Cloning and characterization of an *Arabidopsis thaliana* vacuolar Na\(^+\)/H\(^+\) antiport—AtNHX3

by

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A thesis submitted in conformity with the requirements
For the degree of Master of Science
Graduate Department of Botany
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Master of Science, 2001, Graduate Department of Botany

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**ABSTRACT**

In *Arabidopsis*, overexpression of vacuolar Na⁺/H⁺ antiport, AtNHX1, increases salt tolerance of the plant. Genomic DNA database search indicates that there are four other genes that are homologous to AtNHX1.

In this thesis, the cloning and characterization of AtNHX3 is reported. The ORF of AtNHX3 is about 1.5 kb, coding for 521 amino acids. AtNHX3 protein shares about 35% identical sequence with AtNHX1 and AtNHX2. Western blotting shows that AtNHX3 is located on tonoplast. Northern-blotting and semi-quantitative RT-PCR suggest that AtNHX3 mRNA is in root, stem, leaf and flower, and there is no significant difference for mRNA levels between young seedlings and adult plants. Furthermore, AtNHX3 mRNA levels do not change in response to salt treatment. AtNHX3 can complement a yeast salt sensitive mutant, Δnhx1, and overexpression of AtNHX3 in *Arabidopsis* greatly increases salt tolerance of the plant. Yeast complementation studies suggest that C-terminus of AtNHX3 is necessary for the full function of this antiport.
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## Abbreviations:

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CBBR</td>
<td>Coomassive brilliant blue R-250</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene-diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis-(β-aminoethylether)-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>HKT&lt;sub&gt;1&lt;/sub&gt;</td>
<td>High affinity K&lt;sup&gt;+&lt;/sup&gt; transporter 1</td>
</tr>
<tr>
<td>KDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>KIR</td>
<td>K&lt;sup&gt;+&lt;/sup&gt; inward-rectifying carriers</td>
</tr>
<tr>
<td>mA</td>
<td>Milliampere</td>
</tr>
<tr>
<td>MES</td>
<td>2-(N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td>MMLV RT</td>
<td>Moloney Murine Leukemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>MS</td>
<td>Murashige and Skoog medium</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>NC</td>
<td>Nitrocellulose</td>
</tr>
<tr>
<td>NORC</td>
<td>Non-selective outward-rectifying conductance</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamid gel electrophoresis</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethysulforyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>PVP-40</td>
<td>Polyvinylpyrrrolidne (average MW of 40,000 daltons)</td>
</tr>
</tbody>
</table>
PVPP  Polyvinylpolypyrrolidone
SDS  Sodium dodecyl sulfate
Tris  Tris (hydroxymethyl) aminomethane
VIC  Voltage-insensitive monovalent-cation channels
Table of contents

Abstract
Acknowledgements
List of abbreviations
Table of contents
List of figures

Introduction

1.1. A glance of salt stress in halophytes and glycophytes
1.2. How does NaCl affect plants? 3
   1.2.1. Primary effects of salt stress on plant 3
   1.2.2. Secondary effects of salt stress on plant 4
   1.2.3. Biochemical mechanisms of adaptation to salt stress 4
1.3. Molecular mechanism of Na⁺ influx, efflux and compartmentation in plant cells
   1.3.1. Na⁺ influx into plant cells 6
   1.3.2. Na⁺ extrusion and sequestration
      1.3.2.1. Na⁺ extrusion at plasma membrane 9
      1.3.2.2. Sequestration of Na⁺ in vacuoles 10
1.4. Calcium and salt tolerance
1.5. Two genetic models in salt tolerance research 15

Objectives

Materials and methods

2.1. Plant material and transformation 19
2.2. Cloning of AtNHX3 gene
   2.2.1. 5'-RACE to obtain the 5' end start codon of AtNHX3 21
   2.2.2. 3'-RACE to obtain the full length of AtNHX3 21
2.3. Preparation and purification of anti-X3-GST fusion protein antibody 22
2.4. Electrophoresis
2.5. Protein concentration determination 24
2.6. SDS-PAGE 24
2.7. Electroblotting of protein 25
2.8. Western blot 26
2.9. Cellular membrane protein preparation 26
2.10. RNA preparation from Arabidopsis plant
   2.10.1. Large scale RNA preparation 27
   2.10.2. Small scale RNA preparation 28
2.11. Northern hybridization
   2.11.1. RNA gel electrophoresis 29
   2.11.2. Transfer of RNA to Hybond-N⁺ membrane 30
   2.11.3. DNA probe labeling 30
List of figures:

Fig.1. Common osmolytes 6
Fig.2. NaCl related transport protein on plasma membrane and tonoplast 9
Fig.3. Diagram of SOS pathway 15
Fig.4. The cDNA sequence of AtNHX3 42
Fig.5. The pileup of amino acid sequences of AtNHX3-1 and human NHE6 44
Fig.6. The hydrophobicity of AtNHX3 46
Fig.7. The antibody against AtNHX3-GST fusion protein 48
Fig.8. The subcellular distribution of AtNHX3 50
Fig.9. AtNHX3 mRNA tissue distribution—Northern blot 52
Fig.10. AtNHX3 mRNA tissue distribution—semi-quantitative RT-PCR 54
Fig.11. Salt tolerance test of different yeast transformants 56
Fig.12. The expression of AtNHX3 and AtNHX3-S in yeast 58
Fig.13. Salt tolerance of AtNHX3 transgenic seeds 60
Fig.14. Comparison of salt tolerance of AtNHX3-L2 and WT plant 62
Fig.15. Comparison of AtNHX3 mRNA level in AtNHX3-L2 and WT plant 64
Introduction:

Crop productivity has been seriously affected by salinity stress worldwide. About one third of the world's irrigated land is affected by high salinity (Epstein et al., 1980). Unfortunately, most crop species, such as rice, wheat, corn, barley, and others, are salt sensitive. Intensive research on the mechanisms of salt stress and salt tolerance aimed to increase plant productivity has been carried out in the last two decades.

1.1. A glance of salt stress in halophytes and glycophytes:

Halophytes are plants that are able to grow in high salinity environment. The growth of halophytes require an optimal electrolyte (typically Na⁺ and Cl⁻) concentration (Flowers et al., 1977), but the range of NaCl concentration in which halophytes can survive is quite broad, roughly from 20 to 500 mM NaCl (Hasegawa et al., 2000). No comprehensive classification of halophyte species has yet been made due to the problem in defining the lowest salt concentration at which a plant should be considered as a halophyte (Glenn et al., 1999). Contrarily, glycophytes are those plants that are sensitive to high NaCl concentration and their growth is inhibited by NaCl concentrations as low as 25 mM (Greenway and Munns, 1980).

Research with halophytes has given a clear picture of their mechanisms for salt tolerance. It has been found that although halophytes store high NaCl level inside cells, the concentration of NaCl in the cytosol is still within nontoxic limit: from 10 to 150 mM (Binzel et al., 1988; Cheeseman, 1988). The compartmentation of NaCl in vacuoles is the major mechanism of salt detoxification in halophytes, while salt-sensitive plants mainly rely on the extrusion of Na⁺ at the plasma membrane (Blumwald et al., 2000).
some cases, this could be explained by the absence or relatively low level of vacuolar Na\(^+\)/H\(^+\) antiport activity in glycophytes. For example, in *Plantago* species, the vacuolar Na\(^+\)/H\(^+\) antiport activity is only detected in salt-tolerant *Plantago maritima*, but not in the salt-sensitive *Plantago media* (Staal et al., 1991). In halophytes, the vacuolar Na\(^+\)/H\(^+\) antiport activity is usually upregulated by high NaCl concentration. It has been reported that the vacuolar Na\(^+\)/H\(^+\) antiport activity increases after *Mesembryanthemum crystallium* is exposed to salt treatment (Barkla et al., 1995). However, the mechanisms that regulate the vacuolar Na\(^+\)/H\(^+\) antiport activity or its expression are still unknown. Moreover, halophytic plant cell vacuolar membranes may have modified lipid composition which can prevent the leakage of Na\(^+\) back to the cytosol (Glenn et al., 1999). Some moderate salt tolerant halophytes can also extrude Na\(^+\) and Cl\(^-\) through glands and bladders (Fitzgerald et al., 1992).

Another major difference between halophytes and glycophytes is that halophytes store about 90% of Na\(^+\) in the shoot, and at least 80% in the leaves. Therefore, basically the root parts have a much lower Na\(^+\) concentration than the aerial parts of the plant (Flower et al., 1977), whereas glycophytes restrict ion movement to the shoot by controlling the influx of ions into the xylem (Hasegawa et al., 2000).

Little is known about how NaCl fluxes are regulated into halophytic plant tissues and cells. It has been suggested that in addition to Na\(^+\) and Cl\(^-\) channels, other non-specific gated cation and anion channels or even intracellular vesicles are the pathways for the influx of NaCl (Glenn et al., 1999).

During osmotic stress, both halophytes and glycophytes achieve a common adjustment pathway which is accumulation of organic solutes in the cytosol and organelles (Niu et
al., 1995). Additionally, halophytes also use Na\(^+\) ions stored in vacuoles to drive water into plant cells from salty soil. Therefore, there are two functions of accumulation of Na\(^+\) in vacuoles, avoiding its toxicity to biochemical reactions and using it as an osmoticum in the vacuole.

1.2. How does NaCl affect plants?

At the whole plant level, glycophytic plants under salt stress grow slower, display an increase in root/shoot ratio, and even die (Munns and Termatt, 1986).

At the cellular level, the detrimental effects of salt on glycophytes can be characterized as primary and secondary. The primary effects include water stress (or osmotic stress) and sodium-specific stress (Blumwald et al., 2000). Secondary effects include oxidative damage, decreased CO\(_2\) fixation and photosynthesis, and inhibition of protein synthesis (Hurkman and Tanaka, 1988) or increased turnover of some proteins (Amtmann and Sanders, 1999; Zhu, 2001).

1.2.1. Primary effects of salt stress on plant:

Water stress is caused by the high solute concentration in the salty soil, generating a soil water potential more negative than that of the plant cells, and driving water out of plant cells. The water stress brings the initial growth reduction to the plant. The mechanisms by which water stress inhibits cell division and expansion, as well as accelerates cell death have not been yet elucidated (Yeo, 1998).

Ion-specific stresses include decreased cytosolic K\(^+\)/Na\(^+\) ratios and higher than normal Na\(^+\) and Cl\(^-\) concentrations in the cytosol where many key biochemical reactions occur.
Under typical physiological conditions, plant cells keep a high cytosolic K+/Na+ ratio with K+ concentration between 100-200 mM and that of Na+ between 1-10 mM. In vitro experiments have shown that protein synthesis is inhibited by 100 mM Na+ (Wyn Jones and Pollard, 1983). On the other hand, K+ activates many enzymes through the regulation of protein conformation and also by acting as a co-factor (Raven et al., 1992).

