ROLE OF A VACUOLAR SODIUM HYDROGEN ANTIPORTER IN SALT TOLERANCE IN RICE (Oryza sativa L. spp. indica var. Vikas)

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In

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This is to certify that the thesis entitled "Role of a Vacuolar Sodium Hydrogen Antiporter in Salt Tolerance in Rice" submitted by Mr. Abhishek Mazumder, ID No. PAK 9221, in partial fulfillment of the requirement for the award of the degree of Master of Science (Agriculture) in Plant Biotechnology of the University of Agricultural Sciences, Bangalore is a record of bonafide research work done by him during the period of his study in this university under my guidance and supervision and the thesis has not previously formed the basis of award for the degree, diploma, associateship, fellowship or other similar titles.

Bangalore
SEPTEMBER, 2013

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Abhishek Mazumder
ROLE OF A VACUOLAR SODIUM HYDROGEN ANTIPORTER IN SALT TOLERANCE IN RICE (*Oryza sativa* L. *spp. indica* var. *Vikas*)

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Abstract

Rice is the staple food for more than half of the global human population, including 65% of the population in the Indian subcontinent. Soil salinity, that affects large areas of arable land across the globe, is a major threat to rice production, resulting in significant reductions in yield annually. The Na⁺ toxicity of many crop plants is correlated with overaccumulation of Na⁺ in the shoot. One possible mechanism by which plants could survive salt stress is to sequester sodium ions away from the cytosol. Increasing evidence has demonstrated that vacuolar Na⁺/H⁺ antiporters play a pivotal role in plant salt tolerance. Overexpression of a vacuolar Na⁺/H⁺ antiport from *Pennisetum glaucum* (*PgNHX1*) in paddy plants promote sustained growth and development in hydroponics treated with up to 100 millimolar sodium chloride. This salinity tolerance is correlated with higher than normal levels of a vacuolar Na⁺/H⁺ (sodium/proton) antiport activity in transgenic rice compared with the non-transformed control. These results imply that overexpression of a vacuolar Na⁺/H⁺ antiporter gene in transgenic rice enhances salt-tolerance capacity significantly by reducing the toxic effect of cations in the cytosol. Genetically-engineered salt-tolerant plants therefore could provide an avenue to the amelioration of farmlands lost to agriculture because of salinity and erratic rainfall.

Keywords: Na⁺/H⁺ antiporter . Vacuole . Salt Tolerance. *PgNHX1*. *Pennisetum glaucum*, Transgenic rice
Na⁺ / H⁺

Na⁺ / H⁺

Na⁺ / H⁺
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ABBREVIATIONS

► Butylated Hydroxytolune (BHT)
► Dithiothreitol (DTT)
► Ethylene glycol-bis (β-amino-ethyl Ether)-N,N,N’,N’-tetraacetic acid (EGTA)
► Morpholino-ethane sulphonic acid (MES)
► Morpholino-propane sulphonic acid (MOPS)
► Phenylmethane sulfonyl fluoride (PMSF)
► Pyrophosphate (PPi)
► Polyvinylpyrrolidone (PVP)
Introduction
I INTRODUCTION

The global climate change is feared to promote rapid soil degradations in agricultural land worldwide. Soil salinization is one of the serious soil degradations, which can cause from natural causes and human-mediated activity such as irrigation in arid and semi-arid regions. Moreover, global food requirements are expected to increase by 70-110% by 2050 (Tilman et al., 2011), and as land degradation, urban spread and seawater intrusion are increasing over time, gains in agricultural productivity must come from saline soils. Approximately 20% of the irrigated lands in the world are presumably affected by soil salinization (Yeo, 1999). Salinity stress significantly reduces growth and productivity of glycophytes, which are the majority of agricultural products. The term “salinity” represents all the problems of the soil accumulating excessive salts, which can be categorized into sodic (or alkaline) and saline soils (IRRI, 2011). Sodic soils having a poor soil structure generally spread over arid and semi-arid regions, retaining high concentrations of Na\(^+\) at the exchangeable site of clay particles in the soil, which shows a high pH (greater than 8.5) with a high exchangeable sodium percentage (ESP>15) (IRRI, 2011). Saline soils can generally be found in arid regions, estuaries, and coastal fringes, which are dominated by Na\(^+\) ions with electrical conductivity (EC) more than 4 dS/m which corresponds to approximately 40 mM NaCl (IRRI, 2011). More than 800 million hectares of land throughout the world are salt-affected, either by salinity (39 million ha) or the associated condition of sodicity (434 million ha) (FAO, 2008). This is over 6% of the world’s total land area. Most of this salinity, and all of the sodicity, is natural. Apart from natural salinity, a significant proportion of recently cultivated agricultural land has become saline owing to land clearing or irrigation, both of which cause water tables to rise and concentrate the salts in the root zone. Furthermore, the problem is getting aggravated as sea-level rises because of global warming and expansion of irrigated land worldwide (Kader and Lindberg, 2008).
Production in over 30% of irrigated crops and 7% of dryland agriculture worldwide is limited by salinity stress, (FAO, 2010). Irrigated land accounts for only 15% of total cultivated land, but because irrigated land has at least twice the productivity of rainfed land, it produces one-third of the world’s food (Munns, 2009). In India, the area under salinity is 85,65,000 ha and the state of Karnataka has nearly 4 lakh ha under salinity. Moreover, 0.25-0.50 mha of irrigated lands are lost from production, due to increased salt-level (Kader and Lindberg, 2008). Therefore reducing the spread of salinization and increasing the salt tolerance of high yielding crops are important global issues.

At the molecular level, three mechanisms for salinity tolerance have been proposed (Zhu, 2002): (a) homeostasis that includes ion homeostasis, which is mainly relevant to salt stress and osmotic homeostasis or osmotic adjustment (b) stress damage control and repair or detoxification and (c) growth control.

At the physiological and cellular level, the following mechanisms have been shown to be related to salt tolerance

1. Salt exclusion
   (i) Entry into root
      (ii) Xylem loading
2. Osmoregulation
3. Salt extrusion
   a. Physiological level e.g. Salt glands
   b. Molecular level e.g. Transporters (SOS1, gmCAX1)
4. $K^+$/Na$^+$ discrimination or selectivity
5. Salt stress proteins
6. Membrane proteins
7. Calcium
There are three main strategies for the maintenance of low Na\(^+\) concentration:

(i) Restriction of entry
(ii) Extrusion or efflux
(iii) Compartmentation

Plants use three main strategies for the maintenance of a low Na\(^+\) concentration:

(1) **Exclusion-restricting Na\(^+\) entry at the root cortex cells** (Munns, 2005):

Restricting Na\(^+\) entry at the root cells and then into the transpirational stream is critical to prevent a buildup of toxic levels of salt in the shoot. K\(^+\) starvation induces HKT1 (High affinity K\(^+\) transporter) expression in wheat, indicating that it functions in high affinity K\(^+\) uptake, but it also transports Na\(^+\). In *Arabidopsis*, it’s likely that *ATHKT1* is important in regulating Na\(^+\) and K\(^+\) homeostasis.

(2) **Extrusion of Na\(^+\) from root cells into the soil**:

Pumping of Na\(^+\) from the root cells is mediated by the plasma membrane Na\(^+\)/H\(^+\) antiporters which play an important role in preventing the accumulation of Na\(^+\) in cytosol to toxic levels (Kaur and Gupta, 2005).

(3) **Osmotic adjustment**:

Osmotic adjustment is active accumulation of solutes such as inorganic ions (Na\(^+\) and K\(^+\)) and organic solutes (proline, betaine, polyols and soluble sugars) (Sairam and Tyagi, 2004). Compatible solutes have the capacity to preserve the activity of enzymes that are in saline solutions. So saline tolerance requires compatible solutes which accumulates in the cytosol and organelle where these function in osmotic adjustment and osmoprotection (Munns and Tester, 2008).
Compartmentation (Hasegawa and Bressan, 2000, Yamaguchi and Blumwald, 2005):

The vacuolar sodium sequestration is mediated by the Na\(^+\)/H\(^+\) antiporter at the tonoplast using the proton-motive force generated by the vacuolar H\(^+\) translocating enzymes, H\(^+\) ATPases and H\(^+\) inorganic pyrophosphatase (Xue et al., 2004). Manipulating the vacuolar Na\(^+\)/H\(^+\) antiporter to improve Na\(^+\) homeostasis is recognized as an attractive strategy in plants viz. AtNHX1 has been overexpressed in several dicotyledonous plants, including Arabidopsis (Apse et al., 1999), tomato (Zhang and Blumwald, 2001) and Brassica napus (Zhang et al., 2001). These transgenic plants displayed robust salt tolerance and could grow normally and produced fruits and seeds under high saline conditions. Moreover, AtNHX1 also has been introduced into crop plants such as wheat (Xue et al., 2004) improving salt tolerance. In addition, Ohta et al., (2002) also observed that transgenic rice plant overexpressing AgNHX1 from a halophyte plant (Atriplex gmelini) could survive under conditions of 300 mM NaCl for three days while the wild-type plants could not.

