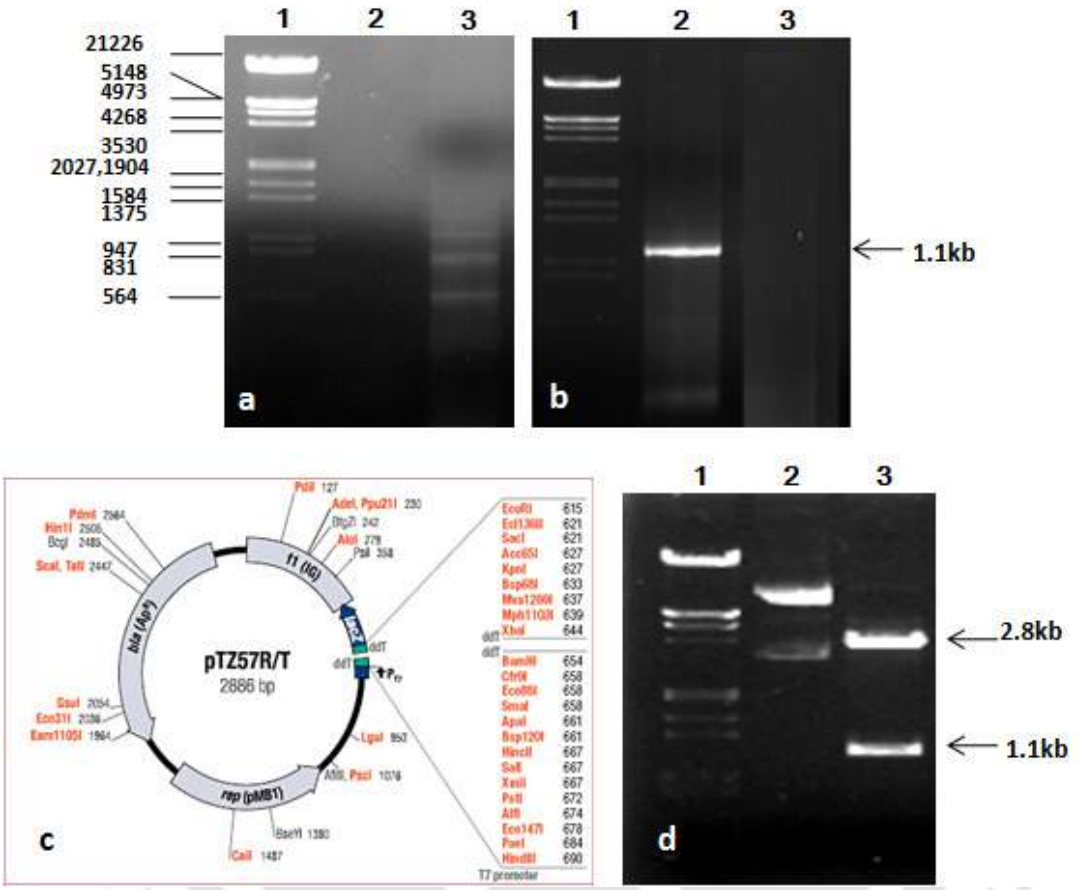


Figure 4.4 Cloning of 3'- untranslated region of *VrNHX1*. (a) Isolation of 3'- UTR fragment of *VrNHX1* gene using GSP4 and AUAP primers. L1: Lambda HindIII/EcoRI DNA Ladder; L2: Blank; L3: PCR amplicons of 1st PCR. (b) Isolation of 3'- UTR fragment of *VrNHX1* gene using nested GSP3 and AUAP primers. L1: Lambda HindIII/EcoRI DNA Ladder; L2: 1.1 kb PCR amplicon of *VrNHX1*; L3: Blank. (c) Vector map of TA cloning vector pTZR/T (2.8 kb). (d) Cloning of 1.1 kb partial fragment of *VrNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder; L2: Uncut plasmid pTZR/T-3'*VrNHX1*; L3: Release of partial fragment from pTZR/T-3'*VrNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI

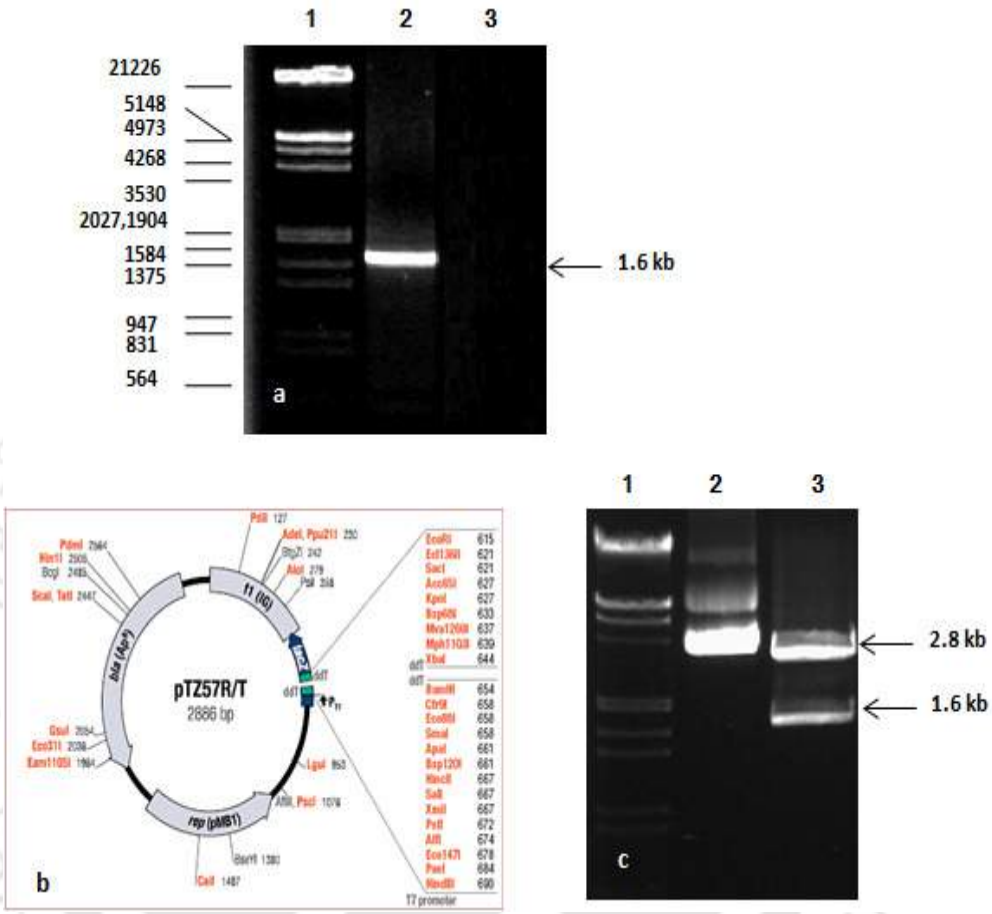


Figure 4.5 Cloning of CDS of *VrNHX1*. (a) PCR amplification of CDS of *VrNHX1* gene using gene specific primers. L1: Lambda HindIII/EcoRI DNA Ladder; L2: 1.6 kb PCR amplicon corresponding to CDS of *VrNHX1*; L3: Blank. (b) Vector map of TA cloning vector pTZR/T (2.8 kb). (c) Cloning of 1.6 kb CDS fragment of *VrNHX1* into TA cloning vector pTZR/T (2.8 kb). L1: Lambda HindIII/EcoRI DNA Ladder; L2: Uncut plasmid pTZR/T*VrNHX1* (4.4 kb); L3: Release of partial fragment from pTZR/T*VrNHX1* plasmid upon disgestion with restriction enzymes XbaI and BamHI

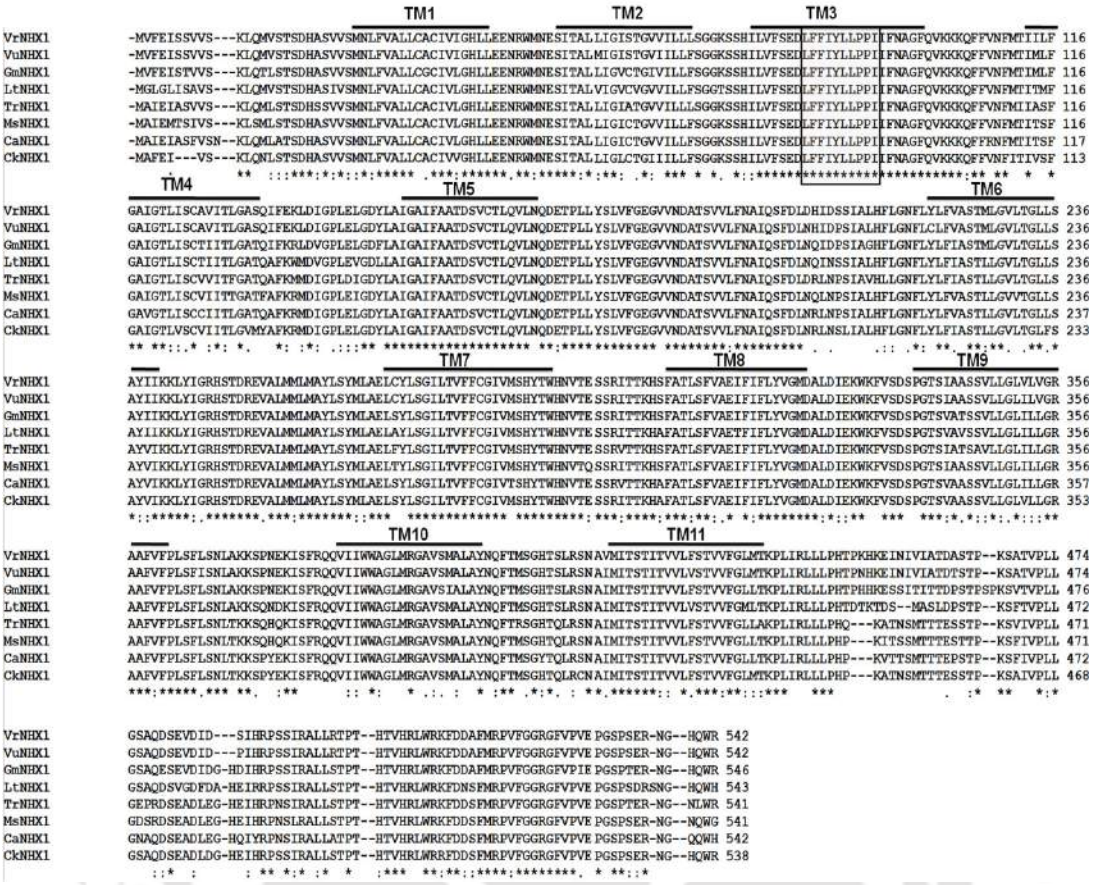


Figure 4.6 Multiple sequence alignment was performed for amino acid sequences of plant NHX proteins using CLUSTAL W. The GenBank Accession numbers for NHX proteins are: VrNHX1 (AEO50758.1), *Vigna radiata*; VuNHX1 (AEO72079.2), *Vigna unguiculata*; GmNHX1 (AAY430061.1), G*lycine max*; CkNHX1 (ABG89337.1), *Caragana korshinskii*; MsNHX1 (AAS84487.1), *Medicago sativa*; CaNHX1 (ADL28385.1), *Cicer arietinum*; TrNHX1 (ABV00895.1), *Trifolium repens*; LtNHX1 (ACE78322.1), *Lotus tenuis*. " * " indicates identical amino acid (AA) residues. ":" indicates conservative AA substitutions and "." represents semi-conservative AA substitutions in the sequence alignment. The transmembrane region of VrNHX1 as indicated by TM1-11 and conserved amiloride binding motif, ^{84}-LFFIYLLPPI-^{93}, a classic inhibitor of the Na^+/H^+ antiporters detected in TM3 region is also shown in the alignment

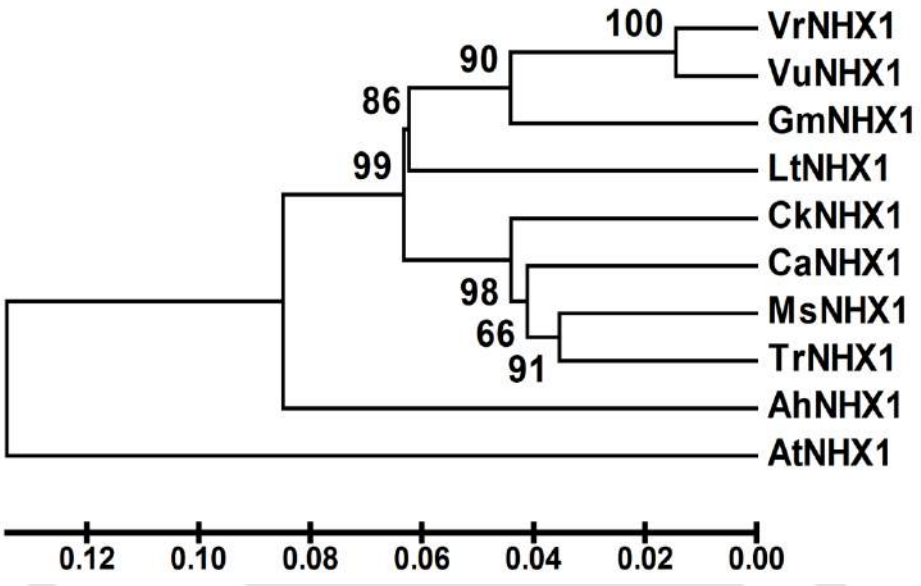


Figure 4.7 The phylogenetic tree for plant Na^+/H^+ antiporters was generated using MEGA4: Tree Explorer software. The evolutionary history was inferred using the neighbor-joining method and analyzed using bootstrap analysis with 500 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method and are in the units of the number of amino acid substitutions per site. The GenBank Accession numbers for NHX proteins used are: VrNHX1 (AEO50758.1), VuNHX1 (AEO72079.2), GmNHX1 (AAY430061.1), CkNHX1 (ABG89337.1), MsNHX1 (AAS84487.1), CaNHX1 (ADL28385.1), TrNHX1 (ABV00895.1), LtNHX1 (ACE78322.1), AhNHX1 (ADK74832.1), AtNHX1 (NM_122597.2)

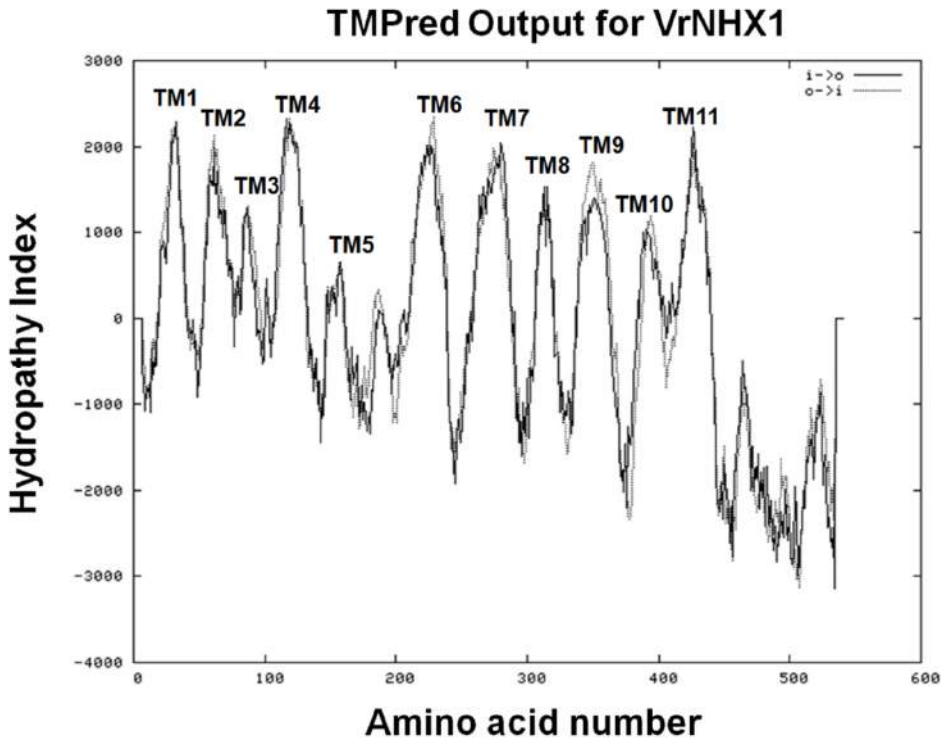
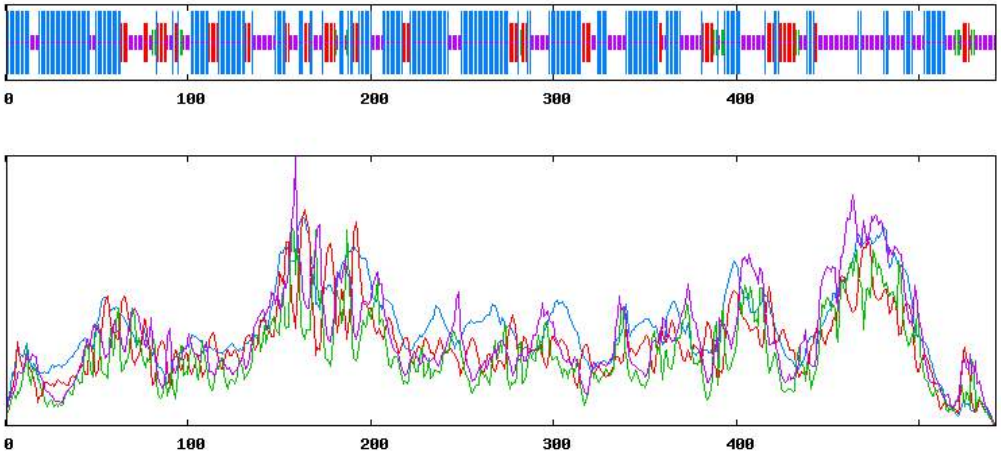


Figure 4.8 Prediction of transmembrane helices of VrNHX1. The hydropathy plot was generated using TMPred online software. The positive values indicate putative transmembrane domains TM1-11

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Sequence length :   542

SOPMA :
   Alpha helix      (Hh) :   262 is  48.34%
   3(10) helix      (Gg) :     0 is   0.00%
   Pi helix         (Ii) :     0 is   0.00%
   Beta bridge      (Bb) :     0 is   0.00%
   Extended strand  (Ee) :    80 is  14.76%
   Beta turn        (Tt) :    18 is   3.32%
   Bend region      (Ss) :     0 is   0.00%
   Random coil      (Cc) :   182 is  33.58%
   Ambigous states  (?)  :     0 is   0.00%
   Other states          :     0 is   0.00%
```

Figure 4.9 Secondary structure prediction of VrNHX1. Secondary structure prediction of *VrNHX1* using SOPMA indicated occurrence of alpha helix (48.34%), extended strand (14.76%), beta turn (3.32%), and random coil (33.58%)

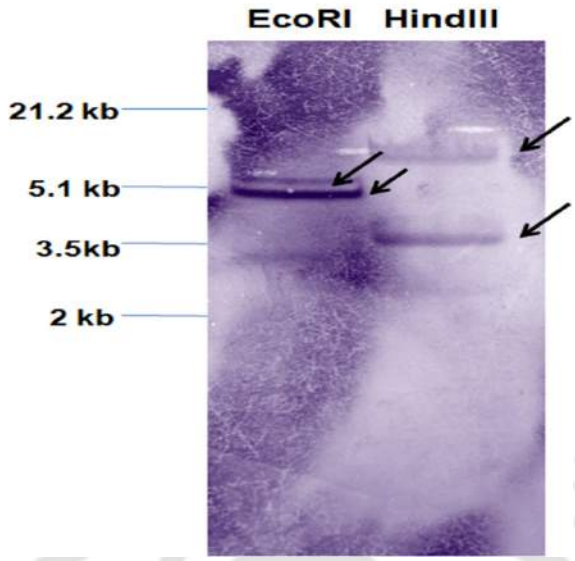

Figure 4.10 Copy number analysis of *VrNHX1* gene in mungbean genome. Mungbean genomic DNA (20µg) was digested with EcoRI and HindIII, and hybridized with DIG-labeled probe (1.6 kb) corresponding to the CDS of *VrNHX1*. The arrows indicate the hybridization signals

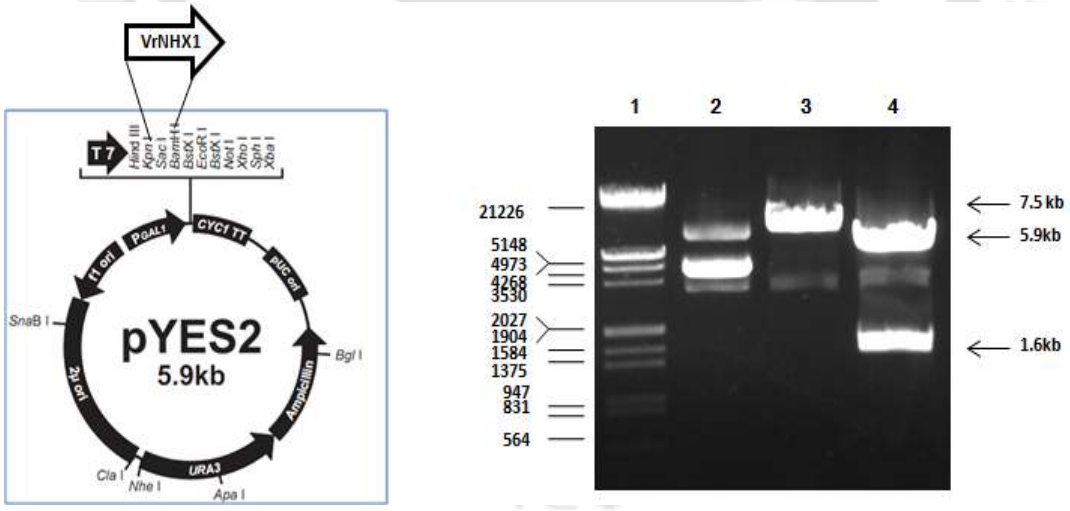

Figure 4.11 Cloning of *VrNHX1* CDS (1.6 kb) into yeast expression vector pYES 2.0 (5.9 kb). L1: Lambda HindIII and EcoRI digested DNA ladder; L2: Uncut pYES*VrNHX1* plasmid (7.5 kb); L3: pYES*VrNHX1* plasmid was linearized with EcoRI; L4: Release of the cloned *VrNHX1* CDS from the vector backbone

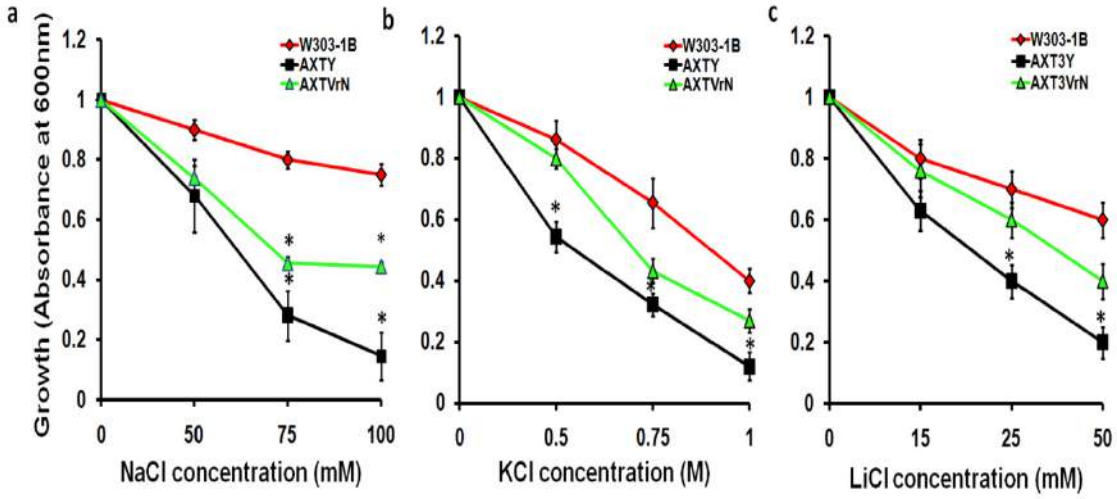

Figure 4.12 Cation sensitivity assay of transformed yeast strains (W303-1B, AXTYES2.0, AXTVrNHX1) under various concentrations of (a) NaCl (0, 50, 75, 100 mM), (b) KCl (0, 0.5, 0.75, 1.0 M), and (c) LiCl (0, 15, 20, 25 mM). Saturated seed cultures for each strain was diluted to an OD_{600} of 0.006 and inoculated to liquid APGal medium (pH 5.5) supplemented with or without above mentioned concentrations of NaCl, KCl, and LiCl. Growth was observed at 30°C after 3 days and absorbance recorded at 600nm. Data are means of 3 independent events (n=3) and standard errors are plotted in the graph. Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

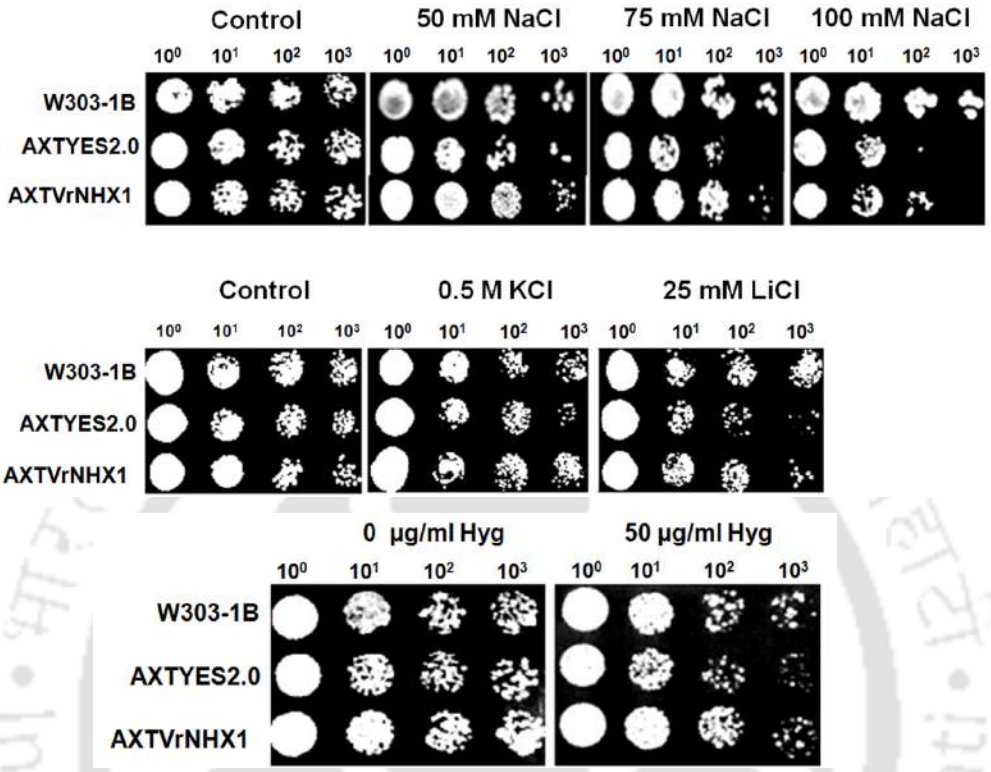

Figure 4.13 Heterologous expression of *VrNHX1* in yeast mutant. Wild type (W303-1B) strain was used as a control, *Δ ena1- 4 Δnha1 Δnhx1* mutant (AXT3) strain was transformed with null pYES2.0 (labeled as AXTYES2.0 strain) and pYES*VrNHX1* recombinant vector (labeled as AXTVrNHX1) were used for complementation assay. 10-fold serial dilutions of saturated seed cultures of each strain were spotted onto APGal media (pH-5.5) supplemented with or without 50, 75 and 100 mM NaCl, 25 mM LiCl, and 0.5 M KCl. Hygromycin sensitivity assay in Wild type (W303-1B), AXTYES2.0 and AXTVrNHX1 strains was performed by spotting 10-fold serial dilutions of saturated seed cultures of each strain onto YPGal media (pH-5.5) supplemented with or without 50 µg/ml Hyg. The plates were incubated at 30°C for 3 days

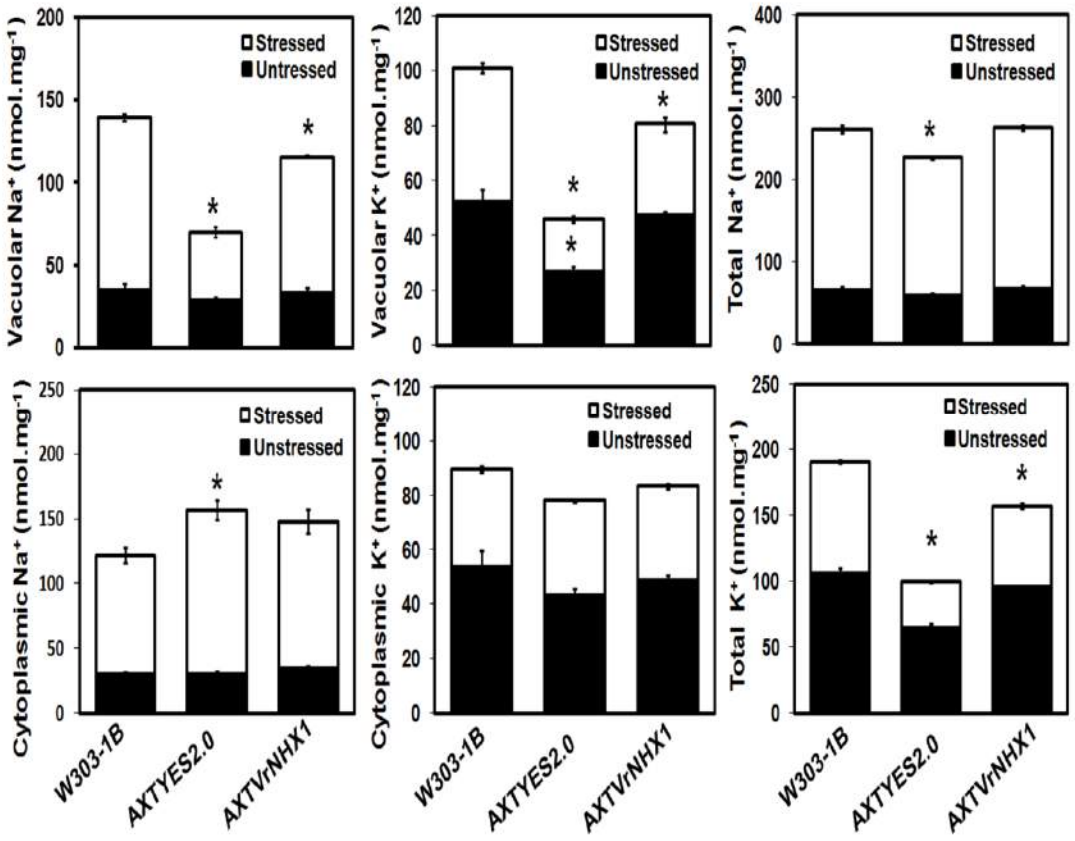

Figure 4.14 Total intracellular ion estimation in yeast strains W303-1B, AXTYES2.0 and AXTVrNHX1. Yeast cells were grown in APG medium (pH 4.0) with 1 mM KCl supplemented in presence (stressed) or absence of 75 mM NaCl (unstressed) and harvested at a cell density of 0.3. Total intracellular, vacuolar and cytoplasmic Na^+ and K^+ content was determined as described in the materials and methods section. Data are means of 3 independent events (n=3) and standard errors are plotted in the graph. Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

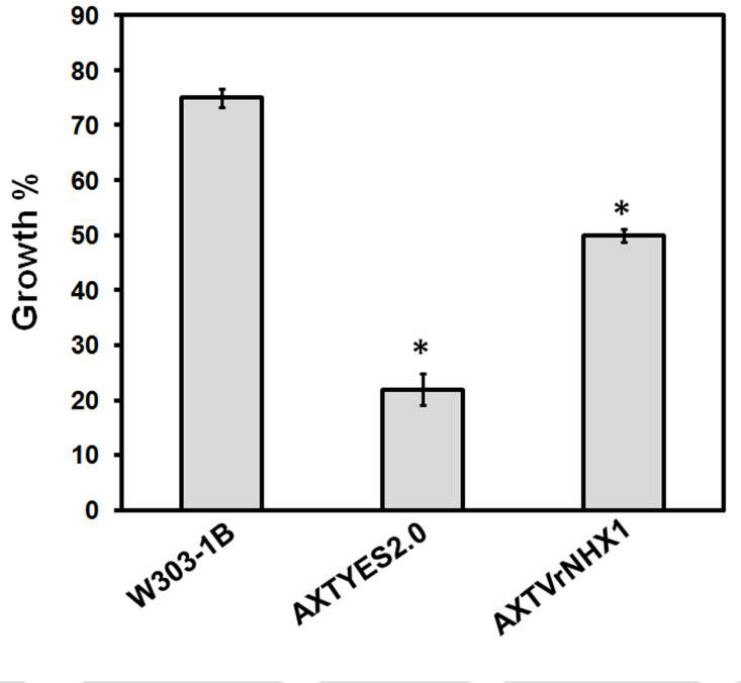

Figure 4.15 Growth measurement of yeast strains under low pH. Yeast strains were grown in synthetic medium APGal (pH 4.0) and absorbance was measured at 600 nm. The data shown above are normalized to growth under normal condition (APGal, pH 7.0). W303-1B:- Wild type strain, AXTYES2.0:- AXT3 mutant harboring null pYES2.0 plasmid, AXTVrNHX1:- AXT3 mutant harboring pYES*VrNHX1* recombinant plasmid. Data represent mean from three independent events (n=3) and standard error plotted in the graph. Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

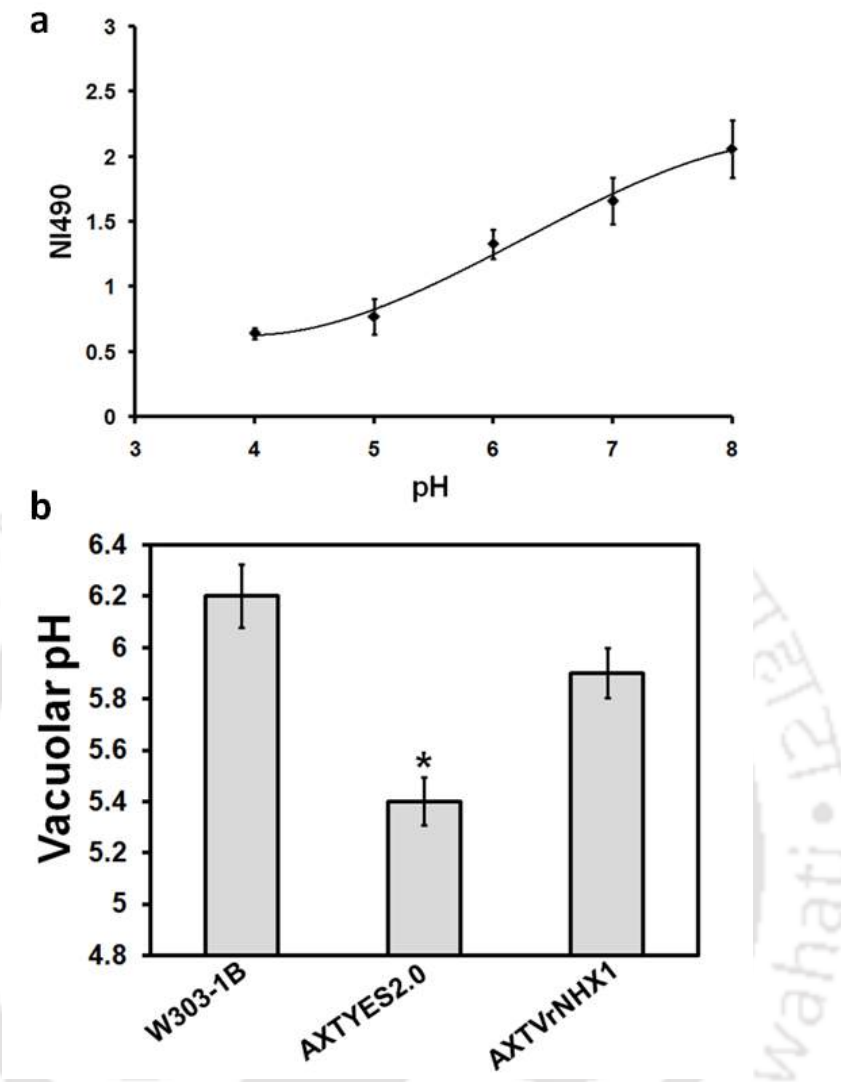

Figure 4.16 Measurement of vacuolar pH in transformed yeast strains. (a) Calibration curve for pH sensitive BCECF fluorescent dye was plotted using standards ranging from pH 4.0-8.0. Yeast strains (W303-1B, AXTYES2.0, AXTVrNHX1) grown in APGal medium (pH 4.0) were loaded with BCECF dye as described in materials and methods, fluorescence intensity was measured at 440 and 490 nm, background values (measured with only cell extract and only BCECF dye) were subtracted and the ratio was plotted for each pH value. The data from the three yeast stains were pooled and mean ratio values were plotted with a fitted non-linear graph. (b) Vacuolar pH was measured for BCECF-AM loaded yeast strains W303-1B, AXTYES2.0 and AXTVrNHX1 as described in materials and methods following the calibration curve (Figure S3). Mean and SEs are plotted for three independent events (n=3) in each case. Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

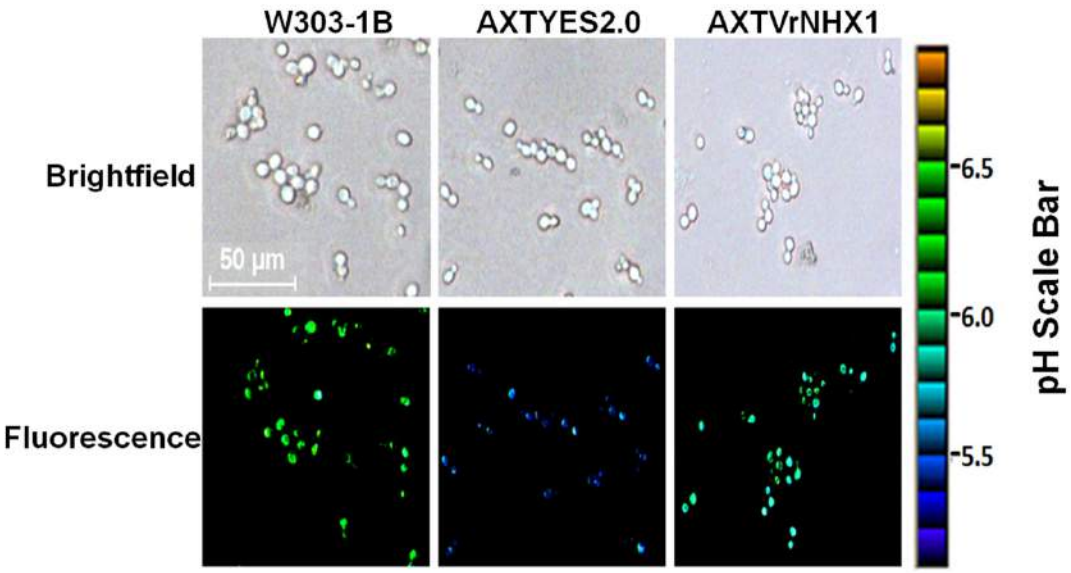

Figure 4.17 Accumulation of pH-sensitive fluorescent BCECF dye in yeast vacuoles. The yeast strains W303-1B, AXTYES2.0 and AXTVrNHX1 were grown in APGal media (pH 5.0), resuspended in minimal medium with BCECF-AM dye for 30 minutes at 30 °C. Yeast cells were visualized by Nikon eclipse Ti-U Fluorescence microscope (Nikon) at excitation wavelength of 440nm. Bar scale, 50 µm

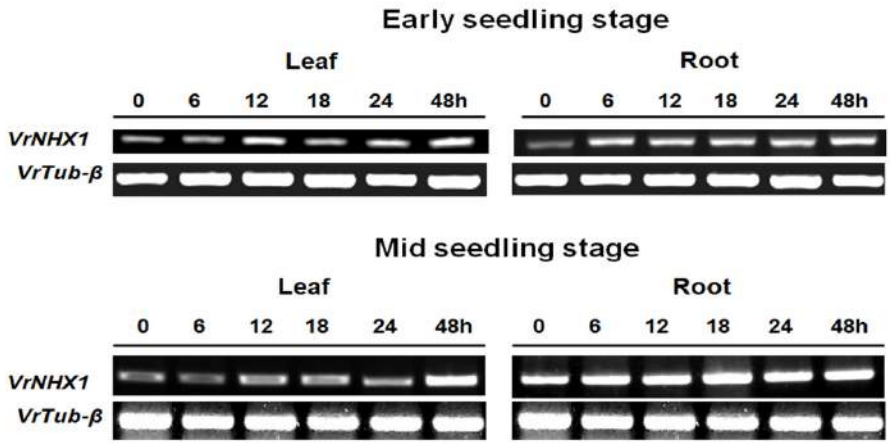

Figure 4.18 Semi-quantitauve RT-PCR for studying expression patterns of *VrNHX1* under salt stress. Total RNA was isolated from leaves and roots of early and mid stage mungbean seedlings under 200 mM NaCl treatment at time intervals of 0, 6, 12, and 24 hrs. PCR fragments of 566 bp and 422 bp size corresponding to *VrNHX1* and *VrTubβ* gene were fractionated electrophoretically on 2% agarose gel stained with 10mg/ml ethidium bromide

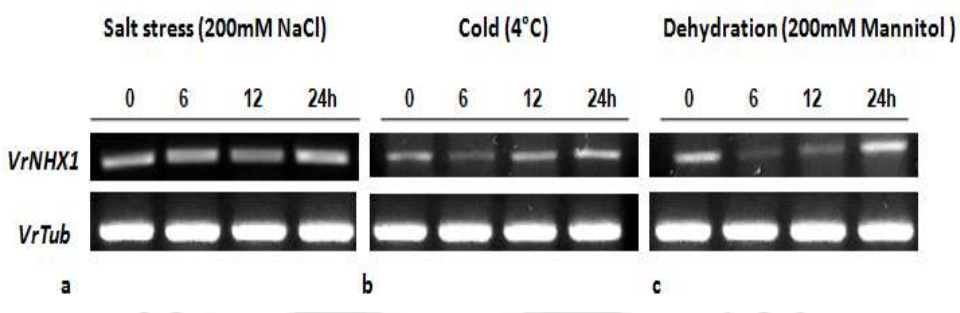

Figure 4.19 Semi-quantitative RT-PCR for studying expression patterns of *VrNHX1* under different abiotic stress conditions such as salt, cold and dehydration stress. Total RNA was isolated from mid stage mungbean seedlings under (a) 200mM NaCl, (b) Cold (4°C), and (c) 200mM Mannitol treatment at time intervals of 0, 6, 12, and 24 hrs. PCR fragments of 566 bp and 422 bp size corresponding to *VrNHX1* and *VrTubβ* gene were fractionated electrophoretically on 2% agarose gel stained with 10mg/ml ethidium bromide

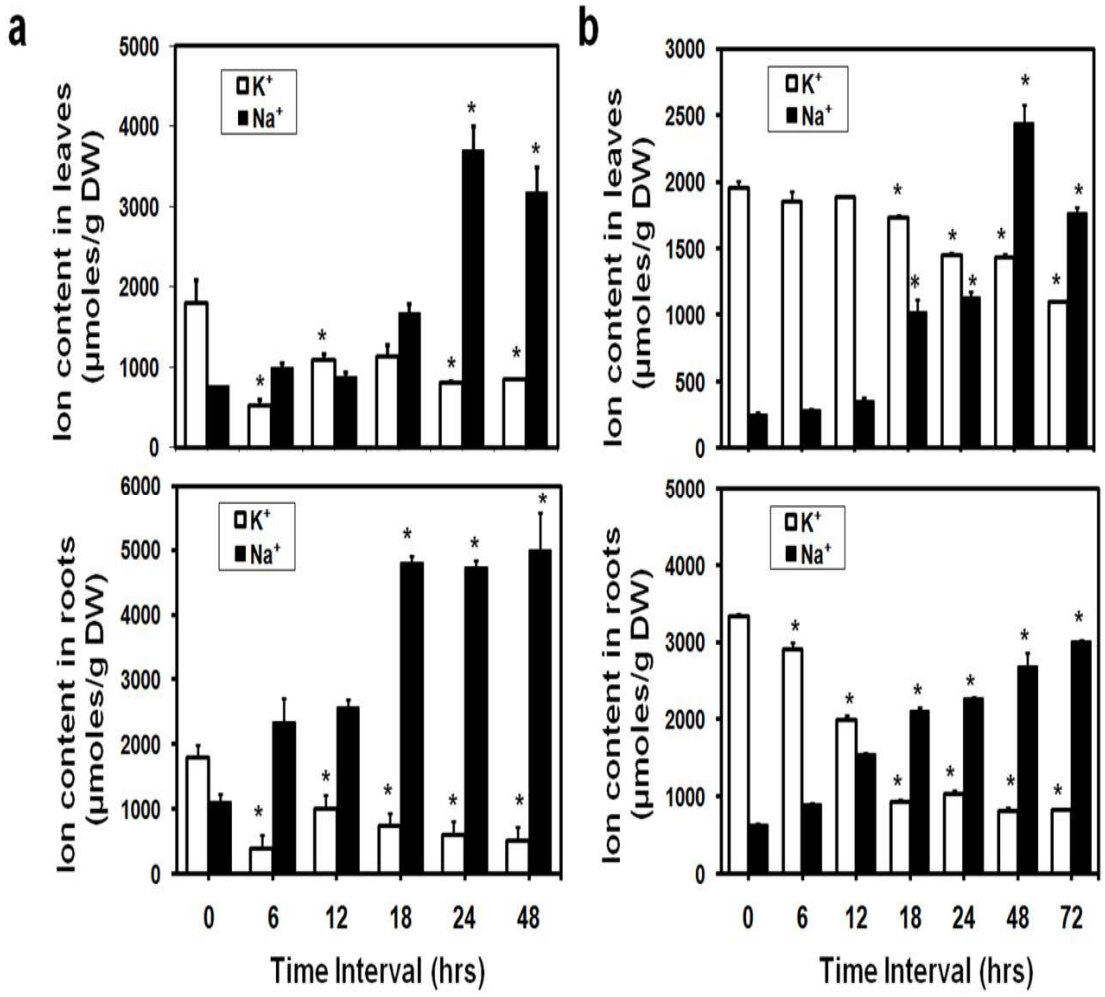

Figure 4.20 Total intra cellular ion measurement in leaves and roots of early and mid stage mungbean seedlings. Na^+ and K^+ content in (a) leaves and (b) roots of unstressed and salt stressed mungbean seedlings harvested at time intervals of 0, 6, 12, 24, 48, and 72 hrs was measured using Flame Photometer. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

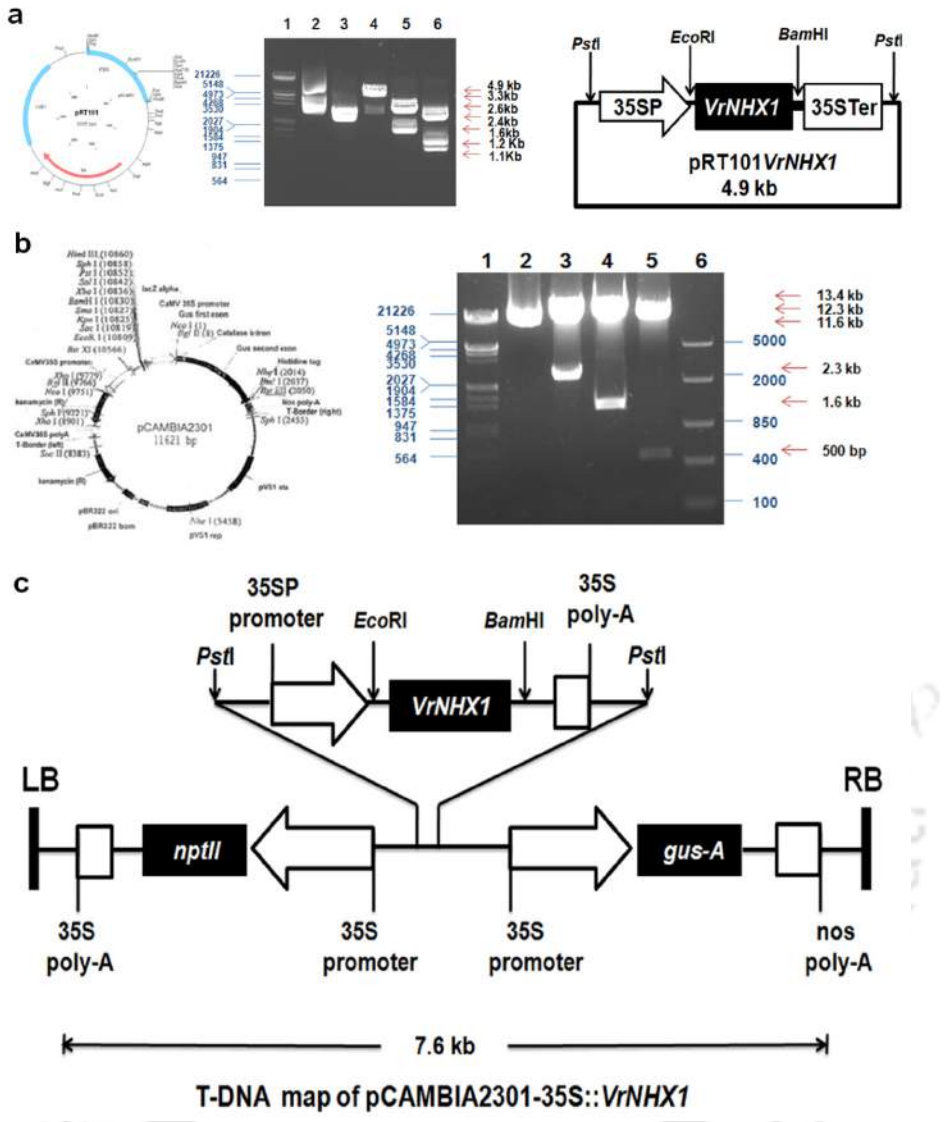

Figure 4.21 Preparation of recombinant pCAMBIA2301-35S::*VrNHX1* plasmid (13.9 kb). (a) The *VrNHX1* CDS was cloned into intermediate pRT101 (3.3 kb) plasmid at *Eco*RI and *Bam*HI sites and the recombinant plasmid pRT101*VrNHX1* (4.9 kb) was generated with the VrNHX1 CDS flanked by CaMV 35S promoter and 35S poly-A. L1: Lamda HindIII/EcoRI DNA ladder; L2: Uncut pRT*VrNHX1* plasmid (4.9 kb); L3: Release of 2.3kb 35P::*VrNHX1*::35Ter cassette from vector backbone; L4: pRT*VrNHX1* plasmid is linearized with BamHI; L4: Release of 1.6kb CDS of *VrNHX1* from vector backbone; L5: Confirmation of orientation of CDS of *VrNHX1* in pRT101. (b) The *Pst*I-35SP::*VrNHX1*::35STer-*Pst*I digested DNA fragment was cloned into plant binary vector pCAMBIA2301 (11.6 kb) and confirmed with restriction

digestion. L1: Lamda HindIII/EcoRI DNA ladder; L2: Uncut pCAMBIA2301-35SP::*VrNHX1* recombinant plasmid (13.9 kb); L3: Release of 2.3kb 35SP::*VrNHX1*::35Ter cassette from vector backbone; L4: Release of 1.6kb CDS of *VrNHX1* from vector backbone; L5: Confirmation of orientation of the 35SP::*VrNHX1*::35Ter cassette in pCAMBIA2301 (11.6 kb). (c) T-DNA region (7.6 kb) of pCAMBIA2301-35S::*VrNHX1* recombinant plasmid (13.9 kb) Restrcition enzyme PstI used for cloning 35SP::*VrNHX1*::35STer cassette (2.3kb) into plant binary vector pCAMBIA 2301 is also highlighted. Abbreviations: LB, left border; RB, right border; 35SPromoter, cauliflower mosaic virus 35S promoter; RD29A promoter, stress indicible AtRD29A promoter; CaMV 35S poly-A, cauliflower mosaic virus 35S terminator; nos poly-A, nopaline transferase terminator; *nptII*, neomycin phosphotransferase; *intron-gus-A*, intron interrupted β-glucuronidase; *VrNHX1*, *Vigna radiata NHX1*

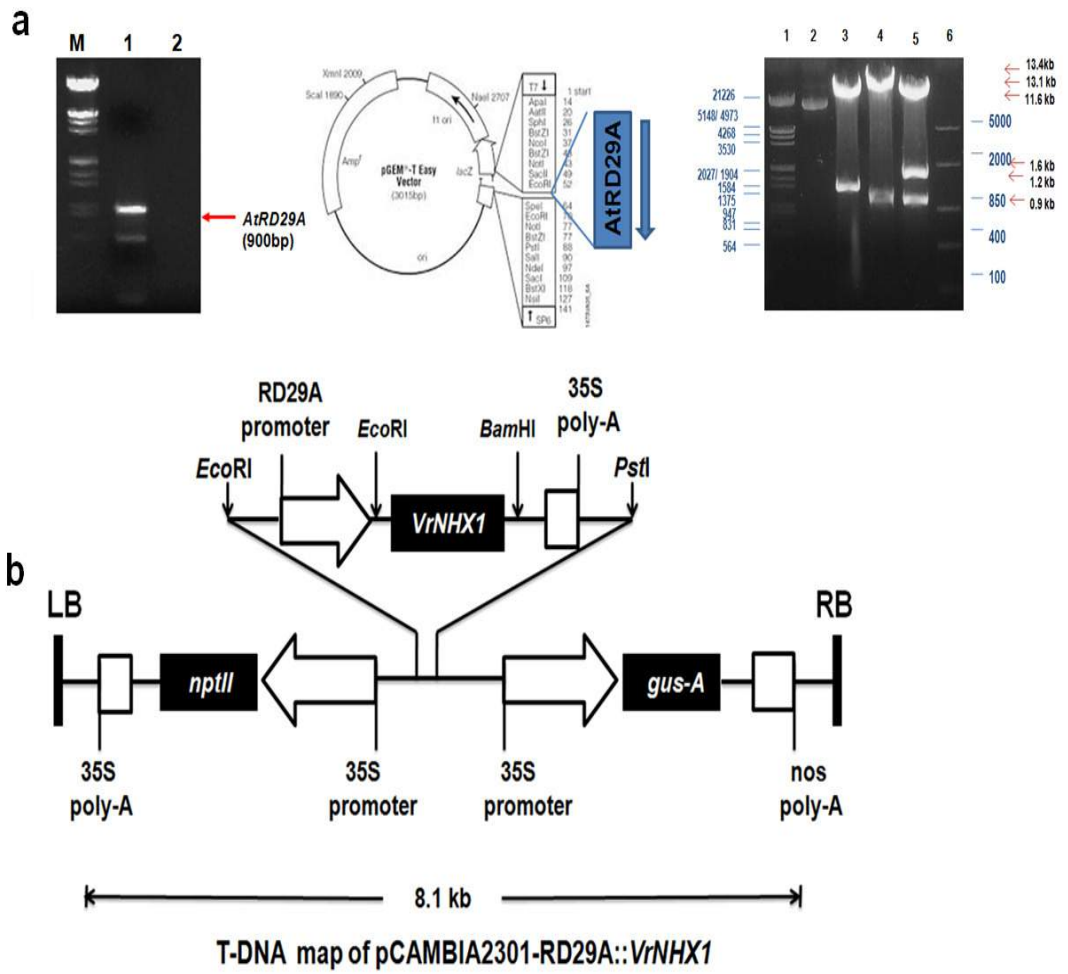

Figure 4.22 Preparation of recombinant pCAMBIA2301-RD29A::*VrNHX1* plasmid (14.4 kb). (a) The first figure depicts the isolation of stress inducible promoter fragment RD29A (0.9 kb) from *Arabidopsis thaliana*. M: Lambda HindIII and EcoRI digested DNA ladder; 1: PCR amplicon for AtRD29A (0.9 kb) promoter fragment; 2: Blank. The second figure depicts the vector map of recombinant pGEMTRD29A plasmid (3.9 kb). The AtRD29A (0.9 kb) promoter fragment was cloned into TA cloning vector pGEMT-easy (3 kb). The third figure depicts the restriction digestion for confirmation of generation of recombinant plant binary vector pCAMBIA2301-RD29A::*VrNHX1* (14.4kb). L1: Lambda HindIII and EcoRI digested DNA ladder; L2: Uncut pCAMBIA2301-RD29A::*VrNHX1* (14.4kb); L3: Checking of orientation of RD29A::*VrNHX1*::35STer in pCAMBIA2301; L4: Release of 0.9kb RD29A promoter from vector backbone; L5: Release of *VrNHX1* and Rd29A promoter with XbaI digestion; L6: Middle Range DNA Ladder

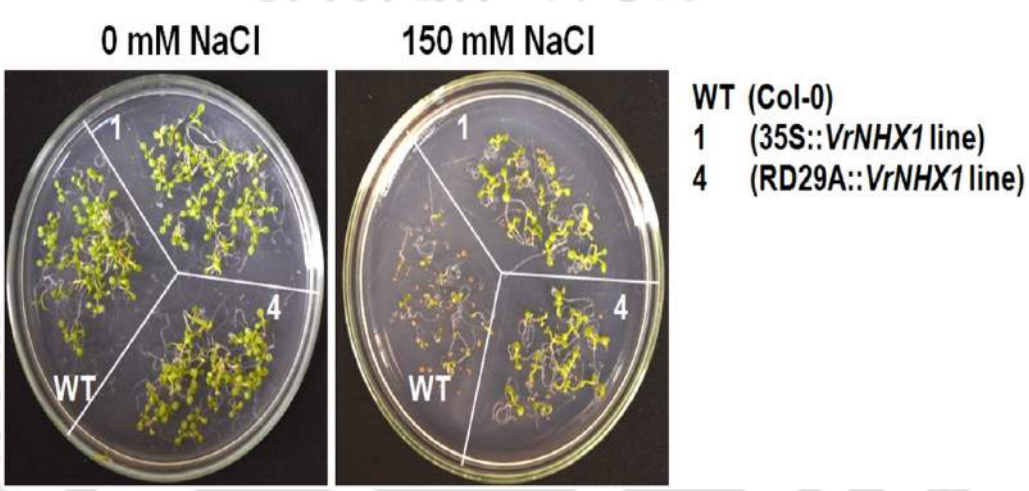

Figure 4.23 Study of seed germination efficiency of transgenic *Arabidopsis* lines under 150 mM NaCl stress. The wildtype (WT, col-0) and transgenic (line 1, 35S::*VrNHX1* and line 4, RD29A::*VrNHX1*) seedlings were observed for germination score after 10 days exposure to salt stress

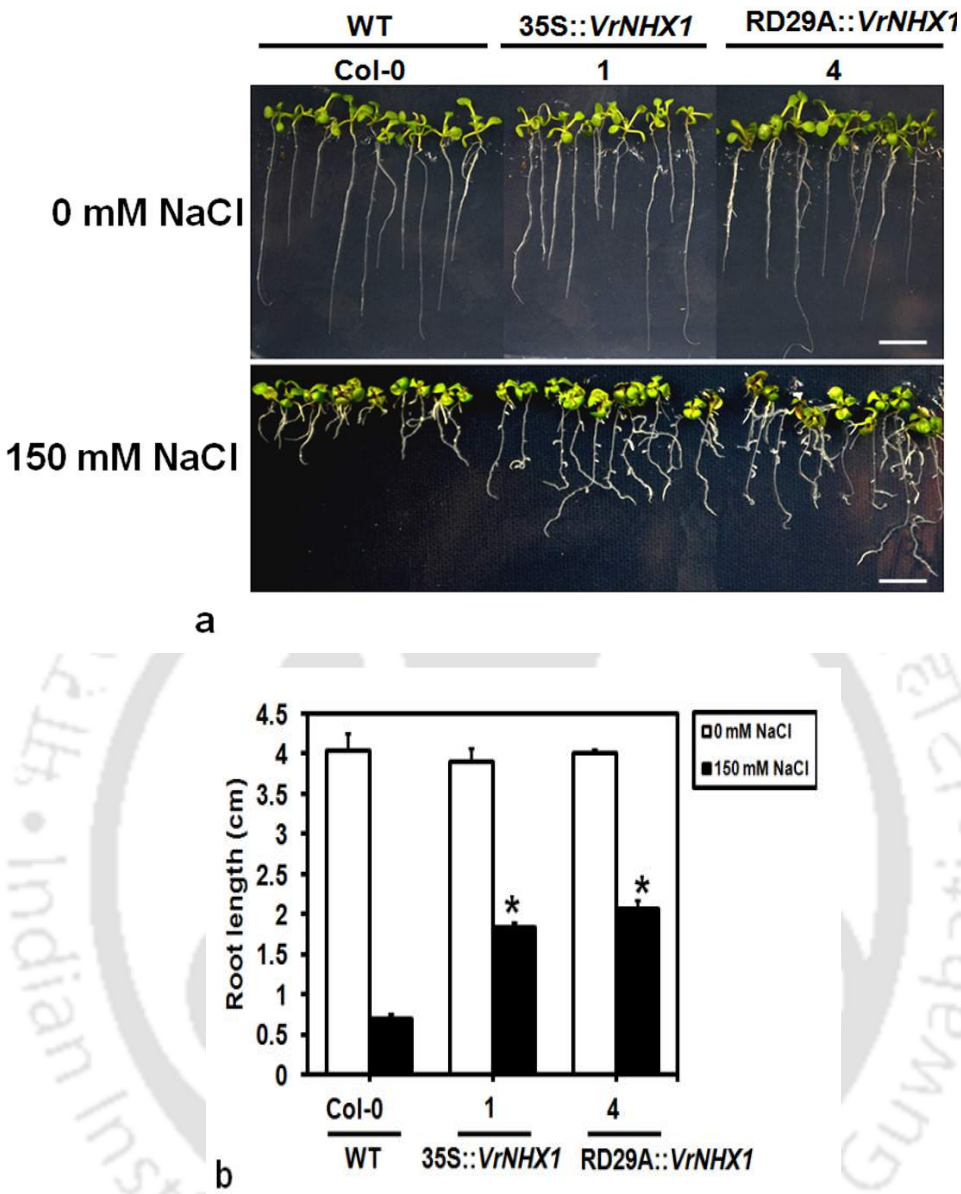

