Chapter 5: Discussion
DISCUSSION

Plant vacuole serves as a reservoir for various toxic elements. The sequestration of Na\(^+\) ions inside the vacuole is mediated by the activity of Na\(^+\)/H\(^+\) antiporter. Vacuolar Na\(^+\)/H\(^+\) antiporters are ubiquitous membrane proteins present throughout the biological kingdom. The V-ATPases and V-Ppiases are pumps present in the tonoplast. They utilize the free energy of hydrolysis of ATP or PPI to transport H\(^+\) into the vacuole. This helps in maintaining an acidic environment inside the vacuole, which favors degradative processes. Under saline environments an active Na\(^+\)/H\(^+\) antiporter utilizes the proton motive force to sequester Na\(^+\) in the vacuole, thereby reducing the toxic effects of Na\(^+\) in the cytosol, simultaneously maintaining turgor in the vacuole. Plants are able to adapt to the saline environments by regulating the uptake of salt and compartmentation of the salt into the vacuoles and thus preventing toxic effects in the cytosol.

It has been reported that several isoforms of Na\(^+\)/H\(^+\) antiporters exist in Arabidopsis, rice and mammalian systems. These isoforms show differences in tissue specificity, expression patterns and regulation. The present study describes the cloning and isolation of two isoforms of NHX from Pennisetum glaucum and their regulation at the transcript level. Functional validation both in yeast as well as plant system was carried out for one of the isoforms (PgNHXI) showing, vacuolar localization and maximum induction under stress.

5.1 Sequence And Structural Analysis Of PgNHXI

The PgNHXI cDNA was found to 1961 b.p in length with an ORF of 1413 b.p. PgNHXI showed more homology to monocot species like rice etc than dicotyledonous species. Prediction of topology of membrane spanning domains using SOSUI program revealed the presence of 5 transmembrane domains in PgNHXI. This is in contrast to Arabidopsis, AtNHX and rice, OsNHX that show 12 and 9 transmembrane spanning domains respectively. However the ORF of these antiporters is approximately 1600 b.p. and thus longer than PgNHXI. The N-terminal region was found to be oriented towards
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the cytosol; however no vacuolar targeting signals were identified. A long hydrophilic C-terminal of PgNHXI was oriented towards the vacuolar lumen side. Comparison of the mammalian antiporter, NHEI with that in Arabidopsis or Pennisetum glaucum antiporter, PgNHX, reveals that the direction of ion transport for these antiporters is reversed. While NHEI mediates Na\(^+\) influx into the cytosol and H\(^+\) efflux from the cytosol, AtNHXI mediates Na\(^+\) influx from the cytosol and H\(^+\) efflux to the cytosol (Yamaguchi et al., 2003). These results indicate that localization of PgNHXI is similar to the Arabidopsis antiporter.

It has been suggested that glycosylation plays an important role in the proper biosynthetic processing of transporter proteins in yeast (Wells et al., 2001). The position of putative glycosylation sites seem to be well conserved in PgNHXI and OsNHX indicating that both the plant antiporters maybe similarly glycosylated (Fukuda et al., 1999). There has been however no report so far to demonstrate glycosylation of plant NHX. In addition to glycosylation sites, coiled-coil domains and ankyrin repeat known to be involved in protein-protein interactions were also identified in PgNHXI. Further several putative phosphorylation and myristoylation sites are predicted.

In mammals, ameloride competitively inhibits Na\(^+\)/H\(^+\) exchange with Ki of 1-100 \(\mu\)M depending on the cell type restricted manner (Counillon et al., 2000). In Arabidopsis thaliana, AtNHXI, and rice OsNHX the motif, LFFIYLLPP has been reported to confer ameloride sensitivity (Darley et al., 2000). However, no such motif was found in PgNHXI, which suggests that there may be functional differences in these antiporters.