Rice is an important cereal crop in India. However its productivity is limited by salinity. Rice is the most salt-sensitive crop among cereals and falls in the group II which includes halophytes and non-halophytes. Although this group also includes Barley in which NaCl concentration of 200 mM inhibits growth by as much as enhanced in rice through genetic engineering, so that the reduction in growth and yield is only 30-40% instead of 80% which would greatly benefit rice productivity in saline areas.

It was demonstrated that the NHX1 gene encoding a vacuolar Na\(^+\)/H\(^+\) antiporter from a highly stress-tolerant millet – Pennisetum glaucum conferred high level of salinity tolerance in rice. Transgenic rice plants overexpressing
*PgNHX1* developed more extensive root system and completed their life cycle by setting flowers and seeds in the presence of 150 mM NaCl (Verma *et al.*, 2007).

In the present study, vacuolar Na\(^+\)/H\(^+\) antiporter from *Pennisetum glaucum* (*PgNHX1*) has been overexpressed in rice (*Oryza sativa* L. *spp. indica* var. *vikas*) under the control of constitutive CaMV35S promoter using *Agrobacterium*-mediated *in planta* transformation approach. Molecular and physiological analysis of relative salt-tolerance capacity of seedlings upto T\(_2\) generation transgenic lines have been performed. The relatively salt-tolerant lines have been selected for analysis in subsequent generations.

**With this background**, the present investigation was carried out with the following objectives,

1. Comparing the transport activity of Na\(^+\)/H\(^+\) antiporter from hydroponically grown T\(_3\) rice (2 months old) transgenic plants (shoots) with that of wild-type under non-stressed condition.
2. Testing the surviving ability of T\(_3\) transgenic rice in comparison with the wild-type under the same condition.
Review of Literature
II REVIEW OF LITERATURE

The ability of plants to survive and maintain their growth under saline conditions is known as salt tolerance. Plants differ greatly in their tolerance of salinity, as reflected in their different growth responses. In terms of their ability to tolerate saline environment, plant species are categorized into two broadly defined groups, i.e., glycophytes and halophytes.

Glycophytes are salt-sensitive plants, including most cultivated species, that do not tolerate long exposure to even mild salinity, whereas halophytes are salt tolerant plants, they effectively manage to convert potentially toxic ions into stable osmolytes, thereby survive in a saline environment (Pardo et al., 2002).

Most agriculturally important plants are glycophytes, so soil salinity represents a significant factor hindering crop yield in large areas of the world. Of the cereals, rice (Oryza sativa) is the most sensitive and barley (Hordeum vulgare) is the most tolerant. Bread wheat (Triticum aestivum) is moderately tolerant and durum wheat (Triticum turgidum ssp. durum) is less so. Tall wheatgrass (Thinopyrum ponticum, syn. Agropyron elongatum) is a halophytic relative of wheat and is one of the most tolerant of monocotyledonous species; its growth proceeds at concentrations of salt as high as in seawater. The variation in salinity tolerance in dicotyledonous species is even greater than in monocotyledonous species (Munns et al., 2008). Some legumes are even more sensitive than rice. Alfalfa or lucerne (Medicago sativa) and halophytes such as saltbush (Atriplex spp.) are very tolerant and continue to grow well at salinities greater than that of seawater. Many dicotyledonous halophytes require quite high concentrations of NaCl (100-200 mM) for optimum growth (Flowers et al., 1997). Arabidopsis, when compared with other species under similar conditions of light and humidity (i.e. at high transpiration rates), is salt-sensitive species.
Fig1. Diversity in the salt tolerance of various species, shown as increase in shoot dry matter after growth in solution or sand culture containing NaCl for at least 3 weeks, relative to plant growth in the absence of NaCl (Munns and Tester, 2008).

2.1 Plant responses to salinity

Salt in soil inhibits plant growth for two reasons. First it reduces the plant ability to take up water, and this leads to slower growth. This is known as osmotic or water deficit response to salinity. Second it may enter the transpirational stream and eventually injure cells in the transpiring leaves, further reducing growth. This is the salt specific or ion-excess effect of salinity.

The two effects give rise to a two phase growth response to salinity:
2.1.1 Osmotic effect:

The first phase of growth response results from effect of salt outside the plant. The salt in the soil solution reduces leaf growth and, to a lesser extent root growth (Munns, 1995). The osmotic component of salinity is caused by excess inorganic ions such as Na\(^+\) and Cl\(^-\) in the environment that decrease the osmotic potential of the soil solution and hence water uptake by the plant root. Uptake of abundantly available Na\(^+\) and Cl\(^-\), therefore, offers a comparatively cheap way to lower the tissue osmotic potential (Mian et al., 2011).

2.1.2 Salt-specific effect:

The second phase of growth response results from toxic effects of salt inside the plant. The salt taken up by the plant concentrates in old leaves: continued transport into transpiring leaves over a long period eventually results in very high Na\(^+\) and Cl\(^-\) concentrations and the leaves die. This is due to the salt load exceeding the ability of the cells to compartmentalize salts in the vacuole. Salt would rapidly build up in the cytoplasm and inhibit enzymatic activity. Alternatively they might build up in the cell walls and dehydrate the cell (Munns, 2005). Potassium toxicity has also been reported in long-term experiments with differentially salinized nutrient solutions, the salt-tolerant *Lycopersicon cheesmanii* was found to be very sensitive to exposure to excessive K\(^+\), much more so when an equivalent amount of Na\(^+\) was added to the nutrient solutions. Chlorosis and slow growth were evident within 3 days after the solution concentration was raised to 50 mM. The plants became progressively more chlorotic and unhealthy in appearance with each K\(^+\) addition, most of them dying when the K\(^+\) concentration of the solution reached 200 mM (Rush and Epstein, 1981).
Figure 2. The growth response to salinity stress occurs in two phases: a rapid response to the increase in external osmotic pressure (the osmotic phase), and a slower response due to the accumulation of Na\(^+\) in leaves (the ionic phase). The solid green line represents the change in growth rate after the addition of NaCl. (a) The broken green line represents the hypothetical response of a plant with an increased tolerance to the osmotic component of salinity stress. (b) The broken red line represents the response of a plant with an increased tolerance to the ionic component of salinity stress. (c) The green-and-red line represents the response of a plant with increased tolerance to both the osmotic and ionic components of salinity stress ( Munns and Tester, 2008).

2.2 K\(^+\)/Na\(^+\) selectivity:

Potassium (K\(^+\)) is an essential macronutrient that fulfills important functions related to enzyme activation, osmotic adjustment and turgor formation, regulation of membrane electric potential, and cytoplasmic pH homeostasis. K\(^+\) is acquired by the roots, redistributed among plant tissues and organs, and stored in large quantities inside vacuoles, and it is the most abundant inorganic cation in plants, comprising up to 10\% of their dry weight (White and Karley, 2010). In contrast,
Na\(^+\) is only essential for a no. of C4 species (for the translocation of pyruvate across the chloroplast envelope) where it functions as a micronutrient. In most other species Na\(^+\) does not act as a nutrient in the sense that it is strictly required for growth, but its addition to the growth medium may promote growth of many plants when the K\(^+\) supply is limited (Flowers and Lauchli, 1983) and in particular the growth of salt tolerant and halophytic plants by contributing to turgor formation.

Although the availability of Na\(^+\) as a cheap osmoticum is generally beneficial, a large excess of Na\(^+\) ions over K\(^+\) is not, for several reasons. Firstly, the similar physicochemical structures of Na\(^+\) and K\(^+\) mean that Na\(^+\) competition at transport sites for K\(^+\) entry into the symplast may result in K\(^+\) deficiency. Secondly, cytoplasmic Na\(^+\) competes for K\(^+\) binding sites and hence inhibits metabolic processes that crucially depend on K\(^+\). Clearly, Na\(^+\) in the cytosol has to be restricted by limiting Na\(^+\) entry and/or operating an efficient system for Na\(^+\) efflux into the vacuole or the apoplast. Therefore, as has been pointed out by many authors (e.g. Yeo, 1998), one of the key elements in salinity tolerance is the capacity to maintain a high cytosolic K\(^+\)/Na\(^+\) ratio.