Figure 4.24 Root growth inhibition study under salt stress in wild-type and transgenic *Arabidopsis* lines. (a) Root growth inhibition in wild type (WT, Col-0) and transgenic *Arabidopsis* (Line 1, 35S::*VrNHX1* and Line 4, RD29A::*VrNHX1*) plants upon salt stress (150 mM NaCl). The 4 days old germinated seedlings were transferred to 150 mM NaCl stress for a period of 7 days, Bar: 1 cm; and (b) root length measured was plotted in graph. Values indicate means ± SE (n=10). Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

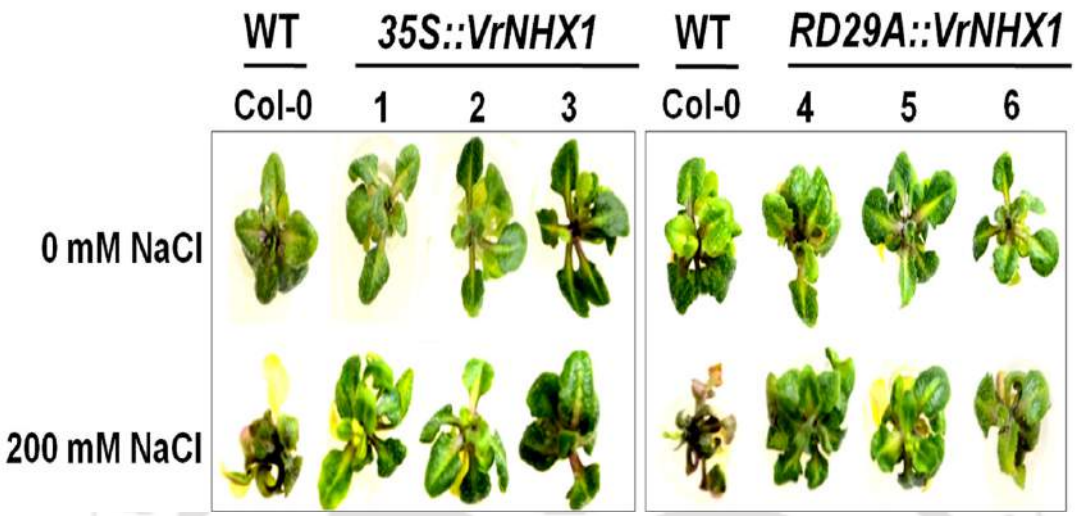

Figure 4.25 Phenotypical effect of salt stress in wild-type and transgenic Arabidopsis lines. Effect of salt stress in wild type (WT, Col-0) and transgenic *Arabidopsis* lines expressing *VrNHX1* constitutively (Lines 1-3, 35S::*VrNHX1*) and inducibly (Lines 4-6, RD29A::*VrNHX1*). NaCl-induced morphological changes was visible in 10 days old WT and transgenic lines after exposure to 200 mM NaCl for 5 days

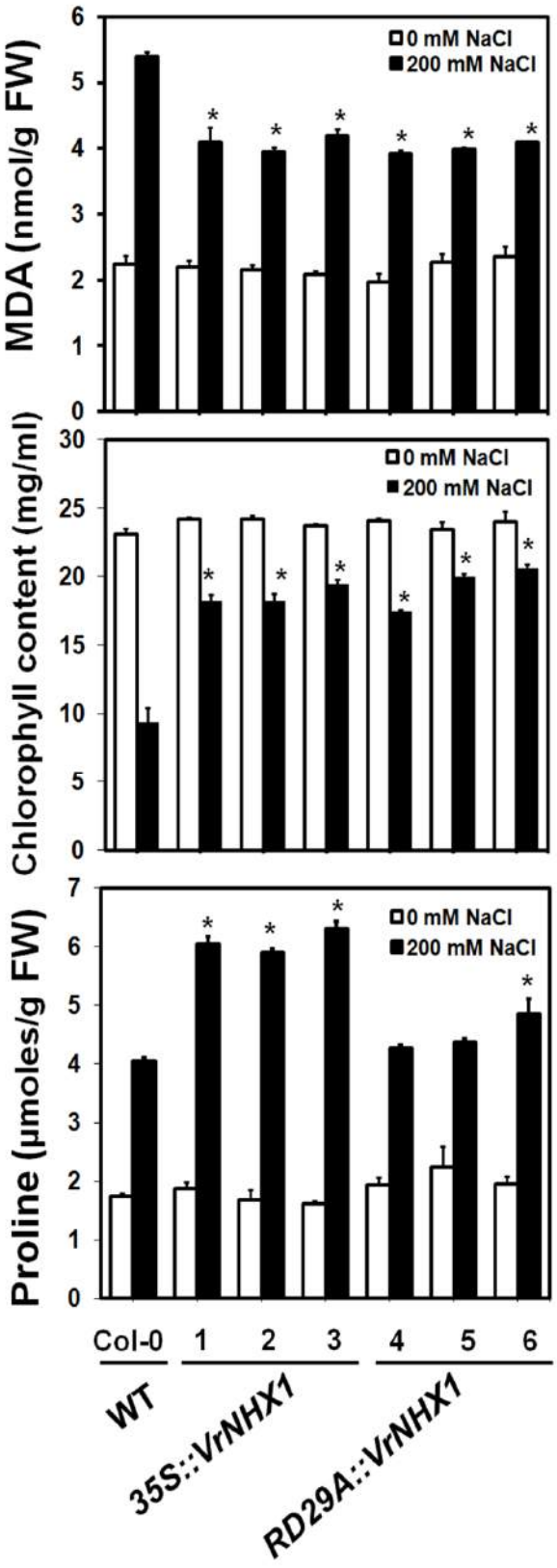

Figure 4.26 Physiological analysis of WT (Col-0) and transgenic *Arabidopsis* plants expressing *VrNHX1* constitutively (Lines 1-3, 35S::*VrNHX1*) and inducibly (Lines 4-6, RD29A::*VrNHX1*) upon salt stress (200 mM NaCl). Changes in chlorophyll, MDA and proline content were estimated and analysed as explained in materials and methods section. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

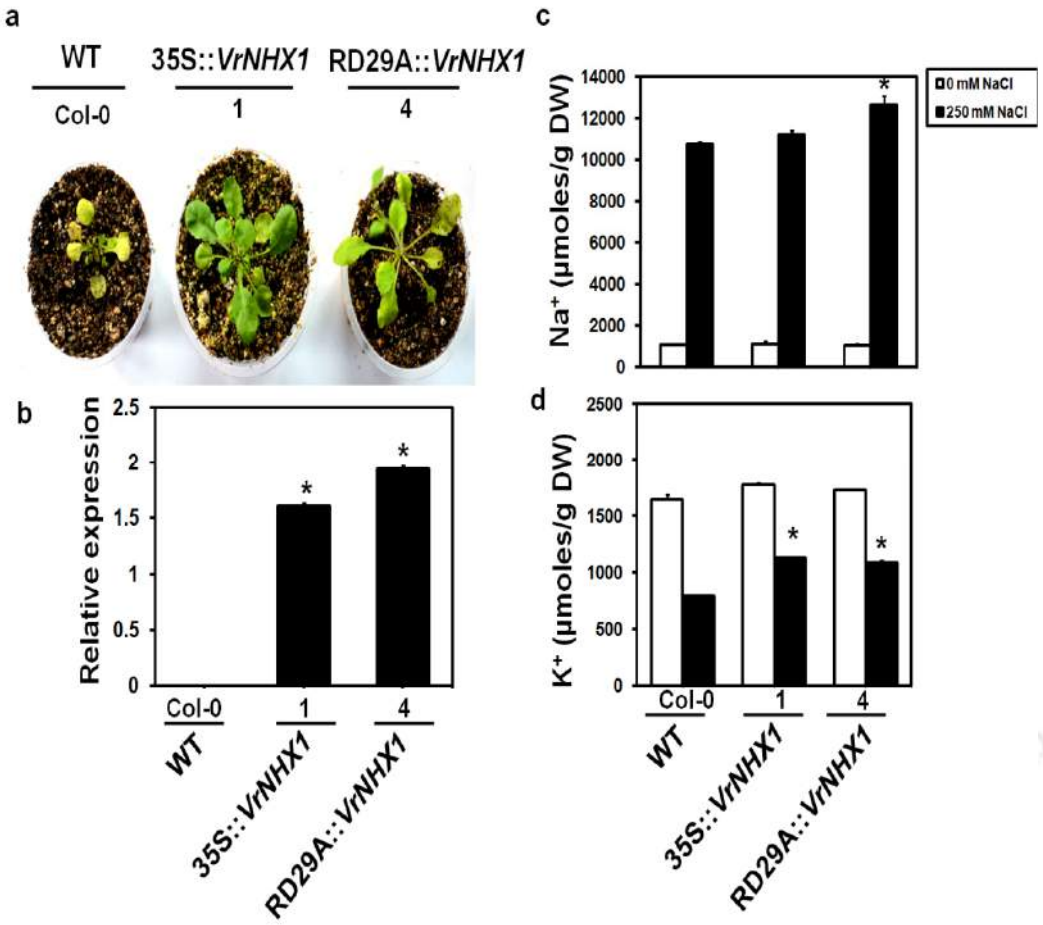

Figure 4.27 Effect of salt stress on mature wild-type and transgenic *Arabidopsis*, measurement of relative transgene expression and ion analysis (a) Salt tolerance assay in wild type (WT, Col-0)and transgenic *Arabidopsis* lines expressing *VrNHX1* constitutively (Line 1, 35S::*VrNHX1*) and inducibly (Line 4, RD29A::*VrNHX1*) subjected to 250 mM NaCl treatment for 2 weeks (b) Relative transgene expression level of *VrNHX1* in transgenic *Arabidopsis* lines. No transgene expression was observed in WT. A 0.283 kb fragment of *VrNHX1*::35SployA and 0.150 kb fragment of *AtUBQ1* was amplified in quantitative RT-PCR analysis (c) Na^+ and (d) K^+ content (µmoles/ g DW) was estimated in leaves of unstressed (0 mM NaCl) and salt stressed (250 mM NaCl) WT and transgenic lines, as described in materials and methods. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as " * ", using Bonferroni analysis

Table 4.1 The putative post-translational modification sites predicted by ScanProsite software for *VrNHX1*

Predicted sites/patterns	Amino acid positions
ASN_GLYCOSYLATION	50-53 293-296
CK2_PHOSPHO_SITE	16-19 250-253 373-376 476-479 480-483 532-535
PKC_PHOSPHO_SITE	250-252 297-299 301-303 379-381 412-414 449-451 465-467 492-494 534-536
MYRISTYL	59-64 71-76 117-122 120-125 153-158 229-234 233-238 283-288 339-344 390-395
LEUCINE_ZIPPER	256-277

ASN_GLYCOSYLATION- N-Glycosylation site, CK2_PHOSPHO_SITE- Casein kinase II phosphorylation site, PKC_PHOSPHO_SITE- Protein kinase C phosphorylation site, MYRISTYL- N-Myristylation site, LEUCINE_ZIPPER- Leucine zipper pattern

CHAPTER 5

Development of salt tolerant transgenic cowpea by ectopic overexpression of *VrNHX1*

5.1. Introduction

Sustainable crop production is threatened by increased salinization in irrigable and cultivable lands worldwide. With the increasing population, the limited availability of cultivable land puts enormous pressure to increase cop production (Yamaguchi and Blumwald 2005). Unlike halophytes, most of crop species are glycophytes which are susceptible to salinity stress. Presence of high Na^+ in soil triggers in plants, osmotic stress due to water deficit, ionic imbalance, altered K^+/Na^+ ratio, membrane dysfunction, excess ROS production, disruption in cellular homeostasis, reduced mineral acquisition, damage to photosynthetic machinery and programmed cell death (Hasegawa et al. 2000; Blumwald et al. 2000; Zhu 2001, 2003; Hu and Schmidhalter 2005). Salt tolerant mechanisms operating in plants include exclusion of toxic Na^+ out of cells, internal detoxification and restricting movement of Na^+ to aerial parts to minimize damage on photosynthetic machinery. Plant NHX exchangers play an important role in detoxification of Na^+ at the cellular and whole plant level besides, controlling the maintenance of K^+ homeostasis, cellular pH regulation, vesicle trafficking and cell expansion are also some of the functions of plant NHX exchangers (Rodríguez-Rosales et al. 2009; Jiang et al. 2010). Compartmentalization of excess Na^+ in vacuoles is known to be mediated in an energy driven process mainly by vacuolar membrane sodium proton antiporter NHX1 in conjuction with vacuolar H^+-ATPases and pyrophosphatases. Further, the sequestered Na^+ in turn serves as an osmoticum to maintain water potential in salt stressed plant cells (Blumwald et al. 2000). Overexpression strategies have been employed to demonstrate the potential of NHX1 antiporter has lead to enhanced salt tolerance in a number of plants.

Cowpea (*Vigna unguiculata* L. Walp.) is one of the major food and fodder legume crops of subsistence, which provides invaluable source of protein, vitamin and minerals for the rural and urban poor, and livestock (Hall et al. 2003; Timko et al. 2007; Omo-Ikerodah et al. 2008). Cowpea is known for its enhanced nitrogen fixing ability and symbiosis with mycorrhizae, ability to restore soil fertility in crop rotation with cereals and better tolerance to wide range of pH variation in soil (Elawad and Hall 1987; Fery 1990; Kwapata and Hall 1985). It is widely cultivated in the marginal areas of semi-arid tropics covering Asia, Africa, and South America.

However, cowpea faces numerous production constraints of which abiotic stress particularly salinity critically affect the productivity by altering several physiological, biochemical and morphological characteristics in plants (Lawlor 2013).

Earlier reports demonstrate the morphological, physiological and biochemical changes witnessed at various stages of cowpea subjected to salt stress assays (West and Francois 1982; Silveira et al. 2001; Murillo-Amador et al. 2006; Lacerda et al. 2006; Chen et al. 2007; Wilson et al. 2006; Gomes-Filho et al. 2008; Guimaraes et al. 2011; Thiam et al. 2013). Classical breeding programs aiming to improve particularly salinity stress tolerance have been hampered by the multigenic nature of the trait and the seemingly scarce natural genetic variability in this important grain legume. Therefore, development of salt tolerant crop plants is of prime importance for maintaining sustainable agriculture for future generations.

Although, cowpea is notoriously recalcitrant to genetic transformation, significant progress has been made recently in improving the efficiency of transformation (Solleti et al. 2008a; Bakshi et al. 2011, 2012a; 2012b; Bakshi and Sahoo 2013) and developing transgenic cowpea with enhanced pest resistance. However, no attempt has been made for the targeted improvement of cowpea for salinity tolerance through transgenic approach.

In the present study, we report development of transgenic cowpea for salt tolerance by overexpression of mungbean Na^+/H^+ antiporter gene, *VrNHX1*. Further, we demonstrate cowpea transgenic plants in T_2 generation surviving high salt stress without any adverse effects on their growth and seed setting. These results demonstrate the potential use of *VrNHX1* in conferring salt tolerance in cowpea.

5.2. Materials and methods

5.2.1. Plant material and explant preparation

Healthy and uniform seeds of commercially grown cultivar of cowpea cv. Pusa Komal (IARI, New Delhi) were used in this study. The seeds were surface sterilized with 0.2% mercuric chloride (w/v) for 5 min, rinsed 4-5 times with sterile deionized water and cultured on MSB_5 medium [MS salts (Murashige and Skoog 1962) and Gamborg B_5 vitamins (Gamborg et al. 1968) supplemented with 3 % sucrose, 0.8 % agar agar (w/v) and 10 µM TDZ. Cotyledonary node explants (5–6 mm) were

prepared from 4 days old *in vitro* raised cowpea seedlings by removing both the cotyledons, and epicotyls and hypocotyls approximately 1 and 3 mm, respectively, from above and below the nodal region (Solleti et al. 2008 a, b).

5.2.2. Plant binary vector and *Agrobacterium* strain

The plant binary vector pCAMBIA2301-35S::*VrNHX1* (13.9 kb) was used for cowpea transformation to generate overexpression lines for *VrNHX1*. The construct preparation is same as explained in section 4.2.8.1. The T-DNA of pCAMBIA2301-35S::*VrNHX1* includes neomycin phosphotransferase gene (*nptII*) as selection marker, β-glucuronidase gene (*gus-A*) as reporter gene and *VrNHX1* CDS driven by CaMV 35S promoter (Fig. 5.1).

5.2.2.1. Mobilization of pCAMBIA230135S::*VrNHX1* to *Agrobacterium tumefaciens*

The recombinant plant binary vectors, pCAMBIA2301-35S::*VrNHX1* (13.9 kb) were transferred into *A. tumefaciens* EHA105 strain by triparental mating. The *A. tumefaciens* strain harboring the overexpression construct was maintained on solid LB medium supplemented with 20 mg/l of rifampicin, and 50 mg/l of kanamycin.

5.2.3. Plant transformation, selection and regeneration of transgenic cowpea lines

Single colony of the bacterial strain was inoculated in 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics, and grown overnight at 28 °C until OD_{600} reached to 1.0. The cells were collected by centrifuging at 5,000 rpm for 5 min and the pellet was resuspended in liquid co-cultivation medium, LCM (MSB_5 medium containing 1 µM BAP, pH adjusted to 5.5) supplemented with 100 µM acetosyringone for inoculation. Prior to the inoculation in bacterial suspension for 30 mins with occasional shaking, the 4-days-old cotyledonary node explants (Fig. 5.2 a) were subjected to mechanical injury with a hypodermic needle. The explants were then blotted on sterile filter paper and co-cultivated in petri dishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone for 3 days under 16 hours photoperiod at 22 °C (Solleti et al. 2008b; Bakshi et al. 2011).

Following co-cultivation, the explants were washed three to four times with LCM and blotted dry on sterile filter paper. The explants were cultured on shoot

induction and selection medium (MSB_5 medium containing 5.0 µM BAP, 0.5 µM kinetin, 150mg/l kanamycin and 500 mg/l cefotaxime). The cultures were transferred after 3 weeks of subculture to shoot induction, elongation and selection medium (MSB medium containing 2.5 µM BAP, 0.5 µM kinetin and 150mg/l kanamycin, 500 mg/l cefotaxime) and cultured for 10 days for optimal induction, elongation and selective regeneration of transformants (Fig 5.2b-f). The elongated shoots were rooted in MS medium supplemented with 2.5 µM IBA and 500 mg/l cefotaxime (Fig 5.2g). The putative transformed plants were established in soil: compost (1:1) and grown to maturity in transgenic greenhouse containment (Fig 5.2h).

5.2.4. Histochemical Gus assay

Transient and stable expression of reporter gene *gus-A* was performed using histochemical gus assays (Jefferson 1987). Transient gus expression was studied in cotyledonary node explants after co-cultivation step (Fig. 5.3 Ia-b). Stable gus expression was detected by immersing flowers, stigma, style, stamen, anthers, and pollen grains of kanamycin selected T_0 transgenic plants in GUS substrate solution for 24 hours at 37 °C. Thereafter, tissues were bleached in 100 % ethanol before examination under microscope (Solleti et al. 2008 a, b) (Fig. 5.3 II) .

5.2.5. Molecular analysis of putative transgenic cowpea plants by PCR

Genomic DNA was isolated from non-transformed and putative transformed cowpea plants using CTAB (cetyl trimethyl ammonium bromide) method (Rogers and Bendich 1988). Polymerase chain reaction (PCR) was performed to detect the presence of *nptII*, *gus-A*, and *VrNHX1* in putative T_0 transformed cowpea plants. 100 ng of genomic DNA was used for PCR analysis. The specific primers: *nptII*-FP 5'-CCACCATGATATTCGGCAAC-3' and *nptII*-RP 5'- GTGGAGAGGCTATT CGGCTA -3' were used to amplify a 0.54 kb fragment of *nptII*. The PCR condition was: 95 °C for 10min; 95 °C for 1 min, 58 °C for 30sec, 72 °C for 30sec and a final extension of 72 °C for 10min for 35cycles. The specific primers: *gus-A* FP 5'-CCCGTGAAATCAAAAAACTC -3' and *gus-A* RP 5'-TTTTCACCGAAGTTCATGC-3' were used to amplify a 1.762 kb fragment of *gus-A*. The PCR condition was: 95 °C for 10 mins; 95 °C for 1 min, 56°C for 1 min, 72 °C for 1 min and 30 sec and a final extension of 72 °C for 10 min for 35cycles. The gene specific primers: G-FP 5'- GCTGTATATTGGAAGGCACTCT -3' and

CaMVpar 35S-RP 5'- GCGAAACCCTATAAGA ACCCTAATTCC -3' were used to amplify a 1.0 kb fragment of 35SP::*VrNHX1*::35STer cassette. The PCR condition was: 95 °C for 10 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension of 72 °C for 10min for 35cycles. Similarly, PCR was performed for the presence of *VrNHX1* in subsequent transgenic progeny lines in T_1, T_2, T_3 generations, using the above specified conditions. The PCR fragments were analyzed on 1% agarose gel and stained with 10mg/ml ethidium bromide. An aliquot (500 ng) of Lambda HindIII/EcoRI DNA ladder (Thermo Fisher Scientific, USA), middle range DNA ruler (Thermo Fisher Scientific, USA, Bangalore Genei, India) were used as molecular weight markers.

5.2.6. Southern hybridization for integration and copy number analysis