The toxicity of Na+ can be explained by the following two reasons. First, since Na+ is smaller than K+, the high charge:mass ratio of Na+ disrupts water structure and lowers hydrophobic interactions within proteins, therefore destabilizing protein structure. Second, Na+ inhibits enzyme function by either binding to inhibitory sites or displacing K+ from its activation sites (Amtmann and Sanders, 1999).

1.2.2. Secondary effects of salt stress on plant:

As a consequence of the primary effects, plants adapted some structural changes, such as stomatal closure to prevent water evaporation. But the closure of stomata decreases the CO2 uptake, which in turn inhibits photosynthesis and generates additional amounts of reactive oxygen species (ROS). Other secondary effects include membrane disorganization, metabolic toxicity, and attenuated nutrient acquisition (Yeo, 1998).

1.2.3. Biochemical mechanisms of adaptation to salt stress:

As mentioned before, plants accumulate some compatible solutes in response to water stress. Frequently observed osmolytes include sugar (mainly sucrose and fructose), sugar alcohols (glycerol, methylated inositol), and complex sugars (trehalose, raffinose, fructans). Other osmolytes such as charged metabolites (glycine betaine, dimethyl-1-4-
carboxyl sulfonium propionate DMSP), proline and ectoin are also observed (Hasegawa et al., 2000). Different species accumulate different metabolites for osmoprotection. For example, Arabidopsis accumulates proline after salt stress (Delauney and Vermas, 1993), while DMSP synthesis is increased after salt treatment in many algae (Trossat et al., 1998). The importance of proline in salt tolerance was demonstrated by several Arabidopsis proline dehydrogenase (ProDH) antisense lines, which showed a higher salt tolerance correlated with a higher proline accumulation than the wild type plant (Nanjo et al., 1999). Osmolytes are usually hydrophilic (Fig.1), which suggests that they could replace water at the surface of proteins, protein complexes, or membranes to act as osmoprotectants without bringing out any effect on normal biochemical reactions.

Another suggested function of osmolytes is the prevention of oxygen radical production or the scavenging of reactive oxygen species (ROS), the product of secondary effect of salt stress. The second function is thought to be even more important than the first one, because sometimes these osmolytes are found in vivo in a concentration too low to regulate the osmotic balance (Noctor and Foyer, 1998). The importance of osmolytes in scavenging ROS during salt tolerance is supported by the obtainment of several salt stress mutants. The catalase-antisense tobacco plants had higher salt sensitivity (Willekens et al., 1997); the ascorbic acid-deficient Arabidopsis plants, soz/ mutant (semi-dominant ozone-sensitive 1), had significantly higher sensitivity to oxidative stress due to much lower accumulation of ascorbate (Conklin et al., 1996). The Arabidopsis pst1 mutant (photoautotrophic salt tolerance 1), which has deficiency in an unknown putative negative regulator of oxidative stress response, is more tolerant to NaCl (Tsugane et al., 1999). Several transgenic Arabidopsis lines overexpressing genes which
are responsible for removing ROS, such as glutathion peroxidase, superoxide dismutase, ascorbate peroxidases and glutathion reductases (Roxas et al., 1997; Allen et al., 1997), also have higher salt tolerance through osmolyte detoxification strategy (Hasegawa et al., 2000).

![Common osmolytes](image)

**Fig.1.** Common osmolytes. (From Hasegawa et al., 2000)

1.3. Molecular mechanisms of Na\(^+\) influx, efflux and compartmentation in plant cells:

1.3.1. Na\(^+\) influx into plant cells:

Na\(^+\) enters plant cells through passive diffusion, which means that the influx of NaCl doesn't need input of energy since the movement is down the electrochemical potential of Na\(^+\). Like other charged ions, Na\(^+\) must be transported into plant cells by some specific
transport proteins in the plasma membrane. These transport proteins are classified as carriers or channels. If the transport proteins need to change their conformation in order to transport ions or other solutes, they are called carriers. If the proteins form pores in the membrane, they are called channels (Taiz and Zeiger, 1998). Transport proteins can be specific to a particular ion, but, sometimes a particular transport protein can also transport a family of related substances. For example, some K⁺ transporters also mediate the flux of Na⁺.

Until now, no Na⁺ specific inward transporter at the plasma membrane has been found in plant cells. Since the ionic radii of Na⁺ and K⁺ are very similar, it is difficult to discriminate between them. It's believed that Na⁺ is transported into plant cells through various K⁺ channels and other cation channels (White, 1999) (Fig. 2). The possible pathways for Na⁺ influx include inward-rectifying K⁺ carriers (KIR), outward-rectifying K⁺ channels (KOR), and voltage-insensitive monovalent-cation channels (VIC) (White, 1997; Amtmann and Sanders, 1999; Schachtman and Liu, 1999; Tyerman and Skerrett, 1999).

There are low- and high-affinity K⁺ transporters. Low-affinity K⁺ carriers, such as AKT1 (Sentenac et al., 1992), have a high K⁺/Na⁺ selectivity at physiological external K⁺ and Na⁺ concentrations. Nevertheless, they could also mediate a significant Na⁺ uptake when external Na⁺ concentration increases. High-affinity K⁺ carriers, such as HKT1 found in wheat root, function as a Na⁺/K⁺ symporter (Rubio et al., 1995). HKT1 has two binding sites, one for Na⁺ and the other for both Na⁺ and K⁺ (Gassmann et al., 1996). It has been found that under high external Na⁺ concentration (millimolar), Na⁺ competes for K⁺ binding site thus blocking the K⁺ uptake. This suggested that HKT1 may be an
important channel for Na\(^+\) influx under salt stress (Rubio et al., 1995). However recently, it has been reported that Arabidopsis HKT1 homolog, AtHKT1, had an altered transport property, which is that AtHKT1 is a Na\(^+\) selective transporter (Uozumi et al., 2000). Uozumi et al (2000) reported that AtHKT1 inhibited the growth of yeast in high salt concentration medium, and it could not complement a yeast mutant deficient in K\(^+\) uptake.

Outward-rectifying K\(^+\) channels could mediate the influx of Na\(^+\) while mediating the efflux of K\(^+\). One example is NORC, non-selective outward-rectifying conductance. NORC doesn't discriminate between cations and is activated by increased cytosol Ca\(^{2+}\) concentration (Wegner and De Boer, 1997).

In addition to these voltage-dependent (inward- and outward-rectifying) cation channels, more and more studies are showing the presence of voltage-independent cation channels (VIC) in the plant plasma membrane (Arntmann et al., 1997; Roberts and Tester, 1997). VICs generally have much lower selectivity for K\(^+\) over Na\(^+\), and they are inhibited by Ca\(^{2+}\) (White and Tester, 1992; White and Lemtiri-Chlieh, 1995). The evidence supporting the idea that VICs are the major pathway for the bulk Na\(^+\) influx into the root cells is that Na\(^+\)-currents in protoplasts from cereal roots and currents-through VICs are identical (Tyerman and Skerrett, 1999). The physiological function of VICs is unknown, but Arntmann and Sanders (1999) suggested that under salinity condition, VICs might mediate voltage-independent cation uptake to quickly adjust the turgor. Until now, none of the VIC genes has been cloned yet.
1.3.2. Na⁺ extrusion and sequestration:

1.3.2.1. Na⁺ extrusion at plasma membrane:

The transport of Na⁺ out of plasma membrane is an active process which is coupled to the downhill transport of H⁺ to the cytosol. In higher plant species, Na⁺ extrusion is fulfilled by the cooperation of two transporters at the plasma membrane, Na⁺/H⁺ antiport and H⁺-ATPase. The Na⁺/H⁺ antiport is the major mediator for transporting Na⁺ out of plasma membrane; H⁺-ATPase is the one which generates the electrochemical H⁺
gradient across the plasma membrane (proton motive force). In algae and yeast, a Na\(^+\)-ATPase is also found at the plasma membrane which can mediate the efflux of Na\(^+\) coupled to the hydrolysis of ATP (Haro et al., 1991). In yeast, the plasma membrane Na\(^-\)-ATPase is responsible for the extrusion of Na\(^+\) under alkaline extracellular conditions. But at acid pH (lower than 7), the Na\(^+\) extrusion is mainly mediated by Na\(^+\)/H\(^+\) antiport (Prior et al., 1996; Banuelos et al., 1998). Until now, only one putative plasma membrane Na\(^+\)/H\(^+\) antiport gene from Arabidopsis thaliana was cloned through seeking salt oversensitive mutants, which is called SOS1 (Shi et al., 2000) (Fig.2).

### 1.3.2.2. Sequestration of Na\(^+\) in vacuoles:

Vacuoles of plant cells have multifunctions. They play important roles in digestion of cytoplasmic constituents and homeostasis of the plant cells. They are involved in the control of cell volume and cell turgor; the regulation of cytoplasmic ions and pH; the storage of metabolites (amino acids, sugars and CO\(_2\)) and the sequestration of toxic ions (Na\(^+\), Cl\(^-\)) (Marty, 1999). Vacuoles are acidic, usually around pH 5.5, but the pH of lemon fruit vacuoles can be as low as 2.5 (Taiz and Zeiger, 1998). Among these functions, the sequestration of toxic ions is crucial to plant salt tolerance. The proteins involved in Na\(^+\) transport across the tonoplast include vacuolar H\(^+\)-ATPase (V-H\(^+\)-ATPase), vacuolar-inorganic pyrophosphatase (V-PPiase), one or more sodium-proton antiport(s) and a chloride channel (Barkla et al., 1995; Gaxiola et al., 1999). V-H\(^+\)-ATPase and V-PPiase hydrolyse ATP and pyrophosphate respectively, and the energy released is used to generate the proton motive force, i.e., the gradient of electrochemical potential of H\(^+\), which is used by the Na\(^+\)/H\(^+\) antiport to transport the Na\(^+\) into vacuoles.
against its electrochemical potential (Wang and Sze, 1985; Rea et al., 1992). There are some evidence showing positive responses of these genes to salt stress. The V-H\textsuperscript{+}-ATPase genes as well as the plasma membrane ATPase genes are salt stress inducible (Binzel, 1995). The expression of Arabidopsis thaliana V-PPiase in yeast salt sensitive mutant, Δenal, confers high salt tolerance to yeast (Gaxiola et al., 1999). Recent study on the cation detoxification in yeast led to the discovery of the role of a chloride channel, Gef1, in salt tolerance. It is believed that the function of Cl\textsuperscript{-} is to neutralize the extra positive charges brought up by the sequestration of Na\textsuperscript{+} ions in vacuoles (Gaxiola et al., 1999). The homologue of Gef1 in Arabidopsis thaliana has also been cloned, which is called At-CLCd (Hechenberger et al., 1996). There is evidence showing that on the plant vacuolar membrane, the proton motive force generating transporters, Cl\textsuperscript{-} channels, and the Na\textsuperscript{+}/H\textsuperscript{+} antiports work together to fulfill the compartmentation of NaCl (Blumwald et al., 2000) (Fig. 2).