The K\(^+\)/Na\(^+\) ratio that ultimately prevails in plant cells will depend on the concerted action of transport system located at plasma and vacuolar membranes. They probably involve K\(^+\) selective, Na\(^+\) selective and non-selective pathways. Since ions are hydrated in solution and do not readily travel the hydrophobic lipid bilayer of membrane and flux across the plasma membrane and tonoplast occurs via specialized transport, which can generally be categorized into three main classes:

2.2.1 Pumps

These transporters are fuelled by metabolic energy and able to transport substrates against electrochemical gradients. Turnover rates are low, around 10\(^2\)
per second. A prime example is the ubiquitous H\(^+\) ATPase. No pumps have been identified in higher plants that directly transport K\(^+\) or Na\(^+\).

### 2.2.2 Carriers

They are transport proteins that undergo specific conformational changes during substrate transport. They generally function in transport of substrates against gradients, are energized via coupling to an electrochemical gradient, and have turnover of 10\(^2\)-10\(^3\) per second. In plants, ‘uphill’ (high affinity) accumulation of K\(^+\) is energized through coupling to the ‘downhill’ transmembrane movement of H\(^+\) proceeding via a H\(^+\)-K\(^+\) symporter;

- e.g. High affinity K\(^+\) transporter (HKTI), Low affinity cation carriers (LCTI)

### 2.2.3 Channels

Cation channels are integral membrane proteins that catalyze passive movement of cations through transmembrane pores with turnover rate of 10\(^6\) -10\(^8\) per second (Demidchik et al., 2002)

- e.g. K\(^+\) inward rectifying channels (KIRC), sK\(^+\) outward rectifying channels (KORC), Voltage independent channel (VIC), Cyclic Nucleotide gated channel (CNGC), Depolarization activated non-selective cation channel (DA-NSCC), Hyperpolarization activated non-selective cation channel (HA-NSCC).

### 2.3 Effect of salinity on plant development

Salinity affects almost all aspects of plant development, including germination, vegetative growth and reproductive development. Soil salinity imposes ion toxicity, osmotic stress, nutrient (N, Ca, K, P, Fe, Zn) deficiency and oxidative stress on plants. Salinity also indirectly limits plant productivity through its adverse effects on the growth of beneficial and symbiotic microbes. High salt
concentrations in soil impose osmotic stress and thus limit water uptake from soil. Sodium accumulation in cell wall can rapidly lead to osmotic stress and cell death (Munns, 2002). Ion toxicity is the result of replacement of K\(^+\) by Na\(^+\) in biochemical reactions, and Na\(^+\) and Cl\(^-\) induced conformational changes in proteins. For several enzymes, K\(^+\) acts as a cofactor and cannot be substituted by Na\(^+\).

High K\(^+\) concentration is also required for binding tRNA to ribosome and thus protein synthesis (Zhu et al., 2002 and Tester et al., 2003). Ion toxicity and osmotic stress cause metabolic imbalance, which in turn leads to oxidative stress (Hernandez et al., 2001). The salt-induced production of ROS such as superoxide radicals (O\(_2\)^\(-\)), hydrogen peroxide (H\(_2\)O\(_2\)) and hydroxyl radicals (OH\(^-\)) is counteracted by different detoxifying enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2). Indeed, transgenic rice overexpressing a yeast Mn superoxide dismutase was shown to have improved salinity tolerance (Tanaka et al., 1999).

Salinity adversely affects reproductive development by inhibiting microsporogenesis and stamen filament elongation, enhancing programmed cell death in some tissue types, ovule abortion and senescence of fertilized embryos. In Arabidopsis, 200 mM Nacl stress causes as high as 90% ovule abortion (Sun et al., 2004).

Plants respond to salinity using two different types of responses. Salt-sensitive plants restrict the uptake of salt and adjust their osmotic pressure by the synthesis of compatible solutes (e.g. proline, glycinebetaine and sugars). Salt-tolerant plants sequester and accumulate salt into cell vacuoles, controlling the salt concentrations in the cytosol and maintaining a high cytosolic K\(^+\)/Na\(^+\) ratio in their cells. Ion exclusion mechanisms could provide a degree of tolerance to relatively
low concentrations of NaCl but would not work at high concentrations of salt, resulting in the inhibition of key metabolic processes and concomitant growth inhibition.

### Table 1. Mechanisms of salinity tolerance, organized by plant processes and their relevance to the three components of salinity tolerance (Munns and Tester, 2008)

<table>
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<th>Process involved</th>
<th>Candidate genes&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>Ionic stress</th>
<th>Tissue tolerance</th>
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<td>Modification of long-distance signaling</td>
<td>Control of net ion transport to shoot</td>
<td>Control of vacuolar loading</td>
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<tr>
<td>Shoot growth</td>
<td>?</td>
<td>Decreased inhibition of cell expansion and lateral bud development</td>
<td>Not applicable&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Delay in premature senescence of old (carbon source) leaves</td>
</tr>
<tr>
<td>Photosynthesis</td>
<td><em>ERA1, PP2C, AAPR, PKN3</em></td>
<td>Decreased stomatal closure</td>
<td>Avoidance of ion toxicity in chloroplasts</td>
<td>Delay in ion toxicity in chloroplasts</td>
</tr>
<tr>
<td>Accumulation of Na&lt;sup&gt;+&lt;/sup&gt; in shoots</td>
<td><em>HKT, SOS1</em></td>
<td>Increased osmotic adjustment</td>
<td>Reduced long distance transport of Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Reduced energy spent on Na&lt;sup&gt;+&lt;/sup&gt; exclusion</td>
</tr>
<tr>
<td>Accumulation of Na&lt;sup&gt;+&lt;/sup&gt; in vacuoles</td>
<td><em>NHX, AVP</em></td>
<td>Increased osmotic adjustment</td>
<td>Increased sequestration of Na&lt;sup&gt;+&lt;/sup&gt; into root vacuoles</td>
<td>Increased sequestration of Na&lt;sup&gt;+&lt;/sup&gt; into leaf vacuoles</td>
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<tr>
<td>Accumulation of organic solutes</td>
<td><em>PCS, OTS, MT1D, M6PR, S6PDH, IMT1</em></td>
<td>Increased osmotic adjustment</td>
<td>Alteration of transport processes to reduce Na&lt;sup&gt;+&lt;/sup&gt; accumulation</td>
<td>Accumulation of high concentrations of compatible solutes in cytoplasm</td>
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</table>
2.4 Sodium uptake

Sodium entry into transpirational stream depends on the amount of Na\(^+\) and nonspecific cation transporters (Fig.3) and the proportion of water entry in the apoplastic/bypass pathway into the xylem. Na\(^+\) from the soil gains the initial entry into cells of the root epidermis and cortex. Transport of Na\(^+\) across the root and into the xylem occurs along symplastic, apoplastic and transcellular pathways from the epidermis to the xylem. However, with the deposition of apoplastic barriers (suberin lamellae/casparian band), a higher selectivity for ion movement (both in and out of the stele) is facilitated. The casparian strip in the endodermis plays a crucial role in preventing apoplastic/bypass pathway into the xylem. This feature may help to reduce the Na\(^+\) entry into the transpirational stream. Recently, It has been demonstrated that in rice roots, Na\(^+\) bypass flow is reduced by the deposition of apoplastic barriers, leading to improved plant survival under salt-stress (Krishnamurthy et al., 2011). Silica deposition and polymerization of silicate in the endodermis and rhizodermis block sodium influx through the apoplastic pathway in the roots of rice. (Yeo,1999).

On the basis of similarity between Ca\(^{++}\) inhibition of radioactive Na\(^+\) influx and Na\(^+\) current through non-selective cation channels (NSCC), the later played a significant role in root Na\(^+\) uptake (Maathuis, 2007). However, the exact proportion and conductance of this pathway may vary substantially. For example, employment of the Na\(^+\) reporter dye SBF1 to directly monitor cellular Na\(^+\) level has shown that NSCCs mediate a significantly greater proportion of overall Na\(^+\) uptake in cells of salt-sensitive rice cultivars (Kader and Lindberg, 2005).

Other potential pathways for the toxic Na\(^+\) influx into the roots are via K\(^+\) channels/transporters and HKT transporters. Plant plasma membrane transporters in the HKT family transport sodium (Na\(^+\)) and potassium (K\(^+\)) and play essential part in salt tolerance. Research in Arabidopsis showed that the ‘class 1’ HKT
transporters are Na\(^+\) selective and protect plant leaves from salinity stress by prohibiting toxic sodium over-accumulation in leaves. Class I HKT transporters are expressed in veins that connect nutrient flux between roots and leaves. These transporters are expressed in the living cells surrounding the xylem. Class I HKT transporters remove excess Na\(^+\) from the xylem in Arabidopsis and rice, thereby keeping Na\(^+\) below toxic levels in the photosynthetic leaf tissues. On the other hand, specific ‘class 2 HKT transporters, together with transporters that sequester sodium and potassium in the vacuole. So, pyramiding HKT transporter traits with vacuolar Na\(^+\) sequestration mechanisms provides a potentially powerful platform for molecular breeding and transgenic approaches to improve the salinity tolerance of crops (Schroeder et al., 2013).