The transgene integration and copy number analysis was studied using 30µg of genomic DNA isolated from non-transformed and randomly selected independent T_3 transgenic cowpea plants, digested with restriction endonuclease, EcoRI. Digested genomic DNA was electrophoretically fractionated on 1% agarose gel and blotted onto Zeta-Probe membrane. The blot was hybridized with DIG-labeled 0.5 kb PCR product, corresponding to the coding region of *nptII* gene prepared according to instructions of the DIG Labeling and Detection system. Prehybridization and hybridization were carried out using hybridization buffer containing 50% deionized formamide, 5X SSC, 0.1% N-lauroylsarcosine, 0.02 % SDS and 1% blocking solution at 42 °C. Washing and detection were performed according to the instruction of the DIG labeling and Detection System.

5.2.7. RNA isolation and expression analysis by semiquantitative RT-PCR

Total RNA was isolated from T_3 southern positive transgenic cowpea lines using RNeasy Plant Mini Kit following the manufacturer's instructions, quantified in Nanovue™ Plus Spectrophotometer and cDNA was prepared. Semi-quantitative RT-PCR was performed using a set of gene specific primers (FP: 5'-GCTGTATATTGGAAGGCACTCT-3' and RP: 5'- CAATGTCCAAGGCATCCATACC -3') for amplifying a 280 bp fragment of *VrNHX1*. The PCR condition was: 95 °C for 10 min; 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 30 sec and a final extension of 72 °C for 10 min for 30 cycles. Housekeeping *VuNSR9* primers (FN: 5'- AGCACAGTTTGGGT ATATTGTGTTG-3' and RN: 5'- TGAGTAAAACTGGCAAAAATTAGAT-3') were used as

an internal control. The PCR conditions are as follows: 95 °C for 10 min; 95 °C for 30 sec, 49 °C for 30 sec, 72 °C for 30 sec and 72 °C for 10 min for 28 cycles. Similarly, a 0.54 kb fragment of *nptII* was amplified using conditions, as described previously in this section. The PCR fragments were analyzed on 1% agarose gel and stained with 10mg/ml ethidium bromide.

5.2.8. Leaf Senescence Assay

The leaf senescence assay was performed with 30 days old non-transformed and T_1 transgenic cowpea lines. Healthy and fully expanded leaves used for the assay were briefly washed in deionized water, 1cm diameter leaf discs prepared for analysis in 0, 150 and 200 mM NaCl solution. The phenotypic changes upon salt stress were monitored for 7 days and total chlorophyll was estimated according to Arnon (1949).

5.2.9. Physiological analysis of transgenic cowpea plants

5.2.9.1. Salt stress assay

One month old cowpea non-transformed and T_2 transgenic cowpea lines grown in soil under green-house condition were initially watered with Hoagland medium supplemented with 50, 100, 150 mM NaCl for 1 week and gradually increased to 200 mM NaCl at a regular interval of 5 days for 3 weeks. The leaves and roots of untreated and salt treated non-transformed and transformed cowpea plants were harvested and used for several physiological and biochemical analysis.

5.2.9.2. Na^+/K^+ estimation in control and salt-treated non-transformed and transgenic cowpea plants

Leaves and roots of untreated and salt-treated non-transformed (wild-type) and *VrNHX1* overexpressing transgenic cowpea lines were harvested for Na^+ and K^+ estimation. Similar method was used as explained in section 3.2.7.

5.2.9.3. Chorophyll ion estimation

Total chlorophyll (Chl a and b) estimation was carried out using 100 mg leaf discs homogenized in 80 % acetone at 4 °C. The lysate was centrifuged at 3,000 rpm for 10 min and absorbance of the supernatant was measured at 652 nm according to

Arnon (1949). The total chlorophyll content was calculated using the formulae:-

$$Chl_{(a+b)}\,(mg/ml) = ((5.248 \times A_{664}) + (22.4 \times A_{648}))$$

5.2.9.4. Measurement of relative water content (RWC)

The relative water content was determined following method as described in Lv et al. (2009). Briefly, the fresh weights (FWs) of cowpea leaves were recorded immediately after excising from plants. After soaking them in deionized water at 4°C overnight, their turgid weights (TWs) were determined. Then their dry weights (DWs) were obtained after oven-drying the leaf samples at 70 °C for 72 hours. The relative water content (RWC) was calculated as:

$$RWC\ (\%) = \frac{(FW-DW)}{(TW-DW)} \times 100\%$$

5.2.9.5. Determination of proline content

Proline colorimetric estimation was performed using method explained in section 3.2.10.2.1.4.

5.2.9.6. Determination of ascorbate content

For determination of total ascorbate content, 0.5 g of leaves were homogenized with 5 ml of phosphoric acid and centrifuged at 17,000 g for 15 min at 4 °C. The reaction mixture contained 2 % sodium molybdate, 0.15N H_2SO_4, 1.5mM Na_2HPO_4 and plant extract. The reaction mixture was incubated at 60 °C in water bath for 40 min, cooled, centrifuged at 3,000 g for 10 min and absorbance measured at 660 nm (Panda et al. 2003). The ascorbate value was calculated using the formulae:-

$$\text{Ascorbate } (\mu g/g.\,FW) = \frac{Abs_{660} \times \text{Standard value } 123.67\,(\mu g/ml)}{\text{Sample } (g/ml)}$$

5.2.9.7. Measurement of lipid peroxidation

Lipid peroxidation was measured according the method explained in section 3.2.10.2.1.3.

5.2.9.8. Histochemical detection of H_2O_2 and O_2^-

In situ detection of hydrogen peroxide (H_2O_2) was done by DAB staining according to method by Ramel et al. (2009) and superoxide radical (O_2^-) by NBT staining following modified method of Rao and Davis (1999). A portion of leaf sample was immersed and infiltrated under vacuum with 1.25 mg/ml 3,3'-diaminobenzidine (DAB) staining solution, pH 7.8 dissolved in H_20 for 6 hours and 3 mg/ml Nitrobluetetrazolium (NBT) staining solution in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM NaN_3 for 30 min at room temperature. Stained leaves were bleached in acetic acid: glycerol: ethanol (1:1:3 v/v) solution at 100 °C for 5 min, stored in glycerol: ethanol (1:4 v/v) solution, till photographed.

5.2.10. Statistical analysis

Statistical comparison between the variances was determined by ANOVA (Analysis of variance) and significant differences between mean values were determined by Bonferroni analysis. Statistically significant mean values were denoted as different letters ($P \leq 0.05$).

5.3. Results and discussion

5.3.1. Generation of transgenic cowpea overexpressing *VrNHX1*

Cotyledonary node explants were used owing to the efficacy of shoot regeneration from axillary meristematic cells. Further, cotyledonary explants were given mechanical injury to facilitate efficient *Agrobacterium* transformation in cowpea as described in Bakshi et al. (2011). A stable transformation efficiency of 3.68 % was observed using a total of 484 explants in 5 different experiments generating 18 independently derived transgenic plants (Table 5.1). Stable gus expression was observed in flower, stamen, anther, pollen grains, carpel, pistil and sepal of all kanamycin resistant T_0 cowpea plants established in greenhouse (Fig. 5.3 II).

5.3.2. Molecular analysis of transgenic cowpea

The amplification of 0.5, 1 and 1.7 kb fragments corresponding to *nptII*, 35SP::*VrNHX1*::35STer cassette and *gus-A*, respectively in PCR analysis, confirmed the presence of transgene in independent T_0 kanamycin-resistant transgenic cowpea plants (Fig. 5.4a-c). No amplification was observed in control non-transformed cowpea plant in all three cases (Fig. 5.4a-c).

5.3.3. Southern hybridization for copy number analysis

Southern hybridization was performed in T_3 progenies of independent T_0 transgenic lines using 0.5 kb *nptII* probe (Fig. 5.4d). Differential integration pattern of transgene was observed in five of six progenies further, single, double integration event was observed in T_3. 2, 4, 6 (Fig. 5.4 d; Lane 3, 5, 7) and T_3. 5 (Fig. 5.4 d; Lane 6) transgenic cowpea lines, respectively, unlike control non-transformed plants not showing any signal (Fig. 5.4 d; Lane 1). More than two transgene integration event was observed in T_3.1 (Fig. 5.4 d; Lane 2). The hybridization signals obtained were greater than 2.1 kb.

5.3.4. Expression analysis of *VrNHX1* in T_3 progenies of transgenic cowpea

Semi-quantitative RT-PCR was performed for *VrNHX1* and *nptII* in non-transformed control and T_3 transgenic cowpea plants. Expression of *VrNHX1* was observed in transgenic plants whereas, owing to 97% similarity of *VrNHX1* with *VuNHX1,* amplification of *VuNHX1* in background was obtained in both control and transgenic cowpea plants (Fig. 5.4 e).

5.3.5. Segregation analysis

The segregation of *VrNHX1* was performed in T_1 plants from four independent T_0 transgenic lines (T_0. 2, 4, 5, 6). PCR amplification of 0.5 and 1 kb fragments of *nptII* and *VrNHX1*, respectively (Fig. 5.5 a, b) in the progeny lines revealed the inheritance of transgene in a Mendelian fashion (Table 5.2). A segregation ratio of 3:1 was observed for transgenic lines T_1. 2 and 4 indicating a single functional locus whereas, a segregation pattern of 1:1 was observed for T_1.6, indicating distortion in segregation of transgene in the progenies which can be explained probably due to chromosomal rearrangement. Further, T_1.1 displayed more than one copy in southern blot but, a 1:1 segregation ratio indicating probability of linked inheritance. A 15:1 segregation ratio was observed in T_1.5 suggesting presence of two independent loci (Table 5.2).

5.3.6. Leaf senescence assay in transgenic T_1 lines

The salt stress assay was performed with 150 and 200 mM NaCl using leaf discs of three independent T_1 cowpea transgenic lines (T_1. 2, 4, 5). It was observed that the control plants showed excessive bleaching under salt stress however, the leaf discs

from transgenic lines appeared comparatively green. The extent of chlorosis was measured with estimation of chlorophyll content according to Arnon (1949) indicating lesser chlorosis (Fig. 5.6a). Under normal physiological condition, no statistically significant difference was observed in non-transformed control (wild-type) cowpea and transgenic cowpea plants. However, under 150 and 200 mM NaCl stress, a statistically significant difference ($P \leq 0.05$) was observed in control and transgenic cowpea lines. No statistically significant difference between the independent transgenic cowpea lines was observed (Fig. 5.6 b).

5.3.7. Salt tolerance of transgenic cowpea overexpressing *VrNHX1*

The effect of salt stress on one month old non-transformed and T_2 transgenic cowpea plants (T_2. 2, 4, 5, 6) overexpressing *VrNHX1* was observed by watering with Hoagland solution supplemented with 200mM NaCl solution for a time period of 3 weeks. Under normal physiological condition, there was no observed difference in growth and morphology between wild-type and transgenic plants. However, under salt stress transgenic plants showed better performance in growth as compared to wild-type plants. Severe growth inhibition, chlorosis, wilting in wild-type plants was observed whereas, in case of transgenic lines, better survival was observed in T_2.2 and T_2.4 (Fig. 5.7). The transgenic plants used in salt stress assay displayed unaltered seed setting after withdrawal of salt stress as compared to salt stressed control cowpea.

5.3.8. Na^+ and K^+ estimation

Total Na^+ and K^+ analysis was performed after the completion of salt stress using the leaves and roots of wild-type and transgenic cowpea plants. Under physiological condition, there was no significant difference in the K^+/Na^+ ratio between wild-type and transgenic lines. On the contradictory, under 200 mM NaCl stress, both, wild-type and transgenic lines exhibited higher [Na^+] in leaves and roots. Transgenic lines showed higher [Na^+] in roots than in leaves as compared to wild-type plants (Fig. 5.8 a, b). Under salt stress, transgenic cowpea lines T_2.2 and T_2.4 displayed statistically significant difference ($P \leq 0.05$) as compared to wild-type and T_2.5 and T_2.6 transgenic lines (Fig. 5. 8a, b). Presence of low [Na^+] in leaves in transgenic lines also reported in *T. aestivum* (Xue et al. 2004), *O. sativa* (Islam et al. 2010; Liu et al. 2010), *Malus X domestica* Borkh. (Li et al. 2010) is indicative of efficient strategy against salt

tolerance by restricting the movement of Na^+ in roots. Transgenic cowpea exhibited higher [K^+] than wild-type in both leaves and roots as compared to wild-type (Fig. 5.8c, d). This was in accordance with earlier reports on transgenic *T. aestivum* (Xue et al. 2004), *O. sativa* (Islam et al. 2010), *M.sativa* (Li et al. 2011), *A. deliciosa* (Tian et al. 2011) but, in contradiction with report on *J. curcas* (Jha et al. 2013) overexpressing *NHX*s.

5.3.9. Measurement of malionaldehyde, proline, ascorbate, RWC, chlorophyll content

5.3.9.1. MDA content

Salinity stress induces oxidative stress in cells leading to damage to cellular machinery including free radical mediated lipid peroxidation. MDA acts as an effective indicator for measuring the degree of lipid peroxidation. Under physiological conditions, no statistically significant difference ($P \leq 0.05$) was observed in MDA content between wild-type and transgenic lines. However, under salt stress, there was a significant increase in MDA content in wild-type plant (2.18 times) as compared to transgenic lines (Fig. 5.9). Transgenic cowpea lines exhibited lesser extent of lipid peroxidation indicative of occurrence of lesser oxidative damage with overexpression of *VrNHX1*. This data was in accordance with reports for on *J. curcas* (Jha et al. 2013), *O. sativa* (Liu et al. 2010), and *A. deliciosa* (Tian et al. 2011).