Although it is not the only mechanism of salt tolerance, sequestration of Na\textsuperscript{+} in vacuoles plays significant roles in Na\textsuperscript{+} detoxification. The vacuolar Na\textsuperscript{+}/H\textsuperscript{+} antiport activity was first measured from cultured Beta vulgaris cells by Blumwald and Poole in 1985. They used the fluorescence quenching of acridine orange, a fluorescent weak base, to monitor the Na\textsuperscript{+}-dependent H\textsuperscript{+} efflux from tonoplast isolated from cultured Beta vulgaris cells. In 1987, Blumwald et al. reported the inhibition of Na\textsuperscript{+}/H\textsuperscript{+}-exchange activity by amiloride and its analogues, which is a common characteristic of Na\textsuperscript{+}/H\textsuperscript{+} exchangers from human cells. Furthermore, H\textsuperscript{+}-dependent Na\textsuperscript{+} fluxes have been monitored by measuring \textsuperscript{22}Na\textsuperscript{+}-uptake in tonoplast vesicles. It has been found that the
Na⁺ movement into the vesicles was dependent on the pH gradient between the interior of tonoplast and the surrounding medium (Barkla et al., 1990).

The cloning of a yeast prevacuolar Na⁺/H⁺ antiport, ScNHX1, confirmed the existence of an intracellular Na⁺/H⁺ antiport in molecular level (Gaxiola et al., 1999). With the aid of Arabidopsis thaliana genomic DNA database, a vacuolar Na⁺/H⁺ antiport gene, AtNHX1 has been cloned for the first time from higher plant species (Gaxiola et al., 1999; Apse et al., 1999). Soon after that, several vacuolar Na⁺/H⁺ antiport genes from Arabidopsis thaliana were cloned (unpublished data, 2001).

The importance of Na⁺/H⁺ antiport in salt tolerance has been demonstrated at the physiological, molecular and genetic levels. First, Blumwald et al. reported that the activity of Na⁺/H⁺ antiport increased upon the treatment of NaCl in cultured Beta vulgaris cells (Blumwald et al., 1987). At the genetic level, the deficiency in the ScNHX1 gene made the yeast strain sensitive to 200 mM NaCl which is not toxic to wild type yeast (Gaxiola et al., 1999). Further evidence is that overexpression of AtNHX1 in wild type Arabidopsis thaliana plant conferred higher salt tolerance (Apse et al., 1999).

1.4. Calcium and salt tolerance:

It is not clear how Na⁺ stress is perceived at the plant cell plasma membrane, but more and more evidence suggest that Ca²⁺ is involved in salt stress signal transduction pathways. It has been reported that in maize root protoplast Ca²⁺ concentration increases in response to salt stress (Lynch et al., 1989). The cytosolic Ca³⁺ concentration elevation upon salinity has also been observed in barley root and wheat cells (Bittisnich et al., 1989; Bush, 1996). A Ca²⁺-binding protein of Arabidopsis (RD20) is induced by high salinity
(Takashashi et al., 2000). Moreover, increased external Ca\(^{2+}\) can ameliorate salt stress (Lauchli, 1990). The Ca\(^{2+}\) elevation can originate from extracellular and intracellular Ca\(^{2+}\) pools. Extracellular Ca\(^{2+}\) enters the cell through calcium channels in the plasma membrane (Gelli and Blumwald, 1997). Intracellular Ca\(^{2+}\) can be released from different organelles. For example, Ca\(^{2+}\) could be released from vacuoles through IP\(_3\)-dependent Ca\(^{2+}\) channels (Knight et al., 1997).

The increased Ca\(^{2+}\) may transduce the salt stress signal through the binding to protein kinases and phosphatases. In yeast, a Ca\(^{2+}\)- and calmodulin-dependent protein phosphatase, calcineurin, is thought to be a secondary signal during the salt stress pathway (Zhu, 2001). The yeast calcineurin has a catalytic A subunit (CnA) and a regulatory B subunit (CnB). CnB has four EF-hand calcium-binding sites and it is required for the activation of A subunit. In yeast, the Na\(^+\), K\(^+\), and Ca\(^{2+}\) homeostasis is regulated by calcineurin (Mendoza et al., 1994). Calcineurin is also required for the transcriptional regulation of Na\(^+\) and Ca\(^{2+}\)-ATPases and cell wall β-1,3 glucan synthase genes (Matheos et al., 1997). Mendoza et al (1994) reported that under salt stress, yeast calcineuin mediates a switch of K\(^+\) uptake from low- to high-affinity mode by regulation of phosphorylation of a high affinity K\(^+\) transporter (TRK1), thereby reducing the influx of Na\(^+\).

The important role of Ca\(^{2+}\) in salt tolerance is enhanced by the research on a salt overly sensitive (sos) Arabidopsis mutant, sos3 (Liu and Zhu, 1998). SOS3 is a homologue of yeast calcineurin B subunit. SOS3 has three EF-hands for Ca\(^{2+}\) binding (Liu and Zhu, 1998). Liu and Zhu also reported that the increased Ca\(^{2+}\) in the growth
medium can suppress the sos phenotype of the mutant probably through increasing K+/Na+ selectivity of the K+ transporter.

In Zhu's lab, another two SOS genes have also been cloned, SOS1 and SOS2. SOS1 is a putative plasma membrane Na+/H+ antiport which is responsible for the extrusion of Na+ out of the plasma membrane. The expression of SOS1 is upregulated by salt, but SOS1 needs both SOS2 and SOS3 for its function (Shi et al., 2000). SOS2 is a serine/threonine protein kinase, and its activity is regulated by Ca2+ (Liu et al., 2000). Moreover, SOS2 physically interacts with SOS3, the calcineuain B-subunit-like protein (Halfer et al., 2000). Based on experimental evidence, Zhu (2000) proposed a new regulatory pathway for ionic homeostasis under salt stress in Arabidopsis (Fig.3). This SOS pathway begins with the binding between SOS3 and Ca2+ whose concentration is somehow increased by salt. Then SOS3 binds with SOS2 to activate the protein kinase. Next, the SOS3-SOS2 complex may regulate the gene expression of SOS1 and other genes at the transcriptional level, or regulate the activities of SOS1 and other transporters at the post-translational level. Therefore, this SOS pathway leads to the homeostasis of Na+, K+ and Ca2+ and consequently plant tolerance to Na+ stress. But how SOS3-SOS2 complex regulates SOS1 or other salt tolerance related genes or proteins is still unknown (Zhu, 2000).
1.5. Two genetic models in salt tolerance research: yeast and Arabidopsis

Most of the favourite plants in salt tolerance research, such as tobacco, ice plant (Mesembryanthemum crystallinum) and tomato, are not good molecular genetic models due to the unavailability of their genomic information. Since the availability of relatively more information on yeast genomic sequence, as well as the similarity in ion transport process between yeast and higher plants, yeast has been used as an alternative model in the study of salt stress at molecular level (Serrano and Gaxiola, 1994; Nass and Rao, 1998).

The emergence of Arabidopsis thaliana provided a plant model in salt tolerance research (Zhu, 2000). Arabidopsis has an obvious advantage over yeast in being a plant which allows the study at the whole-plant level. Although Arabidopsis is a glycophyte, which is not good to study some unique salt-tolerance mechanisms of halophytes, it is expected to have salt tolerance genes that are very similar to those of halophytes. Most important features of Arabidopsis as a genetic model are: its small size, short life cycle,
ability to self-pollinate, high seed number, small genome and ease to be transformed and mutated. Zhu (2000) reported a close relative of Arabidopsis thaliana, Thellungiella halophila, which can survive seawater-level salinity and complete its life cycle in 300 mM NaCl. Thellungiella halophila shares similar growth, development and DNA sequence with Arabidopsis thaliana. This gives the hope of finding a halophytic genetic model for salt tolerance research.

Salt tolerance research in yeast facilitated the molecular characterization of yeast homologous genes and similar mechanisms found in plants. The relatively well-known genetics of yeast and ease of handling genetic manipulation protocols made yeast an important tool to clone and characterize many salt stress related genes, such as the plasma membrane Na⁺-ATPase gene 1-4 (ENA1-4) (Garcia-deblas et al., 1993), plasma membrane Na⁺/H⁺ antiporter, NHA1 gene (Prior et al., 1996; Banuelos et al., 1998), and prevacuolar Na⁺/H⁺ antiport genes, ScNHX1 (Nass et al., 1997), etc. The cloning of several yeast salt tolerance related genes also facilitated the cloning of their homologous in plant, such as the cloning of Arabidopsis vacuolar Na⁺/H⁺ antiport (Gaxiola et al., 1999).

Yeast complementation techniques have a great value in identifying plant salt tolerance related genes. There are several well characterized yeast salt sensitive mutants, such as Δena1 which lacks the plasma membrane Na⁺-ATPase and can't grow in NaCl concentrations which are not harmful to wild type yeast, and Δnhx1 which lacks the prevacuolar Na⁺/H⁺ antiport ScNHX1 and is sensitive to 200 mM NaCl which is not toxic to wild type yeast (Gaxiola et al., 1999). The complementation of corresponding yeast mutant also allows a function to be assigned to a previously cloned plant gene
whose function is difficult to be determined in plant (Bassham and Raikle, 2000). But it is not enough to solely rely on the result of yeast complementation to localize a plant protein because sometimes plant proteins may or may not localize correctly in yeast. For example, AtNHX1 is localized in vacuoles in Arabidopsis, but it can complement the ScNHX1 mutant which is localized to prevacuoles in yeast. Therefore, the results from yeast complementation should be confirmed in plants.

Fortunately, except for gene replacement through homologous recombination, gene transformation, mutagenesis, mutant screening, positional cloning and gene tagging are possible in Arabidopsis (Zhu, 2001). As it was proposed sixteen years ago (Blumwald and Poole, 1985), the overexpression of AtNHX1 in Arabidopsis conferred higher salt tolerance to this glycophyte (Apse et al., 1999). This successful transformation in high plant gave a great hope of generating a whole spectrum of salt tolerant crop plants through genetic modification.
Objectives:

This thesis aimed at contribution to the study of *Arabidopsis thaliana* vacuolar Na\(^+/H^+\) antiport family, including the sequence similarity, tissue distribution, function and response to salt stress.