The characteristic feature of the HKT transporters is the channel-like Na\(^+\) transport activity in the presence of a large amount of Na\(^+\) (Xue et al., 2011), Unlike the case of Arabidopsis plants, HKT transporters are found to form a gene family in rice (Huang et al., 2008). A Na\(^+\) transporter OsHKT2;1, one of seven OsHKT transporters in a japonica rice cultivar Nipponbare, has been demonstrated to mediate Na\(^+\) influx into roots under K\(^+\) starved conditions (Yao et al., 2010). Given that, OsHKT2;4 transporter exhibited a broad cation transport activity including Ca\(^{++}\) in Xenopus laevis oocytes, and that immunological detection localized the OsHKT2 of OsHKT2;4 in Ca\(^{++}\)-linked processes have been proposed (Lan et al., 2010). More recently, OsHKT2;4 was demonstrated to exhibit a strong K\(^+\) selectivity over divalent cations such as Ca\(^{++}\) and Mg\(^{++}\) with an atypical low Na\(^+\) transport activity compared to other HKT transporters (Horie et al., 2011c). It has been reported that, a gene in the Nax2 locus, TmHKT1;5-A, encodes a Na\(^+\) selective transporter located on the plasma membrane of root cells surrounding xylem vessels, which is therefore ideally localized to withdraw sodium from the xylem and reduce transport of Na\(^+\) to the leaves. Field trials on saline soils demonstrate that TmHKT1;5-A significantly reduces leaf [Na\(^+\) ] and
increase durum wheat grain yield by 25% compared to near-isogenic lines without the Nax2 locus (Munns et al., 2012).

2.5 Ion homeostasis

Plants achieve ion homeostasis by restricting the uptake of toxic ions, maintaining the uptake of essential ions and compartmentalization of toxic ions into the vacuole of specific tissue types. In most crop plants, Na\(^+\) is the primary cause for ion toxicity, and hence, management of cellular Na\(^+\) concentration is critical for salt tolerance (Tester and Davenport, 2003). Sodium ions can be kept below the toxic level in the cytosol by

i) Restricting Na\(^+\) entry at the cortex of root cells
ii) Extrusion of Na\(^+\) from root cells into soil,
iii) Storing Na\(^+\) in the vacuole of mature cells, a process known as “compartmentation”
iv) Osmotic adjustment

2.5.1 Restricting sodium entry into roots

Restricting Na\(^+\) entry into root cells and then into the transpirational stream is critical to prevent a buildup of toxic levels of salt in the shoot. Both glycophytes and halophytes must exclude about 97% of the Na\(^+\) present in the soil at the root surface to prevent toxic levels of Na\(^+\) accumulation in the shoots (Munns et al., 2002).

*Control of xylem loading:*

The prevention of sodium accumulation in shoots by the maintenance of low Na\(^+\) concentrations in the xylem can be effected by minimization of Na\(^+\) entry into the xylem from the root symplast, or by maximization of retrieval backout from the xylem before it reaches sensitive tissues in the shoot. It is possible that
xylem loading could take place into living xylem cells in the young parts of the roots (McCully et al., 1987). Management of Na⁺ movements within plants catalyzes transport in a coordinated manner. To minimize Na⁺ delivery to the shoot in the apoplastic compartment of the xylem, cells in the outer half of the roots need to minimize influx from and/or maximize the efflux to the soil solution; whereas in the inner half of the root, cells would need to be manipulated in opposite directions (Tester and Davenport, 2003). Recently, it has been demonstrated using barley cultivars with differences in their salt tolerance that the extent of salt tolerance of the tolerant cultivars investigated are not necessarily associated with Na⁺ accumulations in xylem sap, but rather related to a significantly higher K⁺ loading property into the xylem stream, which maintains a higher K⁺/Na⁺ ratio in xylem sap and thus in the shoots (Shabala et al., 2010).

2.5.2 Sodium extrusion

Sodium efflux from the root cells prevents accumulation of toxic levels of Na⁺ to the shoot. Molecular genetic analysis in Arabidopsis sos mutants have led to the identification of a plasma membrane Na⁺/H⁺ antiporter, SOS1, which plays a crucial role in sodium extrusion from root epidermal cells under salinity. Sodium efflux by SOS1 is also vital for salt tolerance of meristem cells such as growing root tips and shoot apex as these cells do not have large vacuoles for sodium compartmentation (Shi et al., 2000 & 2002). Isolated plasma membrane vesicles from sos1 mutants show significantly less inherent as well as salt-stress induced Na⁺/H⁺ antiporter activity than vesicles from wild-type plants (Qiu et al., 2002). The expression of SOS1 is ubiquitous, but stronger in epidermal cells bordering the xylem. Thus, SOS1 functions as a Na⁺/H⁺ antiporter on plasma membrane. Sodium efflux through SOS1 under salinity is regulated by SOS signaling pathway.
2.5.3 SOS signaling pathway for ion homeostasis

The discovery of SOS (Salt-overly-sensitive) pathway in *Arabidopsis* paved the way for clarification of how Na\(^+\) is sensed in any cellular system (Zhu, 2002) (Fig. 3). A membrane receptor might be involved in sensing of extracellular Na\(^+\), whereas, membrane proteins or any Na\(^+\)-sensitive enzymes in the cytoplasm might sense intracellular Na\(^+\). The plasma membrane Na\(^+\)/H\(^+\) antiporter SOS1 constitutes one of the possible Na sensors, which is also involved in Na\(^+\) efflux from *Arabidopsis* cells (Shi et al., 2000). Residing in the outer surface of the plasma membrane and containing two putative AGP-like (arabino-galactan proteins-like) domains and two alternatively organized fascinin-like domains, SOS5 also seems as a candidate for being a Na\(^+\) sensor, although, limited data is available about the function of this protein (Shi et al., 2003). Genetic evidence suggests that perception of salt stress leads to a cytosolic calcium-signal that activates the calcium sensor protein SOS3. SOS3 has three calcium-binding EF hands and an N-myristylation motif (Liu and Zhu, 1998; Ishitani et al., 2000). The SOS3 gene product transduces a salt-stress elicited calcium signal by activating SOS2 (Guo et al., 2001). Under salt stress, SOS3 binds to FISL motif of SOS2 and activates its substrate phosphorylation (protein kinase) activity (Halfer et al., 2000). Acivated SOS2 then increases the antiporter activity of SOS1. SOS2 also activates the tonoplast Na\(^+\)/H\(^+\) antiporter that sequesters Na\(^+\) into the vacuole. Recently it has been shown that SOS2 phosphorylates SCaBP8 at its C-terminus but does not phosphorylate SOS3. In-vivo, this phosphorylation was induced by salt-stress, occurred at the membrane, stabilized the SCaBP8-SOS2 interaction, and enhances plasma membrane Na\(^+\)/H\(^+\) exchange activity (Lin et al., 2009). Recently, a SOS family member BjSOS3 has been identified in a salinity tolerant *Brassica juncea* var. CS52 Similar to its ortholog from *Arabidopsis thaliana*, the modeled protein shows typical features of a Ca\(^{++}\) binding protein with four conserved EF hands (Kushwaha et al., 2011).
It has been shown that transgenic Arabidopsis plants overexpressing SOS1 have lower Na\(^+\) in the xylem transpirational stream and in shoots compared with wild-type plants. These plants also have shown enhanced salt tolerance measured in terms of their growth, ability to bolt and flower at increasing concentration of salt stress (50-200 mM NaCl); while control plants became necrotic and have failed to bolt (Shi et al., 2003).

Overexpression of an active form of SOS2 could overcome the salt hypersensitivity of sos2 and sos3 mutants and enhanced salt tolerance of transgenic Arabidopsis (Guo et al., 2004). The SOS1 upregulation was also impaired in sos2 and sos3 mutants. Hence, the SOS3-SOS2 signalling pathway positively regulate salt-stress induced SOS1 gene expression and/or the transcript stability and activity of SOS1 transporter (Shi et al., 2003).

Atienza et al., (2007) has identified a rice plasma membrane Na\(^+\)/H\(^+\) exchanger that, on the basis of genetic and biochemical criteria, is the functional homolog of Arabidopsis (Arabidopsis thaliana) salt overly sensitive 1 (SOS1) protein. The Arabidopsis protein kinase complex SOS2/SOS3, which positively controls the activity of AtSOS1, phosphorylated and stimulated its activity in-vivo and in-vitro. Moreover, OsSOS1 suppressed the salt-sensitivity of a sos1-1 mutant of Arabidopsis in-vivo (Atienza, M et al., 2007).