5.2 Localization And Transport Of PgNHXI

Previous studies have reported the localization of S. cerevisiae, ScNhxl-GFP fusion protein to the perivacuolar compartment (Nass et al., 1998). Arabidopsis thaliana, AtNHXI has been shown to be a vacuolar protein (Yokoi et al., 2002) and the Oryza sativa antiporter; OsNHXI also similarly showed tonoplast localization (Fukada et al., 2004). In the present study the expression of the
green fluorescent protein fused to the C-terminus of PgNHXI revealed exclusive localization to the perivacuolar space in a yeast mutant strain, deficient in endogenous antiporter, AXT3K. Further, staining with PgNHXI specific antibodies revealed staining in vacuolar membrane in addition to the vacuolar lumen in antiporter over expressing yeast cells. Although the exact domain involved in localization is not known, the C-terminal of Arabidopsis thaliana antiporter, AtNHXI is implicated in transport to the vacuole. Over-expression of deletion mutant of AtNHXI bearing only the C-terminal domain has been shown to localize to the vacuole suggesting that signals for vacuolar targeting are present in the C-terminal region (Yamaguchi et al., 2003). This kind of a C-terminal vacuolar targeting has been found in other vacuolar genes like chitinase etc. (Vitale et al., 1999). The C-terminal region of PgNHXI showed high degree of homology (80%) to the C-terminal domain of AtNHXI. The present study shows that PgNHXI was folded properly and localized to the vacuoles.

The docking, transport, formation of transport vesicles in plants, mammals and yeast cells is known to be regulated by the family of small GTPases, of the Rab family. The Rab family in Arabidopsis consists of more than 57 members (Pereira-Liel et al., 2001). The Rab GTPases are considerably more diverse in plants and mammal than in yeast (Rutherford et al., 2002). Until recently thought to be primarily involved in vesicle docking and fusion, Rab proteins have emerged as key regulators of vesicle targeting and specificity (Zerial et al., 2001). In fission yeast Schizosaccharomyces pombe mutation in any of the seven members of the Rab genes produces a distinct phenotype, indicating lack of redundancy and existence of a specific function for each gene (Pereira-Liel and Seabra 2001). Among the different members of the Rab protein family, the Rab7 controls delivery of the internalized material into degradative compartments and the acquisition of lysosomal hydrolases (Bruckert et al., 2000). There are suggestions that Rab proteins especially Rab 7 may be involved in transporting NHX to the vacuoles. When yeast, rab7-null mutant, ypt7, is transformed with an intact Rab7 gene, highly fragmented vacuoles seen were replaced with a prominent central vacuole as seen in the wild type.
cells. *Arabidopsis* Rab7 gene shows an increase in the transcript levels after abiotic stress (Bucci et al., 2000). Vacuoles play a critical role in salt tolerance by accumulating sodium, which is removed from the cytosol where it is toxic. In context of the emerging function of Rab7 proteins in vacuolar biogenesis of eukaryotic cells and the role of vacuoles in salt tolerance, a possible interaction between the PgNHXI and Rab-mediated pathway was assessed in this study. Transformation of ypt7 mutant cells with the PgNHXI-GFP construct, however, did not show any defect in PgNHXI localization. In vivo analysis by yeast two-hybrid system also confirmed that there was no direct interaction between the Rab7 and PgNHX. It has recently (Ali et al., 2004) been shown that yeast NHX interacts with a GTPase activating protein, GAP. The results from the present study suggest that vesicular transport of Pg NHXI is most likely independent of interaction with Rab7 and probably mediated by alternate pathways.

Much evidence for a retrograde golgi to ER pathway in plants has come from the use of secretory inhibitor, Brefeldin A (BFA). BFA is a fungal toxin that inhibits egress of proteins from ER. This drug has been often used as an inhibitor of secretion and vacuolar protein transport in plant cells (Nebenfuhr et al., 2002). One of the questions that has received little attention is to unravel the route of NHX transport to vacuole. The transport of proteins to vacuoles occurs either by the direct trafficking to the vacuole or via ER-golgi pathway (Hawes et al., 2005). Various fluorescent protein constructs of Golgi-targeted membrane proteins have been reported to be redistributed into the ER network on exposure to BFA resulting in an almost total loss of golgi fluorescence in the cells (Saint Jore et al., 2002). In PgNHXI-GFP transformed yeast cells (ANT3K) as described earlier a vacuolar GFP localization was observed. Treatments with BFA for a short time period of 5 min. revealed punctuate fluorescence. Following prolonged treatment with BFA there was almost complete loss of fluorescence. It is possible that the observed loss of fluorescence may be due to adverse effects of BFA on GFP synthesis. However it has been reported in other systems that removal of drug results in redistribution of fluorescence to the golgi bodies. Therefore the loss of
fluorescence observed in our system would indicate disruption of PgNHXI trafficking by BFA.