5.3.9.2. Ascorbate content

Under physiological conditions, no statistically significant difference ($P \leq 0.05$) was observed in ascorbate content between wild-type and transgenic lines. Ascorbate, a non-enzymatic antioxidant, a free radical scavenger showed increased concentration in transgenic plants upon salt stress. Similarly, upon salt stress, ascorbate showed an increase of 1.28, 1.56, 1.05, and 1.08 times in transgenic T_2. 2, 4, 5, and 6 cowpea plants, respectively. Whereas, in wild-type plants, a decrease in ascorbate content was observed (Fig. 5.9b).

5.3.9.3. Proline content

Proline, an inert compatible osmolyte leads to osmotic adjustment, stabilization of proteins and protein complexes, protection of sub-cellular structures like

membranes and macromolecules as well as photosynthetic apparatus under osmotic stress, and acts as a free radical scavenger (Ashraf and Foolad 2007; Szabados and Savoure 2010). Proline accumulation upon various environmental stresses has been well documented. Under physiological conditions, no statistically significant difference ($P \leq 0.05$) was observed in proline content between wild-type and transgenic lines. However, under salt stress, proline content observed was higher in transgenic lines as compared to wild-type cowpea plants, indicative of its potent role in osmotic adjustment in transgenic lines. The proline content measured was 1.35, 1.54, 2.74 and 1.7 times higher in T_2. 2, 4, 5, 6, respectively at salt stress condition (Day 21) compared to unstressed condition (Fig. 5.9c). Similar reports have been obtained in *B. napus* (Zhang et al. 2001) and *O. sativa* (Liu et al. 2010), on the contrary, transgenic *M. sativa* overexpressing *SsNHX1* accumulated lesser proline content (Li et al. 2011).

5.3.9.4. Chlorophyll content

Under physiological conditions, no statistically significant difference ($P \leq 0.05$) was observed in chlorophyll content between wild-type and transgenic lines. However, it was significantly higher in transgenic cowpea plants as compared to wild-type under salt stress. A decrease of 66.65% in chlorophyll content, was observed in wild-type plants under salt stress condition (Fig. 5.9d).

5.3.9.5. Relative water content

The relative water content is an indicator for water status in leaves. The difference in relative water content (RWC) content measured under normal physiological condition was insignificant between wild-type and transgenic lines. Under salt stress, transgenic lines maintained a high water status as compared with wild-type which observed a decrease of RWC by 25.6% (Fig. 5.9e). Similar results were obtained with earlier reports (Liu et al. 2010; Gouiaa et al. 2012).

5.3.10. Superoxide radical 0_2^- and hydrogen peroxide H_2O_2 staining

Environmental stresses like salinity and drought are known to trigger the production of reactive oxygen species which affects the cellular machinery. Lack of proper detoxification mechanisms including production of antioxidants (ascorbate, glutathione and ROS-scavengers), compatible osmolytes (glycine betaine, proline)

controlling the upsurge in production of ROS causes oxidative damage to plant cells (Miller et al. 2010). ROS production in control and transgenic salt stressed plants was investigated qualitatively using DAB and NBT histochemical staining. Control and transgenic plants showed low production of 0_2^- and H_2O_2 under normal physiological condition. Under salt stress, transgenic lines T_2.2 and T_2.4 exhibited lower NBT and DAB staining (Fig. 5.10a, b). However, control exhibited higher staining clearly indicating higher generation of ROS under salinity induced oxidative stress.

5.4. Conclusion

Previous reports have demonstrated the efficacy in generation of salt tolerant transgenic plants overexpressing vacuolar Na^+/H^+ antiporter genes. The overexpression strategy aims in reduction of toxic Na^+ from the cytosol by their sequestration in vacuolar compartment to main ionic and cellular homeostasis in transgenic plants. It has been observed that transgenic *A.thaliana* (Apse et al. 1999), *S. lycopersicon* (Zhang and Blumwald 2001), *B. napus* (Zhang et al. 2001), *T. aestivum* (Xue et al. 2004) overexpressing *AtNHX1* have conferred increased salt tolerance. Similarly, overexpression of orthologues of *AtNHX1, OsNHX1* in *Z. mays* (Chen et al. 2007), *L. perenne* (Wu et al. 2005), and *O. sativa* (Liu et al. 2010), *PgNHX1* in *B. juncea* (Rajagopal et al. 2007), and *O. sativa* (Islam et al. 2010) have also yielded similar results.

Constitutive expression of *VrNHX1* in cowpea resulted in increased salt tolerance and lower growth inhibition as compared to non-transformed (wild-type) cowpea plants. Similarly, salt tolerant transgenic lines in legumes have been observed in model legume *Lotus corniculatos* overexpressing *GmNHX1* (Sun et al. 2006), and further, in *A. hypogaea* (Banjara et al. 2012) and *G.max* (Li et al. 2010) overexpressing *AtNHX1*.

The physiological data indicated that *VrNHX1* overexpression was able to mitigate the toxic effects of Na^+ and enabled the transgenic plants to survive better under salinity stress condition. The transgenic lines had reduced ROS production and lesser oxidative damage evident from NBT and DAB staining as compared to control under salt stress. This data was in corroboration with the lower lipid

peroxidation, higher proline, ascorbate and chlotrophyll content obtained for transgenic lines overexpressing *VrNHX1*.

This study demonstrated the successful generation of salt tolerant transgenic cowpea plants by ectopic overexpression of *VrNHX1* using *Agrobacterium* mediated transformation strategy. This is the first attempt on development of transgenic cowpea plant against abiotic stress, such as salinity. Higher level of salt accumulation in vacuole provides an efficient mechanism in sustaining plant growth and development in salt affected areas.

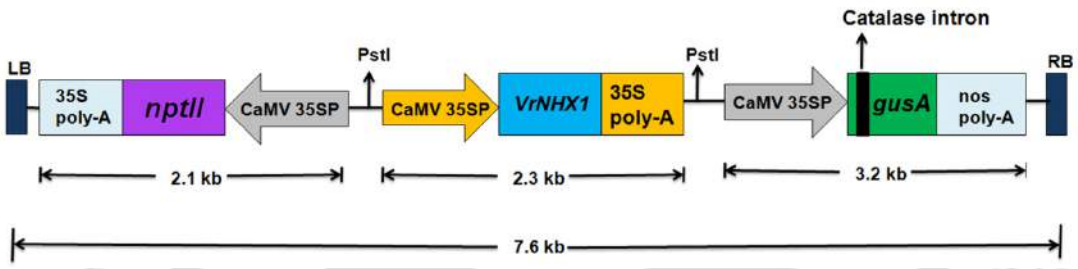

Figure 5.1 T-DNA region (7.6 kb) of pCAMBIA2301-35S::*VrNHX1*. Abbreviations: LB, left border; RB, right border; CaMV 35SP, Cauliflower mosaic virus 35S promoter; CaMV 35S poly-A, Cauliflower mosaic virus 35S terminator; nos poly-A, nopaline transferase terminator; *nptII*, neomycin phosphotransferase; *intron-gus-A*, intron interrupted β-glucuronidase; *VrNHX1*, *Vigna radiata NHX1*. Restrcition enzyme PstI used for cloning 35SP::*VrNHX1*::35STer cassette (2.3kb) into plant binary vector pCAMBIA 2301 is also highlighted

Figure 5.2 Multiple shoot proliferation, regeneration of transgenic cowpea (*Vigna unguiculata* L. Walp cv. Pusa Komal). (a) Cotyledonary node as explants (Bar 1 mm). (b,c,d) Multiple shoot induction from cotyledonary node explant cultured on MSB_5 + 0.5 µM BAP + 0.5 µM kinetin supplemented with 500 mg/L cefatoxime and 150 mg/L kanamycin after 1st, 2nd and 3rd subculture (Bar 6 mm). Kanamycin selected shoots of (e) control and (f) transformed cowpea. (g) Kanamycin selected shoots on rooting medium (Bar 1 cm). (h) Acclimatized plant in greenhouse

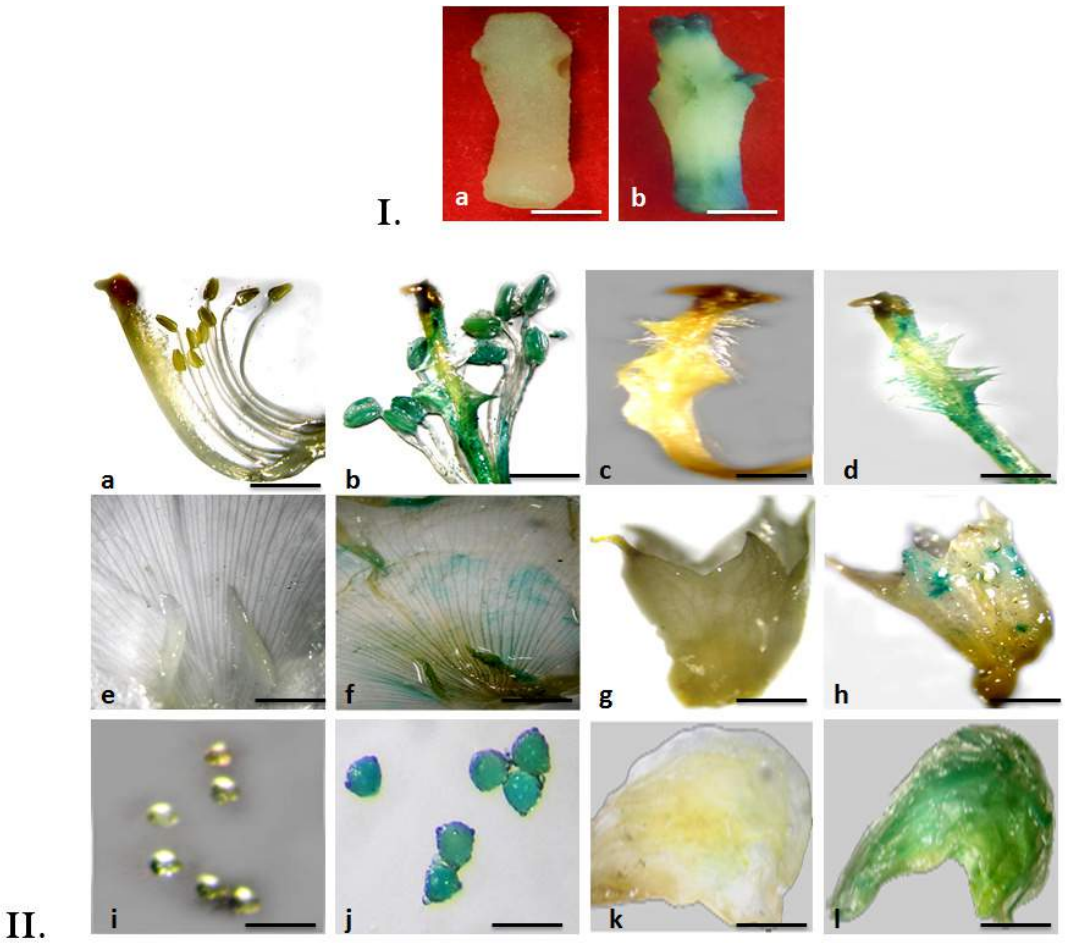

Figure 5.3 Gus assay in untransformed and transformed cowpea. I) Transient GUS assay in (a) non-transformed i.e. control explants not showing GUS activity and (b) transformed explants after 3 days of co-cultivation showing GUS activity (Bar 5 mm). II) Stable GUS expression in stamen, pistil, anthers (b), stigma (d), carpel (f), sepal (h), pollens (j), and flower (l) of T_1 transgenic cowpea plants and no GUS expression in control (a, c, e, g, i, k)

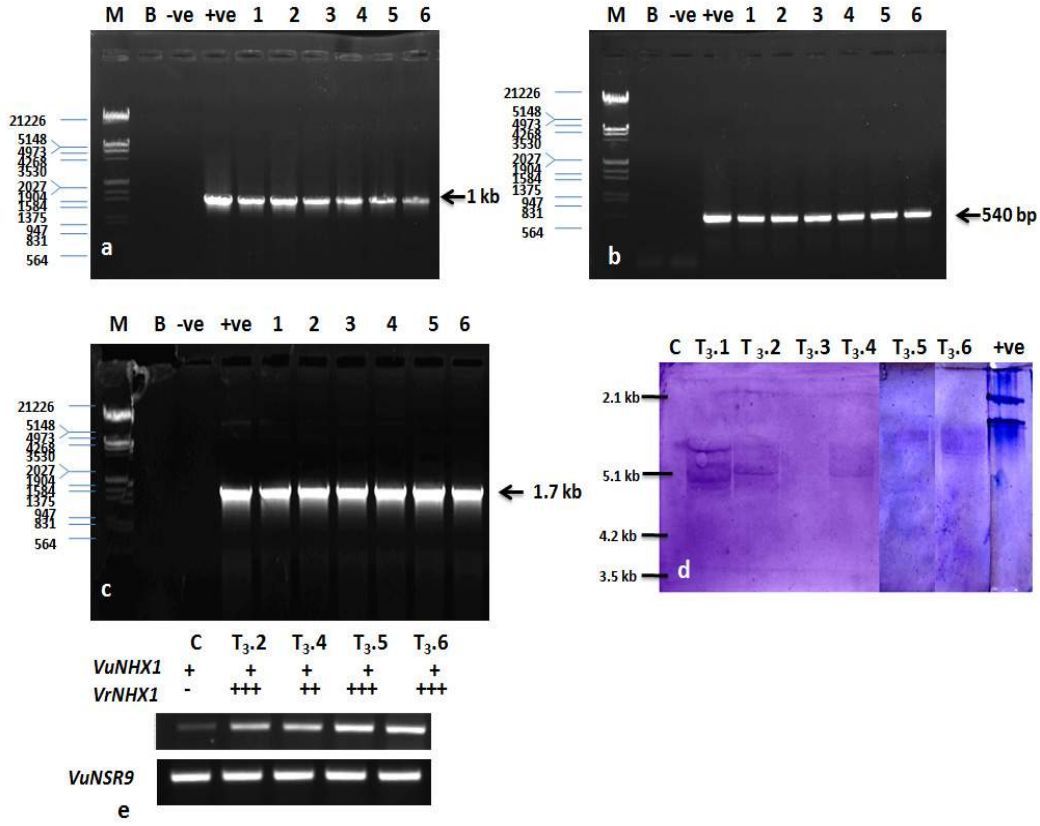

Figure 5.4 Molecular analysis of transgenic plants overexpressing *VrNHX1*. (a-c) Analysis of kanamycin-resistant T_0 transgenic cowpea plants, PCR amplification of (a) 1kb fragment of 35SP::*VrNHX1*::35STer cassette, (b) 0.5kb fragment of *nptII* gene, (c) 1.7 kb fragment of *gus-A* gene. M: Lambda DNA HindIII/EcoRI ladder, -ve: Non-transformed control cowpea, +ve: Plasmid control, 1-6: Putative T_0 trangenic cowpea plants. (d) Southern blot hybridization analysis of six independent T_3 transgenic cowpea lines digested with EcoRI, using a *nptII* probe. C: Non-transformed control cowpea, +ve: Uncut plasmid (pCAMBIA2301-35S::*VrNHX1*), T_3.1-6: Transgenic cowpea lines. (e, f) Expression analysis by semi-quantitative RT-PCR in control (C) and four independent T_3 transgenic cowpea lines (T_3. 2, 4, 5, 6) using *VrNHX1* gene specific primers. *VuNSR9* primers were used as internal control

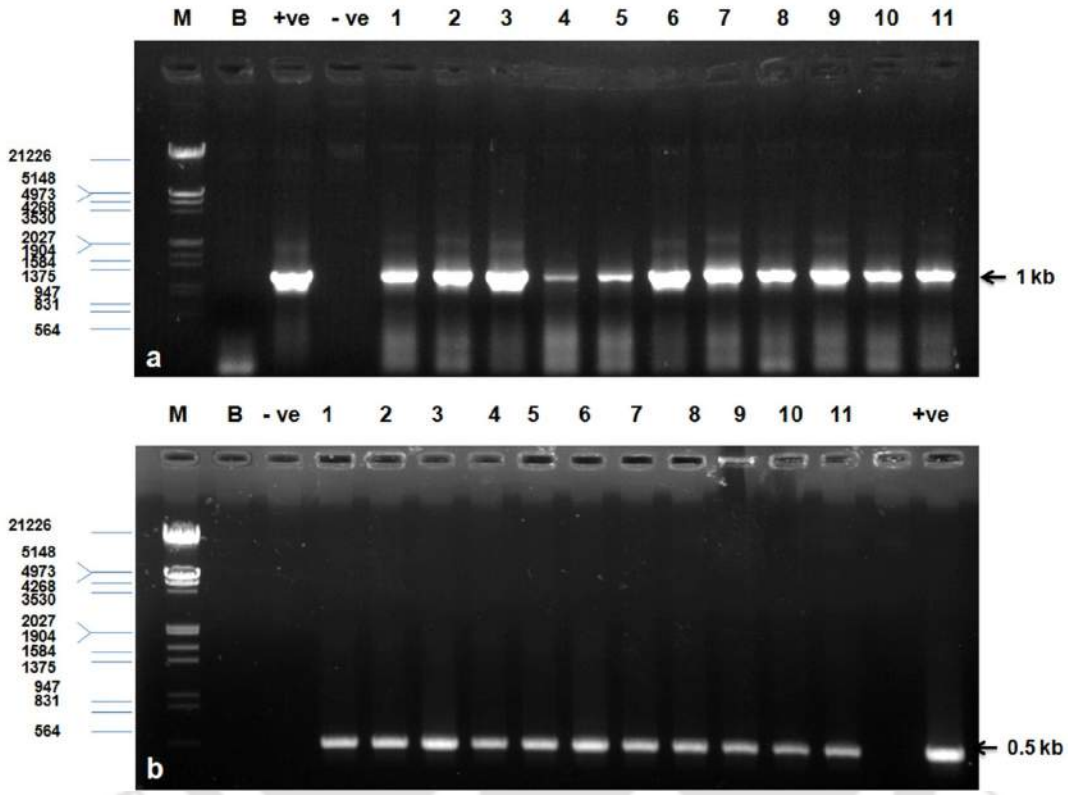

Figure 5.5 PCR amplification of 1 kb and 0.5 kb fragment of 35SP::*VrNHX1*::35STer cassette and *nptII,* respectively in T_1 transgenic cowpea plants. M: Lambda DNA HindIII/EcoRI ladder, -ve: Non-transformed control cowpea, +ve: Plasmid control (pCAMBIA2301-35S::*VrNHX1*), 1-11: T_1 transgenic cowpea plants

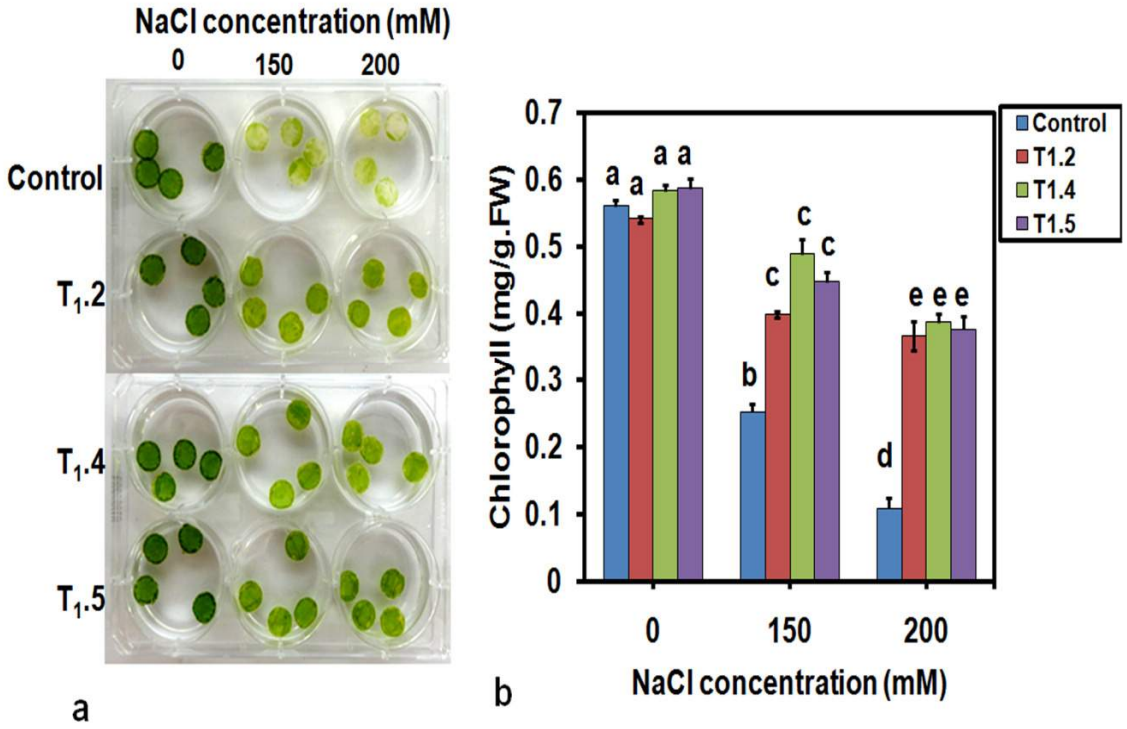

Figure 5.6 Leaf senescence assay and chlorophyll measurement in untransformed and transformed cowpea. (a) Leaf senescence assay was performed using 1mm diameter leaf disc from non-transformed control (C) and T_1 transgenic cowpea plants (T_1. 2,4,5) under 150 and 200 mM NaCl treatment. Higher amount of chlorophyll pigment leaching in leaf disc sections was observed in control than transgenics upon exposure to salt stress for a period of 7 days. (b) Variation in total chlorophyll content in leaf discs exposed to salt stress. Total chlorophyll content was estimated in mg/g FW as described in materials and methods. Data represents mean values ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

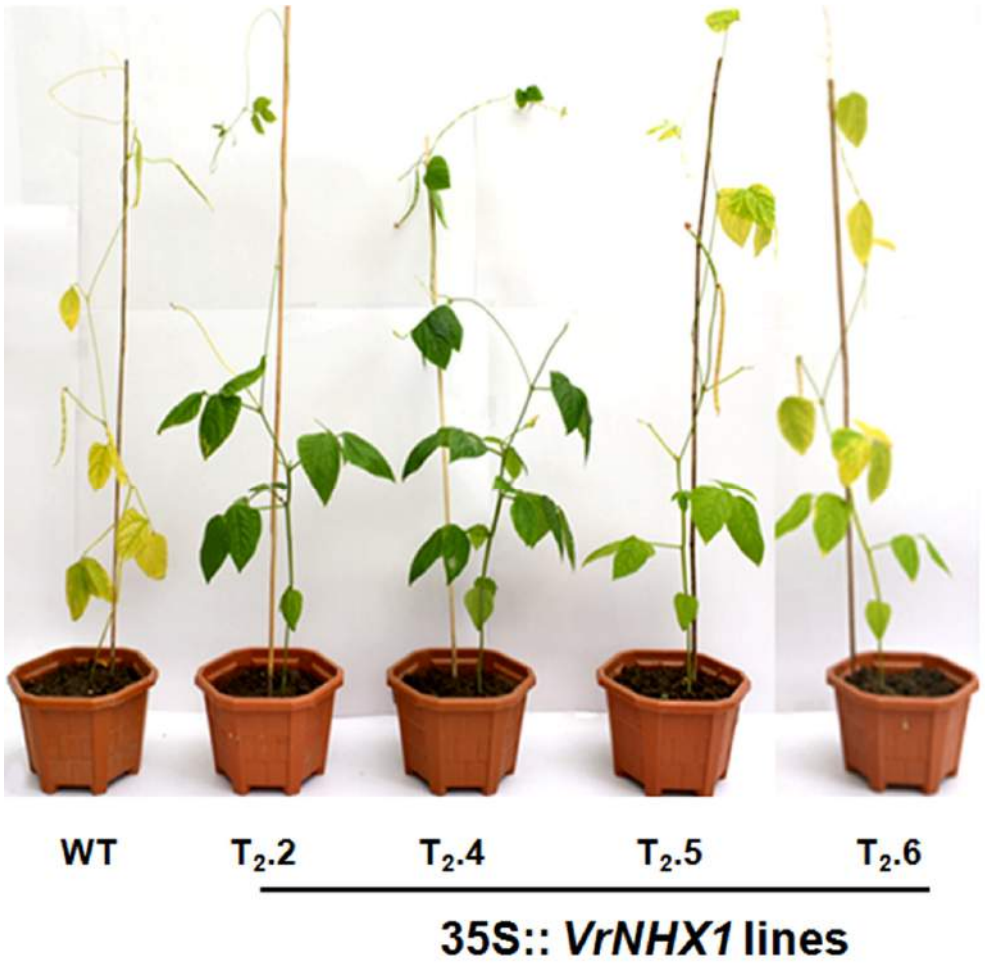

Figure 5.7 Morphological study of salt stress in untransformed and transformed cowpea plants. Salt tolerance was observed by exposing one month old control untransformed (C) and T_2 transgenic cowpea plants (T_2. 2, 4, 5, 6) to 200 mM NaCl for a period of 3 weeks. Control cowpea plant shows extreme bleaching while, T_2 transgenic plants exhibit comparatively better survival under salt stress

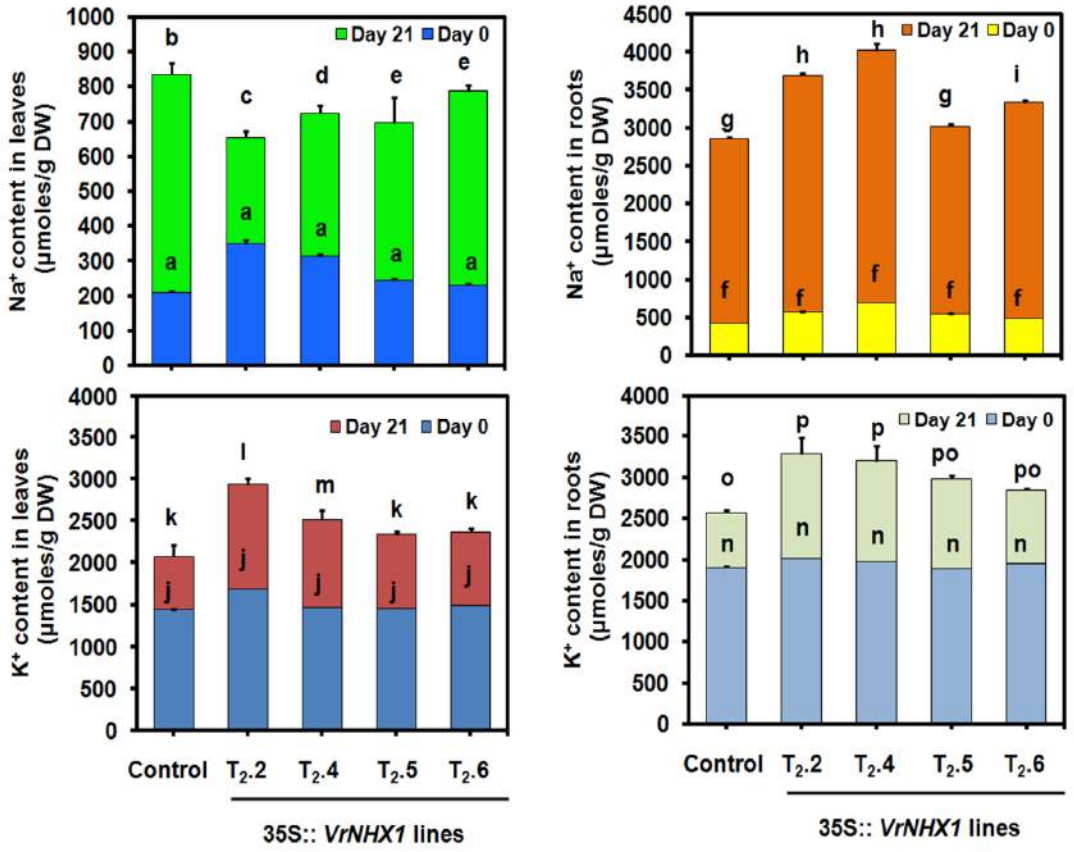

Figure 5.8 Measurement of ion content in untransformed and transformed cowpea plants under salt stress. Total intracellular ion measurement in leaves and roots of control untransformed (C) and T_2 transgenic cowpea plants (T_2. 2, 4, 5, 6) exposed to salt stress as described in materials and methods. Na^+ and K^+ content in (a, c) roots and (b, d) leaves harvested after 3 weeks of salt treatment was measured using Flame Photometer. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

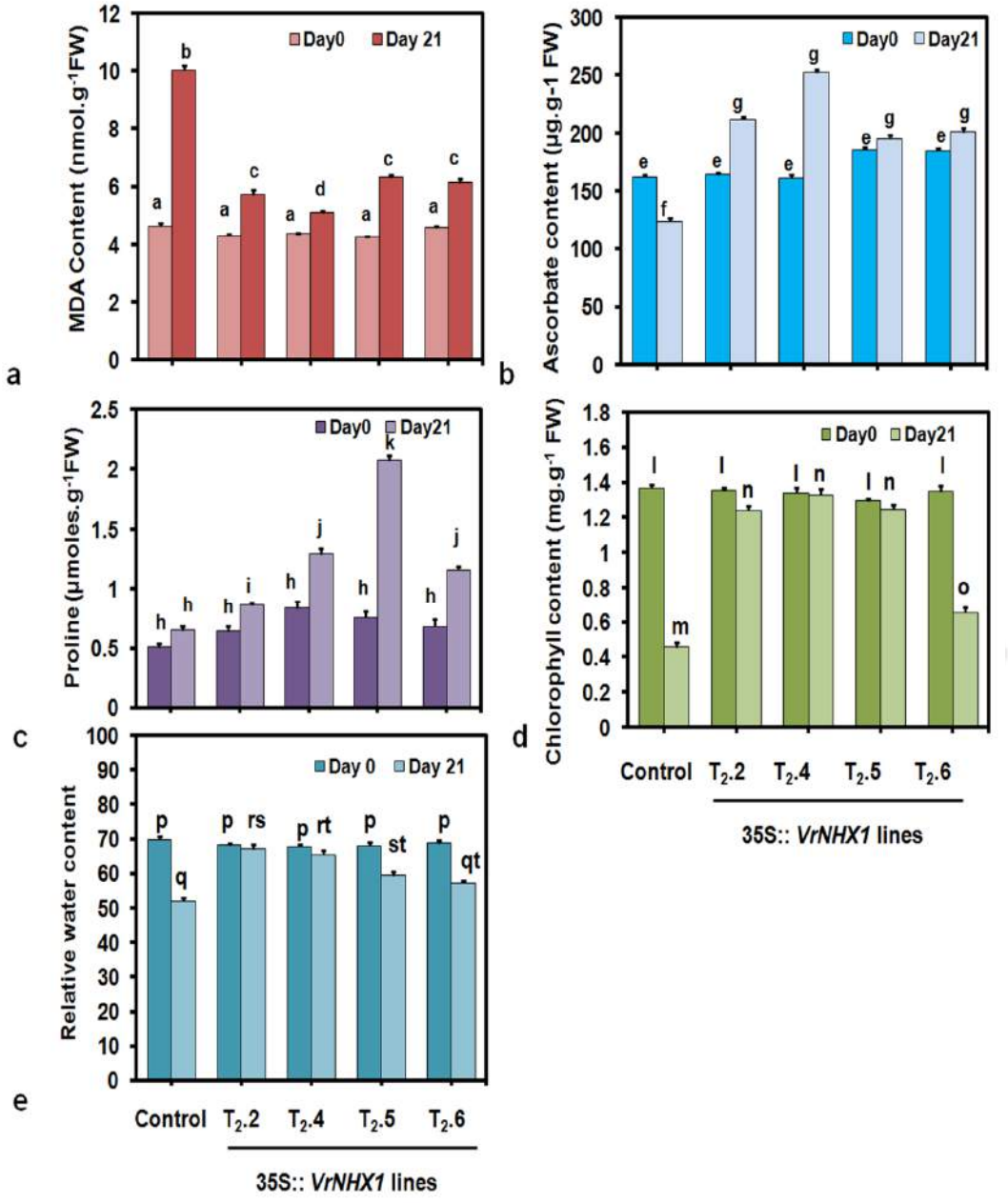

Figure 5.9 Estimation of physiological parameters in untransformed and transformed cowpea plants under salt stress. Measurement of (a) MDA, (b) ascorbate, (c) proline, (d) chlorophyll, and (e) relative water content in leaves of control untransformed (C) and T_2 transgenic cowpea plants (T_2. 2, 4, 5, 6) exposed to salt stress as described in materials and methods. Values indicate means ± SE (n=3). Statistically significant values at $P \leq 0.05$ are indicated as different letters using Bonferroni analysis

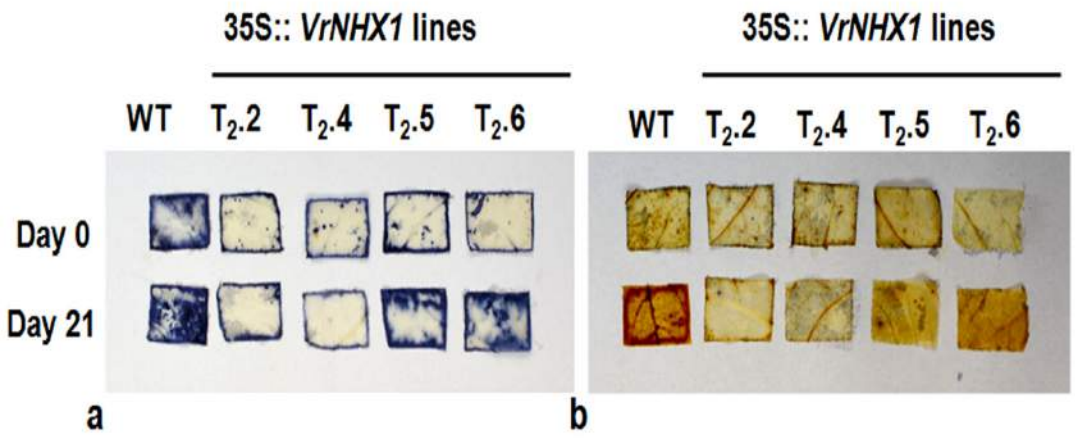

Figure 5.10 Histochemical analysis in untransformed and transformed cowpea plants under salt stress. Histochemical analysis of (a) oxygen radical and (b) hydrogen peroxide in leaves of control untransformed (C) and T_2 transgenic cowpea plants (T_2. 2, 4, 5, 6) exposed to salt stress as described in Materials and methods using NBT and DAB staining, respectively

Table 5.1

Summary of the Agrobacterium-mediated transformation of codyledonary node explants of *Vigna unguiculata* cv. Pusa Komal co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring a binary construct pCAMBIA230135S::*VrNHX1*

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	No. of shoots recovered on selection medium	No. of plants rooted	No. of plants positive for *gus-A, nptII* and *VrNHX1* genes by PCR	Stable Transformation efficiency[a] (%)
1	95	23	08	3	3.15
2	65	22	07	2	3.07
3	115	38	14	5	4.34
4	85	18	09	3	3.52
5	124	33	14	5	4.32
Total/ average	**484[b]**	**134[b]**	**52[b]**	**18[b]**	**3.68[c]**

[a] Number of T_0 plants PCR positive for *nptII*, *gus* and *VrNHX1* divided by total number of explants cocultivated
[b] Total
[c] Average

Table 5.2

Segregation of *VrNHX1* in T_1 progeny of transgenic cowpea plants

T_0 plants	Number of T_1 plants tested for *VrNHX1* and *nptII* [a]			Expected ratio	$\chi 2$ value
	Total	*VrNHX1*, *nptII* +ve	*VrNHX1*, *nptII* -ve		
T_0 1	46	21	25	1:1	0.34
T_0 2	44	32	12	3:1	0.12
T_0 4	38	27	11	3:1	0.31
T_0 6	41	19	22	1:1	0.21
T_0 5	39	36	3	15:1	0.13

[a] PCR was used to analyze presence of *nptII* and *VrNHX1*

CHAPTER 6

Development of a salt screening method for identification of salt tolerant cisgenic cowpea

6.1. Introduction

Cowpea (*Vigna unguiculata* L. Walp) is an important warm season legume primarily grown for food and fodder in the semi-arid tropics including west and central sub-Saharan Africa, parts of Asia, southern Europe, central and south America (Timko et al. 2007). The protein rich grains and stover of cowpea bring invaluable source of income for resource poor farmers of developing nations (Omo-Ikerodah et al. 2009). Despite its economic and social importance in developing parts of the world, cowpea has received relatively little attention for its yield improvement. Cowpea production faces innumerable challenges of which salinity, a major abiotic stress that substantially reduces its yield by altering several physiological, biochemical and morphological characteristics in plants (Thiam et al. 2013). Application of plant breeding and molecular genetics, including genetic engineering in this underexploited legume crop, can bring relatively large genetic gains in improving its productivity under hostile soil conditions. New opportunities for improving cowpea exist by leveraging the emerging genomic tools and knowledge gained through research on other major legume crops and model species (Timko and Singh 2008). Complex multigenic traits, such as salinity stress tolerance, are difficult to introgress and are scarce in the narrow gene pools of pulses, such as cowpea. One promising approach therefore, is to integrate candidate gene(s) controlling salt-tolerant traits through genetic engineering of salt-sensitive cultivars of cowpea.