Specifically, the goals of this thesis are:

1. Cloning AtNHX3 gene
2. Making antibody against the AtNHX3 protein
3. Assessing the subcellular localization of AtNHX3
4. Assessing the tissue distribution of AtNHX3 and response to salt treatment
5. Assessing the function of AtNHX3 by yeast complementation and making AtNHX3 overexpression *Arabidopsis* lines
Materials and methods:

2.1. Plant material and transformation:

Sterilized *Arabidopsis thaliana* seeds (Columbia) were grown either in soil directly or transferred to soil after germinating first on agar plates containing 0.5X Murashige and Skoog (MS) medium. The plants were grown under 12 hour light at a constant 22 °C.

*Agrobacterium*-mediated transformation was used to generate transgenic *Arabidopsis* lines. *Agrobacterium* was resuspended in infiltration solution containing 0.5x MS salt, 0.5g/l MES; 5% sucrose and 0.03% Silwet. Flowering plants with primary bolts reaching 15 cm were dunked into a bacterial solution for 5 min. The plants were re-transformed 12 days later.

Transgenic seeds were screened on 1/2 MS-Kanamycin (25 mg/l) plates (1/2 MS-K25). These plates were placed under 24 hour light at 22 °C. The salt tolerance of the transgenic lines was tested by either germinating the seeds on 1/2 MS-K25 plates - containing 100mM NaCl and 200 mM NaCl or watering the plants in soil with 100 and 200 mM NaCl for two weeks.

*Arabidopsis* seeds were sterilized as following: 50 µl of seeds were washed with 1 ml 70% ethanol alcohol for 2 min, then incubated with 1 ml of sterilization solution with constant shaking for 10 min. The sterilization solution contained 6% bleach and 0.1% Tween 20. Then the seeds were washed 5 times with sterilized ddH₂O. Finally, the seeds were resuspended in 1 ml of 0.1% phytagar and chilled at 4 °C at least overnight to break the dormancy.
2.2. Cloning of AtNHX3

To clone AtNHX3, an Arabidopsis thaliana (Columbia) cDNA library (CD4-15, Kieber et al., 1993) was screened by in situ hybridization (Sambrook et al., 1989). The probe used for the screening was a 730 bp PCR product produced by using CD4-15 library and a pair of primers designed according to the sequence in the Arabidopsis database. The 5'-primer was 5'-TTC GTT CTC GGC CAT GTC C-3'. The 3'-primer was 5'-CGG AGA GAC CAA CAC CTT CTG C-3'. After positive plaques were obtained, the lambda phage was amplified and in vivo excision of the cloned fragment was performed using a Rapid Excision Kit from Stratagene.

5' and 3' Rapid Amplification of cDNA Ends were used to clone the full length of AtNHX3. SMART-RACE cDNA Amplification Kit and Advantage 2 PCR Enzyme System from CLONTECH were used. All reactions were performed according to the manufacturer instructions.

When compared with the conventional reverse transcription reactions, the RACE system provides a better mechanism for generating full-length cDNAs by using both the SMART II oligonucleotide and MMLV reverse transcriptase (RT). This MMLV RT can add 3-5 residues of dC to the 3' end of the first-strand cDNA, and these oligo-dC are harnessed by the dG-residues of SMART II which serves as an extended primer for reverse transcriptase. Since the dc-tailing activity of RT is most efficient only when the enzyme has reached the end of the RNA template, the SMART II sequence is typically added only to complete first-strand cDNA, and this guarantees the formation of cDNA that has a maximum amount of 5'sequence if high quality RNA is used. Furthermore, the Advantage 2 polymerase Mix includes Advan Taq DNA polymerase, a minor amount of
a proofreading polymerase, and Taq Start antibody to provide automatic hot-start PCR, so this enzyme system allows efficient, accurate, and convenient amplification of cDNA.

2.2.1. 5'-RACE to obtain the 5' end start codon of AtNHX3:

For the synthesis of first-strand cDNA (5'-RACE-ready cDNA), 1 µl (1.5 µg) of total RNA, 1 µl of 5'-CDs primer (from the kit), 1 µl of SMART II oligo (from the kit), and 2 µl of ddH2O were mixed in a 0.5-ml PCR tube, and incubated at 70 ºC for 2 min and placed on ice for 2 min. Then 2 µl of 5x first-strand synthesis buffer (from the kit), 1 µl of 20 mM DTT, 1 µl of 10 mM dNTPs and 1 µl of Superscript II (MMLV RT) were added to the tube. Incubation was carried out at 42 ºC for 1.5 hour. The reaction was diluted by adding 100 µl of Tricine-EDTA buffer and the tube was heated at 72 ºC for 7 min.

Tricine-EDTA buffer was used because this buffer can maintain pH at high temperature better than Tris-EDTA buffer. Tris-based buffer can lead to low pH conditions that can degrade DNA.

For the 5'-RACE, an AtNHX3 specific 3'-primer, X3 REV, was designed, which is 5'-CCC CAA CCC CTG CAG ACA TTG AGC CAG C-3'. The PCR reaction was set up by combining the following: 5 µl of 10X advantage 2 PCR buffer, 1 µl of 10 mM dNTPs mix, 1 µl of 50X advantage 2 polymerase mix, 5 µl of 5'-RACE-ready cDNA, 5 µl of 10X UPM, 1 µl of X3REV, and 32 µl of PCR-grade ddH2O. The PCR cycle included 1 cycle of 94 ºC for 2 min; 32 cycles of 94 ºC for 30 sec and 68 ºC for 3 min; 1 cycle of 72 ºC for 5 min. Negative control was done by adding no 5'-RACE-ready cDNA.

2.2.2. 3'-RACE to obtain the full-length of AtNHX3 gene:
3'-RACE-ready cDNA was synthesized similarly to 5'-RACE-ready cDNA, except that 1 μl of 5'-CDs primer and SMART II oligo were replaced by 3'-CDs primer attached with SMART II oligo.

For 3'-RACE, an AtNHX3 specific 5' primer, X3-5'-RACE, was designed, which is 5'-GCT GAA TGG AGG AAG TGA TGA TTT CTC CGG TGG-3'. The 3'-RACE PCR reaction mixture included: 5 μl of 10X advantage 2 polymerase buffer, 1 μl of 10 mM dNTPs, 1 μl of 50X advantage 2 polymerase mix, 5 μl of 3'-RACE-ready cDNA, 1 μl of 5'-RACE primer, 5 μl of 10X UPM, and 32 μl of PCR-grade ddH₂O. Negative controls were obtained by adding no 3'-RACE-ready cDNA. PCR cycle included: 1 cycle of 94 °C for 2 min; 36 cycles of 94 °C for 30 sec and 72 °C for 3 min; 1 cycle of 72 °C for 7 min.

2.3. Preparation and purification of antibodies raised against the X3-GST fusion protein:

A specific region (compared with AtNHX1 and AtNHX2) of 105 amino acids of ATNHX3 was chosen to make the X3-GST fusion protein.

First, the coding sequence of these 105 amino acids was obtained by PCR with AtNHX3 cDNA and a pair of designed primers, GST-X3F (5'-CCC GCG GAT CCG GTG CAC TTA TAT CAG C-3') and GST-X3R (5'-GGC GGA ATT CAC AAC ACT CCA AGT TCT G-3'). Then this piece of DNA was cloned in frame into PGEX-2TK vector in the site of BamHI (5'-end) and EcoRI (3'-end).

E. coli strain BL21 pLysS was used to express the fusion protein under 1 mM IPTG induction. The fusion protein was confirmed by Western blots with the anti-GST
antibody. The E. coli lysate was applied to a polyacrylamide gel, and then the fusion protein was cut off and subjected to electroelution, lyophilization and concentration measurement by dot blot.

For the 1st injection of the rabbit, 200 µl (200µg) of purified fusion protein was mixed by vortex with 200 µl Freund's complete adjuvant (ICN Biochemicals Inc.). For the 2nd, 3rd and 4th time injection, 250 µl (100-150 µg) of purified protein were mixed with equal volume of Freund's incomplete adjuvant (ICN Biochemicals Inc.).

For the purification of the antibody, affinity strip blots were used. For making one PVDF strip, 100 µg purified protein (GST or X3-GST fusion protein) was loaded on a large well of a polyacrylamide gel. The strips were blocked with 5% milk-PBST for 1 or 2 hours and washed with PBST for 5 min three times before placed into the serum tube. The serum was firstly incubated with GST PVDF strips, and then with X3-GST fusion protein strips, at 4 °C overnight. The strips were washed with PBST 5 times with 5 min each time, and then washed with 0.1X PBST three times. The antibody was eluted with 0.2 M glycine (freshly made, pH 2.5), and the pH of the antibody solution was adjusted to 7.5 immediately by adding 2M Tris. Usually in 0.5 ml serum, 0.5 ml 2X PBS was added, and 2 or 3 protein strips were used to bind the antibody. The purified antibody was stored at 4°C.

2.4. Electroelution:

Electroelution was used to purify the X3-GST fusion protein for antibody preparation by using BioRad 422 Electro-Eluter System. Firstly, E. coli lysate was separated on 12.5% SDS-PAGE, and then stained with low fixing CBBR and destained
with 30% methanol. The low fixing CBBR staining was used because it facilitates electroelution. Elution was performed under 8mA/tube for 4-5 h. The electroelution running buffer contained 50 mM NaHCO₃ and 0.1% SDS. After the elution was completed, the eluted protein was sucked out of the chamber and concentrated by lyophilization. The purified protein was then subjected to dot-blots for concentration determination.

2.5. Protein concentration determination:

Protein concentration was measured using the modification of the Bradford dye-binding method available from BioRad (Mississauga, ON). For this blotting assay, the protein samples were diluted 10 or 20 times so that the concentrations of protein obtained would fall within the range of 0.1 to 1 mg/ml. Two µl of each diluted sample was dot blotted onto Whatman No.1 filter paper alongside a set of similarly blotted BSA standards from 0.1 to 1 mg/ml. After air drying for 10 min, the blotted paper was stained in 0.1% CBBR-250, 10% acetic acid, 40% methanol for 20 sec in a microwave oven, and then destained twice in 10% acetic acid, 40% methanol in a microwave oven for 20 sec each time.

2.6. SDS-PAGE:

One-dimensional SDS-PAGE was used to detect the X3 protein and purify X3-GST fusion protein. 12.5% separating gels and 3.5% stacking gels were used.

Two methods for Coomassie blue staining were used. For analytical gels, where the separated proteins were not further processed, a "high fixing" stain was used, which is
staining gels in a solution of 0.1% CBBR in 40% methanol and 10% acetic acid for 20 min or overnight. Destaining was achieved by incubation the gel in 40% methanol and 10% acetic acid. For gels where protein bands were to be cut, the "low fixing" method was used. This consisted of a 10-15 min incubation at room temperature in 0.2% CBBR, 20% methanol and 0.5% acetic acid, followed by a 10 min destaining in 30% methanol.