Events that mediate recruitment of antiporter to the vacuoles are not clearly understood. The present study demonstrates that the transit of PgNHXI to vacuoles occurs via the golgi pathway and in a Rab7-independent manner.

5.3 Functional Analysis Using Yeast Complementation

PgNHXI encodes a predicted 52 kDa hydrophobic polypeptide that shares similarity with the NHE type Na⁺/H⁺ exchangers and Saccharomyces cerevisiae NHX proteins. The NaCl tolerance of yeast is primarily dependent on the activity of ENA1-4 Na⁺ ATPases but deletion of Nhal or NHX genes further increases the NaCl sensitivity of an ena1-4 mutant. Hence the ability of PgNHXI to suppress the Na⁺ sensitivity of yeast mutants defective in endogenous Na⁺/H⁺ antiporters was tested in this mutant background. PgNHXI failed to show co-operativity with the endogenous efflux proteins NHAI and ENA1-4. Instead PgNHXI promoted ion uptake and compartmentation that was commensurate with ScNHXI activity. Comparisons with the ScNHX show that while the endogenous NHX failed to show growth at 70 mM concentrations, the plant PgNHX could confer tolerance upto 70 mM of NaCl. Complementation studies in yeast have been previously done with AtNHX (Quintero et al., 2000) and OsNHX (Fakuda et al., 2004) and cotton (Wu et al., 2004). These antiporter genes have been shown to confer tolerance in yeast upto 50 mM NaCl. However the plasma membrane antiporter AtSOS1 has been shown to confer tolerance upto 70 mM of NaCl (Shi et al., 2002). Our results show that PgNHXI may be more potent in sequestering Na⁺ into the vacuoles (Figure 9).

It has been reported that AtNHXI from Arabidopsis suppresses the NaCl and hygromycin B sensitivity of a yeast nhxl mutant, suggesting that AtNHXI is the plant ortholog of ScNHXI (Darley et al., 2000). Hygromycin is a cationic antibiotic that is driven into the cell or vacuolar lumen in response to a
negative membrane potential or difference in pH. Since Na\(^+\)/H\(^+\) exchange is electro neutral and assuming that the plant antiporter does not directly catalyze the transport of hygromycin, uptake into the vacuoles in the wild type cells must be affected by changes in pH across the vacuolar membrane as a result of Na\(^+\) dependent H\(^+\) exchange (Darley et al., 2000). Thus sensitivity of NHXI mutants to hygromycin probably results from impaired sequestration of this toxic cation into the vacuolar lumen. Similar to AtNHXI, PgNHXI also showed lower tolerance to hygromycin in yeast cells. It is possible that the mechanisms operating in sequestration may be plant specific and different from those operating in yeast cells. Nevertheless, results with yeast complementation point to the identification of a functional plant Na\(^+\)/H\(^+\) antiporter from *P. glaucum* that localizes to the vacuole.

### 5.4 PgNHX Has At Least Two Isoforms

Seven isoforms of Na\(^+\)/H\(^+\) antiporters have been described in the animal cells (Counillon et al., 2000). NHE1-5 localize to the plasma membrane whereas NHE6 to the mitochondria and NHE7 to the Golgi. In *Arabidopsis* six isoforms of the gene, AtNHX1-6, have been reported which are differentially regulated (Yokoi et al., 2002). In rice based on sequence information eight putative isoforms have been reported. Similarly in *Zea mays* six isoforms have been reported (Zorb et al., 2004). These isoforms show variations with respect to their expression patterns.