2.5.4 Compartmentation of Potassium and Sodium

Intracellular compartmentation of toxic solutes is a prerequisite for maintaining cellular integrity. Moreover the compartmentation of Na\(^+\) (and chloride) into the vacuole allows the plants to use NaCl as osmoticum, maintaining an osmotic potential that drives water into the cells. The transport of Na\(^+\) into the vacuoles is mediated by a Na\(^+\)/H\(^+\) antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H\(^+\) translocating enzymes, the H\(^+\)-ATPase and the H\(^+\)-PPiase (Blumwald et al., 1987). In most species, both H\(^+\)
pumps generate pH gradients of 1-2 pH units (acidic inside) and an electrical charge (membrane potential) of 20 to 40 mV that is positive in the vacuolar lumen relative to the cytosol. This fact implies that positively charged K\(^+\) ions are excluded from the vacuole in K\(^+\)-replete cells unless transport is coupled to an energy-dependent uptake mechanism, whereas efflux could be driven by channels that permeate K\(^+\) downhill its electrochemical gradient. Electrophysiological and genetic evidence have shown that upon a decrease in cytoplasmic K\(^+\), slow activating vacuolar (SV) channels release K\(^+\) from vacuoles to assist in the homeostatic partitioning of K\(^+\) between the cytoplasmic and vacuolar pools (Hedrich and Marten, 2011). Since, transport of K\(^+\) into the vacuole in K\(^+\)-replete cells against K\(^+\) electrochemical gradient, a K\(^+\)/H\(^+\) antiporter energized by the pH gradient proceeds across the tonoplast was thought to achieve vacuolar K\(^+\) accumulation (Carden et al., 2003). Scattered evidence indicated that vacuolar Na\(^+\), K\(^+\)/H\(^+\) antiporter (NHX-type exchangers) could serve this critical function in plant cells (Jiang et al., 2010). Recently, it has been reported that the two major isoforms of vacuolar-localized NHX proteins in Arabidopsis, NHX1 and NHX2, play a critical and redundant role in the accrual of K\(^+\) in the vacuole, which in turn, impinges on the ability of plants to generate turgor and sustain osmotic regulation (Barragan et al., 2012). Given the roles of NHX-type transporters in Na\(^+\) and K\(^+\) homeostasis (Apse and Blumwald, 2007), it has been assessed whether the growth of nhx1 nhx2 double knockout mutants was affected by varying the concentrations of K\(^+\) and Na\(^+\) in the growth media. The growth of the wild-type plants in media supplemented with either 30 mM of Na\(^+\) or K\(^+\) or equimolar concentrations of both did not differ significantly. The biomass of nhx1nhx2, however increased at 30 mM Na\(^+\) compared with nhx1 nhx2 grown in control media (i.e. 1 mM Na\(^+\) and 1 mM K\(^+\) ) but remained significantly lower than the biomass of the wild-type plants grown at 30 mM of Na\(^+\). Moreover, in the presence of 30 mM K\(^+\), the growth of nhx1 nhx2 decreased by 30% compared with that of nhx1 nhx2 plants grown in control media, and was only 5% the weight of
the wild-type plants. These results indicate that the ability of \textit{nhx1 nhx2} plants to accumulate K\textsuperscript{+} in favour of Na\textsuperscript{+} was compromised and that these plants were not able to adjust their K\textsuperscript{+} content when challenged with Na\textsuperscript{+}, as seen in wild-type plants, indicating that a high supply of K\textsuperscript{+} was deleterious to \textit{nhx1 nhx2} growth, probably because K\textsuperscript{+} was accumulating to toxic levels in the cytosol (Bassil \textit{et al.}, 2011). Although, little is known about cellular responses to elevated cytosolic K\textsuperscript{+} as changes in cytosolic K\textsuperscript{+} are efficiently modulated by the compartmentation of K\textsuperscript{+} into vacuoles (Leigh, 2001).

The vacuolar Na\textsuperscript{+} sequestration also protects essential enzymatic reactions in the cytoplasm from excess Na\textsuperscript{+} levels while maintaining turgor (Glenn \textit{et al.}, 1999). Therefore compartmentation of Na\textsuperscript{+} into the vacuoles provides an efficient mechanism to prevent the deleterious effects of Na\textsuperscript{+} in the cytosol by maintaining a higher ratio of K\textsuperscript{+}/Na\textsuperscript{+}. Moreover, salt-tolerant barley cultivars were found to exhibit more efficient Na\textsuperscript{+} sequestration in leaves than sensitive cultivars suggesting that Na\textsuperscript{+}/H\textsuperscript{+} antiport activity in leaves could be more predominant mechanisms for barley plants to resist salinity stress (Shabala \textit{et al.}, 2010).
Fig 3. Regulation of ion homeostasis by SOS signaling pathway for salt stress adaptation (Zhu, 2003)
2.5.5 Osmotic adjustment

Soil salinity decreases soil water potential, which leads to osmotic stress. To maintain water uptake during osmotic stress, plants have evolved a mechanism known as osmotic adjustment. Osmotic adjustment is active accumulation of solutes such as inorganic ions (Na\(^+\) and K\(^+\)) and organic solutes (proline, glycinebetaine, polyols and soluble sugars). These molecules play a role in turgor maintenance and osmotic balance, but they are also involved in protection of cell structure from stress.

Many organic osmolytes are presumed to be osmoprotectants, as their levels of accumulation are insufficient to facilitate osmotic adjustment. An osmolyte role has been suggested for glycinebetaine accumulation in maize; comparison of near-isogenic lines with contrasting glycinebetaine showed that the lines were homozygous for the Bet I (glycine betaine accumulation) gene and had a 10-20% higher saline conditions (Saneoka et al., 1995).

Accumulation of these compatible solutes, such as proline and mannitol, also occurs under drought stress and sometimes under other stresses that reduce growth, such as low temperature (Munns et al., 2008). Many studies of genes controlling the synthesis or metabolism of these solutes have indicated their essential role in tolerance to abiotic stresses (Chen et al., 2002). For example, the lower expression of gene encoding proline dehydrogenase (PDH) may contribute to the higher tolerance of Thellungiella halophila compared with its salt-sensitive relative Arabidopsis thaliana (Kant et al., 2006). Enhancement of mannitol accumulations in Arabidopsis by overexpression of a mannose-6-phosphate reductase from celery caused substantial and sustained increases in growth rate and photosynthesis in saline treatment but not drought, suggesting that mannitol protects the chloroplast against salt (Sickler et al., 2007).
A common feature of compatible solutes is that these compounds can accumulate to high levels without disturbing intracellular biochemistry (Bohnert and Jensen, 1996).

Compatible solutes have the capacity to preserve the activity of enzymes that are in saline solutions. So saline tolerance requires compatible solutes which accumulate in the cytosol and organelle where these function in osmotic adjustments and osmoprotection.

2.6 Endosomal exchangers of the NHX family

Phylogeny and subcellular localization.

The Arabidopsis AtNHX1 protein was the first Na\(^+\)/H\(^+\) exchanger identified in plants (Gaxiola et al., 1999). Since then, the number of homologous NHX transporters identified has grown dramatically. DNA sequences encoding NHX proteins from more than 60 plant species, including gymnosperms and dicots and monocot angiosperms, have been deposited in the different datasets of GenBank. With the sole known exception of yeast, where a single NHX1 gene exists, NHE/NHX exchangers are present as multiple isoforms in all genomes sequenced to date. However, luminal localization of the C-terminal domain of the Arabidopsis vacuolar exchanger AtNHX1 has been reported (Yamaguchi et al., 2003). The highest sequence homology among NHXs occurs in the N-terminal part that forms the membrane pore, whereas C-terminal domains are more dissimilar.

On the basis of protein sequence similarity, the NHE/NHX subfamily can be classified in two major groups that have been named plasma membrane (PM) and intracellular (IC) according to their subcellular localization (Brett et al., 2005). The PM group is exclusively present in animal cells, whereas members of the IC group can be found in animals, plants and fungi, with the exception of NHE8-like
exchangers that are found only in animals and would have appeared later in evolution to fulfill specific physiological needs of animal cells (Brett et al., 2005). All plant NHXs characterized to date are assigned to the IC group and can be further divided into two main groups that will be denoted herein as class I and class II. In Arabidopsis, members of the class-I category (AtNHX1-4) are 56-87% similar to each other, whereas AtNHX5 and 6 (class II) are 79% similar but only 21-23% similar to class-I isoforms (Yokoi et al., 2002). All NHX proteins of class I characterized to date are localized in the vacuolar membrane and form a separate clade within the IC group that is composed exclusively of plant exchangers. By contrast, class-II members are found in endosomal vesicles of plants and homologous proteins with various endosomal localizations are also present in animals and fungi. In Arabidopsis and rice, where whole genomic sequence is known, the size of the NHX gene family is almost identical. These data indicate that divergence between class-I and class-II exchangers in plants occurred before the separation of dicots and monocots.