Compartmentalization of Na^+ into vacuoles by vacuolar Na^+/H^+ antiporter (NHX) is a critical process in salt adaptation, which allows maintenance of ion homeostasis and cellular turgor (Blumwald et al. 2000; Hasegawa et al. 2000). The genes encoding for NHX proteins have been discovered in more than 60 plant species (Xu et al. 2013). Overexpression of vacuolar NHX genes suppressed the salt sensitive phenotype of a yeast mutant defective for endosomal/vacuolar NHX antiporters and conferred salt tolerance to transgenic plants (Yokoi et al. 2002; Zhang and Blumwald 2001). Several reports on improvement of salt tolerance through overexpression of vacuolar NHX genes in agriculturally important but, glycophytic crops implicate a pivotal function of NHX genes in intracellular compartmentalization of Na^+ and salt tolerance (Rodriguez-Rosales et al. 2009; Schroeder et al. 2013).

However, one of the major concerns of general public about transgenic crop relates to the mixing of genetic materials between species that cannot hybridize by natural means (Gaskell and Bauer 2001; Bauer and Gaskell 2002; Lassen et al. 2002). This reservation is often linked to a notion of preserving genetic diversity of species and also appears to be interlinked with fears for potential health risks and for spreading of new gene combinations in the environment (Holme et al. 2013). Recent surveys show higher public acceptance of cisgenic crops as the gene pool exploited by cisgenesis is identical to the gene pool available for conventional breeding thus, expecting less comprehensive regulatory measures (Holme et al. 2013). Therefore, cisgenic approach which imply that plants must only be transformed with genetic material derived from the species itself or from closely related species capable of sexual hybridization will be of major significance for future plant breeding.

Cisgenic cowpea plants for salt tolerance were developed by introduction of a vacuolar NHX gene, *VrNHX1* from mungbean (GenBank Accession No. JN656211). As cowpea and mungbean genetic pool are cross compatible as evident from successful F1 hybrid generated through interspecific hybridization between the mungbean and cowpea (Win et al. 2011), therefore, transferring genes from mungbean to cowpea or vice versa well fall within the gambit of cisgenic domain.

Conventionally, genetic transformation procedure established in legumes in general, and cowpea in particular, use seedling explants with pre-existing meristem that presents an attractive target for *Agrobacterium*-mediated transformation, taking into consideration efficiency of T-DNA delivery to axillary meristem and regeneration of shoots via axillary proliferation (Solleti et al. 2008a,b; Bakshi et al. 2011; 2012a; 2012b; Bakshi and Sahoo 2013). The primary transgenic plants (T_0 generation) raised through antibiotic or herbicide selection by *in vitro* culture are often penalized with improper vegetative and reproductive growth, and reduced seed yield due to several passages of culture under selection pressure. The growth and yield penalty in T_0 plants due to culture and selection pressure induced stress are restored in subsequent generations (T_1 generation onwards) and therefore, detection of stable expression of introduced trait and phenotyping of plants under stress are preferably carried out in T_1 plants or descendants. Moreover, T_0 transgenic plants generated in legumes are often heterozygous in nature with

inconsistent expression of introduced trait. This underlines the importance of detection of stable expression and phenotype of the introduced trait in T_1 plants or descendants, through screening of seeds from primary transgenic plants in lethal dose of selection agent (antibiotic or herbicide). However, as per previous experimental evidences, seeds of legumes in general, and cowpea and mungbean in particular, during germination, show intrinsic tolerance to high dose of both antibiotic and herbicide and thereby, limits the use of seed screening procedure in antibiotic or herbicide for selection of stable expressing lines or homozygous lines. Selection of stably expressing lines has traditionally been performed at the phenotypic level which requires, first growing plants in a greenhouse, and then assessing them for introduced traits such as antibiotic/herbicide resistance, drought/salinity tolerance, or yield parameters. The process is not only time consuming, cumbersome and not feasible for large-scale experiments, but often limited by available greenhouse space. Therefore, development of an efficient alternative screening method during seed germination stage under salt stress, could select the stable expressing T_1 plants or descendants thereof, eliminate those with target mRNA instability, and additionally minimize the task of growing all plants, and enable the identification of high expressor lines and homozygous ones.

In the present study, we report salt screening as a basis for identification of cis-genic cowpea plants stably expressing a mungbean vacuolar NHX gene. First, the cis-genic cowpea plants were developed by introducing a mungbean vacuolar NHX gene (*VrNHX1*) through *Agrobacterium*-mediated transformation procedure. The T_0 cisgenic plants were confirmed by detection of introduced genes by PCR analysis, visualization of stable expression of reporter gene in vegetative and reproductive tissues, and detection of transcript accumulation. Second, we optimized and adapted a salt selection procedure for selecting cisgenic cowpea seedlings stably expressing introduced *VrNHX1*, during germination of seeds of T_0 plants, and tested the growth parameters, seed yield and survival of cisgenic cowpea plants for four consecutive generations. In summary, we demonstrate, for the first time in any grain legume, a highly efficient salt screening procedure for selection and characterization of salt-tolerant cisgenic cowpea stably expressing *VrNHX1*.

6.2. Materials and methods

6.2.1. Plant material, explant preparation, vector construction and plant transformation

Healthy and uniform seeds of commercially grown cultivar of cowpea (*Vigna unguiculata* L. Walp) cv. Pusa Komal (IARI, New Delhi) were used in this study. The cotyledonary node explant preparation is same as described in section 5.2.1.

The plant binary vector pCAMBIA230135S::*VrNHX1*, mobilized into *Agrobacterium* strain EHA 105 and maintained on solid LB medium supplemented with 20 mg/l of rifampicin, and 50 mg/l of kanamycin was used for the experiment as described in section 5.2.2.

The plant transformation method is same as described previously in section 5.2.3. The seeds from the T_0 putative cisgenic cowpea plants were collected for future analysis.

6.2.2. Histochemical GUS assay

Histochemical GUS assay was performed in cotyledonary node explants after co-cultivation to study transient gus expression and, stable gus expression in flower, stamen, anthers, pollens, and stigma of kanamycin selected T_0 cisgenic plants as previously described in section 5.2.4.

6.2.3. Molecular analysis of putative cisgenic cowpea plants

6.2.3.1. PCR analysis

Genomic DNA was isolated from non-transformed and putative T_0 transformed cowpea plants using modified CTAB (cetyl trimethyl ammonium bromide) method (Solleti et al. 2008a). Polymerase chain reaction (PCR) was performed to detect the presence of *nptII*, and *VrNHX1* genes in putative T_0 transformed cowpea plants. The specific primers for amplification of 0.54 kb fragment of *nptII* and PCR condition was same as described previously in section 5.2.5. The gene specific primers: VrNFP 5'-GTATTTCCACTGG CGTAGTCATTTTGC -3' and VrNRP 5'-GCGAAACCCTATAAGAACCCTAATTCC -3' were used to amplify a 1.5 kb fragment of 35SP::*VrNHX1*::35STer cassette. The PCR condition was: 95 °C for 10 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 30 sec, and a final extension of 72 °C for 10

min for 35 cycles. The PCR fragments were analyzed on 1% agarose gel and stained with 10mg/ml ethidium bromide.

6.2.3.2. Southern hybridization

The transgene integration and copy number analysis was studied using 30µg of genomic DNA isolated from non-transformed and randomly selected independent T_0 cisgenic cowpea plants digested with restriction endonuclease, EcoRI. Digested genomic DNA was electrophoretically fractionated on 1% agarose gel and blotted onto Zeta-Probe membrane. The blot was hybridized with DIG-labeled 0.5 kb PCR product, corresponding to the coding region of *nptII* as explained previously in section 5.2.6. Southern hybridization was performed according to instructions of the DIG Labeling and Detection system.

6.2.3.3. RNA Isolation and Semiquantitative RT-PCR

Total RNA was isolated from T_0 southern positive cisgenic cowpea lines using RNeasy Plant Mini Kit, following the manufacturer's instructions and cDNA was prepared. Semi-quantitative RT-PCR was performed using a set of gene specific primers (SMFP: 5'-GTATTTCCACTGGCGTAGTCATTTTGC -3' and SMRP: 5'-GAACTGATTAT ATGCAAGTGCC -3') for amplifying a 1 kb fragment of *VrNHX1*. The PCR condition was: 95 °C for 10 min; 95 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min and, a final extension of 72 °C for 10min for 28 cycles. *VuNSR9* was used as an internal control. The PCR conditions are same as previously described in section 5.2.7. The PCR fragments were analyzed on 1% agarose gel and stained with 10mg/ml ethidium bromide.

6.2.4. Identification of stable cowpea cisgenic lines overexpressing *VrNHX1* using salt screening

6.2. 4.1. Salt stress assay in control cowpea seedlings

Seedling screening under salt stress was optimized using seedlings (T_1) germinated from seeds of control untransformed wild type cowpea plants (WT). The seeds were placed on filter paper for 2 days in dark followed by transferring the germinated seedlings to Hoagland's nutrient medium (Hoagland and Arnon 1950) supplemented with different concentrations of NaCl (0, 125, 150, 175, and 200 mM) and maintained in a growth chamber for 4 days at 25 °C and 70 % relative humidity with

a 16hour/8hour photoperiod and photosynthetic flux intensity of 300 µmol $m^{-2}s^{-1}$. For seedling survival assay, salt stressed and unstressed seedlings were shifted to Hoagland's nutrient medium in absence of NaCl for 2 weeks and the number of seedlings surviving salt stress were counted.

6.2.4.2. Salt stress assay in cisgenic cowpea seedlings

The seeds from Southern positive T_0 cisgenic cowpea plants (#1-3; 35S::*VrNHX1* independent lines) were subjected to salt screening at 150 mM NaCl concentration, and T_1 seedlings were shifted to Hoagland's nutrient medium in absence of NaCl for 14 days for revival following the procedure similar to as described for control WT cowpea seedlings. The survived plantlets were transferred to soil and established in green house.

6.2.4.3. Southern blotting

The efficiency of salt screening based selection method was verified by subjecting the surviving T_1 plantlets to PCR analysis using primers specific for *nptII* and CaMV35S::*VrNHX1*::35ST cassette as described earlier in section 6.2.3.3. The inheritance of *VrNHX1* in T_1 generation cisgenic cowpea plants was studied based on plants showing PCR amplification of *VrNHX1*. The stably expressing descendants of T_1 salt tolerant cisgenic cowpea plants (T_2, T_3 and T_4) were selected following the optimized salt stress screening method and its efficiency was verified by subjecting the salt stress-surviving cisgenic descendants (T_4) to PCR analysis as described previously. To verify, the fidelity of the PCR amplicons, the fragments specific to *nptII* and CaMV35S::*VrNHX1*::35ST obtained from T_4 salt selected cisgenics were resolved on the agarose gel and transferred onto Zeta-Probe membrane and the blot was hybridized with DIG-labeled 0.5 kb and 0.7 kb PCR product, corresponding to the coding region of *nptII* (as described previously in section 5.2.6) and *VrNHX1* gene respectively. The *VrNHX1* gene specific primers: SHFP: 5'-GATTACCTAGCAATTGGTGCAATATT-3' and SHRP: 5'-GAACTGATTATATGCAA GTGCC-3' were used to amplify a 0.7 kb fragment of *VrNHX1*. The PCR condition was: 95 °C for 10 min; 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and 30 sec, and a final extension of 72 °C for 10min for 35cycles. Southern hybridization was performed according to instructions of the DIG Labeling and Detection system. The salt selected plantlets (T_4) were used for further stress tolerance assays.

6.2.4.4. Scanning electron microscope analysis of stomatal opening in unstressed control and salt selected T_4 cisgenic cowpea

The cowpea control seedlings (2 days old) were transferred to Hoagland medium for 96 hours without salt stress (WT, unstressed). Similarly, leaves were also collected from 96 hour salt selected T_4 cisgenic cowpea plants. The leaves were fixed by 2.5 % glutaraldehyde in 0.1M phosphate buffer (pH 7.2) at 4 °C for 24 hours, followed by washing twice in 0.1 M sodium phosphate buffer for 10 min, and immersed in ascending range of ethanol (50-100%) sequentially for 10 min each. After vacuum drying, the samples were coated with gold for scanning electron microscopy (Leo VP-45, Leo Scanning Electron Microscopy Ltd., Cambridge, UK) analysis.

6.2.4.5. Measurement of lipid peroxidation

The malondialdehyde (MDA) content was determined with thiobarbituric acid (TBA) following the protocol of Heath and Packer (1968), using the leaves of unstressed control wild type and T_4 cisgenic cowpea seedlings sampled after 96 hour of stress period, as described in section 3.2.10.2.1.3.

6.2.4.6. Analysis of growth and yield parameters in salt screened cisgenic cowpea

The *VrNHX1* cisgenic cowpea plants of T_4 generation selected through salt stress screening were used for stress tolerance experiments. To study the growth and yield parameters, the plant height, surface area of leaf (1^{st}, 2^{nd}, 3^{rd} and 4^{th} mature leaves) with corresponding chlorophyll content (Arnon 1949), pod number, pod length and total seed yield per plant were measured in both control wild-type (WT, unstressed) and T_4 cisgenic plants post 30 days of their transfer to green house.

6.2.4.7. Statistical analysis

Mean data was collected from 10 progeny lines (T_4) from 3 independent salt selected T_3 cisgenic cowpea plants. Statistical comparison between the variances was determined by ANOVA (Analysis of variance) and significant differences between mean values were determined by Student's t-test and Bonferroni analysis at $P \leq 0.05$ and $P \leq 0.001$.

6.3. Results

6.3.1. Generation of *VrNHX1*-cisgenic cowpea lines

Cotyledonary node explants of cowpea were transformed with a binary construct containing *VrNHX1* driven under CaMV35S promoter, *nptII* as selectable marker and *gus-A* as reporter (Fig. 6.1), using *Agrobacterium* mediated transformation method. Explants co-cultivated with *Agrobacterium* (Fig. 6.2a) induced shoot formation on shoot multiplication medium containing 150 mg/l kanamycin (Fig. 6.2b, c). The putative transformed shoots remained green and grew healthy (Fig. 6.2d) while the untransformed shoots bleached on medium containing lethal dose of kanamycin (Fig. 6.2e). The transformed shoots were selected over a period of 30-40 days and were rooted *in vitro* in medium devoid of kanamycin (Fig. 6.2f) and finally transferred to the greenhouse to set seeds (Fig. 6.2g). Neither the putative transformants nor their subsequent generations (T_1, T_2, T_3 and T_4) exhibited any phenotypical abnormalities compared with wild-type (WT) control plants. Three independent transformation experiments were performed and resulted in the regeneration of a total of eight cisgenic lines (T_0) out of 226 explants (Table 2). The overall transformation efficiency was 3.51% (Table 6.1). Five out of eight cisgenic lines were randomly selected for molecular analysis.

6.3.2. Histochemical Gus assay

The cotyledonary node explants cocultivated with *Agrobacterium* showed transient GUS activity, predominantly in the regenerating sites (Fig. 6.3a) while no GUS activity was detected in untransformed control explants (Fig. 6.3b). The flower, stamen, anther, pollen grains, and stigma of kanamycin resistant T_0 cisgenic cowpea plants (#1-3, 35S::*VrNHX1* independent lines) showed characteristic blue color typical to GUS expression indicating stable expression of reporter gene in reproductive tissues (Fig. 6.3c-l).

6.3.3. Molecular analysis of cisgenics

6.3.3.1. PCR analysis

All 5 cisgenic T_0 lines showed the expected amplification product of 0.5 kb and 1.5 kb determined by PCR using the primers specific for internal sequence of *nptII* and CaMV35SP::*VrNHX1*::35STer cassette respectively (Fig. 6.4 a, b), indicating that they

were true transformants. No amplification was observed in control wild type plants in either case (Fig. 6.4 a, b).

6.3.3.2. Southern hybridization

The three PCR positive cowpea T_0 cisgenic lines (#1-3, 35S::*VrNHX1* independent lines) analyzed by Southern hybridization to check integration of introduced gene to cowpea genome showed single copy insertion in all three T_0 cisgenic lines (Fig. 6.4c; Lane 2-4) tested, while no hybridization signal was detected in lane corresponding to wild type (WT) non-transformed plants (Fig. 6.4c; Lane 1). The hybridization signals of size greater than 2.1 kb (35SP::*nptII*::35Spoly-A cassette) signified that the signals were due to genuine T-DNA integration and not *Agrobacterium* contamination. Further, the fact that all the three randomly selected T_0 PCR positive cisgenic lines showing differential integration events, confirmed that these plants were derived from independent transformation events (Fig. 6.4c).

6.3.3.3. Expression analysis

A comparative semi-quantitative RT-PCR was performed for detection of expression of *VrNHX1* in cisgenic cowpea lines. Due to high homology between *VuNHX1* and *VrNHX1*, a faint band was observed in WT owing to *VuNHX1* amplification. However, amplification of *VrNHX1* obtained after normalization with internal primers indicated relatively higher expression in the three independent cisgenics lines (T_0.#1-3) as compared to *VuNHX1* amplification in WT thus, confirming gene integration (Fig. 6.4 d), into cowpea genome and also stabilized expression. However, there was variability to some extent on the expression levels among the lines. The expression level of 35S::*VrNHX1* cisgenic lines #1 and #3 was high and on the other hand, line #2 showed relatively low expression (Fig. 6.4 d).

6.3.4. Selection of stably expressing T_1 cisgenic cowpea lines and their descendants by salt screening approach

6.3.4.1. Effect of salt selection on control cowpea seedlings

To determine the salt concentration effective for selection of stably expressing *VrNHX1* T_1 cisgenic lines and their subsequent generations, the control wild type seedlings (2-days old) were grown in Hoagland's nutrient medium supplemented with different concentrations of NaCl (0, 125, 150, 175 and 200 mM) for a period of

96 hours and growth of seedlings was recorded. Under control unstressed condition, emergence of primary leaves and development of secondary root was observed within 3 days of culture of seedlings in stress medium. However, under salt stress, the hypocotyl started turning brown within 24 hours indicating cell death, and further, growth and proliferation of root was not observed in any of the salt treatments indicating growth inhibition (Fig. 6.5a). In salt stress treatments (150, 175 and 200 mM), the cotyledons displayed a distorted morphology and turned brown with increase in treatment duration (Fig. 6.5a). Furthermore, a constriction at the junction between the hypocotyl and root tip was observed in seedlings at these salt treatments (150, 175 and 200 mM). The salt concentrations below 125 mM (25, 50, 75 and 100 mM) were not taken into account as preliminary experiments revealed the growth of cowpea seedlings was not affected significantly in salt concentrations below 125 mM. The salt stressed seedlings were transferred to nutrient medium (Hoagland medium without NaCl concentrations) and maintained for 2 weeks for revival and thereafter, the efficiency of revival was recorded. None of the control wild type seedlings showed any sign of revival upon shifting from salt stress treatments as the cotyledons and hypocotyls completely turned brown and emergence of primary leaves was also not observed (Fig. 6.5b).

6.3.4.2. Effective salt selection on cisgenic cowpea lines

Based on the observations concluded in control wild type seedlings under salt stress, initially the cisgenic T_1 seedlings were given 200 mM salt stress (maximum stress concentration) and their survival was monitored. The T_1 cisgenic seedlings (#4_1-4; progenies of T_0 #4, 35S::*VrNHX1* line) showed emergence of primary leaves and poor growth of primary root and exhibited no secondary root growth (Fig. 6.6). However, on shifting to nutrient medium, only 25% of seedlings showed recovery with well formed primary leaves and roots (Fig. 6.6). The results were verified by subjecting the seedlings, recovered from 200 mM salt stress, to PCR analysis. PCR analysis confirmed the presence of *VrNHX1* in seedlings (#4_1-4) (Fig. 6.7).

6.3.4.3. Segregation analysis

The segregation of *VrNHX1* in T_1 progenies, generated from three independent T_0 cisgenic events (#1-3, 35S::*VrNHX1* independent lines) and further, selected on 150 mM salt stress, performed by PCR analysis of *VrNHX1*, showed that progenies of

events T_0.#1 and T_0.#2 segregated following a monogenic ratio of 3:1 (Fig. 6.8) indicating a single functional locus whereas, the progenies of event T_0.#3 revealed a segregation pattern of 1:1 (Fig. 6.8) possibly due to chromosomal rearrangement following T-DNA insertion.

6.3.4.4. Selection of salt tolerant T_4 cisgenic cowpea lines

The selection criterion was reduced to 150 mM NaCl to avoid loss of cisgenic seedlings with low expression of integrated candidate gene, *VrNHX1.* The salt screening of cisgenic seedlings was therefore performed at 150 mM NaCl for four consecutive generations.The emergence of primary leaves and secondary roots was observed in T_4 cisgenic seedlings after 48 hours of salt stress (Fig. 6.9a). The secondary leaves emerged within 7 days of revival in all salt tolerant seedlings (Fig. 6.9b). The cisgenic seedlings showed complete revival after salt stress with well formed root system (Fig. 6.9b). After 2 weeks revival in hydroponics culture, the salt tolerant cisgenic plants were transferred to soil: compost mixture in pot and established in green house for further analysis (Fig. 6.9c).

6.3.4.5. Molecular analysis

The PCR analysis of T_4 cisgenic plants (#1_1.41-44, #2_1.41-43, #3_1.41-42; 35S::*VrNHX1* lines) selected through salt stress screening method confirmed that the selection method's efficiency is 100% as no escapes had emerged. The Southern hybridization performed to validate the fidelity of the PCR amplification of T_4 cisgenic plants selected through salt stress screening method (Fig. 6.10) further, reconfirmed 100% efficiency of salt stress screening method.

6.3.5. Stomatal closure in T_4 cisgenic cowpea lines under salt stress

The lower leaf surface of unstressed WT and salt stress selected T_4 cisgenic plants (#1_1.41-410, #2_1.41-410, #3_1.41-410; 35S::*VrNHX1* progenies of independent lines) were used to study stomatal behavior under scanning electron microscope. The WT unstressed cowpea plants exhibited wider stomatal aperture as compared to the salt selected T_4 cisgenics exhibiting structural differences and a comparatively less wider aperture, unlike complete closure as an expected phenomena for salinity induced oxidative stress (Chaves et al. 2009) (Fig. 6.11).

6.3.6. Comparison of malondialdehyde (MDA) content between wild type and T_4 cisgenic plants

Stomatal closure is often associated with ROS production (Cavalcanti et al. 2004) and can be accounted as a factor involved in lipid peroxidation during salinity stress. Since MDA is an end-product of free radical chain reactions and lipid peroxidation in biomembranes, MDA content can represent the extent of lipid peroxidation and membrane injury that has occurred. Therefore, the MDA contents of T_4 cisgenic (#1_1.41-410, #2_1.41-410, #3_1.41-410; 35S::*VrNHX1* progenies of independent lines) plants were measured after 96 hours of salt stress (150 mM NaCl) and compared with MDA content obtained for wild type unstressed cowpea plants (Fig. 6.12). However, no statistically significant difference ($P \leq 0.05$) was observed in extent of lipid peroxidation thus, indicating reduced oxidative damage in cisgenic plants overexpressing exogenous *VrNHX1*.

6.3.7. Analysis of growth and yield parameters in salt screened cisgenic lines

Wild-type and *VrNHX1* T_4 cisgenic plants (#1_1.41-410, #2_1.41-410, #3_1.41-410; 35S::*VrNHX1* progenies of independent lines) were compared for their performance under salt stress to further investigate the role of *VrNHX1* in conferring salt tolerance in cisgenic plants. The cisgenic plants showed no significant difference in plant height as compared to unstressed wild type (WT) cowpea, indicating the expression of *VrNHX1* conferred normal plant growth in cisgenic plants under salt stress (Fig. 6.13). The leaf surface area (LSA) measured by grid method (Griffiths and Orians 2003) in fully explanded leaves revealed a significant difference in LSA of 1st and 2nd mature true leaves in cisgenics as compared to unstressed WT cowpea, with an observed reduction of 31-35%, 20-34%, 7-12% respectively in 1st, 2nd and 3rd mature leaves of T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41 plants. Stabilization in growth parameter LSA was observed with 4th mature leaves (Fig. 6.14a). Chlorophyll estimation revealed a 10-18%, 20-30% and 5-7% reduction in 1st, 2nd, and 3rd mature leaves in salt selected cisgenics in comparison with unstressed control wild type. However, no significant difference in chlorophyll content was observed in 4th mature leaves (Fig. 6.14b). The pod number and seed yield in cisgenic plants were consistent and not affected at early seedling stage except an exception observed in pod length for T_4.#3_1.41 cisgenic plant (Table 6.2).

6.4. Conclusion

The artificial intermixing of genetic materials between species incapable of hybridization naturally, has been considered as a mass concern in development of transgenic crops (Bauer and Gaskell 2002; Gaskell and Bauer 2001; Lassen et al. 2002). This reservation is often linked to a notion of respect for nature and also appears to be interlinked with fears for potential health risks along with spreading of new gene combinations in the environment. To meet this concern, the two transformation concepts cisgenesis and intragenesis were developed as alternatives to transgenic crop development. Both concepts imply that plants must only be transformed with genetic material derived from the species itself or from closely related species capable of sexual hybridization. Several surveys show higher public acceptance of intragenic/ cisgenic crops compared to transgenic crops. This is in contrast to transgenesis, where genes and DNA sequences can be moved between any species. The gene pool exploited by intragenesis and cisgenesis is accordingly identical to gene pool available for traditional breeding. Moreover, as the gene pool exploited by intragenesis and cisgenesis are identical to that available for conventional breeding, less comprehensive regulatory measures are expected. It is therefore possible that the intragenic/cisgenic route will be of major significance for future plant breeding. Both intragenesis and cisgenesis have great potential to overcome some of the limitations of classical breeding as both can be used as a fast tool to transfer genes between related plants (Holme et al. 2013). While this transfer can also be performed through classical breeding, the success and length of these breeding programs depend on the propagation system of the crop. Furthermore, the intragenesis/ cisgenesis approach avoids potential 'linkage drags' associated with classical backcross breeding. Genetic material encoding for inferior properties are sometimes so tightly linked to the gene of interest that recombination between this gene and the unwanted genetic material is almost impossible. In consequence, this 'linkage drag' may render the backcrossed line useless. The intragenic/cisgenic concepts can also overcome limitations of classical breeding when it comes to improving traits with limited natural allelic variation within the sexually compatible gene pool (Holme et al. 2013). Higher expression level of a trait can be obtained by re-introducing gene of the trait with its own promoter and terminator (cisgenesis) or with a promoter and terminator isolated from the sexually compatible gene pool

(intragenesis). Lower expression levels can be obtained through different silencing constructs (intragenesis). Intragenic/cisgenic approaches were successfully implemented initially in commercially important crops like potato, apple, strawberry and grapevine, known to be recalcitrant towards classical breeding methods (Haverkort et al. 2009; Vanblaere et al. 2011; Dhekney et al. 2011; Han et al. 2011). Silencing of unwanted gene activities and enhancement towards disease resistance approach have been practiced for achieving improvement in cisgenic crops (Holme et al. 2013).

Till date, no report is available on development of cisgenic plants in any legumes. We report, for the first time, development of cisgenic plants of cowpea by overexpressing a mungbean vacuolar NHX1 gene. As cowpea and mungbean genetic pool are cross compatible as evident from successful F1 hybrid generated through interspecific hybridization between the mungbean and cowpea (Win et al. 2011), therefore, transferring genes from mungbean to cowpea or vice versa fits well the criteria of cisgenic.