Screening of the salt stressed root library lead to the identification of another isoform denoted as PgNHXII. This indicated that at least two isoforms exist in *Pennisetum* showing 52% similarity at the nucleotide level. Interestingly, the two isoforms showed differences in their topological organization. PgNHXII revealed the presence of four transmembrane domains. A region in PgNHXII that is cytosolic is found to be membrane associated in PgNHXI. This may account for the lesser number of transmembrane domains in PgNHXII. Another distinguishing feature was the reverse orientation of the C-terminal hydrophilic region. While the C-terminal of PgNHXI seems oriented towards...
the vacuolar lumen side, it was cytosolic facing in PgNHXII. Further, as compared to PgNHXII the C-terminus region is 262 b.p. longer in PgNHXI. These observations indicate that the regulation of the two isoforms may be unique.

5.5 Real Time Transcript Analysis Of PgNHX Isoforms

Regulation of the PgNHX isoforms at transcript level in response to various abiotic stress conditions was studied by real time PCR. PgNHXII showed a higher basal level of transcript expression in shoots as compared to PgNHXI. This increased expression level ~100 fold was independent of any stress treatment indicating that a strong promoter drives PgNHXII expression. The PgNHX isoforms were expressed in all the tissues, such as root, old leaf, panicles with maximum expression in shoots. The physiological significance of the high-observed basal level is unclear, but it is possible that the PgNHXII maybe involved in influx of K⁺ into the cytoplasm and thus required to be constitutively expressed at higher levels. The ubiquitous and relatively high basal level of expression indicates that NHX genes have important physiological roles even in the absence of stress. It has been shown that AtNHX1 can mediate both Na⁺ and K⁺ coupled transport to vacuoles (Zhang and Blumwald, 2001) and the vacuolar Na⁺/H⁺ antiport has been shown to regulate vacuolar pH (Yamaguchi et.al., 2001). Also vacuoles isolated from leaves of nhxl plants had a much lower Na⁺/H⁺ and K⁺/H⁺ exchange activity. nhxl plants also show an altered leaf development with reduction in the frequency of large epidermal cells and a reduction in overall leaf area compared to wild type plants (Apse et al., 2003). Maintenance of K⁺ levels in leaves is important for growth of the plant under salt and ABA stress and the high basal levels of expression especially in shoot tissues may indicate the importance of this gene in K⁺ homeostasis (Venema et al., 2003). Another reason may be the inherent high expression levels in Pennisetum itself. All these indicate that PgNHXII may be involved in essential house-keeping functions.
Treatment of shoot tissues with NaCl for different lengths of time show an induction of the transcripts for both isoforms, with PgNHXI showing a greater induction than the transcript levels for PgNHXII where induction observed was modest. The basal expression of PgNHXII was always found to be 100-fold higher than PgNHXI even though the induction levels were low. Thus both the isoforms seem to have functions in tolerance of osmotic stress and it seems that while PgNHXI is the stress responsive isoform, PgNHXII is the constitutively expressed form. In comparison to untreated plants, NaCl treatment caused an up-regulation of the transcript levels within 2h. Faster turnover rates for PgNHXI transcript or effects of NaCl on the stability of the PgNHXI mRNA could be the possible reasons. An up-regulation of transcript after NaCl treatment has been observed for AtNHXI (Shi et al., 2002), OsNHX (Fakuda et al., 2004) and cotton (Wu et al., 2004). It has been suggested that NaCl increases the stability of AtSOSI mRNA and it seems possible that such a stabilization effect of NaCl on PgNHXI transcript may occur. In moderately halophytic archaean, *Haloferax mediterranei*, gas vesicles are synthesized when grown in presence of 17-30% (w/v) NaCl while, cultures grown in low 15% salt medium are gas vesicle free (Jager et al., 2002). The gas vesicle consists of two proteins, the hydrophobic gas vesicle protein (GvpA) constituting the vesicle wall and the hydrophilic GvpC located on the outer surface. While the stability of most of the gvp transcripts is independent of external salt concentration in the medium, gvpA mRNA decays about twice as fast in cultures grown at 18% salt compared to cultures grown at 25% salt. It has been suggested that an mRNA secondary structure located at the 3' end of gvpA mRNA that protects 3' to 5' exonucleases action is stabilized by high salt concentration (Jager et al., 2002). The notion that PgNHXI transcript stability may correlate with its relative abundance through mechanisms promoting inherent and induced stability needs further analysis.