The selectivity for ion substrate seems to be an additional distinctive feature of each subgroup. Vacuolar exchangers of class-I catalyze Na\(^+\)/H\(^+\) or K\(^+\)/H\(^+\) exchange with equal affinity (Venema et al., 2002; Apse et al., 2003), whereas the endosomal class II shows a preference for K\(^+\) over Na\(^+\) as substrate (Venema et al., 2003). Recently, it has been shown that, unlike single knockouts nhx1 and nhx2, double knockout nhx1 nhx2 vacuoles were more acidic and accumulated only 30% of the wild-type K\(^+\) concentration, highlighting the roles of NHX1 and NHX2 in mediating vacuolar K\(^+\)/H\(^+\) exchange (Bassil et al., 2011b). All these differential characteristics suggest that vacuolar and endosomal exchangers play distinct roles in planta.
Fig.4: Phylogenetic tree of intracellular NHE/NHX exchangers (Pardo et al., 2006)
Recently, it has been demonstrated that, unlike other intracellular NHX proteins, NHX5 and NHX6 are associated with punctuate, motile cytosolic vesicles, that colocalize to golgi and trans-golgi network markers (Bassil et al., 2011a). A novel plant vacuolar Na\(^+\)/H\(^+\) antiporter gene AtNHXS1 has been identified recently in Arabidopsis and expression of AtNHXS1 in yeast showed that the antiporter localized to the vacuolar membrane and its expression improved the tolerance of yeast to NaCl, KCl, LiCl and hygromycin B. Measurements of ion transport activity across the intact yeast vacuole demonstrated that the AtNHXS1
protein showed higher \( \text{Na}^+ / \text{H}^+ \) exchange activity and a slightly improved \( \text{K}^+ / \text{H}^+ \) exchange activity (Xu et al., 2010).

### 2.7 Overexpression studies for salt tolerance

Transgenic manipulations of ion homeostasis have developed the possibilities of genetically engineering salt-tolerant crop plants. Genes for salt tolerance have been overexpressed in many plants and results have shown that transgenic plants were performing better under salt-stress compared to wild-type plants. Transgenic *Arabidopsis* plants overexpressing *AtNHX1* have shown significantly higher salt (200 mM NaCl) tolerance than wild-type plants (Apse et al., 1999).

Since tomato is a highly salt-sensitive crop, an effort has been made to improve its salt-tolerance by overexpressing *AtNHX1*. These tomato transgenics grow and produce fruits in presence of very high salt concentrations (200 mM NaCl). Yield and fruit quality of transgenic tomato plants under salinity are equivalent to those of control plants grown under non-stress conditions (Zhang and Blumwald, 2001). Recently, salt-stress tolerant transgenic tomato plants (*Solanum lycopersicum* cv. PED) have been developed by overexpression of the wheat \( \text{Na}^+ / \text{H}^+ \) antiporter gene *TaNHX2* and transgenic plants have substantial amount of relative water content and chlorophyll content under salt stress compared to the wild-type plants and cope better with salt-stress (Yarra et al., 2012). Similar results have been reported for transgenic canola (*Brassica napus* L.) overexpressing *AtNHX1* (Zhang et al., 2001).

Increasing evidence has demonstrated that vacuolar \( \text{Na}^+ / \text{H}^+ \) antiporters play a crucial role in plant salt tolerance. The *Suaeda salsa* vacuolar \( \text{Na}^+ / \text{H}^+ \) antiporter *SsNHX1* has been overexpressed in transgenic rice to investigate whether this can increase the salt tolerance in rice, and to study how overexpression of this gene affected other salt-tolerant mechanisms. It was found that transgenic rice plants
showed markedly enhanced tolerance to salt stress and to water deprivation compared with non-transgenic controls upon salt stress imposition under outdoor conditions. Furthermore, shoot V-ATPase hydrolytic activity was dramatically increased in transgenics compared to that of non-transformed controls under salt-stressed conditions. Physiological analysis also showed that the photosynthetic activity of the transformed plants was higher whereas the same plants had reduced reactive oxygen species generation (Zhao et al., 2006). Recently, it has been reported that the effective expression of SsNHX1 (Na\(^+\)/H\(^+\) antiporter from Salsola soda) confers salt tolerance in transgenic alfalfa (Medicago sativa L.) which could grow in high concentrations of NaCl (upto 400 mM) over 50 days (Li et al., 2011).

Overexpression of AVP1, a vacuolar H\(^+\) pyrophosphatase in Arabidopsis enhanced sequestration of Na\(^+\) into the vacuole and maintained higher relative content in leaves. These plants also show higher salt and drought-stress tolerance than that of wild-type (Gaxiola et al., 2001). Recently, an Arabidopsis vacuolar H\(^+\) pyrophosphatase gene (AVP1) has been expressed in cotton and tested under high-salt and reduced irrigation conditions. The AVP1-expressing cotton plants showed more vigorous growth than wild-type plants under 200 mM NaCl under hydroponic growth conditions (Pasapula et al., 2010). Overexpression of PgNHX1 conferred high level of salinity tolerance in rice. Transgenic rice plants overexpressing PgNHX1 developed more extensive root system and completed their life cycle by setting flowers and seeds in the presence of 150 mM NaCl (Verma et al., 2007). Recently, co-expression of Pennisetum glaucum vacuolar Na\(^+\)/H\(^+\) antiporter and Arabidopsis H\(^+\) pyrophosphatase in tomato plants have enhanced salt tolerance to the transformed tomato plants compared to the AVP1 and PgNHX1 single-gene transgenic plants and the wild-type. These transgenic plants grew well in presence of 200 mM NaCl while wild-type plants exhibited chlorosis and died within 3 weeks. The transgenic line co-expressing AVP1 and PgNHX1 retained more chlorophyll and accumulated 1.4 times more proline as a
response to stress than single gene transformants. Moreover, these transgenic plants accumulated a 1.5 times higher Na\(^+\) content in their leaf tissue than the single gene transformants (Bhaskaran and Savithramma, 2011).

To develop a salt-tolerant upland rice cultivar (*Oryza sativa* L.), *OsNHX1*, a vacuolar type Na\(^+\)/H\(^+\) antiporter gene from rice was transferred into the genome of an upland rice cultivar. T\(_2\) generation plants exhibited increased salt tolerance, showing delayed appearance and development of damage or death caused by salt-stress, as well as improved recovery upon removal from this condition. Several physiological traits such as increased Na\(^+\) content, and decreased osmotic potential in transgenic plants grown in high saline concentrations, further indicated that the transgenic plants had enhanced salt tolerance (Chen *et al.*, 2007). It has also been reported that overexpression of the Arabidopsis SOS1 gene, which encodes a plasma membrane Na\(^+\)/H\(^+\) antiporter, improves plant salt tolerance in *Arabidopsis thaliana*. Transgenic plants overexpressing SOS1 accumulate less Na\(^+\) in the xylem transpirational stream than in the shoot (Shi *et al.*, 2003).