2.7. Electroblotting of protein:

Both nitrocellulose membranes and PVDF membranes were used for blotting proteins by using semidry electroblotting system (Pharmacia). For analytical western blots, protein was transferred to nitrocellulose membrane (GIBCOBRL). The transfer sandwich included, from anode to cathode, one sheet of 3mm paper soaked in buffer 1 (20% methanol, 0.3 M Tris), one sheet of 3mm paper soaked in buffer 2 (20% methanol, 25mM Tris), 1 sheet of nitrocellulose membrane soaked in buffer 2, polyacrylamide gel, and two sheets of 3mm paper soaked in buffer 3 (20% methanol, 25 mM Tris, 2.62g caprioic acid per 500 ml). A Pasteur pipette was used to roll on the sandwich to drive away bubbles. Transfer was executed at 70 mA per minigel for 60 min. For the purification of antibodies, PVDF membrane (HELIXX Technologies, INC.) was used to blot the protein because of its higher protein binding capacity compared with nitrocellulose membrane. For blotting with PVDF, all of the three buffers mentioned above didn't contain methanol. PVDF membrane was wet in 100% methanol briefly, and then placed into buffer 2. After transfer, the membranes were stained with Ponceau S to verify the transfer result, and destained by distilled water.
2.8. Western Blots:

Firstly antibodies were raised against X3-GST fusion protein in rabbit (Dept. of Zoology, U of T). Secondary antibody was goat anti rabbit IgG conjugated with horse-radish peroxidase (Sigma). Incubation of the membrane with antibodies was done at room temperature for one or two h. ECL Western blot analysis kit (Amersham) and autoradiography were used to visualize protein bands.

2.9. Cellular membrane protein preparation:

This procedure is based on the method described by Blumwald and Poole (1985).

Seventy five grams of plant tissue without roots were ground with sand in 500 ml of cold homogenization buffer in cold mortar and pestle. The homogenization buffer contained 30 mM Tris, 10% glycerol, 2.5% soluble PVP-10, 2.5% insoluble PVPP, 5mM EGTA, 5mM MgSO4, 250mM Manitol, 1mM Caproic acid, 1 mM PMSF, 2mM DTT, 0.5 mM BHT, 25 mM dibucaine, 26 mM K-metabisulfite, 1 mM benzamindine, pH8.0. This buffer had components which can maintain the integrity of the cellular membranes and their component proteins: polyvinylpyrrolidine (PVP) binds the released vacuolar phenolic componds; EGTA is a Ca^{2+} chelator and also helps to prevent membrane aggregation; butylated hydroxytoluene (BHT) inhibits lipid degradation; metabisulfite protected against oxidation; dibucaine inhibits phosphatase A activity; and PMSF, caproic acid, and benzamidine are protease inhibitors.

The homogenate was filtered through 4 layers of cheesecloth and then centrifuged for 12-14 min at 2000rpm to pellet cell debris. The supernatant was centrifuged for 20 min at 12,000 g to obtain the mitochondria and nuclei. The supernatant was centrifuged in a
Beckman Type 35 rotor for 47 min at 33,000 rpm. The resulting microsomal pellets were resuspended in a hand-held tissue homogenizer with 10 ml of resuspension buffer (250 mM mannitol, 10% glycerol, 6 mM Tris-MES pH 8.0 and 0.2 mM DTT).

The microsomal membrane suspension was layered onto a discontinuous sucrose gradient containing, from top to bottom, 9 ml 16% (W/V) sucrose, 8 ml of 32% sucrose, and 8 ml of 40% sucrose, in resuspension buffer. After centrifugation for 2 h at 23,000 rpm in a Beckman SW28 rotor, the tonoplast-enriched membrane fraction was collected from the 0/16% interface; Golgi and ER membrane fraction were collected from the 16%/32% interface; and plasma membrane was collected from 32%/40% interface. These membrane fractions were pelleted with resuspension buffer by centrifugation at 45,000 rpm for 36 min in a Beckman Ti 60 rotor. The pellets were resuspended in 100 μl of resuspension buffer and then were subjected to protein concentration measurement by dot blot. The membrane proteins were stored at -80 °C.

2.10. RNA preparation from Arabidopsis plants:

Two methods were used to prepare RNA from Arabidopsis plants. For large scale RNA preparation, the hot-phenol protocol was used (Jebanathirajah, 2001; Verwoerd et al, 1989). For small scale preparation, TRI Zol Reagent (GIBCOBRL) was used.

2.10.1. Large scale RNA preparation:

Five grams of frozen tissue (leaf, stem, flower, root) were grounded in liquid nitrogen in a cold mortar, and the powder was transferred to a 50-ml Falcon tube. 15 ml of hot (80 to 90 °C) extraction buffer were added to the tube. The tube was vortexed for 30 sec,
then incubated at 80 to 90 °C in a water bath for 90 sec. Hot extraction buffer contained equal volumes of phenol and LiCl buffer (0.1 M LiCl, 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). 7.5 ml of CHCl₃:isoamyl alcohol (24:1) was added and the tube was vortexed briefly. The extraction was centrifuged at 3000 rpm for 15 min at 4 °C, and the upper layer transferred to a new tube. An equal volume of CHCl₃:IAA or CHCl₃: Butanol (4:1) was added to extract the RNA again. RNA was precipitated by adding an equal volume of 4M LiCl and 50 μl Chloroform at -20 °C overnight. RNA was recovered by centrifugation at 3000 rpm for 30 min at 4 °C. The RNA pellet was washed with 70% ethanol (in DEPC-H₂O) once. The RNA was dissolved in EDPC-H₂O and the OD₂₆₀/₂₈₀ ratio was measured and the RNA concentration was calculated by [RNA]=40ug/ml X time of dilution. RNA sample was stored at -80 °C.

2.10.2. Small scale RNA preparation:

One hundred mg of frozen tissue were grounded with liquid nitrogen, and 1 ml of TRI Zol reagent was added and the material was homogenized further. The mixture was transferred to a 1.5-ml Eppendorf tube, and incubated at room temperature for 5 min. The tube was centrifuged in a bench top centrifuge for 10 min at 4 °C and the debris was discarded. The supernatant was mixed with 0.2 ml of chloroform and incubated at room temperature for 5 min. After centrifugation at 12,000rpm for 5 min at 4 °C, the upper phase was transferred to a new tube and an equal volume of CHCl₃:butanol (4:1) was added for RNA extraction. The tube was centrifuged at 12,000rpm for 5 min at 4 °C. The upper phase was transferred to a new tube and 0.5 ml of isopropanol was added to precipitate RNA by incubation at RT for 10 min. RNA was pelleted by centrifugation at
12,000 rpm for 10 min at 4 °C. The RNA was washed with 75% ethanol once, and dried in air briefly, and finally dissolved in DEPC-H₂O with incubation in 60 to 65 °C water bath for 10 min. The OD₂₅₀/₂₈₀ ratio of the RNA sample was measured and the RNA concentration was calculated by [RNA]=40μg/ml X OD₂₅₀/ time of dilution. RNA sample was stored at -80 °C.

2. 11. Northern hybridization:

2. 11. 1. RNA gel electrophoresis:

RNA gel electrophoresis was performed according to the formaldehyde denaturing gel protocol in the handbook of Molecular Cloning (Sambrook et al., 1989) with a few changes.

The 1.2% formaldehyde denaturing agarose gel was made as below: Firstly, 1.2 grams of agarose was dissolved in 77.1 ml of DEPC-H₂O in microwave, then 5 ml of 20x formaldehyde running buffer and 17.9 ml of formaldehyde were added. The 20X formaldehyde running buffer contained 0.4 M MOPS (pH 7.0), 160 mM sodium acetic acid and 20 mM EDTA (pH8.0).

RNA samples were treated as follow: 6.5 μl RNA (maximum 30 μg), 0.625μl of 20X formaldehyde running buffer, 4.38 μl of formaldehyde, 12.5 μl formamide, and 1 μl of EB (10μg/μl) were mixed together and incubated at 65 °C for 15 min, and then chilled on ice. The RNA ladder from 0.24 to 9.5 Kb (GIBCOBRL) was used as molecular weight standard. Before loading onto the gel, 5 μl of 6X loading buffer was added to each sample. The 6X loading buffer contained 0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (W/V) sucrose in DEPC-H₂O. The running condition was 4.5V/cm for 2.5 h
with 1x formaldehyde running buffer. The running buffer was mixed once during running.

2.11.2. Transfer of RNA to Hybond-N* membrane:

The gel was washed in DEPC-H₂O for 15 min (to remove the formaldehyde), and then transferred to Hybond-N* membrane by Capillary elution (Sambrook et al., 1989) at RT overnight. Transfer buffer was 20x SSC. After transferal, UV cross-linking was applied to the membrane. The membrane was stored at RT in Saran-wrap.

2.11.3. DNA probe labeling:

DNA probes were labeled by use of T7 Quick labeling Kit (Amershan) and α-³²P-dCTP (Amersham-Pharmacia Biotech. Inc.). The labeling reaction was carried out by following the manufacturer instructions. Fifty ng of DNA in 34 µl ddH₂O was denatured by boiling for 5 min and chilled on ice immediately. Then 10 µl of Reagent Mix provided by the Kit, 5 µl of α-³²P-dCTP and 1 µl of T7 DNA polymerase provided by the Kit were added. The labelling mixture was incubated at 37 °C for 15 min, and the labelled probe was denatured by boiling and chilling.

2.11.4. Hybridization:

Pre-hybridization and hybridization buffer, UltraHybTM, was purchased from Ambion. The whole hybridization was carried out according to the manufacturer's instructions. Both the buffer and the hybridization tube were heated to 42 °C, then the RNA membrane was placed into the tube. Pre-hybridization was carried out for 1-2 h at
42 °C. Then labeled and denatured probe were added to the tube. The hybridization was carried out overnight at 42 °C. The membrane was washed with 2X and 0.2X SSC for 15 min twice, respectively. The membrane was exposed to a phospho-Imager.

2.12. Yeast complementation:

Yeast complementation was performed to test the function of AtNHX3. AtNHX3 ORF sequence was first obtained by PCR with AtNHX3 cDNA and a pair of primers, 5'-X3-yeast-new (5'-CGC TCC CCC GGG ATG GAG GAA GTG ATG ATT TCT CC-3') and 3'-X3-yeast-new (5'-GGA CGC GTC CTA CTC CCC ATC TCC ATC TCC-3'). The yeast mutant, Δnhx1 (nutrition type: trp-), was transformed with AtNHX3 ORF sequence cloned in a yeast expression vector pYPGE15 at the SmaI (5') and SalI (3') sites. The wild type yeast and Δnhx1 mutant transformed with empty pYPGE15 vector, were also used as controls.