Treatment with ABA resulted in rapid and high levels of induction for both isoforms upto 4h followed by a rapid degradation of the transcript. Interestingly, unlike SOSI (Shi et al., 2002) AtNHXI has been reported to be induced by ABA treatment, which suggests that different signal transduction
pathways may be involved in the regulation of plasma membrane Na⁺/H⁺ antiporter, SOSI and vacuolar Na⁺/H⁺ antiporter, AtNHXI. This kind of a response to ABA and NaCl is typical of osmotic stress responsive genes. The induction of ABA is an important response to stress and mediates the expression of several proteins that function towards alleviating deleterious effects of osmotic stress. While an up regulation of transcripts after ABA treatment has been reported for AtNHXI (Shi et al., 2002) and Gossypium (Wu et al., 2004), detailed time kinetics has not been done. The degradation of transcript after prolonged treatment has been shown in these isoforms. It is possible that long exposures lead to saturation of sensors and in the absence of any NaCl stress may lead to the reduction in the transcript levels. This also indicates that mRNA is unstable in the absence of ionic stress after 4h time point.

Low temperature treatment and drought stress induced by water withdrawal for different time periods lead to a drastic reduction of the transcript levels of both the PgNHX isoforms. Interestingly PgNHXII showed no detectable transcript upon cold treatment. Infact even basal levels were not detected. It is possible that osmotic stress may either directly induce mRNA destabilization and/or some of the factors required for maintenance of mRNA stability may either be inactivated or not induced. Cold treatment at 4°C for 24h and dehydration for 30 min did not alter transcript levels (Shi et al., 2002).

Both the PgNHX isoforms respond to salt and ABA treatment. ABA levels are induced during salt and drought conditions which lead to induction of ABRE-responsive genes. PgNHX isoforms do not respond to drought treatment and it may be proposed that the response of these genes is ionic stress induced. Further the factors involved in providing stability to the mRNA during NaCl treatment or conversely the factors involved in transcript destabilization need further analysis.
5.6 Analysis Of Cis-Regulatory Elements Of PgNHXI

Stress responses primarily include transcriptional regulation of gene expression and this depends on the interaction of transcription factors with cis-regulatory sequences. Interactions with upstream regulatory sequences frequently determine the rate of transcription and activation and/or repression of promoter in response to environmental conditions. It is interesting to note that the PgNHX isoforms respond to all kinds of stresses and show a marked induction of transcript levels 2h post NaCl and ABA treatment. With the aim of understanding the cis-elements that function in response to abiotic stress a 1500 b.p. fragment upstream and corresponding to the PgNHXI promoter was isolated. This is the first report of isolation of NHX promoter from a monocot plant. AtNHXI is the only other plant promoter so far isolated and analyzed. Analysis of the promoter for putative sites showed the presence of several sites including, G-box, ABRE, MYB, MYC, light responsive elements and also low temperature responsive elements (Figure 13). Both ABA-dependent as well as independent pathways have been identified in plants.

The ABA-dependent pathways are thought to mediate the gene expression through an ABRE element and b-ZIP transcription factors (Busk et al., 1998), while the other pathway is through MYC and MYB elements and transcription factors (Yamaguchi-Shinozaki., 1993). The observation of reduced salt stress mediated up-regulation of AtNHXI in the ABA-insensitive and ABA-absent mutants, abi-1, aba2-1 and aba3-1 mutants suggest a partially ABA-dependent regulation of AtNHXI expression (Shi and Zhu, 2002). The presence of a putative ABA-responsive element within the promoter region of PgNHXI gene suggested regulation by ABA-mediated pathway. Analysis of PgNHXI promoter revealed the presence of five putative ABRE elements with typical AGCT motif present within a 200 b.p. region whereas AtNHXI has only one ABRE. Thus due to the presence of multiple functional ABREs it is possible that PgNHX may be more strongly regulated by ABRE than AtNHX. The core element of ABREs consists of the CACGTG motif also known as G-box motif, which functions in the regulation of plant genes stimulated by a variety of
environmental signals and nucleotides flanking the ACGT core specify the DNA-binding interactions and subsequent gene activation (Williams et al., 1992). It was found that an oligo corresponding to the core sequence and flanking sequences could bind the nuclear extract indicating the presence of active ABRE. Coupling elements which are active in concert with a consensus ABRE but have no activity on their own have been reported (Shen et al., 1996). CEI element that is necessary for ABRE function was also present in PgNHXI. In promoters of the barley HVA22 and HVA1, the coupling elements CEI and CE3 (ACGCGTGTCCTC) are necessary for activation by ABA.