Microarray expression profiles of wild-type plants, a T-DNA insertion knockout mutant of *AtNHX1* (nhx1), and a ‘rescued’ line (*NHX1:: nhx1*) were exposed to both short (12h and 48h) and long (one and two weeks) durations of a non-lethal salt stress to identify key gene transcripts associated with the salt response that are influenced by *AtNHX1*. It was evident that only a small number of other salt-responsive membrane transporter transcripts appeared significantly influenced by *AtNHX1* (Sottosanto *et al.*, 2007).
**Fig 5. Schematic representation of Na\(^+\) transport in plant cells.** Electrogenic H\(^+\) transport in plant cells. Electrogenic H\(^+\) transport (H\(^+\)-ATPase in the plasma membrane and vacuolar membrane) generates gradients of pH and electrical potential difference across the cell and vacuolar membranes. Na\(^+\) ions enter the cell via different channels (AKT1,NORC,NSCC) or carriers (HKT1) and can be translocated out of the cell or into the vacuole by the action of a plasma membrane Na\(^+\)/H\(^+\) antiporter (SOS1) or a vacuolar Na\(^+\)/H\(^+\) antiporter (NHX1) respectively (Yamaguchi and Blumwald, 2005).
Table 3. Salt tolerance in transgenic plants expressing genes involved in ion transporters (Yamaguchi and Blumwald, 2005)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene product</th>
<th>Source</th>
<th>Cellular role(s)</th>
<th>Target plant</th>
<th>Parameter studied</th>
<th>Refs</th>
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<td>Arabidopsis</td>
<td>Na(^+) vacuolar sequestration</td>
<td>Arabidopsis</td>
<td>Biomass</td>
<td>[38]</td>
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<td>Na(^+) vacuolar sequestration</td>
<td>Tomato</td>
<td>Biomass, fruit yield</td>
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<td>Arabidopsis</td>
<td>Na(^+) vacuolar sequestration</td>
<td>Brassica napus</td>
<td>Biomass, oil production</td>
<td>[38]</td>
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<td>Na(^+) vacuolar sequestration</td>
<td>Maize</td>
<td>Biomass</td>
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<td>Arabidopsis</td>
<td>Na(^+) vacuolar sequestration</td>
<td>Wheat</td>
<td>Biomass, grain yield</td>
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<td>GhNHX1</td>
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<td>Cossyrium hirsutum</td>
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<td>Tobacco</td>
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<td>[41]</td>
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<td>Rice</td>
<td>Growth, ion content</td>
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<td>Brassica napus</td>
<td>Na(^+) vacuolar sequestration</td>
<td>Tobacco</td>
<td>Growth, seed yield</td>
<td>[44]</td>
</tr>
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<td>Arabidopsis</td>
<td>Biomass, photosynthesis</td>
<td>[34]</td>
</tr>
<tr>
<td>nhaA</td>
<td>Plasma membrane Na(^+)/H(^+) antiporter</td>
<td>Escherichia coli</td>
<td>Na(^+) extrusion</td>
<td>Rice</td>
<td>Biomass, ion content</td>
<td>[35]</td>
</tr>
<tr>
<td>AVP1</td>
<td>Vacuolar H(^+)-pyro phosphatase</td>
<td>Arabidopsis</td>
<td>Vacuolar acidification</td>
<td>Arabidopsis</td>
<td>Biomass</td>
<td>[46]</td>
</tr>
</tbody>
</table>
Materials and Methods
III MATERIALS AND METHODS

3.1 Plant material

Seeds (Oryza sativa L. spp. indica var. vikas) of rice (T₃ generation) wild-type and transgenic (constitutively overexpressing sodium-hydrogen antiporter in tonoplast membrane) were surface sterilized with 0.1% Bavistin (w/v) and soaked in distilled water overnight. Overnight soaked seeds were allowed to germinate in petriplates. The germinated seedlings were transferred into Styrofoam cups (250 ml, having small bottom outlets, each containing 5 seedlings) filled with soil-like mix (Perlite, coarse grade, 2 to 6 mm) and placed on a sandbed with regular watering for 2 weeks.

3.2 Plant growth in Hydroponics

Two weeks old rice seedlings in cups were placed in plastic trays (5 lit) and suspended over half-strength Hoagland nutrient solution consisting of 1 M KNO₃, 0.69 M Ca(NO₃)₂.4H₂O, 0.48M MgSO₄.7H₂O, 1M KH₂PO₄, 0.55M FeSO₄.7H₂O with the following micronutrients: 9 mM H₃BO₃, 2 mM MnCl₂.4H₂O, 0.1 mM ZnCl₂ and 0.07 mM CaCl₂.2H₂O, dissolved in deionized water. The aerated solution was changed every 2 days interval upto a period of two months.

3.3 Salt stress treatment of rice

After transferring the cups in Hoagland solution, the 2-week-old rice seedlings were subjected to salt stress treatment under the same conditions. NaCl was dissolved in half-strength Hoagland nutrient solution and loaded in plastic trays (the solution was changed every 2 days interval). NaCl concentrations were stepped up in 25 mM/7 days increments with an initial concentration of 25mM until the final concentration (100mM) was achieved. This concentration
was maintained up to 60 days and the stressed seedlings were used for subsequent analysis.

### 3.4 Isolation of Tonoplast Vesicles

Tonoplast vesicles were isolated using two-phase partitioning. All steps have been carried out at 4°C or ice. Leaves were homogenized in isolation buffer containing 250 mM D-Sorbitol powder, 1.5% (wt/vol) PVP, 2.5 mM Potassium metabisulphite, 10 mM Sodium-β-glycerophosphate, 0.45 mM Butylated Hydroxytolune, 50 mM MOPS-KOH (pH=7.6), 5 mM EGTA and 1 mM PMSF. Four ml of homogenization buffer has been used per gram of tissue. The homogenate was filtered through filter cloth and centrifuged at 3600×g for 15 min. The supernatant was then centrifuged at 1,50,000×g for 40 min to obtain a microsomal pellet that was resuspended in a buffer containing 2.7 mM Sucrose, 10 mM Potassium Phosphate (pH=7.80), 1 mM EGTA and 2 mM DTT. An overlay buffer consisting of 250 mM D-Sorbitol powder, 167 mM EGTA, 1 mM DTT and 5 mM MOPS-KOH (pH=7.3) has been added onto the top of the suspension to obtain a two-phase system and centrifuged at 1,20,000×g for 60 min. The interface was collected, diluted with the above-described overlay buffer and centrifuged at 1,50,000 ×g for 30 min. The resulting pellet was resuspended with resuspension buffer [250 mM D-Sorbitol powder, 2 mM DTT and 10 mM Tris-MES (pH=7.5)], aliquot, frozen instantly in liquid nitrogen and stored at -80°C.

### 3.5 Na⁺/H⁺ Exchange Assay

The membrane identity and transport competence of the vesicles were assessed with measurement of the H⁺ transport activity of the tonoplast H⁺ pyrophosphatase. An inside acid pH gradient (ΔpH) has been established in the vesicles by the activity of the H⁺ pump and measured as a decrease (quench) in the fluorescence of acridine orange (a pH sensitive fluorescent dye). Assays (1.7 ml) contained 5μM acridine orange, 1 mM PPi, 100 mM NaCl, 25 mM (NH₄)₂SO₄,
100mM KCl, 1mM MgSO₄, 5mM MOPS-KOH(pH=7.6), 250mM Sorbitol, 50µl of tonoplast vesicle. Reactants were mixed by stirring several times and then placed in a dark chamber in a fluorescence spectrophotometer (HORIBA Job-in Yvon). Reactions were equilibrated in the dark with stirring for 5 min before beginning fluorescence readings, and all additions to reactions were made in a darkened room. Assay was initiated with the addition of PPI and formation of ΔpH was measured at excitation and emission wavelengths of 492 and 525 nm, respectively. Na⁺/H⁺ exchange activity was measured as a Na⁺-induced dissipation of pH gradient (i.e. a Na⁺-induced increase in fluorescence of the dye). When the maximum ΔpH has been formed (reached steady state), NaCl was added to initiate Na⁺ transport. Complete recovery of the fluorescence has been achieved by addition of (NH₄)₂SO₄, once Na⁺ induced increase in fluorescence has reached its maximum (steady state).
Experimental Results
IV RESULTS

The present investigation was carried out to study the Na⁺/H⁺ exchange activity in wild-type and NHX1 transgenic plants using purified tonoplast vesicles. The results obtained from these experiments are presented in this chapter.

4.1 Rice Two-phase Membrane Vesicles are Transport-Competent and Enriched in Tonoplast membrane

Measurement of H⁺ transport in membrane vesicles isolated from shoots of wild-type and transgenic rice using aqueous two-phase partitioning illustrate that the vesicles were transport-competent and enriched in tonoplast. The result indicates that the PPi-induced fluorescence quench was the result of the transport of H⁺ and reflects the formation of a ΔpH in both side of the membrane.

4.2 Wild-type Rice Plants have no Tonoplast Na⁺/H⁺ Exchange Activity

Na⁺ /H⁺ Exchange Activity was examined by using purified tonoplast vesicles. As shown in Fig.6A, a dissipation of the ΔpH was not induced by the addition of Na⁺ to vesicles that had been isolated from wild-type plants grown under non-stress control condition. Hence, no activity could be measured when Na⁺ was added to vesicles isolated from wild-type plants.