2.12.1. Transformation of Yeast:

The lithium acetate transformation method was used for the transformation of yeast. Yeast mutant strain, Δnhx1, was streaked on a try-SD (Synthetic medium with 2% Dextrose, Difco) plate and incubated at 30°C overnight. Several colonies were picked and resuspended in 1 ml of sterile ddH2O. The yeast cells were centrifuged at 3500 rpm for 2 min. Yeast cells were washed with 300µl of 100 mM LiAc, and finally resuspended in 150 µl of 100 mM LiAc (yeast competent cells). For transformation, 75 µl of the yeast competent cells were mixed with 5 µl of plasmid, 5 µl of 10 µg/µl of denatured single-stranded salmon sperm DNA and 300 µl of 40% PEG (in LiAc/TE), and then incubated
at 30°C for 30 min without shaking. The yeast cells were heat-shocked at 42°C for 15 min to let the DNA go inside. Half the mixture was plated on a trp⁻-ura⁻-SD plate and the plate was incubated at 30°C for 2 days.

2.12.2. Salt tolerance test of the transformants:

For testing the salt tolerance of the transformants, the yeast cells were plated on APG medium with NaCl concentrations ranging from 200 to 500 mM, and pH 4.5 and 5.5. As controls, wild type yeast \(\Delta nhx1\) mutant and transformed with an empty pYpGE15 vector and plated on the same medium plate with equal amount of cells. In order to plate an equal amount of cell of every kind of yeast strains, all of the transformed yeast strains were grown in 2 ml of trp⁻-ura⁻-2% glucose-SD medium for 3 days at 30°C, because at this point, all yeast cultures were saturated. Then dilutions of 10x, 100x and 1000x were made, and then 3 µl of each culture were plated on APG plates containing 200 mM, 300 mM, 400 mM, and 500 mM NaCl at pH 4.5 and pH 5.5, respectively. The plates were incubated at 30°C. APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, and trace minerals and vitamins.

2.12.3. Preparation of yeast tonoplast:

For preparation of tonoplasts from yeast, 500 ml of AtNHX3 transformed yeast \(\Delta nhx1\) and control strain, pYpGE15 empty vector transformed \(\Delta nhx1\) strain, were grown at 30°C overnight respectively. The same protocol for preparation of plant tonoplast was used to prepare the yeast tonoplast with a few changes, both PVP and PVPP were omitted (see
section 2.9), and 22% sucrose was used to separate the tonoplast from other membrane proteins (Nass and Rao, 1998). The tonoplast samples were subjected to Western blot to detect the expression of AtNHX3.

2.13. Semi-quantitative RT-PCR:

RT-PCR is a highly sensitive and rapid method of detecting mRNA levels of a gene. It has been shown to be thousands of times more sensitive than the traditional RNA blot techniques (Chirgwin et al., 1979; Chomczynski and Sacchi, 1987). Thus RT-PCR is more useful in analysis of single-copy genes or lowly expressed genes. RT-PCR involves two enzymatic reactions: a reverse transcription by usually MMLV RT, and polymerase chain reaction. But it is difficult to get quantitative information with this technique due to the exponential nature of PCR. Under ideal or theoretical conditions, the amount of product always doubles after each cycle, but the rate of production will reach a plateau stage after a certain number of cycles. There are several factors which may affect the amplification efficiency, such as the impurity of the RNA sample and the length of the amplified sequence. There may be additional factors that cause the tube-to-tube variation even when a master mix of reaction components is used. So, in order to get accurate information, several strategies have been used in a semi-quantitative RT-PCR, including using an appropriate amount of initial template, appropriate numbers of PCR cycles, and an endogenous sequence which is known to be expressed constantly as a internal control. The internal control is amplified using a second pair of specific primers. Usually, a mini-southern blot is applied to detect the bands of the PCR products. The ratios of the
amount of the target products and that of the endogenous control represent the mRNA level of a gene, and this is called normalization.

Usually, 2 μg of total RNA and 25 PCR cycle are used for semi-quantitative RT-PCR. The RT-PCR beads from Amersham Pharmacia Biotech. Inc. was used for this thesis. To each RT-PCR bead (provided in a tube), 45 μl of DEPC-H2O were added and dissolved on ice. Then 1 μl of Oligo dT (0.5 μg/μl), 0.5 μl of each of the four following primers, actin7F, actin7R, X3-GSTF and X3-GSTR were added. Finally, 2 μl of 1 μg/μl RNA sample was added. The total volume of the PCR reaction was 50 μl.

The first cDNA strand synthesis was carried out at 42 °C for 30 minutes. Then the tube was heated to 95 °C to inactivate the MMLV RT. then the normal PCR was started. The PCR conditions were: 25 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

The sequence of the actin7F prime was 5'-GGT GAG GAT ACC CAG CCA CTT GTC TG-3', and that of the actin7R was 5'-TGT GAG ATC CCG ACC CGC AAG ATC-3'.

For detecting the RT-PCR product by mini-southern blot, the PCR product was equally loaded in two agarose gel wells and transferred to hybond-N+ membrane by using 0.4 M NaOH overnight at room temperature.

For hybridization, one set of the membrane was probed with α-32P labelled actin 7 probe and the other set was probed with α-32P labelled AtNHX3 specific probe. The RT-PCR reactions were repeated several times, and all the results were subjected to statistical analysis.
3.1. Cloning AtNHX3 cDNA

Through the screening of an *Arabidopsis* cDNA library, only a partial sequence of AtNHX3 was obtained, which encoded for 350 amino acids. Compared with the predicted AtNHX3 amino acid sequence in *Arabidopsis* database, this 350 amino acid peptide lacks 31 amino acids in the N-terminus and 140 amino acids in the C-terminus. According to this partial sequence, an AtNHX3 specific 3'-primer, X3 REV was designed for 5'-RACE to obtain the 5'-end, and then an AtNHX3 specific 5'-primer, X3-5'-RACE, was designed for 3'-RACE to obtain the full length of AtNHX3 (Materials and Methods).

A strong band around 1.8 kb was produced by 3'-RACE, and BLAST search results showed that the sequence of this band was a putative *Arabidopsis* vacuolar Na+/H+ exchanger. This 1.8 Kb AtNHX3 cDNA contained 5 bp in the upstream of the start codon ATG, while a long enough 3'-UTR was cloned, including the polyA tail (Fig. 4).

The open reading frame of AtNHX3 was a 1563-bp fragment which coded for 521 amino acids. The deduced amino acid sequence from this AtNHX3 ORF matched with the predicted one in Arabidopsis database, and it shared about 35% identical sequence with AtNHX1 and AtNHX2 (Fig. 5). One amiloride binding site (FFLFLLPPPII) was located in the N-terminus of AtNHX3. A cation binding region (FGESVLNDA) was also present (Fig. 5). The topology prediction (using TopPred 2) showed that the full length AtNHX3 protein has 11 transmembrane domains (Fig. 6).
3.2. Preparation and purification of antibody against AtNHX3

In order to detect AtNHX3 protein, the polyclonal antibody against the central part of AtNHX3 was prepared and purified. An AtNHX3 specific and also hydrophilic fragment of 105 amino acids (Fig. 5) was chosen to make the AtNHX3-GST fusion protein which was expressed in E. coli BL21 pLysS and purified by electroelution (Fig. 7A). Then the fusion protein was used to inject rabbit (Materials and Methods). Although the serum has strong reaction with the fusion protein, in order to reduce the background of future Western-blot, the antibody was still purified using affinity strip blot (Materials and Methods). Figure 7B shows the Western-blotting results done with 1:500 dilution of the unpurified (panel B1) and purified antibody (panel B2). On both gels, equal amounts of purified fusion protein (left lane) and GST (right lane) were loaded. The secondary antibody was goat-anti-rabbit alkaline-phosphatase. The membranes were developed by adding substrates NBT and BCIP. The result shows that the purified antibody has a stronger affinity with the fusion protein and a weaker affinity with GST than unpurified antibody.

3.3. The natural subcellular distribution of AtNHX3

In order to detect the subcellular location of AtNHX3, wild type Arabidopsis cell membrane fractions, including tonoplast, mitochondria, plasma membrane, and ER, were prepared using sucrose gradient sedimentation, and 40 μg of each kind of membrane protein was subjected to Western-blotting using antibody raised against AtNHX3. The result shows a strong band around 45 KDa exclusively located on tonoplast (Fig. 8, lane1). The molecular weight of the band was smaller than expected (56 KDa). This
could be due to specific cleavage of the protein by the plant, or protein degradation, or abnormal migration caused by the interference of lipid component from the membrane.

3.4. The expression pattern of AtNHX3 and its response to salt treatment

The tissue distribution of AtNHX3 was detected at mRNA level by both Northern-blotting and semi-quantitative RT-PCR. RNA samples from mature Arabidopsis thaliana flowers, leaves, stem and roots were prepared (Materials and Methods). Thirty μg of each kind of RNA sample was subjected to a formaldehyde denatured agarose gel electrophoresis and then transferred to Hybond-N\(^+\) membrane (Materials and Methods). The membrane was probed with α-\(^{32}\)P labeled AtNHX3 cDNA. Phosphor-image results showed a weak band around 2.1 kb in leaf, stem and root tissues, but not in the flower tissue, and no signal was detected in salt-treated leaf tissue (Fig. 9). Compared with Northern-blot of Arabidopsis RNA using AtNHX1 (data not shown), the AtNHX3 band was much much weaker. This may suggest a very low AtNHX3 expression.

In order to confirm the Northern-blotting results, semi-quantitative RT-PCR was performed. Actin-7 was used as an internal control (Materials and Methods). The expected size of actin7 band was 550 bp, and that of AtNHX3 was 315 bp. The semi-quantitative RT-PCR results suggested that AtNHX3 was expressed in a slightly lower level in flower than in the other tissues, but the difference was not statistically significant (Fig. 10).

To answer the question of how AtNHX3 responds to salt treatment, RNA sample from leaf tissue of mature Arabidopsis plant treated with 200 mM NaCl for 3 days was prepared and subjected to both Northern blot (Fig. 9) and semi-quantitative RT-PCR
(Fig. 10). Similar to the flower sample, salt treated leaf RNA sample didn't show a band on the Northern blot, and the semi-quantitative RT-PCR result suggested there was no statistically significant difference at mRNA level between salt-treated leaves and non-salt-treated samples.