Salt stress inducible basic helix-loop-helix type transcription factors as well as MYC2 and MYB2 regulate ABA-responsive gene expression in Arabidopsis. Constitutive overexpression of AtMYC2 and AtMYB2 results in constitutive expression of RD22 and levels are increased upon ABA treatment. Transgenic Arabidopsis plants over expressing MYC2 and MYB2 show higher osmotic stress tolerance as measured by electrolyte leakage from cells (Abe et al., 2003), particularly drought. Reduction in the transcript levels and the absence of DRE lead us to study the functionality of MYB-site. Gel mobility shift assay with the MYB-consensus binding sequence showed existence of functional MYB-binding sequence upstream of PgNHX gene. Functional PgNHXI specific MYB and MYC indicates that these cis-elements may be involved in ABA/NaCl response and not dehydration stress. Thus it is plausible that osmotic stress regulated stress tolerance determinant, PgNHXI antiporter, may function following activation by MYC/MYB through an ABA/NaCl dependent pathway. Different signaling pathways are however known to be responsible for imparting salt tolerance in plants providing an indication that antiporters may infact be regulated through multiple pathways.

5.7 PgNHXI Interacting Proteins

The importance of Ca\(^{2+}\) in mediating plant responses to external stimuli of biotic and abiotic origin is now a subject of interest. Mechanisms that govern the generation and translation of Ca\(^{2+}\) induced signals into appropriate cellular
responses support the role of calcium as an important second messenger in both animal as well as plant cells (Sanders et al., 1999). The current hypothesis is that spatial and temporal changes in the intracellular levels of second messengers such as calcium initiate cellular responses to a given stimulus (Bressan et al., 1998). PgNHX antiporter is rapidly activated in response to osmotic stress. Results discussed earlier reveal that addition of Ca\(^{2+}\) positively influenced/ enhanced the ability of PgNHX to impart salt tolerance in yeast cells. Interestingly this Ca\(^{2+}\) dependent enhancement was diminished upon treatment with divalent cation chelator, EGTA. Experiments with leaf discs of transgenic plants over expressing PgNHX\(1\) also showed enhanced tolerance on addition of extracellular Ca\(^{2+}\), suggesting the influence of Ca\(^{2+}\) on response to salt stress (Figure 44). In order to get an insight into the mechanism of PgNHX activation the role of calcium as a second messenger was investigated.