In cowpea, previous experience in transgenic development (Solleti et al. 2008a, b; Bakshi et al. 2011, 2012a; 2012b; Bakshi and Sahoo 2013) demonstrate that the primary transgenic plants (T_0) raised through antibiotic selection penalized with improper vegetative and reproductive growth, and reduced seed yield due to several passages of culture under selection pressure. The growth and yield penalty are subsequently restored in next generations (T_1 generation onwards) enabling detection of stable expression of introduced trait and stress phenotyping preferably in T_1 plants or descendants thereof. Moreover, frequent heterozygous nature of T_0 transgenic plants in legumes resulted in inconsistent expression of introduced trait thereby, limiting the effective screening of seeds of primary transgenics in lethal dose of selection agent (antibiotic or herbicide). Moreover, seeds of legumes in general, and cowpea and mungbean in particular, during germination, show high intrinsic tolerance to antibiotic resulting in seed screening in antibiotic ineffective. Selection of stably expressing lines has traditionally been performed at the phenotypic level which requires first growing plants in a greenhouse, and then assessing them for introduced traits such as antibiotic/herbicide resistance, drought/salinity tolerance, or yield parameters. The process is not only time

consuming, cumbersome and not feasible for large-scale experiments but, often limited by available greenhouse space. To overcome these limitations, in the present work, an efficient alternative screening method during seed germination stage in salt stress for selection of T_1 plants or descendants thereof, is reported. The stably expressing cigenic lines eliminate the chance of target mRNA instability, and additionally minimize the task of growing all plants. Further, it enables the identification of high expressor lines and homozygous ones. The cowpea cisgenic lines overexpressing *VrNHX1* were selected over four generations by employing salt screening approach and validated with PCR detection of introduced gene(s). No escapes were observed through salt screening approach. Under salt stress, the cisgenics displayed open stomata with narrow aperture, unlike the closed stomata observed in salt stressed plants. The stomata closure is a phenomenon in plants to reduce the water loss under physiological drought condition. However, regulation of stomatal aperture by plants is the deciding factor for measuring biomass accumulation in coherence with CO_2 assimilation (Shabala 2013). Therefore, it can be also observed that the salt tolerant cisgenic lines could combat the hypersalinity and induced oxidative stress without compromising in metabolic activities. The stomatal closure is often associated with ROS production (Cavalcanti et al. 2004) and can be accounted as a factor involved in lipid peroxidation during salinity stress. However, no significant difference was observed in extent of lipid peroxidation thus, indicating the reduced oxidative damage in cigenic cowpea lines overexpressing *VrNHX1.* Earlier reports indicated reduction of vegetative growth, seed yield and pod number in cowpea (wild-type) upon salt stress (West and Francois 1982; Silveira et al. 2001; Gomes-Filho et al. 2008; Taffouo et al. 2009). In the contrary, salt treatment on cisgenic cowpea seedlings overexpressing *VrNHX1* did not cause any changes in growth and yield factors. Soil salinity is a major concern worldwide and development of salt tolerant crops reduces the cost requirement in resurrecting the saline affected cultivable lands. The cisgenic cowpea plants overexpressing *VrNHX1* conferred enhanced tolerance to salt stress. The cisgenic approaches reported here will pave way for development and implementation of cisgenics in different grain legumes for tolerance to abiotic stresses.

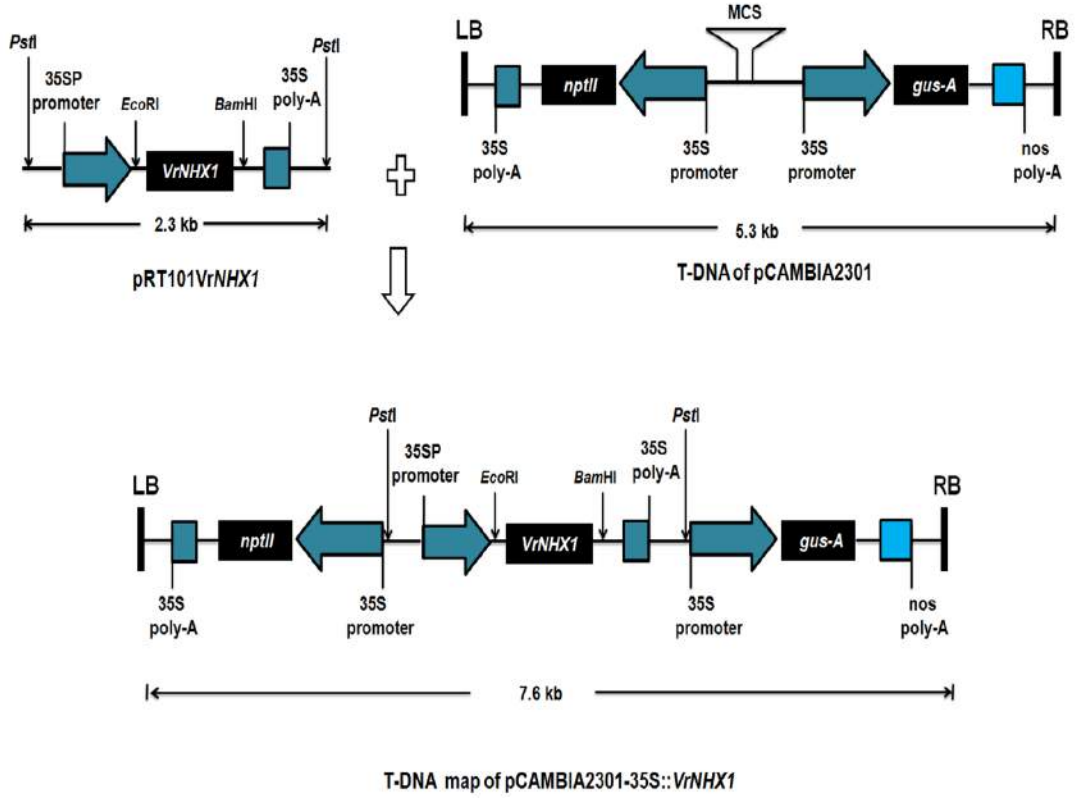

Figure 6.1 Cloning strategy of *VrNHX1* in plant binary vector pCAMBIA 2301. Abbreviations: LB, left border; RB, right border; CaMV 35SP, Cauliflower mosaic virus 35S promoter; CaMV 35S poly-A, Cauliflower mosaic virus 35S terminator; nos poly-A, nopaline transferase terminator; *nptII*, neomycin phosphotransferase; *intron-gus-A*, intron interrupted β-glucuronidase; *VrNHX1*, *Vigna radiata NHX1*. The T-DNA of pCAMBIA2301-35P::*VrNHX1*::35Ter (7.6 kb) is shown

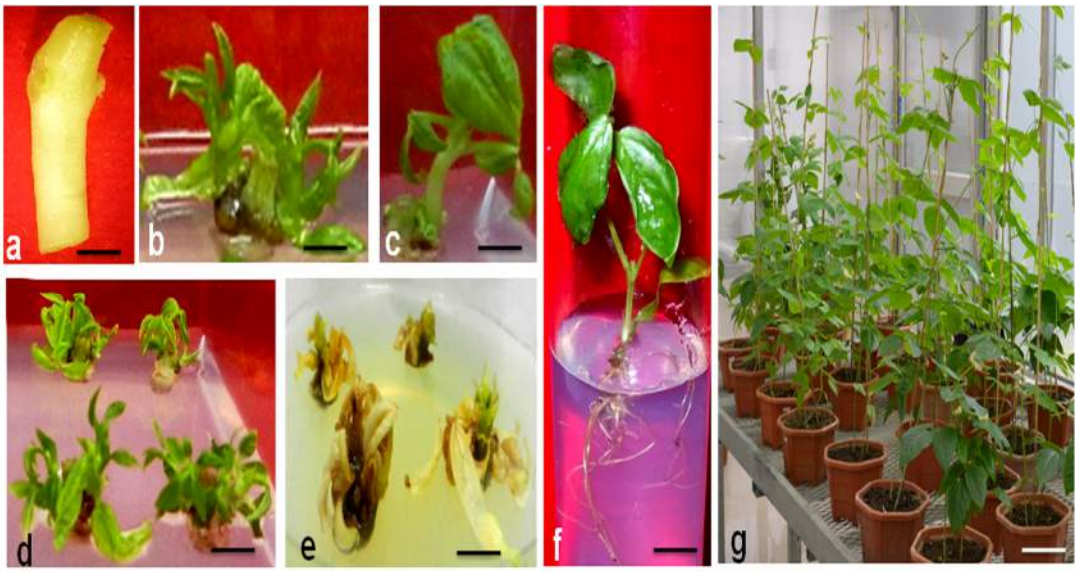

Figure 6.2 Multiple shoot proliferation, regeneration of cisgenic cowpea (*Vigna unguiculata* L. Walp cv. Pusa Komal) plants and transient gus expression patterns. Transient GUS assay in non-transformed i.e. control explants (a) not showing GUS activity and transformed explants (b) after 3 days of co-cultivation showing GUS activity (Bar 6 mm). (c) Cotyledonary node as explants (Bar 1 mm). (d) Multiple shoot induction from cotyledonary node explant cultured on MSB_5 + 0.5 µM BAP + 0.5 µM Kinetin supplemented with 500 mg/L cefatoxime and 150 mg/L kanamycin (Bar 5 mm). (e) Kanamycin selected shoots transferred to elongation medium (f) Control cowpea shoots selected on selection medium with 150 mg/lL kanamycin (Bar 6 mm). (g) Kanamycin selected cowpea transformed shoots (Bar 6 mm). (h) Kanamycin selected shoots on rooting medium (Bar 1 cm). (i) Acclimatized plant in greenhouse

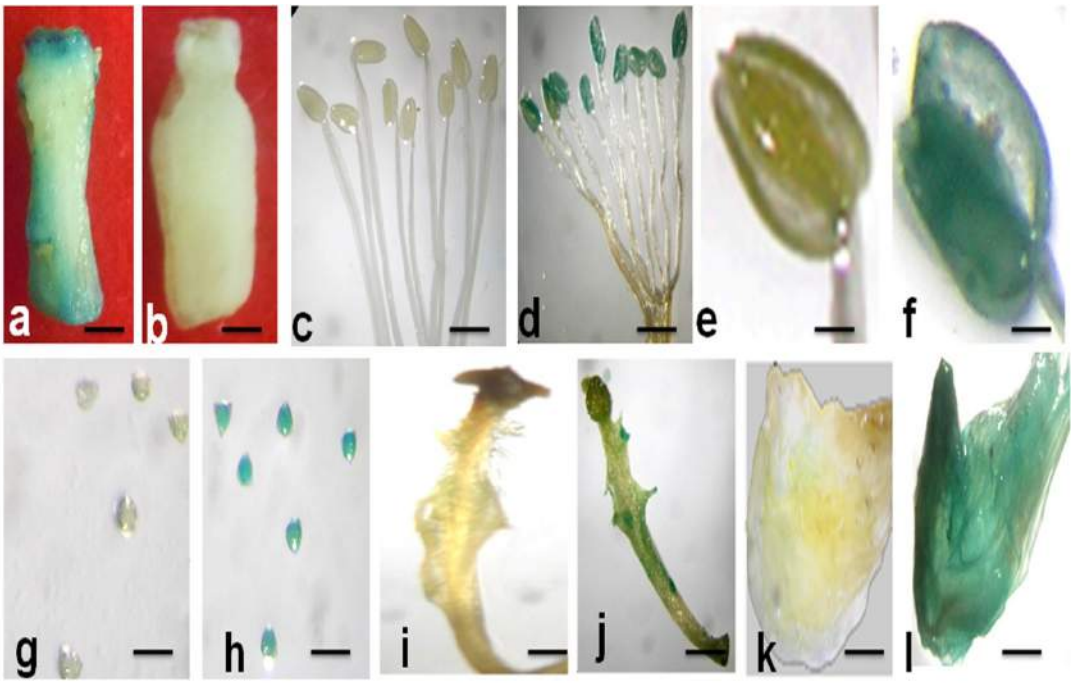

Figure 6.3 Transient and stable GUS assay in cisgenic cowpea plants. Transient GUS assay in (a) transformed explants after 3 days of co-cultivation showing GUS activity (Bar 6 mm). (b) Non-transformed control explants not showing GUS activity. Stable GUS expression observed in (d) stamen, (f) anthers, (h) pollens, (j) stigma and, (l) flower of T_1 cisgenic 35S::*VrNHX1* cowpea plants. (c, e, g, i, k) No gus pattern was observed in control non-transformed cowpea plant

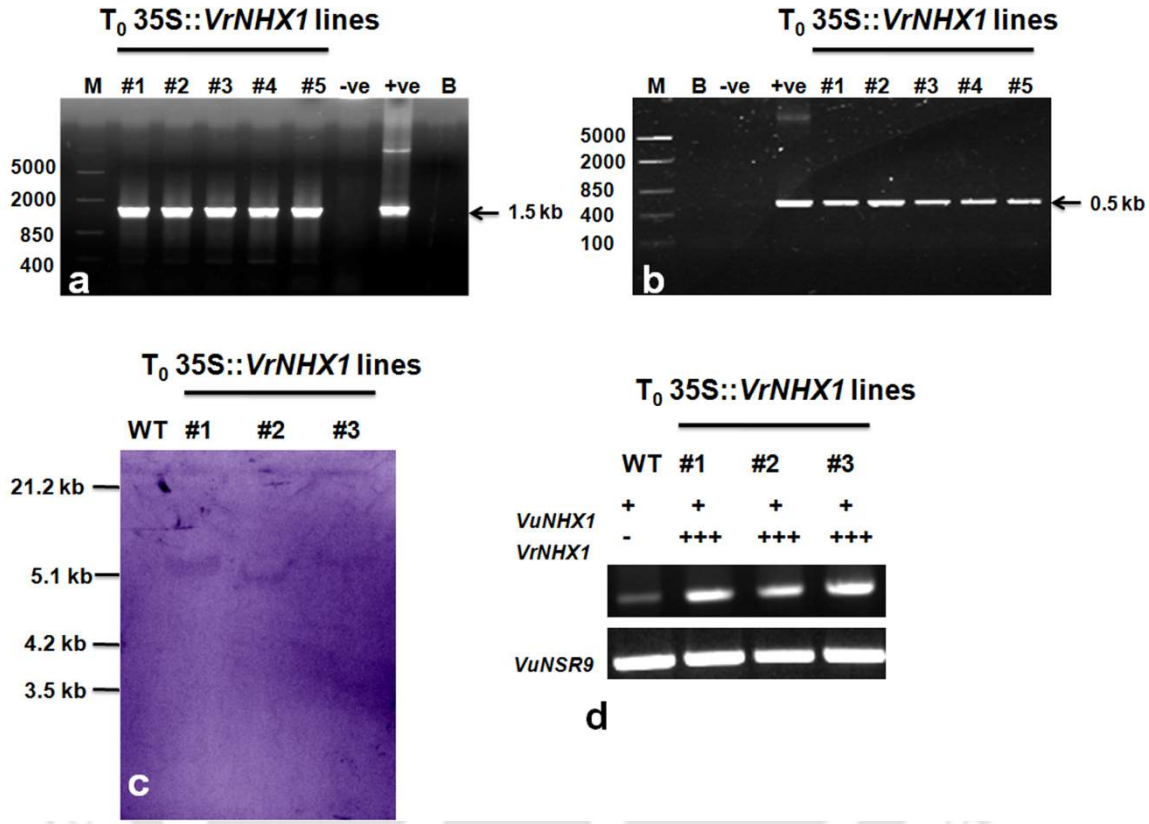

Figure 6.4 Molecular analysis of cisgenic 35S::*VrNHX1* cowpea lines overexpressing *VrNHX1*. PCR amplification of (a) 1.5 kb fragment of 35SP::*VrNHX1*::35STer cassette. (b) 0.5kb fragment of *nptII* gene. M: Lambda DNA HindIII/ EcoRI ladder, -ve: Non-transformed control WT cowpea. +ve: Plasmid control, #1-5: Putative independent T_0 35S::*VrNHX1* cowpea lines. (c) Southern blot hybridization analysis of three independent T_0 cisgenic cowpea lines (T_0.#1-3) digested with EcoRI, using a *nptII* probe. (d) Expression analysis by semi-quantitative RT-PCR in control and three independent T_0 cisgenic 35S::*VrNHX1* cowpea lines (T_0.#1-3) using *VrNHX1* gene specific primers. *VuNSR9* primers were used as internal control

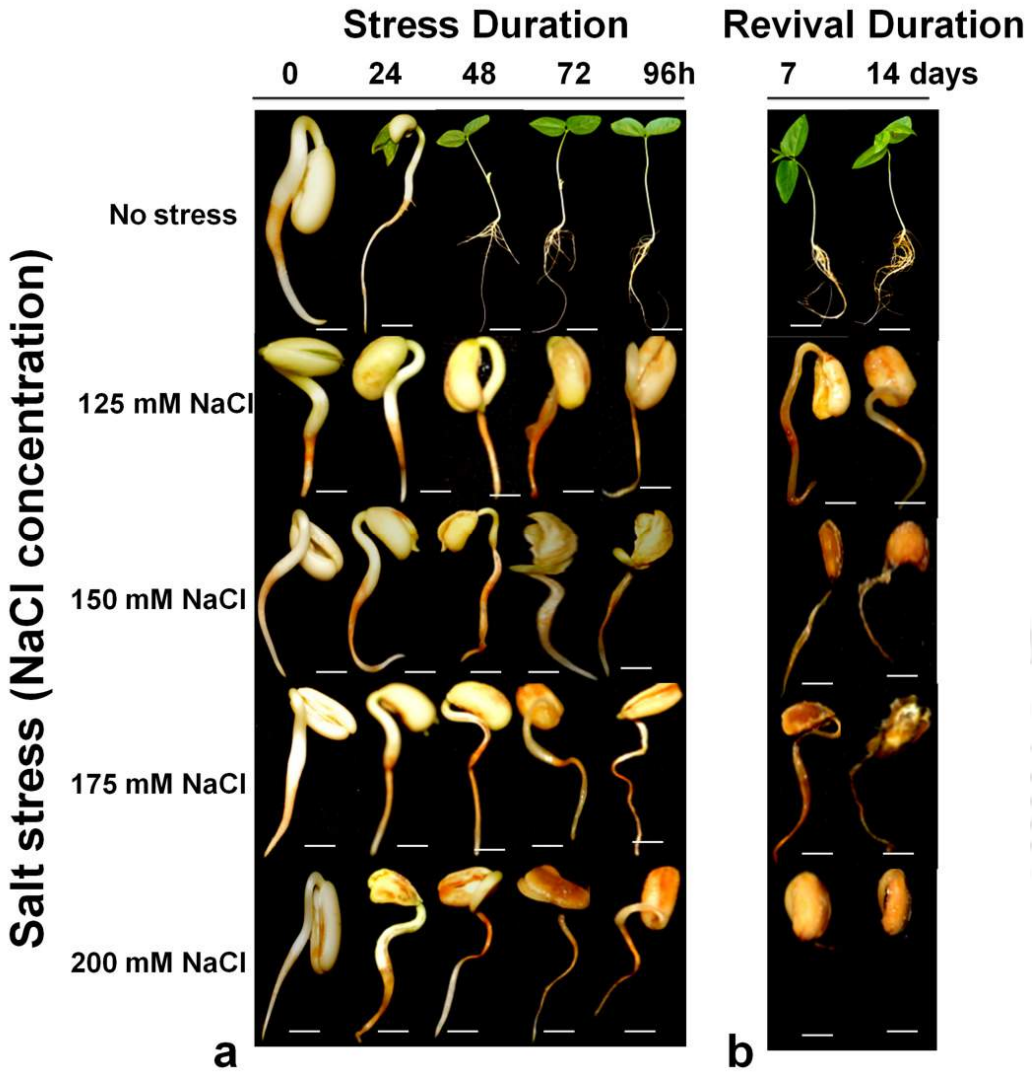

Figure 6.5 Salt sensitivity assay in control cowpea seedlings. (a) The 2 days old germinated control cowpea seedlings were exposed to increasing concentrations of NaCl (0, 125, 150, 175, 200 mM) treatment supplemented in Hoagland's nutrient solution for 96 hours and (b) subjected to revival medium (Hoagland's nutrient solution) and observed for 7 and 14 days. Bar: 5mm for 0-96 h salt stress (125, 150, 175, 200 mM) and 5 cm for revival time period (7-14 days); 5 mm for control seedling under no stress and 1 cm for 24 hours-14 days time period

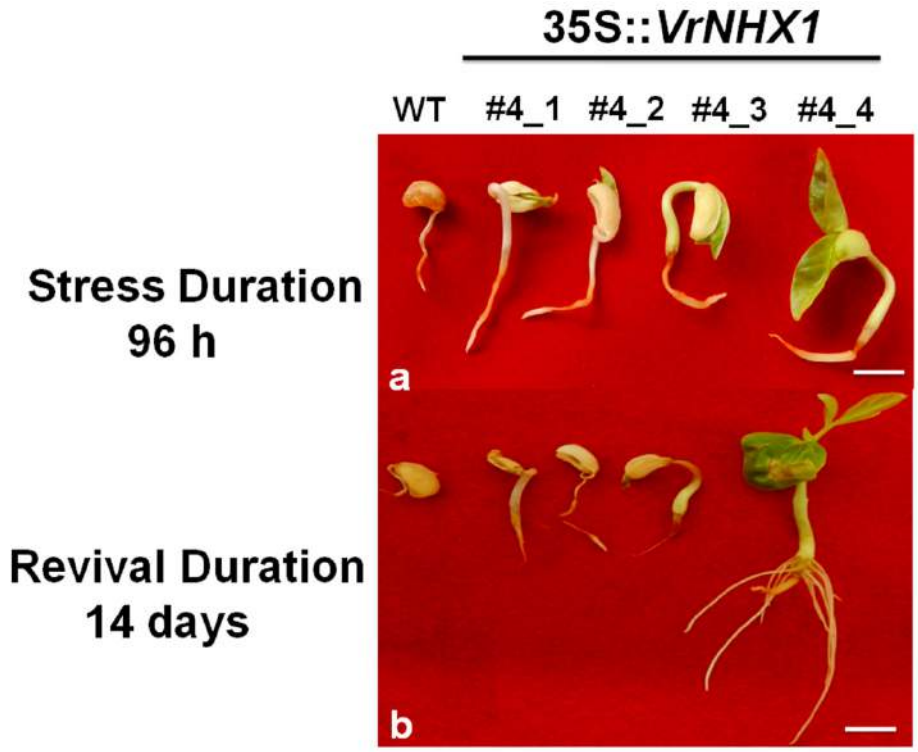

Figure 6.6 Salt screening of cowpea 35S::*VrNHX1* cisgenic seedlings in T_1 generation. The progenies of cisgenic cowpea (T_0.#4) labeled as T_1.#4_1-4 were subjected to salt screening assay as described in materials and methods. **a** The effect of salt stress on 2 days old germinated control and T_1 cisgenic cowpea seedlings grown in Hoagland's solution supplemented with 200 mM NaCl after 96 hours of salt treatment. Bar: 1 cm. **b** The salt screened control and cisgenic seedlings were exposed to revival condition (Hoagland's nutrient solution). Bar: 3 cm

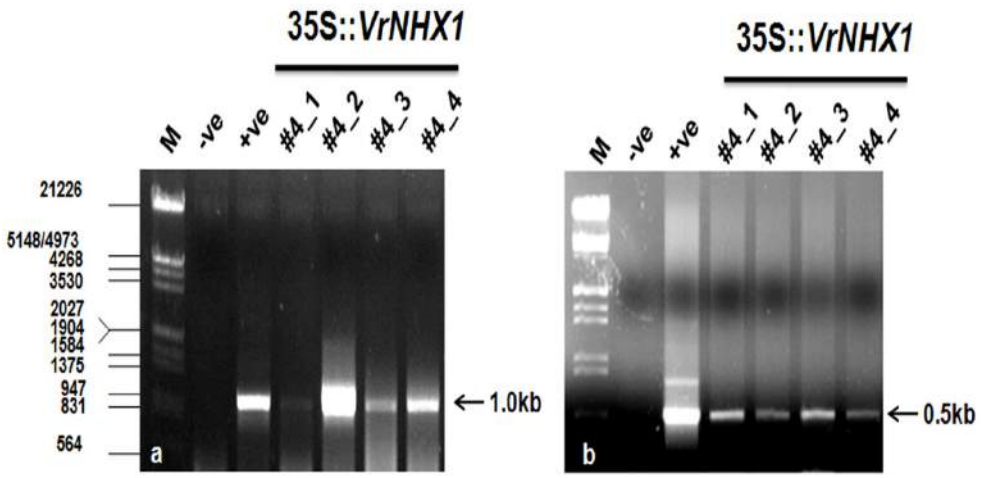

Figure 6.7 Molecular analysis of 35S::*VrNHX1* cisgenic plants overexpressing *VrNHX1*. Analysis of kanamycin-resistant T_1 cisgenic cowpea plants, PCR amplification of **a** 1.0 kb fragment of 35SP::*VrNHX1*::35STer cassette, **b** 0.5kb fragment of *nptII* gene. M: Lambda DNA HindIII/EcoRI ladder, -ve: Non-transformed control cowpea, +ve: Plasmid control, #4_1-4: PCR amplicons corresponding to progenies of an independent cisgenic 35S::*VrNHX1* cowpea line (T_0.#4)

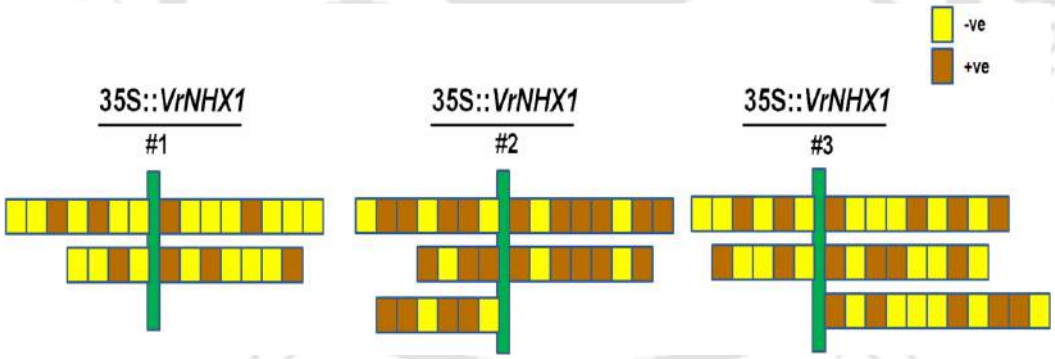

Figure 6.8 Segregation analysis of *VrNHX1* in T_1 35S::*VrNHX1* cisgenic cowpea plants. The salt screened cisgenic cowpea plants were confirmed for the presence of *VrNHX1* by PCR analysis. The diagrammatic representation includes the segregation pattern in the progenies (T_1 generation) of three independent southern positive T_0 cowpea cisgenic lines (T_0.#1-3). #1-3: Independent T_0 cowpea cisgenic lines overexpressing *VrNHX1*; Bar: Pods bearing seeds in T_0 cisgenic cowpea lines; Solid dark boxes: PCR confirmed and salt screened T_1 plants positive for presence of *VrNHX1*; Solid light boxes: PCR negative and salt sensitive cowpea lines lacking presence of *VrNHX1*

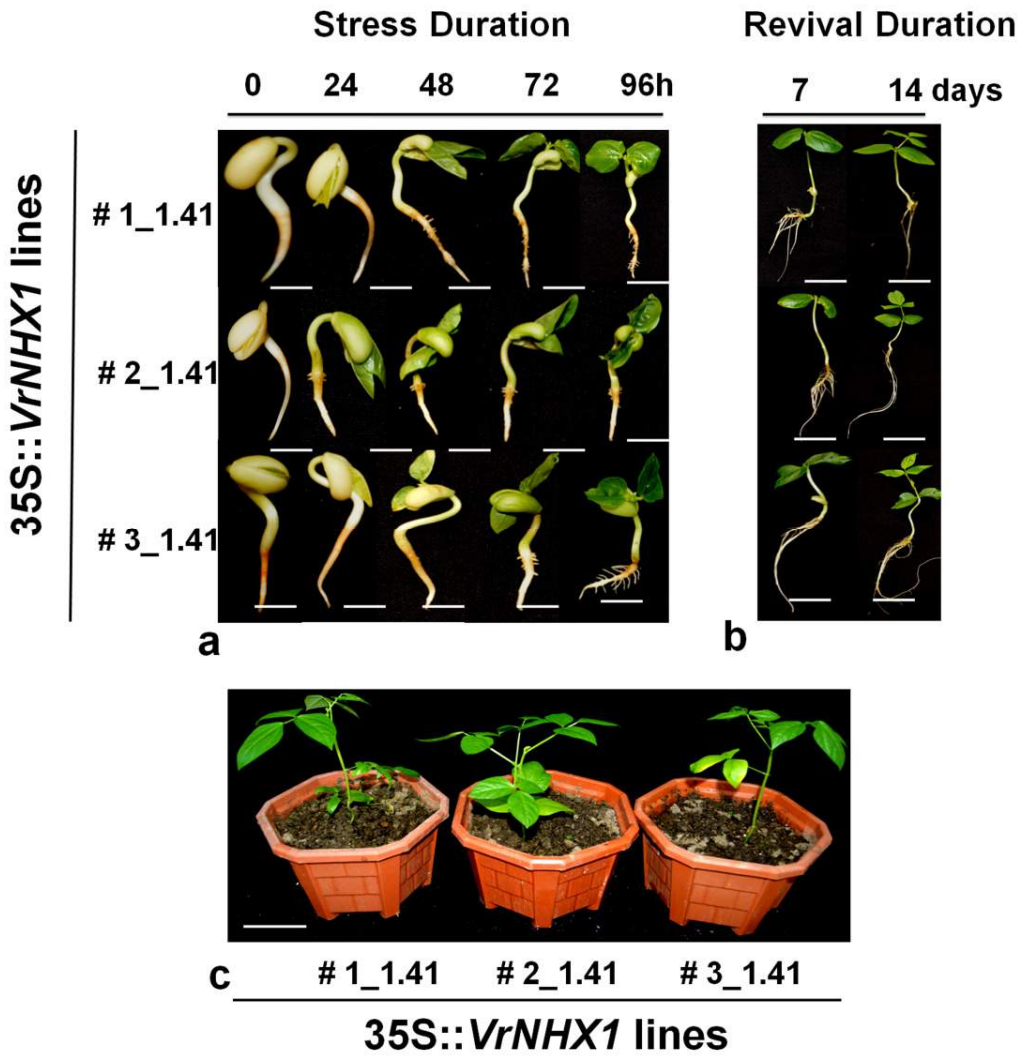

Figure 6.9 Salt screening of T_4 cisgenic 35S::*VrNHX1* cowpea plants (#1_1.41, #2_1.41, #3_1.41). (a) The 2 days old germinated seedlings were grown in Hoagland's nutrient solution supplemented with 150 mM NaCl for 96 hours and subjected to (b) revival medium (Hoagland's nutrient solution) and observed for 2 weeks. Bar: 5mm for 0-h salt stress,1 cm for 24-96 h and 5 cm for salt stress revival time period (7-14 days). (c) The salt selected cisgenic cowpea plants were transferred to soil in grown in greenhouse. The figure represents the salt selected cisgenics after 2 weeks of growth in green house. Bar: 8 cm

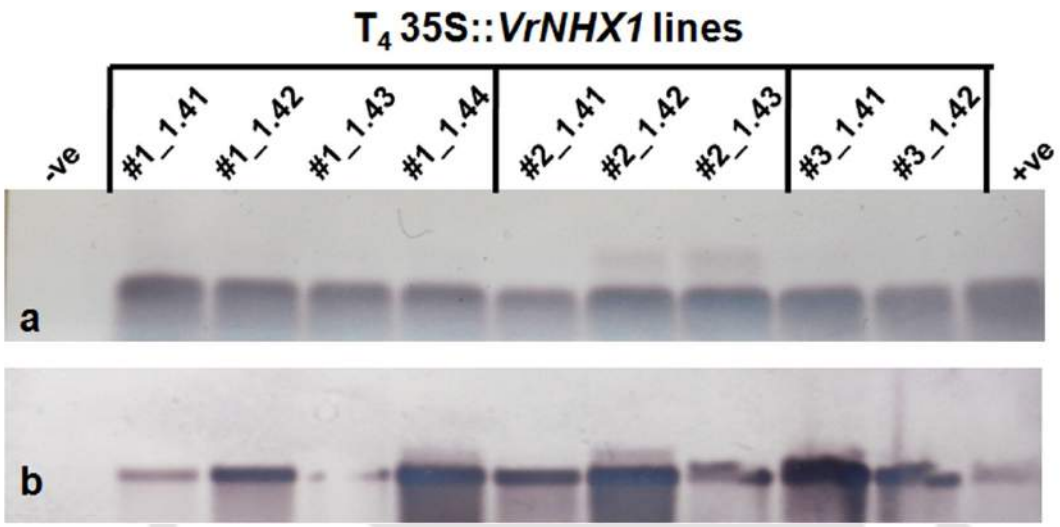

Figure 6.10 Southern blot analysis of T_4 cisgenic cowpea plants overexpressing *VrNHX1*. The PCR amplicons corresponding to 0.5 kb and 1.5 kb fragments of *nptII* and 35SP::*VrNHX1*::35STer cassette, were run on 1% agarose gel and cross-examined with southern hybridization using non-radioactive probes for *nptII* and *VrNHX1*, respectively. Southern blot confirming **a** *nptII* and **b** *VrNHX1* in cisgenic cowpea plants. Lane 1: -ve, Untransformed cowpea plant as control; Lane 2-5: T_4 cisgenic progenies from T_3.#1_1 35S::*VrNHX1* line (labeled as #1_1.41-44); Lane 6-8: T_4 cisgenic progenies from T_3.#2_1 35S::*VrNHX1* line (labeled as #2_1.41-44); Lane 9-10: T_4 cisgenic progenies from T_3.#3_1 35S::*VrNHX1* line (labeled as #3_1.41-44); Lane 11: PCR amplification from positive plasmid

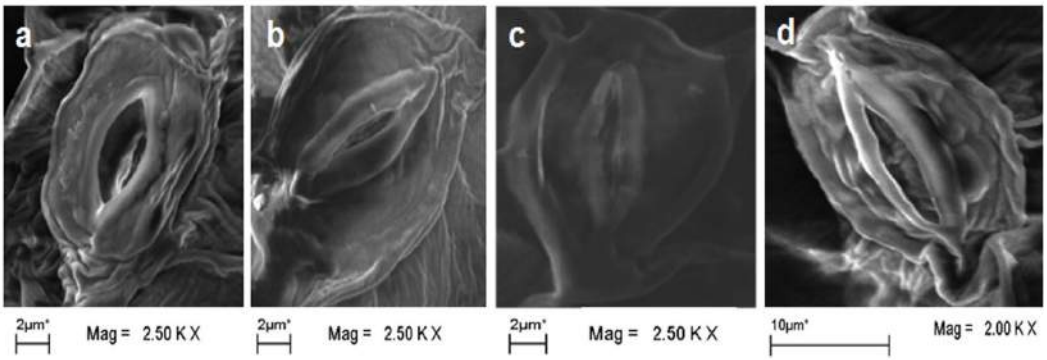

Figure 6.11 Scanning electron microscope analysis of stomatal aperture in control (unstressed) and salt selected T_4 cisgenic cowpea plants (T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41). SEM images for control untransformed plants with **a** no salt stress; **b-d** salt screened T_4 cisgenic cowpea plants T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41, respectively.