To detect the expression of AtNHX3 at different developmental stages, RNA sample from 3-week old seedlings grown on MS-agar plates was prepared and subjected to semi-quantitative RT-PCR, and the results were compared with those from mature plants (Fig. 10). Again, there was no statistically significant difference between the seedling sample and the mature samples at the AtNHX3 mRNA level.

These results suggest that AtNHX3 might be expressed constantly from young seedling to adult stage albeit at low levels, and that the expression of AtNHX3 was insensitive to salt stress.

3.5. The function of AtNHX3

The function of AtNHX3 was tested by both yeast complementation and overexpression in Arabidopsis.

3.5.1. Yeast complementation

For yeast complementation experiments, the ORF of AtNHX3 was cloned into a yeast expression vector, pYpGE 15, under a PGK promoter, and the resulting construct (AtNHX3-pYpGE15) and the empty pYpGE15 vector were used to transform a yeast salt sensitive mutant, Δnhx1, by the lithium acetate method (Materials and Methods). As a control, wild type yeast strain was also transformed with an empty pYpGE15 vector. The
salt tolerance of the different transformants was tested by plating equal amount of yeast
cells on APG plates containing 200, 300, 400 and 500 mM NaCl at pH 4.5 and 5.5,
respectively. The result showed that the growth of Δnhx1 with empty pYpGE15 vector
was inhibited by 200 mM NaCl under both pH, but AtNHX3 could suppress the salt
sensitivity of the Δnhx1 mutant strain (Fig. 11).

In order to test the effect of C-terminus of AtNHX3, a short version of AtNHX3 ORF
(called AtNHX3-S) which encodes the first 350 amino acids was also used in yeast
complementation experiment. The results showed that AtNHX3-S could complement
the mutated ScNHX1 function, but not as well as the full ORF of AtNHX3 did. This
results suggest that the C-terminus of AtNHX3 might have a regulatory function (Fig.
11).

Western-blotting was used to detect the expression in yeast of both the full and short
length of AtNHX3. Tonoplast samples from transformants with full ORF and with short
ORF of AtNHX3, as well as from Δnhx1 with the empty pYpGE15 strain were prepared,
and 60 µg of each sample was used for Western blots. The results show a 57 KDa band
for the full ORF transformants (Fig. 12A), and a 40 KDa band for the short ORF
transformants (Fig. 12B). Based on the results described above, AtNHX3 may have the
same function as ScNHX1.

3.5.2. AtNHX3 transgenic Arabidopsis plant

To make AtNHX3 overexpression Arabidopsis lines, the ORF of AtNHX3 was cloned
into a plant expression vector (pBISN1) under the supermas promoter, with Kanamycin
selection marker, at 5' SalI site and 3' SmaI site. Then agrobacteria-mediated
transformation protocol was applied (Materials and Methods).

T₀ transgenic seeds were first screened on MS-K25-agar plates. In total, 15 lines
survived. Then T₁ seeds of two lines (AtNHX3-L1 and AtNHX3-L2) were plated on
MS-K25-agar plates containing 50, 100, 150, and 200 mM NaCl. As controls, WT seeds
were also plated on MS-agar plates containing the same NaCl concentrations, but they
couldn’t survive. There were several seedlings from each of the two lines, which
survived on 50 and 100 mM NaCl plates, but not on the 150 mM NaCl and above plates
(Fig. 13). Then the surviving seedlings from the 100 mM NaCl MS-K25-agar plates
were transferred to soil. Ten days after the transfer, a set of three plants of each line were
watered with 0, 100, and 200 mM NaCl solutions every other day. As controls, a set of
wild type plants were also treated the same way. After watering with 200 mM NaCl for
2 weeks, the wild type plants were almost dead (Fig. 14B), while one transgenic line,
AtNHX3-L2, did not show any stress symptoms (Fig. 14A). Unfortunately, the
AtNHX3-L2 started to wilt after watering with salt for 3 weeks due to the accumulation
of NaCl in the pot. As a result of this, the NaCl concentration is well above the 200 mM
level and the transgenic plant died.

In order to test the effect of C-terminus of AtNHX3 in plant, the short version of
AtNHX3 (AtNHX3-S) used in the yeast complementation experiment was also used to
make Arabidopsis transgenic lines. There are three lines of AtNHX3-S transgenic
Arabidopsis, AtNHX3-S-6, AtNHX3-S-10 and AtNHX3-S-18, which can germinate on
MS-K25-Agar plates of 100 mM NaCl (data not shown). The mature plants of these
three lines can survive 100 mM NaCl treatment, but they died at 200 mM NaCl (data not shown).

The mRNA level of AtNHX3 in both AtNHX3-L2 line and wild type were tested. RNA samples from young seedlings of AtNHX3-L2 line and wild type (from MS plates) were prepared and subjected to semi-quantitative RT-PCR. The same AtNHX3 specific primers and actin7 control primers used for detecting the tissue distribution of AtNHX3 were used here. The results show that AtNHX3 mRNA was about 4 times higher in AtNHX3-L2 line than in wild type (Fig. 15). The AtNHX3-S mRNA level in AtNHX3-S transgenic lines was not tested.

Therefore, overexpression of AtNHX3 in Arabidopsis can confer a higher salt tolerance to the plants. However, the effect of the C-terminus of AtNHX3 in plant needs to be tested further.
Fig. 4. The cDNA sequence of *AtNHX3*.

A 1.8 kb fragment of *AtNHX3* cDNA was obtained by 3'-RACE using the specific X3-5'-RACE primer. At the 5'-end, 5 bp before the start codon ATG were cloned. At the 3'-end, long enough 3'-UTR was obtained including polyA tail. The start codon ATG and stop codon TAG were framed.
Fig. 5. The pileup of amino acid sequences of AtNHX3, AtNHX1, AtNHX2 and human NHE6.

AtNHX3 shares about 35% similarity with AtNHX1 and AtNHX2. The first conserved domain 'FFLFLPPII' (framed) is the putative amiloride binding site. The second frame shows the cation binding region (FGESVLNDA). The AtNHX3 part from 156 to 261 (underlined) was used to make AtNHX3-GST fusion protein for preparation of antibody.
**Fig. 6. The hydrophobicity of AtNHX3.**

Total of 11 transmembrane domains are predicted in AtNHX3 protein by TopPred2.
Fig.7. The antibody against AtNHX3-GST fusion protein

**A: The induction of X3-GST fusion protein.** Lane1: protein molecular weight marker; Lane2: BL21pLysS/pGEX2TK- vector without IPTG induction; Lane3: BL21pLysS/pGEX2TK-vector induced with 1mM IPTG (the fat band is GST); Lane4: BL21pLysS/X3-pGEX2TK without IPTG induction; Lane5-7: BL21pLysS/X3-pGEX2TK induced with 0.1, 0.5 and 1 mM IPTG respectively.

**B: The purification of anti-AtNHX3-GST fusion protein.** Both B1 and B2 membranes contain the same amount of purified X3-GST fusion (left) and purified GST protein (right). B1 was blotted with unpurified anti-X3-GST fusion protein antibody. B2 was blotted with purified anti-X3-GST fusion protein antibody. For both cases, the antibody was diluted at 1:500.
Fig. 8. The subcellular distribution of AtNHX3.

In both A and B, 40 μg protein of each sample was loaded. Lane 1. tonoplast Lane 2. mitochondria Lane 3. plasma membrane Lane 4. ER/Golgi.

For A, the gel was stained with CBBR. For B, the membrane was blocked with anti-AtNHX3-GST fusion protein.

Figure is representative of 3 independent experiments.
Fig. 9. AtNHX3 mRNA distribution --- Northern blot

A: RNA gel electrophoresis shows equal loading of five RNA samples: salt treated leaf (L-s), non-salt treated leaf (L-w), stem (ST), root (R), and flower (F). 30 µg total RNA of each sample was loaded.

B: Northern blotting of panel A. The membrane was probed with α-32P labelled AtNHX3 probe.

Figure is representative of 3 independent experiments.
Fig. 10. AtNHX3 mRNA tissue distribution ---semi-quantitative RT-PCR.

Results are the mean ± SD (n=4).
Fig. 11. Salt tolerance test of different yeast transformants.

On both A and B plates, equal amounts of yeast cells of different transformants were loaded at 10X, 100X and 1000X dilutions. From top to bottom: WT yeast transformed with empty pYpGE15 vector (WT + pYpGE15); Δnhxl transformed with empty pYpGE15 vector (Δnhxl +pYpGE15); Δnhxl transformed with AtNHX3-pYpGE15 construct (Δnhxl+ AtNHX3-pYpGE15); and Δnhxl transformed with the short version of AtNHX3 cloned in pYpGE15 vector (Δnhxl+ AtNHX3-S-pYpGE15).

Figure is representative of 3 independent experiments.
A. APG-ura plate containing 0 mM NaCl, pH 5.5.

B. APG-ura plate containing 200 mM NaCl, pH 5.5.
Fig. 12. The expression of AtNHX3 and AtNHX3-S in yeast.

A: 60 μg of tonoplast samples from Δnhx1+AtNHX3 yeast line (lane1) and Δnhx1+ pYpGE15 vector line (lane2) were blocked with anti X3-GST fusion protein antibody.

B: 60 μg of tonoplast samples from Δnhx1+AtNHX3-S yeast line (lane1) and Δnhx1+ pYpGE15 vector line (lane2) were blocked with anti X3-GST fusion protein antibody.
Fig. 13. Salt tolerance of AtNHX3 transgenic seeds.

T1 seeds of AtNHX3 transgenic Arabidopsis line 1 and 2 (AtNHX3-L1 and AtNHX3-L2) germinate on MS-Kanamycin 25-100 mM NaCl plate.
Fig. 14. Comparison of salt tolerance of AtNHX3-L2 and WT Arabidopsis.

A. A set of three AtNHX3-L2 plants were watered with 0, 100 and 200 mM NaCl respectively (from left to right).

B. A set of three WT Arabidopsis plants were watered with 0, 100 and 200 mM NaCl respectively (from left to right).

Experiments were performed in triplicates.
Fig. 15. Comparison of AtNHX3 mRNA level in AtNHX3-L2 line and WT Arabidopsis.

The plot was done using mean± SD (n=4).