Ca\(^{2+}\) dependent modulation of cellular processes occurs via intracellular Ca\(^{2+}\) binding proteins also known as Ca\(^{2+}\) sensors of which calmodulin, CaM, is one of the best characterized. Although CaM has no catalytic activity of its own it activates numerous target proteins upon Ca\(^{2+}\) binding (Snedden et al., 2001). In addition to the evolutionarily conserved form of CaM, plants posses an external family of CaM isoforms and CaM-like proteins, which have been implicated in stress responses (Pardo et al., 1998). Analysis of the PgNHX\(1\) revealed the presence of a CaM binding site at position 302, which extends into the vacuolar lumen. Confocal microscopy using CaM-specific monoclonal antibodies revealed co-localization with PgNHX. Using yeast two hybrid system in vivo positive interaction was observed between PgNHX and CaM suggesting the role of Ca\(^{2+}\)/CaM-signaling pathway in PgNHX activity response to osmotic stress. PgNHX\(1\) is known to be localized to the vacuole; however CaM is cytosolic in location. In view of the above, the results showing co-localization of CaM and PgNHX as well as the direct in vivo interaction are intriguing. Recent analysis of the vacuole proteome (Carter et al., 2004), has revealed the existence of a large number of proteins that are primarily associated with ER including 40S and 60S ribosomal proteins, lumenal binding
protein, calreticulin, calnexin and CaM. The presence of these proteins in the dense vesicles coated with ribosomes (ER bodies/protease precursor vesicles) that bud directly from the ER and undergo fusion with the vacuole tonoplast might explain the localization of some cytosolic proteins to the vacuole. Deletion of the hydrophilic C-terminus, facing the vacuolar lumen has been reported to cause a drastic increase in the relative rate of Na\textsuperscript{+}/H\textsuperscript{+} transport in *Arabidopsis*. The ratio of Na\textsuperscript{+}/K\textsuperscript{+} transport was observed to be twice that of the unmodified AtNHX1 (Yamaguchi *et al.*, 2003). These observations indicate that deletions of the C-terminal where the CaM binding site is present, result in alteration of NHX activity. It has been reported in mammalian systems that mutations in the CaM binding region (region A, amino acid 636-656 of NHE1) render NHE1 (Wakabayashi *et al.*, 2000) constitutively active (Betrand *et al.*, 1994). These mutations abolished the ionomycin-induced activation of NHE1. Region A, when unbound by CaM, functions as an auto-inhibitory domain and the ionomycin induced increase in Ca\textsuperscript{2+} activates the exchanger by permitting CaM to bind region A, thus preventing it from exerting the inhibitory effect (Wakabayashi *et al.*, 1994). The auto inhibitory effect of the CaM binding site has been postulated for other CaM binding proteins such as the plasma membrane Ca\textsuperscript{2+} pump. It is possible that CaM interaction with PgNHX may have a role to play in regulation and/or modulation of antiporter activity. It has been shown previously that overexpression of CaM imparts salinity tolerance. It is possible that this occurs by relieving of NHX auto-inhibitory domain. The physical interaction of PgNHX/CaM thus indicates the possibility of regulation of NHX activity through CaM binding similar to that occurring in the NHE1. Activation of the tonoplast resident cation exchanger has been reported to mediate downstream events that induce localized alterations in Ca\textsuperscript{2+} levels and regulate antiporter through a Ca\textsuperscript{2+}/calmodulin dependent pathway (Cheng *et al.*, 2004). Thus a complex interplay between various components maybe involved in regulation of antiporter activity.

It has been reported that the activity of AtNHX diminished in sos2 mutants, which shows a salt sensitivity phenotype (Yokoi *et al.*, 2002). It was with this notion that the possible PgNHX and CIPK, Ca\textsuperscript{2+} induced protein kinase, was
assessed. Confocal microscopy indicated co-localization of CIPK with PgNHXI and antibodies specific to CIPK immunoprecipitated PgNHXI suggesting a physical association of the two proteins. Further, antibodies to CIPK co-immunoprecipitate PgNHXI from extracts of antiporter over-expressing transgenic plants. Using yeast two-hybrid an in vivo interaction between CIPK and PgNHXI was observed. Together these results conclusively show the interaction of PgNHXI and CIPK in vivo and also in planta (Figure 21). The question of how CIPK is recruited to the vacuolar location is not clear. SOS3 targets SOS2/CIPK to the plasma membrane to allow activation of SOS1 Na\(^+\)/H\(^+\) antiporter. Several SOS3 homologs of Arabidopsis have been identified and it seems possible that interaction of an SOS3 homologue may function in recruitment of SOS2/CIPK to the tonoplast (Guo et al., 2001) and CIPK could modulate PgNHX activity in vivo by phosphorylation. Several putative phosphorylation sites exist in PgNHXI.

Calnexin is involved in the proper folding of proteins and acts as a chaperone. PgNHXI was found to co-localize with calnexin in yeast cells. Calnexin-specific polyclonal antibodies could immunoprecipitate PgNHXI, showing an association of the two proteins in vivo. The interaction was confirmed using the yeast two-hybrid system. These results for the first time show interactions and possible regulation of PgNHX by calmodulin, calnexin and CIPK. The various pathways by which calcium maybe regulating PgNHX is shown in Figure IV.
Figure IV: Hypothetical model indicating possible mechanisms by which calmodulin, calnexin and SOS2 may regulate PgNHX activity. The arrows in blue show results of the present investigation. The arrows in black are earlier published reports.