4.3 Transgenic Plants overexpressing Nhx1 constitutively have Enhanced Tonoplast Na⁺/H⁺ Exchange Activity

To determine whether Nhx1 has been overexpressed in rice tonoplast, Na⁺ induced dissipation of ΔpH was examined in tonoplast vesicles isolated from the shoots of Nhx1 transgenic rice grown in non-stress control condition. As shown in Fig.6B, a dissipation of the ΔpH was induced by the addition of Na⁺ to vesicles. Hence the transport activity has been found to be higher in transgenics compared to that of wild-type.
Fig. 6 Vesicles isolated from wild-type and transgenic plants are transport-competent and enriched in tonoplast vesicles. (A) Vesicle from wild-type rice does not show a dissipation of ΔpH upon addition of NaCl (100 mM). (B) When the ΔpH formation has reached a steady state, NaCl (100 mM) dissipated the existing ΔpH in transgenic rice tonoplast.
Fig. 7  **Wild-type (non-stress) plants**
WT- wild-type, T- transgenic

Fig. 8 Rice (60 days old) in hydroponics
Fig. 9  Extent of Survival of salt-stressed (100 mM NaCl) plants (2 months old)
Discussion
By 2050, global population is expected to reach from the current 6.7 to 9.2 billion. To feed those people with current crop yields and farming practices, we need to clear, fertilize, and spray vast amounts of wild land. Many of the world’s poorest people farm in areas that are away from ideal, and freshwater sources are decreasing in quantity and quality throughout the world. Clearly, there must be a better way to enhance production through the development of superior varieties of crops that can withstand adverse environmental conditions (Ronald P, 2008). Salinity is one of the major threats in agricultural productivity which is affecting plant growth and development in large terrestrial areas of the world. The detrimental effect of salt accumulation in agricultural soils has influenced ancient and modern civilizations from decades. Approximately 7% of the world’s land including agricultural lands is affected by either salinity or sodium toxicity (FAO, 2010). Therefore, increasing the yield of crop plants in normal soils and in less productive lands, including salinized lands, is an absolute requirement for feeding the world. The development and use of crops that can tolerate high levels of salinity in the soils would be a practical contribution towards addressing the problem.

Efforts to improve crop performance under environmental stresses have not been fruitful because the fundamental mechanisms of stress tolerance in plants remain to be completely understood. The existence of salt-tolerant plants (halophytes) and differences in salt tolerance between genotypes within salt-sensitive plant species (glycophytes) indicates that there is a genetic basis to salt response. Two basic genetic approaches that are currently being used to improve stress tolerance include: (i) exploitation of natural genetic variations, either through direct selection in stressful environments or through the mapping of quantitative trait loci and subsequent marker assisted selection, and (ii) generation
of transgenic plants to introduce novel genes or to alter gene expression levels of the existing genes to affect the degree of salt stress tolerance.

Transgenic approach to engineer salt tolerance can be through various mechanisms. One mechanism involves removal of Na\(^+\) from the cytoplasm by transporting it into the vacuole via Na\(^+\)/H\(^+\) exchangers driven by the electrochemical gradient of protons (H\(^+\)) generated by the tonoplast H\(^+\)-ATPase (V-ATPase) and H\(^+\) pyrophosphatase (V-PPase) (Niu et al., 1995; Qiu et al., 2004). In plants Na\(^+\)/H\(^+\) antiporters catalyze the exchange of Na\(^+\) for H\(^+\) across membrane and have a variety of functions, including maintenance of cellular ion homeostasis and regulation of cytoplasmic pH and cell turgor (Horie and Schroeder, 2004). Increasing evidence has demonstrated that vacuolar Na\(^+\)/H\(^+\) antiporters play a crucial role in plant salt tolerance. Improvement in salt tolerance evoked by overexpression of AtNHX1 was observed in Arabidopsis, Tomato, Brassica, wheat and Tobacco (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001; Xue et al., 2004). Increased salt tolerance was also observed in transgenic rice carrying OsNHX1 (NHX1 from Oryza sativa) and AgNHX1 (NHX1 from Atriplex gmelini) (Fukuda et al., 2004; Ohta et al., 2002). Results indicated that expression of a single Na\(^+\)/H\(^+\) antiporter gene in plants can be effective in reducing Na\(^+\) toxicity. However, thus far the mechanism underlying the enhancement of salt tolerance in Na\(^+\)/H\(^+\) antiporter transformed plants are not yet clear (Tester and Davenport, 2003).

It was envisaged that the NHX1 gene from a highly stress-tolerant source – Pennisetum glaucum in the present study may result in high level of salinity tolerance in rice (Verma et al., 2007). In order to assess whether PgNHX1 has been overexpressed in rice (Oryza sativa spp. indica var. vikas) tonoplast or not and to examine whether it provides a Na\(^+\)/H\(^+\) exchange function, Na\(^+\)-dependent H\(^+\) movements were measured in vacuoles isolated from shoots of wild-type plants and plants overexpressing PgNHX1. Na\(^+\)/H\(^+\) antiport activity was very low in
wild-type plants. In contrast, the activity was higher in vacuoles from transgenic plants. The Na\(^+\) induced dissipation of the proton-gradient (\(\Delta p\)H) formed by quenching of fluorescence of acridine orange, is the evidence of a Na\(^+\)/H\(^+\) antiport mechanism. Complete recovery of the fluorescence was achieved when (NH\(_4\))\(_2\)SO\(_4\) was added in the reaction mixture after Na\(^+\) induced dissipation of \(\Delta p\)H has reached a steady state. The degree of formation of \(\Delta p\)H is inversely related to the extent of leakiness of the vacuole. The observation suggests that in wild-type plants grown under non-stress condition, OsNHX1 function may be repressed. Overexpression of \(PgNHX1\) in transgenic rice may overcome this endogenous repression mechanism.

It has been shown previously that vacuoles isolated from transgenic \(A.thaliana\) leaves overexpressing AtNHX1 displayed increased AtNHX1 Na\(^+\)/H\(^+\) exchange activity (Apse \textit{et al.}, 1999) and that the increase in activity was higher than the relative increase in AtNHX1 protein abundance. Apse \textit{et al.}, (Apse \textit{et al.}, 1999) suggested that, in wild-type plants, under normal growth conditions, AtNHX1 function was repressed and that the overexpression of AtNHX1 helped to overcome the endogenous repression mechanism. It is possible to speculate that AtCAM15, bound to AtNHX1 repressed the Na\(^+\)/H\(^+\) exchange activity in the wild-type plants and that the increase in AtNHX1 protein provided the increase in vacuolar Na\(^+\)/H\(^+\) exchange activity seen in the transgenic plants (Yamaguchi \textit{et al.}, 2005). Results show that the increase in vacuolar Na\(^+\)/H\(^+\) exchange activity of transgenic Arabidopsis overexpressing \(AtNHX1\) was much higher than the relative increase in AtNHX1 abundance (Apse \textit{et al.}, 1999).

**Future line of work**

Future work will focus on examining the Na\(^+\)/H\(^+\) antiport activity of T\(_3\) transgenic rice tonoplast under salt-stress condition and compare that with the wild-type under similar condition. The stability of the transgene expression has to be validated in subsequent generations.
Summary
VI SUMMARY

In the present study, an attempt was made to compare the \(\text{Na}^+ / \text{H}^+\) antiport activity of \(T_3\) transgenic rice plants which constitutively overexpress a vacuolar \(\text{Na}^+ / \text{H}^+\) antiporter (\(Pgnhx1\)) from a salt-tolerant millet (\(Pennisetum glaucum\)) with that of wild-type plants. Constitutive gene expression was employed to engineer vacuolar \(\text{Na}^+\) compartmentation capacity of a salt-sensitive cultivar (\(Oryza sativa\) spp. \(indica\) var. Vikas) and hence \(\text{Na}^+\) exclusion from cytosol to reduce \(\text{Na}^+\) toxicity and to increase retrieval of \(\text{Na}^+\) from transpirational stream, thereby, enhancing salt tolerance of the plants.

The experiments have shown a higher than normal activity of \(\text{Na}^+ / \text{H}^+\) antiporter in transgenic shoots than that of wild-type plants grown hydroponically under non-stress control condition. This result indicates that in wild-type plants, the activity of the endogenous antiporters may be repressed. To test the effect on salinity tolerance of constitutive expression of \(PgNHX1\); plants were grown hydroponically and salt-stress treatment was imposed with a stepwise increase of Nacl concentration every week from 25 mM to 50, 75 and 100 mM for 2 months. Wild-type plants displayed progressive chlorosis, and stunted growth when grown in this condition. These inhibitory effects increased with increase in Nacl concentration in culture solution. In contrast, growth of transgenics were not severely compromised as that of wild-type and transgenic plants have shown a much higher survival percentage than that of wild-type plants when treated in the same way.

These results clearly demonstrate that transgenic rice plants overexpressing \(PgNHX1\), a vacuolar \(\text{Na}^+ / \text{H}^+\) antiporter, confer better salt tolerance. This could have been mediated by sequestration of excess \(\text{Na}^+\) from cytosol into the vacuole which is driven by \(NHX1\) and thereby reducing the toxic effects of \(\text{Na}^+\) in the cell. Since rice (\(Oryza sativa\) spp. \(indica\) var. vikas) is a glycophytic plant with a
sensitivity to salt similar to most other crop plants, the findings suggest the feasibility of genetically engineering crop plants with improved salt tolerance.
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