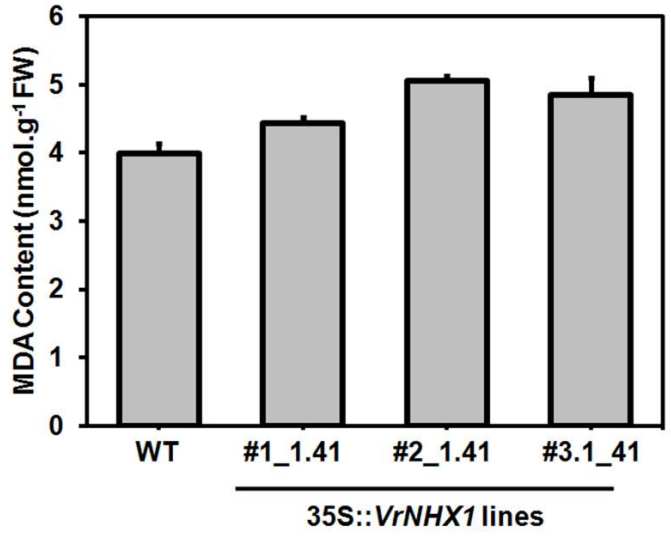

Figure 6.12 Measurement of lipid peroxidation in control WT and salt selected T_4 cisgenic cowpea plants (T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41) by estimation of malondialdehyde content (MDA). Statistically significant difference between mean values ± SE, n=3 performed by Student's t-test are marked as " * " for $P \leq 0.05$ and " ** " for $P \leq 0.001$

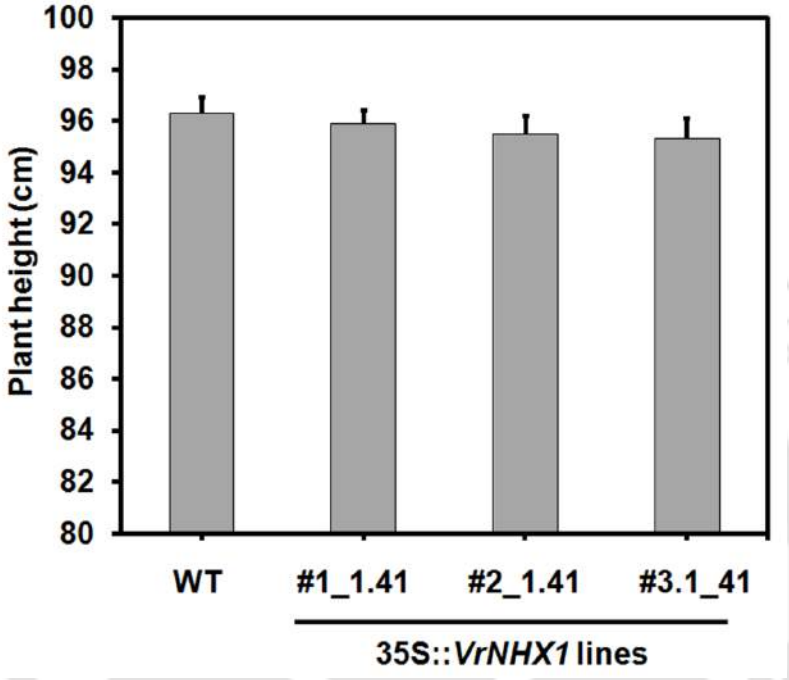

Figure 6.13 Measurement of plant height (cm) of unstressed control WT and salt selected T_4 cisgenic cowpea plants (T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41). No statistically significant difference was observed between mean values ± SE, n=10 (Student's t-test, $P \leq 0.05$ and $P \leq 0.001$)

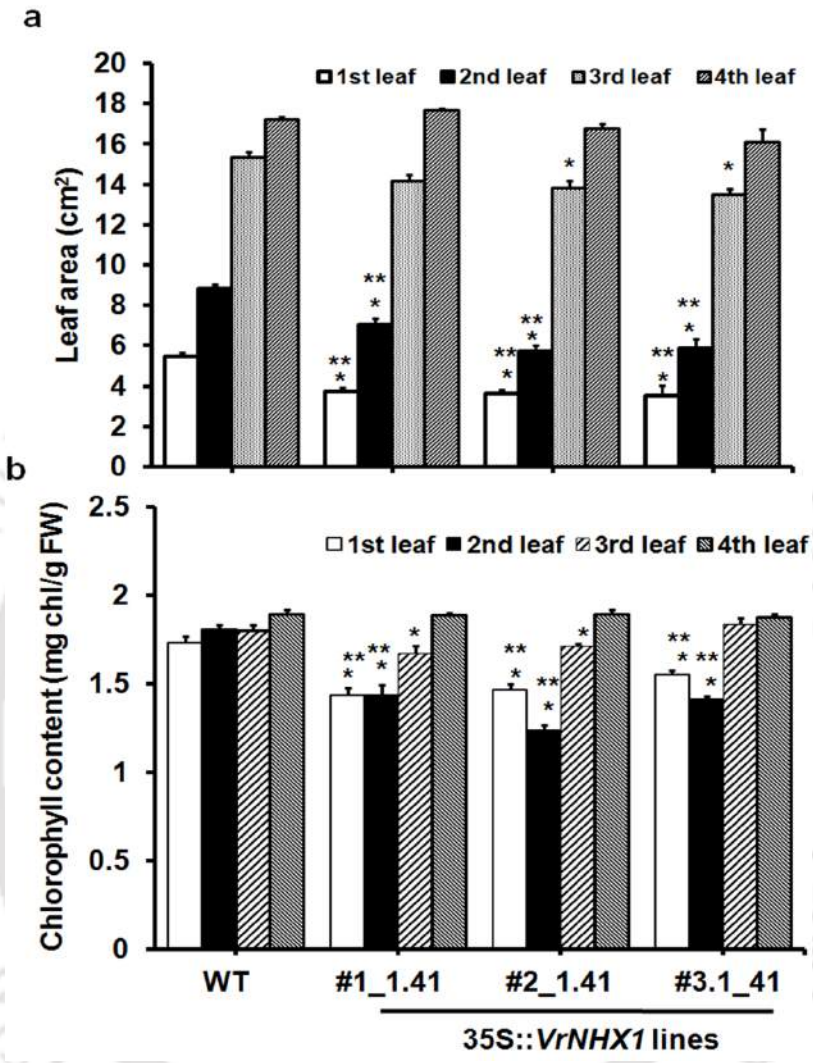

Figure 6.14 Measurement of leaf surface area (cm^2) andchlorophyll content of 1st, 2nd, 3rd and 4th mature leaves from unstressed control WT and salt selected T_4 cisgenic cowpea plants (T_4.#1_1.41, T_4.#2_1.41, T_4.#3_1.41). (a) The leaf area (cm^2) was measured using grid method and (b) chlorophyll content was measured as described in materials and methods. Statistically significant difference between mean values ± SE, n=10 performed by Student's t-test are marked as " * " for $P \leq 0.05$ and " ** " for $P \leq 0.001$

Table 6.1

Summary of the *Agrobacterium*-mediated transformation of cotyledonary node explants of *Vigna unguiculata* cv. Pusa Komal co-cultivated with *Agrobacterium tumefaciens* strain EHA105 harboring a binary construct pCAMBIA2301-35S::*VrNHX1*

Exp. no.	No. of explants inoculated in *Agrobacterium* suspension	No. of shoots recovered on selection medium	No. of plants rooted	No. of plants positive for *nptII* and *VrNHX1* by PCR	Stable Transformation efficiency[a] (%)
1	85	28	11	3	3.52
2	63	21	9	2	3.17
3	78	34	14	3	3.84
Total/ average	**226[b]**	**83[b]**	**34[b]**	**8[b]**	**3.51[c]**

[a] Number of T_0 plants PCR positive for *nptII*, *gus* and *VrNHX1* divided by total number of explants cocultivated

[b] Total

[c] Average

Table 6.2

Measurement of yield parameters in unstressed wild-type (WT, control) and salt selected T_4 cisgenic cowpea plants (T_4 #1_1.41, T_4 #2_1.41, T_4 #3_1.41)

Plant	Pod number	Pod length (cm)	Total seed
WT	5.22±0.249	10.57±0.833	41.22±0.817
#1_1.41	5.0±0.394	9.28±0.657	37.55±0.766
#2_1.41	4.66±0.326	11.55±0.499	37.33±1.714
#3_1.41	5.0±0.348	8.66±0.718* **	39.55±1.790

Statistically significant difference between mean values ± SE, n=10 performed by Student's t-test are marked as " * " for P≤0.05 and " ** " for P≤0.001.

CHAPTER 7

Concluding Remarks

7.1 Significance and salient features of the study

Soil salinity poses a serious threat to agricultural productivity worldwide owing to loss of nearly 1 % of cultivable lands each year. Cowpea (*Vigna unguiculata* L. Walp) and mungbean (*Vigna radiata* L. Wilczek) are two important grain legumes widely grown in tropical and subtropical regions of Indian subcontinent. Salinity is recognized as major constraint in the production of cowpea (Patel et al. 2010) and mungbean (Hasanuzzaman et al. 2013). Enhanced production of cowpea and mungbean in salinity inflicted areas will rely on innovative agronomic practices coupled with use of genetically improved varieties. In saline soil, Na^+ is the most predominantly toxic ion. Excess accumulation of Na^+ in plants leads to increase in cytoplasmic osmotic stress and ion (mainly Na^+)-specific toxicity (Zhu 2001). Sequestration of Na^+ to vacuoles by the activity of vacuolar Na^+/H^+ antiporters (NHX) is key mechanism in plants to withstand salt stress (Zhu 2003). The gene encoding for NHX has been reported from many plant species. Overexpression of vacuolar NHX genes has conferred salt tolerance in agriculturally important crops. However, no efforts have been made till date to clone genes involved in salt tolerance in cowpea and mungbean, and generate transgenics exhibiting salt tolerance.

In the present study, we cloned the orthologue of the vacuolar Na^+/H^+ antiporter gene, *VuNHX1* from cowpea and *VrNHX1* from mungbean. We studied the function of *VuNHX1* and *VrNHX1* by complementation in salt sensitive yeast mutant and overexpression in model plant *Arabidopsis thaliana* which indicated the potent role of *VuNHX1* and *VrNHX1* in salt tolerance mechanisms. We generated cisgenic/transgenic cowpea plants overexpressing *VrNHX1* that conferred enhanced salt tolerance to transgenic cowpeas. We established an efficient salt selection strategy for identification of stable salt tolerant cisgenic cowpea lines for four successive generations. This strategy reduced the labor and time involved in screening of high expressor salt tolerant cisgenic lines with no penalty on seed setting, plant growth and vigor.

The salient features of the present study are summarized below:

- The vacuolar Na^+/H^+ antiporters *VuNHX1* and *VrNHX1* were isolated from salt stressed roots of cowpea and mungbean, respectively.
- In-silico analysis showed that both, VuNHX1 and VrNHX1 share 97% similarity with each other and 92 % similarity with soybean NHX1.
- The hydrophobicity plot predicted for VuNHX1 and VrNHX1 suggested 11 putative transmembrane domains, and a conserved amiloride binding motif was also found in TM3 for both legume NHX proteins.
- The expression analysis of salt stressed cowpea and mungbean indicated increased transcript accumulation of *VuNHX1* and *VrNHX1* in both leaves and roots, while comparatively higher induction in roots.
- The concomitant increase in Na^+ accumulation in roots than leaves of salt stressed cowpea and mungbean seedlings at different time period indicated the lower transport of Na^+ to aerial parts of plants suggesting this mechanism possible for salt tolerance in cowpea and mungbean.
- The studies showed that both the crops could tolerate salinity up to 100 mM NaCl.
- A single copy of NHX1 gene detected in genome of both legume crops, and the presence of single copy might be attributed to insufficient salt tolerance in these crops mediated by the vacuolar Na^+/H^+ antporter.
- The yeast complementation assay performed with different concentrations of Na^+, K^+ and Li^+ stress, and measurement of accumulated sodium and potassium ions in vacuolar and cytosol indicated that, *VuNHX1* and *VrNHX1* could only complement for the loss of yeast *NHX1*.
- The functional validation of *VuNHX1* and *VrNHX1* carried out in model plant *Arabidopsis thaliana* under the regulation of constitutive (CaMV35S) and stress-inducible (AtRD29A) promoter showed improved salt tolerance (upto 200 mM NaCl stress) in transgenic *Arabidopsis* lines
- Transgenic cowpea lines overexpressing *VrNHX1* exhibited enhanced salt tolerance and better growth and yield under salt stress.
- We reported for the first time the generation of cisgenesis cowpea

overexpressing *VrNHX1* and identification of stable cisgenic lines through an efficient salt screening strategy.

- The efficient salt screening method during early seedling stage enabled rapid and precise selection of T_1 plants and their descendants stably expressing *VrNHX1* which is otherwise a labor-intensive and time consuming task.
- Cisgenic cowpea plants overexpressing *VrNHX1* conferred enhanced tolerance to salt stress. The cisgenic cowpea selected through salt selection strategy showed no penalty on growth and yield factors (plant height, seed yield, leaf area and chlorophyll content).

7.2 Future prospects

In spite of the progress in genomics and development of genetic resources in model as well as crop legumes, there is still a long way to go in the generation of "super-crops" exhibiting improved tolerance to various environmental stresses. The functional genomics and information generated from various omics approaches like transcriptomics, metabolomics, and proteomics in genetic resources challenged to various abiotic stress conditions such as salinity, drought, and extreme temperatures is expected to accelerate the understanding of the complex and integrated stress tolerance mechanism in crop plants. The improvement in genetic transformation technology in recalcitrant crops will assist in generating large number of overexpression as well as knock out lines for understanding of novel gene functions in crop legumes. Further, generation of improved cultivars cowpea and mungbean through manipulation of genes involved in intricate stress responses and cross-talks by both molecular breeding and transgenic approaches will bring revolution in sustainable production of these orphan grain legumes.

Activation tagging, promoter trapping through T-DNA insertion could lead to generation of gene mutants in cowpea and mungbean, identification of regulatory elements specific for root nodules and root systems and identification of novel genes responsible for abiotic stress tolerance in these legume crops.

The successful generation of stable transgenic/cisgenic cowpea lines overexpressing *VrNHX1* and faithful inheritance of the transgene to successive generations can greatly facilitate their limited field trial for their eventual deployment in salt-afflicted regions worldwide.

CHAPTER 8

References

Ali S, Khan MI, Yasmin K, Mushtaq N, Peoples MB, Herridge DF (1997) Surveys of N_2 fixation of summer legumes in farmers' fields in the Potwar, Punjab, Pakistan. In: Rupela OP, Johansen C, Herridge DF (eds) Extending Nitrogen Fixation Research to Farmers' Fields. ICRISAT, Patancheru, India, pp 345–351

Ali R, Brett CL, Mukherjee S, Rao R (2004) Inhibition of sodium/ proton exchange by a Rab-GTPase- activating protein regulates endosomal traffic in yeast. J Biol Chem 279:4498-4506

Amtmann A, Beilby MJ (2010) The role of ion channels in plant salt tolerance. In: Demidchik V, Maathuis F (eds) Ion Channels and Plant Stress Responses, Springer, Berlin, Heidelberg, pp 23-46

An BY, LuoY, Li JR et al (2008) Expression of a vacuolar Na^+/H^+ Antiporter Gene of Alfalfa Enhances Salinity Tolerance in Transgenic Arabidopsis. Acta Agron Sin 34:557-564

Apse MP, Aharon GS, Snedden WA, Blumwald E (1999) Salt tolerance conferred by overexpression of a vacuolar Na^+/H^+ antiporter in *Arabidopsis*. Science 285:1256-1258

Apse MP, Blumwald E (2007) Na^+ transport in plants. FEBS Lett 581:2247-2254

Arnon DI (1949) Copper enzymes in isolated chloroplasts Polyphenoloxidase in *Beta vulgaris.* Plant Physiol 24:1-10

Arumuganathan K, Earle ED (1991) Nuclear DNA content of some important plant species. Plant Mol Biol Rep 9:208-218

Ashraf M, Rasul E (1988) Salt tolerance of mung bean (*Vigna radiata* (L.) Wilczek) at two growth stages. Plant Soil 110:63-67

Ashraf MPJC, Harris PJC (2004) Potential biochemical indicators of salinity tolerance in plants. Plant Sci 166:3-16

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environ Exp Bot 59:206-216

Atkinson NJ, Lilley CJ, Urwin PE (2013) Identification of genes involved in the response of *Arabidopsis* to simultaneous biotic and abiotic stresses. Plant Physiol 162:2028-2041

Bakshi S, Sadhukhan A, Mishra S, Sahoo L (2011) Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration. Plant Cell Rep 30:2281-2292

Bakshi S, Roy NK, Sahoo L (2012a) Seedling preconditioning in thidiazuron enhances axillary shoot proliferation and recovery of transgenic cowpea plants. Plant Cell Tiss Org Cult 110:77-91

Bakshi S, Saha B, Roy NK, Mishra S, Panda SK, Sahoo L (2012b) Successful recovery of transgenic cowpea (*Vigna unguiculata*) using phosphomannose isomerase gene as alternative selectable marker. Plant Cell Rep 31:1093-1103

Bakshi S, Sahoo L (2013) How relevant is recalcitrance for the recovery of transgenic cowpea: implications of selection strategies. J Plant Growth Reg 32:148-158

Baltierra Q, Castillo M, Gamboa MC et al (2012) Molecular characterization of a novel Na^+/H^+ antiporter cDNA from *Eucalyptus globules*. Biochem and Biophys Res Commun 430:535-540

Banjara M, Zhu L, Shen G, Payton P, Zhang H (2012) Expression of an Arabidopsis sodium/proton antiporter gene (*AtNHX1*) in peanut to improve salt tolerance. Plant Biotechnol Rep 6:59-67

Bao-Yan AN, Yan L, Jia-Rui LI et al (2008) Expression of a vacuolar Na^+/H^+ Antiporter gene of alfalfa enhances salinity tolerance in transgenic *Arabidopsis*. Acta Agron Sin 34:557-564

Bao AK, Wang YW, Xi JJ, Liu C, Zhang JL, Wang SM (2014) Co-expression of xerophyte *Zygophyllum xanthoxylum ZxNHX* and *ZxVP1-1* enhances salt and drought tolerance in transgenic *Lotus corniculatus* by increasing cations accumulation. Funct Plant Biol 41:203-214

Barragán V, Leidi EO, Andrés Z et al (2012) Ion exchangers NHX1 and NHX2 mediate active potassium uptake into vacuoles to regulate cell turgor and stomatal function in *Arabidopsis*. Plant Cell 24:1127-1142

Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant Soil 39:205-207

Bauer MW, Gaskell G (2002) The biotechnology movement. In: Bauer MW, Gaskell G (eds) Biotechnology-the making of a global controversy. Cambridge University Press, UK: 379-404

Becana M, Dalton DA, Moran JF, Iturbe-Ormaetxe I, Matamoros MA, Rubio MC (2000) Reactive oxygen species and antioxidants in legume nodules. Physiol Plantarum 109:372-381

Berthomieu P, Conéjéro G, Nublat A, Brackenbury WJ, Lambert C, Savio C, Uozumi N, Oiki S, Yamada K, Cellier F et al (2003) Functional analysis of *AtHKT1* in *Arabidopsis* shows that Na^+ recirculation by the phloem is crucial for salt tolerance. EMBO J 22:2004–2014

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12:431-434

Blumwald E, Aharon GS, Apse MP (2000) Sodium transport in plant cells. Biochim Biophys Acta Biomembr 1465:140-151

Bohnert HJ, Su H, Shen B (1999) Molecular mechanisms of salinity tolerance. In: Shinozaki K (ed) Cold, drought, heat and salt stress: Molecular responses in higher plants. RGlandes, Austin, Texas, pp 29-60

Bor M, Özdemir F,Türkan I (2003) The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. Plant Sci 164: 77-84

Botella M, Rosado A, Bressan RA, Hasegawa PM (2005) Plant adaptive responses to salinity stress. In: Jenks MA, Hasegawa PM (eds.) Plant Abiotic Stress. Blackwell Publishing, USA, pp 37-70

Bowers K, Levi BP, Patel FI, Stevens TH (2000) The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. Mol Biol Cell 11:4277-4294

Brady NC, Weil RR (2008) Soil water: Characteristics and behavior. In: Brady NC, Weil RR (eds) The nature and properties of soils. Prentice Hall, New Jersey, pp 177–217

Brett CL, Tukaye DN, Mukherjee S, Rao R (2005) The yeast endosomal $Na^+(K^+)/H^+$

exchanger Nhx1 regulates cellular ph to control vesicle trafficking. Mol Biol Cell 16:1396-1405

Britto DT, Kronzucker HJ (2008) Cellular mechanisms of potassium transport in plants. Physiol Plantarum 133:637-650

Bui EN (2013) Soil salinity: A neglected factor in plant ecology and biogeography. J Arid Environ 92:14-25

Byrt CS, Platten JD, Spielmeyer W et al (2007) *HKT1; 5*-like cation transporters linked to Na^+ exclusion loci in wheat, Nax2 and Kna1. Plant Physiol 143:1918-1928

Cao D, Hou W, Liu W, Yao W, Wu C, Liu X, Han T (2011) Overexpression of *TaNHX2* enhances salt tolerance of 'composite' and whole transgenic soybean plants. Plant Cell Tiss Org Cult 107: 541-552

Cavalcanti FR, Oliveira JTA, Martins-Miranda AS, Viégas RA, Silveira JAG (2004) Superoxide dismutase, catalase and peroxidase activities do not confer protection against oxidative damage in salt-stressed cowpea leaves. New Phytol 163: 563-571

Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. Ann Bot 103:551-560

Chen C, Tao C, Peng H, Ding Y (2007) Genetic analysis of salt stress responses in asparagus bean (*Vigna unguiculata* (L) ssp Sesquipedalis Verdc). J Hered 98:655-665

Chen M, Chen QJ, Niu XG, Zhang R, Lin HQ, Xu CY, Wang XC, Wang GY, Chen J (2007) Expression of *OsNHX1* gene in maize confers salt tolerance and promotes plant growth in the field. Plant Soil Environ 53:490-498

Chen T, Cai X, Wu X, Karahara I, Schreiber L, Lin J (2011) Casparian strip development and its potential function in salt tolerance. Plant Signal Behav 6:1499-1502

Chen H, He H, Yu D (2011) Overexpression of a novel soybean gene modulating Na^+ and K^+ transport enhances salt tolerance in transgenic tobacco plants. Physiol Plantarum 141:11-18

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672-3676

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16:735-743

Conway G (2012) One Billion Hungry: Can We Feed the World? Cornell Univ Press, NY, USA

Darley CP, van Wuytswinkel OCM, van der Woude K, Mager WH, de Boer AH (2000) *Arabidopsis thaliana* and *Saccharomyces cerevisiae NHX1* genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers. Biochem J 351:241-249

Das S, Bhat PR, Sudhakar C, Ehlers JD, Wanamaker S, Roberts PA, Cui X, Close TJ (2008) Detection and validation of single feature polymorphisms in cowpea (*Vigna unguiculata* L. Walp) using a soybean genome array. BMC Genomics 9:107-119

Dassanayake M, Oh DH, Haas JS et al (2011a) The genome of the extremophile crucifer *Thellungiella parvula*. Nat Genet 43:913-918

Dassanayake M, Oh DH, Hong H, Bohnert HJ, Cheeseman JM (2011b) Transcription strength and halophytic lifestyle. Trends Plant Sci 16:1-3

de Almeida Costa GE, da Silva Queiroz-Monici K, Pissini Machado Reis SM, de Oliveira AC (2006) Chemical composition, dietary fibre and resistant starch contents of raw and cooked pea, common bean, chickpea and lentil legumes. Food Chem 94:327-330

Demetriou G, Neonaki C, Navakoudis E, Kotzabasis K (2007) Salt stress impact on the molecular structure and function of the photosynthetic apparatus—the protective role of polyamines. BBA Bioenergetics 1767: 272-280

Dhekney SA, Li ZT, Gray DJ (2011) Grapevines engineered to express cisgenic *Vitis vinifera* thaumatin-like protein exhibit fungal disease resistance. In Vitro Cell Dev Biol Plant 47:458–466

Diouf D (2011). Recent advances in cowpea [*Vigna unguiculata* (L.) Walp.] "omics" research for genetic improvement. Afr J Biotechnol 10:2803-2810

Du Y, Hei Q, Liu Y, Zhang H, Xu K, Xia T (2010) Isolation and Characterization of a Putative Vacuolar Na^+/H^+ Antiporter Gene from *Zoysia japonica* L. J Plant Biol 53: 251-258

Duh PD, Du PC,Yen GC (1999) Action of methanolic extract of mung bean hulls as

inhibitors of lipid peroxidation and non-lipid oxidative damage. Food Chem Toxicol 37:1055-1061

Duzdemir O Unlukara A, Kurunc A (2009) Response of cowpea (*Vigna unguiculata*) to salinity and irrigation regimes. New Zealand J Crop Hort Sci 37:271-280

Elawad HOA, Hall AE (1987) Influences of early and late nitrogen fertilization on yield and nitrogen fixation of cowpea under well-watered and dry field conditions. Field Crop Res 15:229-244

Fang J, Chao CCT, Roberts PA, Ehlers JD (2007) Genetic diversity of cowpea [*Vigna unguiculata* (L.) Walp.] in four West African and USA breeding programs as determined by AFLP analysis. Genet Res Crop Evol 54:1197-1209

Feki K, Quintero FJ, Khoudi H, Leidi EO, Masmoudi K, Pardo JM, Brini F (2014). A constitutively active form of a durum wheat Na^+/H^+ antiporter SOS1 confers high salt tolerance to transgenic Arabidopsis. Plant Cell Rep 33:277-288

Fery RL (1990) The cowpea: production, utilization, and research in the United States. Hort Rev 12:197-222

Flowers T, Troke PF, Yeo AR (1977) The mechanism of salt tolerance in halophytes. Ann Rev Plant Physiol 28:89–121

Flowers T, Yeo A (1986) Ion relations of plants under drought and salinity. Aust J Plant Physiol 13:75–91

Flowers TJ (2004) Improving crop salt tolerance. J Exp Bot 55:307-319

Flowers TJ, Flowers SA (2005) Why does salinity pose such a difficult problem for plant breeders? Agric Water Manage 78:15-24

Flowers TJ, Colmer TD (2008) Salinity tolerance in halophytes. New Phytol 179:945-963

Fougère F, Le Rudulier D, Streeter JG (1991) Effects of salt stress on amino acid, organic acid, and carbohydrate composition of roots, bacteroids, and cytosol of alfalfa (*Medicago sativa* L.). Plant Physiol 96:1228-1236

Fukuda A, Nakamura A, Tanaka Y (1999) Molecular cloning and expression of the Na^+/H^+ exchanger gene in *Oryza sativa*. Biochim Biophys Acta 1446:149-55

Fukuda A, Nakamura A, Tagiri A, Tanaka H, Miyao A, Hirochika H, Tanaka Y (2004) Function, intracellular localization and the importanc in salt tolerance of a vacuolar Na^+/H^+ antiporter from rice. Plant Cell Physiol 45:146-159

Gamborg OL, Miller R, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158

Gaskell G, Bauer MW (2001) *Biotechnology in the years of controversy: a social scientific perspective.* In: Gaskell G, Bauer MW (eds) Biotechnology 1996-1999: the years of controversy. Science Museum Press, London, pp 3-14

Gattiker A, Gasteiger E, Bairoch A (2002) ScanPROSITE: a reference implementation of a PROSITE scanning tool. Appl Bioinformatics 1:107-108

Gaxiola RA, Rao R, Sherman A, Grisafi P, Alper SL, Fink GR (1999) The Arabidopsis thaliana proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. Proc Nat Acad Sci 96:1480-1485

Geourjon C, Deleage G (1995) SOPMA: significant improvements in protein secondary structure prediction by consensus prediction from multiple alignments. Comput Appl Biosci 11:681-684

Gietz D, St. Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20:1425

Gomes-Filho E, Lima CRFM, Costa JH, da Silva ACM, Lima MDGS, de Lacerda CF, Prisco JT (2008) Cowpea ribonuclease: properties and effect of NaCl-salinity on its activation during seed germination and seedling establishment. Plant Cell Rep 27:147-157

Gouiaa S, Khoudi H, Leidi EO et al (2012) Expression of wheat Na^+/H^+ antiporter *TNHXS1* and H^+-pyrophosphatase *TVP1* genes in tobacco from a bicistronic transcriptional unit improves salt tolerance. Plant Mol Biol 79:137-155

Grattan SR, Grieve CM (1999) Salinity-mineral nutrient relations in horticultural crops. Sci Hort 78:127-157

Griffiths ME, Orians CM (2003) Responses of common and successional heathland species to manipulated salt spray and water availability. Am J Bot 90:1720-1728

Guan B, Hu YZ, Zeng YL, Wang Y, Zhang FC (2010) Molecular characterization and functional analysis of a vacuolar Na^+/H^+ antiporter gene (*HcNHX1*) from *Halostachys caspica*. Mol Biol Rep 38:1889-1899

Guimaraes FVA, de Lacerda CF, Marques EC, de Maranda MRA, de Abreu CEB, Prisco JT, Gomes-Filho E (2011) Calcium can moderate changes on membrane structure and lipid composition in cowpea plants under salt stress. Plant Growth Regul 65:55-63

Hafeez FY, Aslam Z, Malik KA (1988) Effect of salinity and inoculation on growth, nitrogen fixation and nutrient uptake of *Vigna radiata* (L.) Wilczek. Plant Soil 106:3-8

Hall AE, Cisse N, Thiaw S et al (2003) Development of cowpea cultivars and germplasm by the Bean/Cowpea CRSP. Field Crops Res 82:103-134

Hall AE (2004) Breeding for adaptation to drought and heat in cowpea. Eur J Agron 21: 447-454

Hamada A, Shono M, Xia T, Ohta M, Hayashi Y, Tanaka A, Hayakawa T (2001) Isolation and characterization of a Na^+/H^+ antiporter gene from the halophyte *Atriplex gmelini*. Plant Mol Biol 46: 35-42

Harris C, Fliegel L (1999) Amiloride and the Na^+/H^+ exchanger protein: mechanism and significance of inhibition of the Na^+/H^+ exchanger. Int J Mol Med 3: 315-321

Hasanuzzaman M, Ali MR, Hossain M, Kuri S, Islam MS (2013) Evaluation of total phenolic content, free radical scavenging activity and phytochemical screening of different extracts of Averrhoa bilimbi (fruits).Int Curr Pharmaceut J 2: 92-96

Hasegawa PM, Bressan RA, Zhu JK, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Biol 51:463-499

Hasegawa PM (2013) Sodium (Na^+) homeostasis and salt tolerance of plants. Environ Exp Bot 92:19-31

Hauser F, Horie T (2010) A conserved primary salt tolerance mechanism mediated by HKT transporters: a mechanism for sodium exclusion and maintenance of high K^+/Na^+ ratio in leaves during salinity stress. Plant Cell Environ 33:552-565

Haverkort AJ, Struik PC, Visser RGF, Jacobsen E (2009) Applied biotechnology to

combat late blight in potato caused by *Phytophthora infestans*. Potato Res 52:249–264

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetic and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125:189-198

Hernandez A, Jiang X, Cubero B, Nieto PM, Bressan RA, Hasegawa PM, Pardo JM (2009) Mutants of the *Arabidopsis thaliana* cation/H^+ antiporter *AtNHX1* conferring increased salt tolerance in yeast: the endosome/prevacuolar compartment is a target for salt toxicity. J Biol Chem 284:14276-85

Hoagland DR, Arnon DI (1950) The water-culture method for growing plants without soil. Calif Agric Exp Stn Circ 347

Hofmann K, Stoffel W (1993) A database of membrane spanning proteins segments. Biol Chem 374:166

Holme IB, Wendt T, Holm PB (2013) Intragenesis and cisgenesis as alternatives to transgenic crop development. Plant Biotechnol J 11:395-407

Horie T, Hauser F, Schroeder JI (2009) HKT transporter-mediated salinity resistance mechanisms in *Arabidopsis* and monocot crop plants. Trends Plant Sci 14:660-668

Hu YC, Schmidhalter U (2005) Drought and salinity: A comparision of their effects on the mineral nutrition of plants. J Plant Nutr Soil Sci 168:541-549