Results are the mean ± SD (n=4).
Discussion:

4.1. The sequence of AtNHX3

The putative AtNHX3 amino acid sequence reported in this thesis has 11 transmembrane domains, which is a characteristic of Na⁺/H⁺ antiport found in mammalian cells (Yun et al., 1995). AtNHX3 showed about 35% identity at amino acid level with AtNHX1 and AtNHX2. Among the five cloned Arabidopsis vacuolar Na⁺/H⁺ antiporters in our lab, namely, AtNHX1-3, X5 and X8 (unpublished data), AtNHX3 is the most divergent one. The other four antiporters share about 60% similarity to each other. A similar situation was also observed in mammalian NHEs family, where NHE1-NHE5 shared 34% -60% amino acid identity, but NHE6 only shared about 20% identity to the others (Orlowski and Grinstein, 1997). Neither the N- nor the C-terminus of these Arabidopsis antiporters share high degree similarity with that of human NHE6 (Fig.5). AtNHX3, as well as the other four Arabidopsis Na⁺/H⁺ antiporters, has one putative amiloride binding site (FFLFLLPPPII) similar to that in human NHE6 (DVFFFLFLPPPI) (Gaxiola et al., 1999). In mammalian cells, the competitive inhibition of the antiporter activity by amiloride is another characteristic of Na⁺/H⁺ antiports (Yun et al., 1995). However, this amiloride inhibition was not observed when AtNHX1 was expressed in yeast (Gaxiola et al., 1999). In fact, one mammalian Na⁺/H⁺ exchanger, NHE5, was also reported to be amiloride insensitive (Raley-Susman et al., 1991). However, in sugar beet, it was reported that amiloride analogs could inhibit the Na⁺/H⁺ antiport activity (Blumwald et al., 1987). In E. coli, NhaA is inhibited by amiloride, while NhaB is not (Pinner et al., 1995).
The deduced molecular mass of AtNHX3 is 56 KDa, but Western blots showed a 45 KDa band. Similar results were reported with AtNHX1, where a 47 KDa band, instead of 58 KDa, was detected (Apse et al., 1999). The other AtNHXs family members have not been tested by Western blotting yet. However, the expressions of both the full length of AtNHX3 protein and a short version of AtNHX3 in yeast gave exactly the expected sizes of products, which are 56 KDa and 40 KDa, respectively. One possible explanation is that the AtNHX3 protein is specifically cleaved in the plant. The evidence for the protein cleavage is that in Arabidopsis transgenic lines overexpressing the AtNHX1 full length, increased amounts of the 47 KDa band instead of an extra 58 KDa band were detected (Apse et al., 1999). When expressed in yeast, these Arabidopsis antiporters are foreign proteins, and yeast may not be able to recognize their cleavage sites, so the expected protein sizes were detected. Another possibility is lipid modifications of the antiport which may not be denatured fully during the SDS-PAGE. Anyway, the question about the size of AtNHX3 protein remains unsolved. Western blotting using antibodies against different parts of the N-terminus of the antiport may provide some answers.

4.2. The subcellular localization of AtNHX3

Western blots with a polyclonal antibody against the central part of AtNHX3 showed that AtNHX3 was exclusively located at tonoplast (Fig. 8). Human NHE6 is located at mitochondrial inner membrane (Orlowski and Grinstein, 1997). The other mammalian Na+/H+ exchangers, NHE1-5, are localized at both plasma and mitochondrial membranes (Grinstein et al., 1989; Garlid, 1988). The subcellular localization of
AtNHX1 appears to be more complex. Except for being mainly localized at tonoplast, AtNHX1 protein was also detected in Golgi/ER-enriched fractions (Apse et al., 1999). Apse et al (1999) explained this as either the contamination of the Golgi/ER fraction with tonoplast fraction or the presence of AtNHX1 in prevacuolar compartments. Yeast NHX1 is reported localized at prevacuoles (Nass and Rao, 1998).

4.3. AtNHX3 expression pattern and response to salt treatment

In our group, five Arabidopsis vacuolar Na⁺/H⁺ antiport genes have been cloned already. An obvious question is that why Arabidopsis, a glycophyte, needs so many isoforms of the vacuolar Na⁺/H⁺ antiporter. We have tried to answer this question by detecting the tissue distribution, expression level and salt response of these five different antiports.

Northern blots showed that AtNHX3 mRNA was in stem, leaf and root tissues, but not in flowers. It is much more difficult to detect AtNHX3 mRNA than AtNHX1 mRNA by Northern blots under the same experimental conditions. This may suggest that AtNHX3 is expressed at lower levels than AtNHX1. Semi-quantitative RT-PCR indicated that AtNHX3 was also present in flowers at lower levels than in other tissues. Combining the Northern blot result with that of semi-quantitative RT-PCR, we could propose that AtNHX3 is equally expressed in leaf, stem and root tissues at a low level, and it is expressed in flower at an even lower level. It has been reported that AtNHX1 is equally expressed in leaf, stem, flower and root tissues (Apse et al., 1999). The tissue distributions of other AtNHXs family members are: AtNHX2 has been detected mainly in flowers; AtNHX5 has been detected in all tissues but not stems; AtNHX8 has been
detected in flowers, stems and roots, but it is not detectable in leaves (unpublished data). Based on the above information, we can conclude that the members of this AtNHXs family are regulated spatially in different tissues. The differential expression of Na+/H+ antiport isoforms was also reported in mammalian cells. It has been reported that NHE1 is expressed in all tissues and cells (Fliegel and Frohlich, 1993). NHE2, 3, and 4 have a limited expression pattern, and they are usually found in the gastrointestinal tract and in the kidney (Orlowski et al., 1992; Wang et al., 1993; Tse et al., 1993). NHE5 is found in several nonepithelial tissues with the declining order: brain>spleen>testis>skeletal muscle) (Klanke et al., 1995). NHE6 is expressed at the highest levels in human brain and skeletal muscle (Orlowski and Grinstein, 1997).

Semi-quantitative RT-PCR with RNA from young seedlings showed a slightly higher expression level of AtNHX3 in young seedlings than in adult plants, but, again, the difference is not statistically significant. Therefore, it is plausible to conclude that there is no significant difference at the AtNHX3 mRNA level in young seedlings and adult plants.

As for the response of AtNHX3 to salt treatment, Northern blotting showed that AtNHX3 mRNA level decreased upon salt stress. However, semi-quantitative RT-PCR indicated a slight increase of AtNHX3 mRNA level, although the difference is not statistically significant. Taken together, our results indicate that AtNHX3 does not response to salt treatment significantly. This is similar to the study of AtNHX1 (Apse et al., 1999), where no increase in AtNHX1 transcript level in response to NaCl was detected. However, Gaxiola et al (1999) reported that AtNHX1 mRNA level increases 4.2-fold upon salt treatment, which resembles the response of yeast NHX1 (Nass and
Rao, 1998). The different results of AtNHX1 mRNA level upon salt stress may be caused by different experimental conditions, such as the age of the plant. However, the expression of a rice (*Oryza sativa*) Na\(^+/+\) antiport, OsNHX1, was reported to be upregulated by salt-stress (Fukuda et al., 1999).

4.4. The function of AtNHX3

Yeast complementation showed that AtNHX3 could partially suppress a yeast salt sensitive mutant, Δnhx1. This suggests that AtNHX3 may have the same function as the yeast NHX1. AtNHX1 and AtNHX8 could also complement Δnhx1 (unpublished data, 2001). Western blots with the tonoplast of AtNHX3 transgenic Δnhx1 strain showed that the expressed AtNHX3 protein was localized at tonoplast; nevertheless, whether AtNHX3 locates on prevacuolar membranes is not clear yet. Ros et al. (1998) found that when bacterial NhaA was expressed in yeast, 30% of the expressed protein was at plasma membrane, while 70% at tonoplast. These results could be due to the possible mislocalization of the Na\(^+/+\) exchanger in yeast.

The yeast complementation with a short version of AtNHX3, AtNHX3-S, lacking the 150 amino acids in the C-terminus of AtNHX3, could partially rescue the Δnhx1 mutant from salt stress, but not as well as the full length of AtNHX3 did. This suggests that the C-terminus is required for the full function of AtNHX3. However, the role of C-terminus in regulating antiport activity needs to be demonstrated. The observation that AtNHX1 C-terminus can specifically interact with a calmodulin-like protein, CaMX, suggests that C-terminus may regulate the Na\(^+/+\) antiport activity by interaction with
Calmodulin (Aharon and Blumwald, 2000). The similar Ca\(^{2+}\)-regulation of Na\(^{+}/H^{+}\) exchanger was also reported with the mammalian NHE1 (Bertrand et al., 1994). The C-terminus of NHE1 contains two calmodulin binding domains, CaM-A and CaM-B, and the CaM-A domain has high affinity with CaM which is thought to be import in transport regulation. There is evidence suggesting that under basal [Ca\(^{2+}\)] levels, CaM-A domain is unoccupied and the activity of the exchanger is inhibited (Bertrand et al., 1994).

It was also proposed that the animal Na\(^{+}/H^{+}\) exchanger activity can be regulated through phosphorylation and dephosphorylation (Goss et al., 1996). The evidence is that a constitutively phosphorylated calcineurin B homolog protein (CHP) was found to interact with the cytosolic tail of NHE1 (Lin and Barber, 1996), and this binding has an inhibitory effect on NHE1, while the dephosphorylation of CHP can activate NHE1. A calcineurin B like protein has also been found in plant, and a pathway of the regulation of plasma membrane Na\(^{+}/H^{+}\) antiport activity through Ca\(^{2+}\) was proposed (Zhu, 2001).

Interaction between the C-terminus of NHE1 and hsp 70 (heat shock protein) was also reported, and it is speculated that this interaction may reflect an intermediate stage of NHE1 biosynthesis and also account for the ATP dependence of the Na\(^{+}/H^{+}\) exchanger (Silva et al., 1995).

The transgenic Arabidopsis line with AtNHX3 has a significantly higher salt tolerance than WT plant, so do the AtNHX1 overexpression lines (Apse et al., 1999) and AtNHX2 overexpression lines (unpublished data). These results give the hope of generating high salt tolerant crops through genetic modification. It would be interesting to know whether a transgenic line overexpressing both AtNHX1 and AtNHX3 (or AtNHX2) will be more tolerant to salt stress than the transgenic lines that only overexpresses either one of these
Na\textsuperscript{+}/H\textsuperscript{+} antiporters. The AtNHX1 and AtNHX3 (or AtNHX2) double overexpression lines could be obtained by crossing the AtNHX1 overexpression lines with AtNHX3 overexpression lines.
Future work:

Future work should aim at characterizing the function of AtNHX3 and the other members of AtNHXs family in Arabidopsis. This should include:

1. GUS-staining of transgenic lines expressing AtNHX3 promoter-GUS construct. The GUS expression patterns may give clues about the expression of AtNHX3 at different developmental stages.

2. In situ hybridization. mRNA levels may provide clues on function of AtNHX3.

3. AtNHX3 knockout lines and AtNHX3 antisense lines. Although these transgenic lines may or may not show any significant developmental difference, phenotypical difference could provide clues about the role of AtNHX3 in key physiological processes, in particular, the role of AtNHX3 in turgor (i.e. leaf expansion) and vacuolar pH regulation (i.e. regulation of K⁺ vacuolar contents).
Reference:


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