Huang S, Spielmeyer W, Lagudah ES, James RA, Platten JD, Dennis ES, Munns R (2006) A sodium transporter (HKT7) is a candidate for *Nax1*, a gene for salt tolerance in durum wheat. *Plant Physiol* 142:1718–1727

Islam SMT, Tammi RS, Singla-Pareek SL, Seraj ZI (2010) Enhanced salinity tolerance and improved yield properties in Bangladeshi rice Binnatoa through *Agrobacterium*-mediated transformation of *PgNHX1* from *Pennisetum glaucum.* Acta Physiol Plant 32:657-663

Jacoby B (1999) Mechanisms involved in salt tolerance of plants. In: Pessarakli M (ed) Plant and Crop Stress, pp. 97–123

Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol 204: 387-405

Jha B, Mishra A, Jha A, Joshi M (2013) Developing transgenic Jatropa using the *SbNHX1* gene from an extreme halophyte for cultivation in saline wasteland. PloS One 8:e71136

Jiang XY, Leidi EO, Pardo JM (2010) How do vacuolar NHX exchangers function in plant salt tolerance? Plant Signal Behav 5:792–795

Kader MA, Lindberg S (2010) Cytosolic calcium and pH signaling in plants under salinity stress. Plant Signal Behav 5:233-238

Kim T-H, Böhmer M, Hu H, Nishimura N, Schroeder JI (2010) Guard cell signal transduction network: Advances in understanding abscisic acid, CO_2, and Ca^{2+} signaling. Annu Rev Plant Biol 61:561–591

Kinclova-Zimmermannova O, Flegelova H, Sychrova H (2004) Rice Na^+/H^+- Antiporter Nhx1 partially complements the alkali-metal-cation sensitivity of yeast strains lacking three sodium transporters. Folia Microbiol 49:519-525

Knight H, Trewavas AJ, Knight MR (1997) Calcium signalling in Arabidopsis thaliana responding to drought and salinity. Plant J 12:1067-1078

Koyro HW, Hussain T, Huchzermeyer B, Khan MA (2013) Photosynthetic and growth responses of a perennial halophytic grass *Panicum turgidum* to increasing NaCl concentrations. Environ Exp Bot 91:22-29

Kronzucker HJ, Britto DT (2011) Sodium transport in plants: a critical review. New Phytol 189:54-81

Kwapata MB, Hall AE (1985) Effects of moisture regime and phosphorous on mycorrhizal infection, nutrient uptake, and growth of cowpeas [*Vigna unguiculata* (L) Walp]. Field Crop Res 12:241-250

Lacerda CF, Junior JOA, Filho LCA et al (2006) Morpho-physiological responses of cowpea leaves to salt stress. Braz J Plant Physiol 18:455-465

Lambot, C (2002). Industrial potential of cowpea. In: Fatokun CA, Tarawali SA, Singh BB, Kormawa PM, Tamo M (eds) Challenges and opportunities for enhancing sustainable cowpea production, IITA, Ibadan, Nigeria, pp 367-375

Lambrides CJ, Godwin ID (2007) Pulses, Sugar and Tuber Crops. Springer, Berlin,

Heidelberg

Lassen L, Allansdottir A, Liakopoulos M et al (2002) Testing times- the reception of Round-up Ready soya in Europe. In: Bauer MW, Gaskell G (eds) Biotechnology- The Making of a Global Controversy. Cambridge University Press, UK, pp 279-312

Lawlor DW (2013) Genetic engineering to improve plant performance under drought: physiological evaluation of achievements, limitations, and possibilities. J Exp Bot 64:83-108

Lee BH, Zhu JK (2010) Phenotypic analysis of *Arabidopsis* mutants: germination rate under salt/hormone-induced stress. Cold Spring Harb Protoc 2010:pdb-prot4969

Li WYF, Wong FL, Tsai SN, Phang TH et al (2006) Tonoplast-located *GmCLC1* and *GmNHX1* from soybean enhance NaCl tolerance in transgenic bright yellow (BY)-2 cells. Plant Cell Environ 29:1122-1137

Li J, Jiang G, Huang P, Ma J, Zhang F (2007) Overexpression of the Na^+/H^+ antiporter gene from *Suaeda salsa* confers cold and salt tolerance to transgenic *Arabidopsis thaliana*. Plant Cell Tiss Organ Cult 90:41-48

Li TX, Zhang Y, Liu H, Ting WY, Li WB, Zhang HX (2010) Stable expression of *Arabidopsis* vacuolar Na^+/H^+ antiporter gene *AtNHX1*, and salt tolerance in transgenic soybean for over six generations. Chinese Sci Bull 55:1127-1134

Li Y, Zhang Y, Feng F, Liang D, Cheng L, Ma F, Shi S (2010) Overexpression of a Malus vacuolar Na^+/H^+ antiporter gene (*MdNHX1*) in apple rootstock M26 and its influence on salt tolerance. Plant Cell Tiss Org Cult 102:337-345

Li W, Wang D, Jin T, Chang Q, Yin D, Xu S, Liu B, Liu L (2011) The vacuolar Na^+/H^+ antiporter gene *SsNHX1* from the halophyte *Salsola soda* confers salt tolerance in transgenic alfalfa (*Medicago sativa* L.). Plant Mol Biol Rep 29: 278-290

Lichtenthaler HK (1987) Chlorophyll fluorescence signatures of leaves during the autumnal chlorophyll breakdown. J Plant Physiol 131:101-110

Liu S, Zheng L, Xue Y, Zhang Q, Wang L, Shou H (2010) Overexpression of *OsVP1* and *OsNHX1* Increases Tolerance to Drought and Salinity in Rice. J Plant Biol 53:444-452

Liu L, Fan XD, Wang FW et al (2013). Coexpression of *ScNHX1* and *ScVP* in Transgenic

Hybrids Improves Salt and Saline-Alkali Tolerance in Alfalfa (*Medicago sativa* L.). J Plant Growth Reg 32:1-8

Lv SL, Lian LJ, Tao PL, Li ZX, Zhang KW, Zhang JR (2009) Overexpression of *Thellungiella halophila* H^+-PPase (*TsVP*) in cotton enhances drought stress resistance of plants. Planta 229:899-910

Ma XL, Zhang Q, Shi HZ, Zhu JK, Zhao YX, Ma CL, Zhang H (2004) Molecular cloning and different expression of a vacuolar Na^+/H^+ antiporter gene in *Suada salsa* under salt stress, Biol Plantarum 48: 219-225

Maathuis FJM, Amtmann A (1999) K^+ nutrition and Na^+ toxicity: the basis of cellular K^+/Na^+ ratios. Ann Bot 84:123–133

Maas EV, Poss JA (1989) Salt sensitivity of cowpea at various growth stages. Irri Sci, 10:313-320

Mahajan S, Tuteja N (2005) Cold, salinity and drought stresses: an overview. Arch Biochem Biophys 444:139-158

Manchanda G, Garg N (2008) Salinity and its effects on the functional biology of legumes. Acta Physiol Plant 30: 595-618

Mäser P, Eckelman B, Vaidyanathan R, Horie T, Fairbairn DJ, Kubo M, Yamagami M, Yamaguchi K, Nishimura M, Uozumi N et al (2002) Altered shoot/root Na^+ distribution and bifurcating salt sensitivity in *Arabidopsis* by genetic disruption of the Na^+ transporter *AtHKT1.* FEBS Lett 531: 157–161

Maxted N, Mabuza-Dlamini P, Moss H, Padulosi S, Jarvis A, Guarino L (2004) An Ecogeographic Survey: African *Vigna.* Systematic and Ecogeographic Studies of Crop Genepools. Vol 10, IPGRI, Rome, pp 1-468

Miller GAD, Suzuki N, Ciftci-Yilmazi S, Mittler R (2010) Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ 33:453-467

Misra AN, Murmu B, Singh P, Misra M (1996) Growth and proline accumulation in mungbean seedlings as affected by sodium chloride. Biol Plantarum 38:531-536

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant

Sci 7:405-410

Mittler R, Blumwald E (2010) Genetic engineering for modern agriculture: challenges and perspectives. Ann Rev Plant Biol 61:443-462

Mohammadkhani N, Heidari R (2008) Drought-induced accumulation of soluble sugars and proline in two maize varieties. World Appl Sci J 3:448-453

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651-681

Munns R, Wallace PA, Teakle NL, Colmer TD (2010) Measuring soluble ion concentrations (Na^+, K^+, Cl^-) in salt-treated plant. In: Sunkar R (ed) Plant Stress Tolerance: Methods and protocols. Humana Press, pp. 371-382

Munns R, James RA, Xu B et al (2012) Wheat grain yield on saline soils is improved by an ancestral Na^+ transporter gene. Nat Biotechnol 30:360-364

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15: 473-497

Murillo-Amador B, Troyo-Dieguez E, Garcia-Hernandez JL et al (2006) Effect of NaCl salinity in the genotypic variation of cowpea (*Vigna unguiculata*) during early vegetative growth. Scien Horticul 108:423-431

Nair RM, Yang RY, Easdown WJ, Thavarajah D, Thavarajah P, Hughes JDA, Keatinge JDH (2013) Biofortification of mungbean (*Vigna radiata*) as a whole food to enhance human health. J Sci Food Agric 93:1805-1813

Negrão S, Courtois B, Ahmadi N, Abreu I, Saibo N, Oliveira MM (2011) Recent updates on salinity stress in rice: from physiological to molecular responses. Crit Rev Plant Sci 30:329-377

Niu X, Bressan RA, Hasegawa PM, Pardo JM (1995) Ion homeostasis in NaCl stress environments. Plant Physiol 109:735

Oh DH, Lee SY, Bressan RA, Yun DJ, Bohnert HJ (2010) Intracellular consequences of SOS1 deficiency during salt stress. *J Exp Bot* 61:1205-1213

Omo-Ikerodah EE, Fawole I, Fatokun CA (2008) Genetic mapping of quantitative trait loci (QTLs) with effects on resistance to flower bud thrips (*Megalurothrips sjostedti*)

identified in recombinant inbred lines of cowpea (*Vigna unguiculata* (L) Walp). Afr J Biotechnol 7:263-270

Panda SK, Singha LB, Khan MH (2003) Does aluminium phytotoxicity induce oxidative stress in greengram (*Vigna radiata*)? Bulg J Plant Physiol 29:77-86

Pardo JM, Quintero FJ (2002) Plants and sodium ions: keeping company with the enemy. Genome Biol 3:1017-1

Pardo JM, Cubero B, Leidi EO, Quintero FJ (2006) Alkali cation exchangers: roles in cellular homeostasis and stress tolerance. J Exp Bot 57:1181-1199

Pasquet RS (2000) Allozyme diversity of cultivated cowpea *Vigna unguiculata* (L.) Walp. Theor Appl Genet 101:211-219

Pastori GM, Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of "redox" and abscisic acid-mediated controls. Plant Physiol 129:460-468

Patel PR, Kajal SS, Patel VR, Patel VJ, Khristi SM (2010) Impact of saline water stress on nutrient uptake and growth of cowpea. Braz J Plant Physiol 22:43-48

Phillips RD, McWatters KH, Chinnan MS et al (2003) Utilization of cowpeas for human food. Field Crops Res 82:193-213

Plaut Z, Grieve CM, Federman E (1989) Salinity effects on photosynthesis in isolated mesophyll cells of cowpea leaves. Plant Physiol 91:493-499

Plaut Z, Grieve CM, Maas EV (1990) Salinity effects on CO_2 assimilation and diffusive conductance of cowpea leaves. Physiol Plantarum 79:31-38

Plett DC, Møller IS (2010) Na^+ transport in glycophytic plants: what we know and would like to know. Plant Cell Environ 33:612-626

Qiao WH, Zhao XY, Li W et al (2007) Overexpression of *AeNHX1*, a root-specific vacuolar Na^+/H^+ antiporter from *Agropyron elongatum*, confers salt tolerance to *Arabidopsis* and *Festuca* plants. Plant Cell Rep 26:1663-1672

Qingxia Z, Xuefeng X, Wang Y, Tianzhong L, Jin K, Zhenhai H (2009) Isolation and preliminary function analysis of a Na^+/H^+ antiporter gene from *Malus zumi*. Afr J Biotechnol 8:4774-4781

Quintero FJ, Blatt MR, Pardo JM (2000) Functional conservation between yeast and plant endosomal Na^+/H^+ antiporters. FEBS Letters 471:224-228

Rachie KO, Roberts LM (1974) Grain legumes of the lowland tropics. Adv Agron 26:1

Rajagopal D, Agarwal P, Tyagi W et al (2007) *Pennisetum glaucum* Na^+/H^+ antiporter confers high level of salinity tolerance in transgenic *Brassica juncea*. Mol Breed 19:137-151

Ramel F, Sulmon C, Bogard M, Couee I, Gouesbet G (2009) Differential patterns of reactive oxygen species and antioxidative mechanisms during atrazine injury and sucrose-induced tolerance in Arabidopsis thaliana plantlets. BMC Plant Biol 9:28

Rao MV, Davis KR (1999) Ozone-induced cell death occurs via two distinct mechanisms. The role of salicyclic acid. Plant J 17:603-614

Rao TP, Yano K, Iijima M, Yamauchi A, Tatsumi J (2002) Regulation of rhizosphere acidification by photosynthetic activity in cowpea (*Vigna unguiculata* L. Walp.) seedlings. Ann Bot 89:213-220

Reguera M, Bassil E, Blumwald E (2014) Intracellular NHX-type cation/H^+ antiporters in plants. Mol Plant 7:261-263

Ren ZH, Gao JP, Li LG, Cai XL, Huang W, Chao DY, Zhu MZ, Wang ZY, Luan S, Lin HX (2005) A rice quantitative trait locus for salt tolerance encodes a sodium transporter. Nat Genet 37: 1141–1146

Rockström J, Steffen W, Noone K, Persson Å et al (2009) A safe operating space for humanity. Nature 461:472-475

Rodriguez-Navarro A, Ramos J (1984) Dual system for potassium transport in *Saccharomyces cerevisiae*. J Bacteriol 159:940-945

Rodríguez-Rosales MP, Gálvez FJ, Huertas R, Aranda MN, Baghour M, Cagnac O, Venema K (2009) Plant NHX cation/proton antiporters. Plant Signal Behav 4:265-276

Rogers SO, Bendich AJ (1988) Extraction of DNA from plant tissues. In: Gelvin SB, Schilperoort RA (eds.) Plant Molecular Biology Manual. Kluwer, Netherlands, pp1-10

Sahoo L, Sugla T, Jaiwal PK (2003) In vitro regeneration and transformation of cowpea, mungbean, urdbean and azukibean. In: Biotechnology for the improvement of

legumes Part-B. Kluwer Acad. Publishers, Netherlands, pp 89–120

Saijo Y, Hata S, Kyozuka J, Shimamoto K, Izui K (2000) Over-expression of a single Ca^{2+}-dependent protein kinase confers both cold and salt/drought tolerance on rice plants. Plant J 23:319-327

Salekdeh GH, Reynolds M, Bennett J, Boyer J (2009) Conceptual framework for drought phenotyping during molecular breeding. Trends Plant Sci 14:488-496

Sambrook KJ, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York.

Schachtman D, Liu W (1999) Molecular pieces to the puzzle of the interaction between potassium and sodium uptake in plants. Trends Plant Sci 4:281-287

Schachtman DP, Schroeder JI (1994) Structure and transport mechanism of a high-affinity potassium uptake transporter from higher plants. Nature 370:655 - 658

Schroeder JI, Delhaize E, Frommer WB, Guerinot ML, Harrison et al (2011) Using membrane transporters to improve crops for sustainable food production. Nature 497: 60-66

Schroeder JI, Delhaize E, Frommer WB, Guerinot ML, Harrison MJ et al (2013) Using membrane transporters to improve crops for sustainable food production. Nature 497:60-66

Sehrawat N, Bhat KV, Sairam RK, Jaiwal PK (2013) Identification of salt resistant wild relatives of mungbean (*Vigna radiata* (L.) Wilczek). Asian J Plant Sci Res 3:41-49

Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? Plant Cell Environ 25:333-341

Serrano R, Rodriguez-Navarro A (2001) Ion homeostasis during salt stress in plants. Curr Opin Cell Biol 13:399-404

Shabala S, Hariadi Y, Jacobsen SE (2013) Genotypic difference in salinity tolerance in quinoa is determined by differential control of xylem Na^+ loading and stomatal density. J Plant Physiol 170:906-914

Shi H, Ishitani M, Kim C and Zhu JK (2000) The Arabidopsis thaliana salt tolerance gene SOS1 encodes a putative Na^+/H^+ antiporter. Proc Nat Acad Sci 97:6896-6901

Shi H, Quintero FJ, Pardo JM, Zhu JK (2002) The putative plasma membrane Na^+/H^+ antiporter *SOS1* controls long-distance Na^+ transport in plants. Plant Cell 14:465-477

Shi H, Zhu JK (2002) Regulation of expression of the vacuolar Na^+/H^+ antiporter gene *AtNHX1* by salt stress and abscisic acid. Plant Mol Biol 50:543-550

Shi H, Wu S-J, Zhu JK (2003) Overexpression of a plasma membrane Na^+/H^+ antiporter improves salt tolerance in Arabidopsis. Nat Biotechnol 21:81–85

Silveira JAG, Melo ARB, Viegas RA, Oliveira JTA (2001) Salinity-induced effects on nitrogen assimilation related to growth in cowpea plants. Environ Exp Bot 46:171-179

Singh BB (2005) Cowpea [*Vigna unguiculata* (L.) Walp]. In: Singh RJ. Jauhar PP (eds) Genetic resources, chromosome engineering, and crop improvement: Grain legumes, vol 1, CRC Press, Boca Raton, Florida, USA, pp 117-162

Singh DP, Singh BB (2011) Breeding for tolerance to abiotic stresses in mungbean. J Food Legumes 24:83-90

Smith AM, Stitt M (2007) Coordination of carbon supply and plant growth. Plant Cell Environ 30:1126-1149

Solleti SK, Bakshi S, Sahoo L (2008a) Additional virulence genes in conjuction with efficient selection scheme and compatible culture regime enhance recovery of stable transgenic plants in cowpea via *Agrobacterium tumefaciens*- mediated transformation. J Biotechnol 135:97-104

Solleti SK, Bakshi S, Purkayastha J, Panda SK, Sahoo L (2008b) Transgenic cowpea (*Vigna unguiculata*) seeds expressing a bean α-amylase inhibitor1 confer resistance to storage pests, bruchid beetles. Plant Cell Rep 27:1841-1850

Sousa MF, Campos FAP, Prisco JT, Enéas-Filho J, Gomes-Filho E (2003) Growth and protein pattern in cowpea seedlings subjected to salinity. Biol Plantarum 47: 341-346

Sun Y, Wang D, Bai Y, Wang N, Wang Y (2006) Studies on the overexpression of the soybean *GmNHX1* in *Lotus corniculatus*: The reduced Na^+ level is the basis of the increased salt tolerance. Chinese Sci Bull 51:1306-1315

Sunarpi, Horie T, Motoda J, Kubo M, Yang H, Yoda K, Horie R, Chan WY, Leung HY, Hattori K (2005) Enhanced salt tolerance mediated by AtHKT1 transporter-induced Na^+ unloading from xylem vessels to xylem parenchyma cells. Plant J 44: 928–938

Szabados L, Savouré A (2010) Proline: a multifunctional amino acid. Trends Plant Sci 15: 89-97

Taffouo V, Meguekam L, Kenne M, Magnitsop A, Akoa A, Ourry A (2009) Salt stress effects on germination, plant growth and accumulation of metabolites in five leguminous plants. Afr Crop Sci Conf P 9:157-161

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596-1599

Tang R, Li C, Xu K et al (2010) Isolation, Functional Characterization, and Expression Pattern of a Vacuolar Na^+/H^+ Antiporter Gene *TrNHX1* from *Trifolium repens* L. Plant Mol Biol Rep 28: 102-111

Teakle NL, Amtmann A, Real D, Colmer TD (2010) *Lotus tenuis* tolerates combined salinity and waterlogging: maintaining O_2 transport to roots and expression of an *NHX1*-like gene contribute to regulation of Na^+ transport. Physiol Plant 139:358-374

Tester M, Davenport R (2003) Na^+ tolerance and Na^+ transport in higher plants. Ann Bot 91:503-527

Tharanathan RN, Mahadevamma S (2003) Grain legumes- a boon to human nutrition. Trends Food Sci Tech 14:507-518

Thiam M, Champion A, Diouf D, Oureye SY M (2013) NaCl Effects on In Vitro Germination and Growth of Some Senegalese Cowpea (*Vigna unguiculata* (L) Walp) Cultivars. ISRN Biotechnol 2013

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgin DG (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882

Tian N, Wang J, Xu ZQ (2011) Overexpression of a Na^+/H^+ antiporter gene *AtNHX1* from *Arabidopsis thaliana* improves the salt tolerance of kiwifruit (*Actinidia deliciosa*). S Afr J Bot 77:160-169

Timko MP, Ehlers JD, Roberts PA (2007) Cowpea. In: Kole (ed) Genome Mapping and Molecular Breeding in Plants, vol 3, Pulses, Sugar and Tuber Crops, SpringerVerlag, Berlin Heildelberg, pp 49-67

Timko MP, Rushton PJ, Laudeman TW et al (2008) Sequencing and analysis of the gene-rich space of cowpea. BMC Genom 9:103

Timko MP, Singh BB (2008) Cowpea, a multifunctional legume. In: Moore PH, Ming R (eds) Genomics of tropical crop plants. Springer, New York, pp 227-258

Tuteja N (2007) Mechanisms of high salinity tolerance in plants. Methods Enzymol 428:419-438

Uozumi N, Kim EJ, Rubio F, Yamaguchi T, Muto S, Tsuboi A, Bakker EP, Nakamura T, Schroeder JI (2000) The *Arabidopsis HKT1* gene homolog mediates inward Na^+ currents in *Xenopus laevis* oocytes and Na^+ uptake in *Saccharomyces cerevisiae.* Plant Physiol 122:1249–1259

Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plants. Curr Opin Plant Biol 9:189-195

Vanblaere T, Szankowski I, Schaart J, Schouten H, Flachowsky H, Broggini GAL, Gessler C (2011) The development of a cisgenic appleplant. J Biotechnol 154:304–311

Van Hoorn JW, Katerji N, Hamdy A, Mastrorilli M (2001) Effect of salinity on yield and nitrogen uptake of four grain legumes and on biological nitrogen contribution from the soil. Agric Water Manage 51:87-98

Venema K, Belver A, Marin-Manzano MC, Rodgriguez-Rosales MP, Donaire JP (2003) A novel intracellular K^+/H^+ antiporter related to Na^+/H^+ antiporters is important for K^+ ion homeostasis in plants. J Biol Chem 278: 22453-22459

Wang J, Zuo K, Wu W, Song J, Sun X, Lin J, Li X, Tang K (2003) Molecular cloning and characterization of a new Na^+/H^+ antiporter gene from *Brassica napus*. DNA Seq 14: 351-358

Wang SM, Zhang JL, Flowers TJ (2007) Low-affinity Na_+ uptake in the halophyte *Suaeda maritima*. Plant Physiol 145:559-571

Wang S, Zhang YD, Perez PG et al (2011) Isolation and Characterization of a vacuolar

Na^+/H^+ antiporter gene from *Cucumis melo* L. Afr J Biotechnol 10:1752-1759

Weinberger K (2005) Assessment of the nutritional impact of agricultural research: the case of mungbean in Pakistan. Food Nutri Bull 26:287-294

West DW, Francois LE (1982) Effects of Salinity on Germination, Growth and Yield of Cowpea. Irrig Sci 3:169-175

Wilkinson S, Davies WJ (2010) Drought, ozone, ABA and ethylene: new insights from cell to plant to community. Plant Cell Environ 33:510-525

Wilson C, Liu X, Lesch SM, Suarez DL (2006) Growth response of major USA cowpea cultivars: II. Effect of salinity on leaf gas exchange. Plant Sci 170:1095-1101

Win KT, Oo AZ, Nwe KL, San Thein M, Yutaka H (2011) Diversity of Myanmar cowpea accessions through seed storage polypeptides and its cross compatibility with the subgenus Ceratotropis. J Plant Breed Crop Sci 3:87-95

Wu SJ, Wang JS, Lin CC, Chang CH (2001) Evaluation of hepatoprotective activity of legumes. Phytomedicine 8:213-219

Wu YY, Chen QJ, Chen M, Chen J, Wang XC (2005) Salt-tolerant transgenic perennial ryegrass (*Lolium perenne* L) obtained by *Agrobacterium tumefaciens*-mediated transformation of the vacuolar Na^+/H^+ antiporter gene. Plant Sci 169:65-73

Wu C, Gao X, Kong X et al (2009) Molecular cloning and functional analysis of a Na^+/H^+ antiporter gene *ThNHX1* from a halophytic plant *Thellungiella halophila*. Plant Mol Biol Rep 27: 1-12

Wu GQ, Xi JJ, Wang Q et al (2011) The *ZxNHX* gene encoding tonoplast Na^+/H^+ antiporter from the xerophytes Zygophyllum xanthoxylum plays important roles in response to salt and drought. J Plant Physiol 168: 758-767

Xin L, Zan W, XueMin W, HongWen G, XiaoFang C, Jie D, Bo X (2009) Cloning and analysis of a vacuole Na^+/H^+ antiporter gene in *Galega orientalis*. Plant Physiol Commun 45: 444-448

Xing J, Wang B, Jia K, Wan S, Meng J, Guo F, Li X (2011) Isolation of *Arachis hypogaea* Na^+/H^+ antiporter and its expression analysis under salt stress. Afr J Biotechnol 10:14302-14310

Xu Y, Zhou Y, Hong S et al (2013) Functional Characterization of a Wheat NHX Antiporter Gene *TaNHX2* That Encodes a K+/H+ Exchanger. PloS One 8:e78098

Xue ZY, Zhi DY, Xue GP, Zhang H, Zhao YX, Xia GM (2004) Enhanced salt tolerance of transgenic wheat (*Triticum aestivum* L) expressing a vacuolar Na^+/H^+ antiporter gene with improved grain yields in saline soils in the field and a reduced level of leaf Na^+. Plant Sci 167:849-859

Yadav S, Irfan M, Ahmad A, Hayat S (2011) Causes of salinity and plant manifestations to salt stress: a review. J Environ Biol 32:667–685

Yamaguchi T, Blumwald E (2005) Developing salt-tolerant crop plants: challenges and opportunities. Trends Plant Sci 10:615-620

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol 57:781-803

Yang Q, Wu M, Wang P, Kang J, Zhou X (2005) Cloning and expression analysis of a vacuolar Na^+/H^+ antiporter gene from Alfalfa. Mitochon DNA 16:352-357

Yang DH, Song LY, Hu J et al (2012) Enhanced tolerance to NaCl and LiCl stresses by over-expressing *Caragana korshinskii* sodium/proton exchanger 1 (*CkNHX1*) and the hydrophilic C terminus is required for the activity of *CkNHX1* in *Atsos3-1* mutant and yeast. Biochem Biophys Res Commun 417: 732-737

Yokoi S, Quintero FJ, Cubero B, Ruiz MT, Bressan RA, Hasegawa PM, Pardo JM (2002) Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J 30:529-539

Yoo CY, Pence HE, Hasegawa PM, Mickelbart MV (2009) Regulation of transpiration to improve crop water use. Crit Rev Plant Sci 28:410-431

Yue Y, Zhang M, Zhang J, Duan L, Li Z (2012) *SOS1* gene overexpression increased salt tolerance in transgenic tobacco by maintaining a higher K^+/Na^+ ratio. J Plant Physiol 169:255-261

Zayed MA, Zeid IM (1997) Effect of water and salt stresses on growth, chlorophyll, mineral ions and organic solutes contents, and enzymes activity in mung bean seedlings. Biol Plantarum 40:351-356

Zhang HX, Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat Biotechnol 19:765-768

Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc Nat Acad Sci 98:12832-12836

Zhang GH, Su Q, An LJ, Wu S (2008) Charcterization and expression of a vacuolar Na^+/H^+ antiporter gene from the monocot halophyte *Aeluropus littoralis*. Plant Physiol Biochem 46: 117-126

Zhang YM, Liu ZH, Wen ZY, Zhang HM, Yang F, Guo XL (2012) The vacuolar Na^+/H^+ antiport gene *TaNHX2* confers salt tolerance on transgenic alfalfa (*Medicago sativa*). Funct Plant Biol 39: 708-716

Zhang H, Liu Y, Xu Y, Chapman S, Love AJ, Xia T (2012) A newly isolated Na^+/H^+ antiporter gene, *DmNHX1*, confers salt tolerance when expressed transiently in *Nicotiana benthamiana* or stably in *Arabidopsis thaliana*. Plant Cell Tiss Org Cult 110: 189-200

Zhou G, Guan R, Li Y, Chang R, Qiu L (2009) Molecular characterization of *GmNHX2*, a Na^+/H^+ antiporter gene homolog from soybean, and its heterologous expression to improve salt tolerance in *Arabidopsis*. Chinese Sci Bull 54:3536-3545

Zhu JK (2001) Plant salt tolerance. Trends Plant Sci 6:66-71

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247

Zhu JK (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441-445

Zorb C, Noll A, Noll A, Karl S, Leib K, Yan F, Schubert S (2005) Molecular characterization of Na^+/H^+ antiporters (*ZmNHX*) of maize (*Zea mays* L.) and their expression under salt stress. J Plant Physiol 162: 55-65