5.8 Over Expression Of PgNHXI In Brassica juncea

Over-expression of Na$^+$/H$^+$ antiporter results in concomitant expression of proton pumps V-ATPases and Ppiases, which create high acidic environments required for transport of Na$^+$ into the vacuoles (Apse et al., 1999). Sequestration of Na$^+$ into the vacuoles leads to cell expansion. PgNHXI conferred salinity tolerance to plants over-expressing the gene. Tolerance of germinating seed and seedlings to sodium was first assessed. Seeds of PgNHXI over-expressing plants were able to germinate in high NaCl concentration at which wild type seeds failed to germinate. Over-expression of PgNHXI had no adverse effects on seedling establishment in the absence of NaCl. These results show for the first time the ability of PgNHXI to confer...
tolerance to high NaCl concentrations at the seedling stage. A factor that could contribute to enhanced germination of PgNHXI over expressing lines could be the supply of nutrients to developing embryo and the antiporter might contribute to the transport of essential cationic species like K⁺/ Na⁺. It has been demonstrated that more than 90% of water transported into the expanding fruit of tomato occurs through the phloem (Zhang et al., 2001). Thus, the ability to maintain a high cytosolic K⁺/Na⁺ concentrations ratio along the symplastic pathway may be important for seedling vigor. It has been shown that AtNHXI mediates K⁺ and pH homeostasis in cells and confers salinity tolerance to plants (Apse et al., 2003) and it not only counters salinity but is also involved in developmental process.

Regeneration of plants from cotyledons under high NaCl concentrations in the medium was studied as another parameter to assess stress tolerance. PgNHXI over-expressing Brassica juncea plants were able to regenerate whereas the wild type plants failed to show any regeneration in presence of 100 mM NaCl in the medium (Figure 39). It has been shown that the regeneration frequency in salt-stressed callus of sensitive cultivars of rice was lower as compared to control and salt tolerant cultivars of rice such as CSR10 and Pokkali showed better growth in presence of salt (Shankhdhar et al., 2000), thus showing that NaCl stress interferes with the normal metabolism of the cell. The ability of PgNHXI over expressing lines to normally regenerate under saline conditions is also suggestive of the important role in vacuole expansion. It has been observed that leaf area was reduced in mutant NHXI plants, nhxl and this difference was evident in young leaves and persisted to fully expanded leaves (Apse et al., 2003). Effect of the antiporter on ionic homeostasis, has an impact on the ability of cells to undergo expansion. The large central vacuole effectively reduces the surface to volume ratio in plant cells and provides the turgor necessary for cell expansion (Mimura et al., 2003). Thus ability of PgNHXI over-expressing lines to be able to regenerate under salt stress could be by utilizing vacuole expansion to maintain growth.
Growth is often used as a parameter to assess tolerance, as it is the endpoint of metabolic process. One-month-old transgenic plants were able to grow well when continuously watered with 300 mM NaCl stress. These results corroborate with the earlier findings showing the tolerance of plants over-expressing AtNHX1 in various systems such as *Arabidopsis* (Apse et al., 1999), and *Brassica napus* (Zhang et al., 2001). However, these transgenics differed with respect to their tolerance to concentration of NaCl stress. Our studies show that *B. juncea* plants over-expressing the monocot gene from *Pennisetum* could resist up to 300 mM of NaCl at the whole plant level. Further the leaf disc senescence analysis (Figure 40) as well as the germination tests demonstrate that PgNHX was more potent in imparting salinity tolerance. Based on the above results it can be hypothesized that overexpression of PgNHX1 would enable pumping of Na⁺ into the vacuoles and impact cellular osmolarity. This would increase the concentration of Na⁺ in the vacuole and additionally help in removal of potentially toxic Na⁺ from the cytoplasm. Higher ionic concentration in the vacuole would promote inflow of water leading to an increase in vacuolar volume and thereby cause cell growth.

Plants over-expressing Na⁺/H⁺ antiporter showed higher levels of K⁺ under non-stress conditions when compared to wild type plants. This is consistent with reported literature where AtNHX1 T-DNA insertional mutants show altered Na⁺/H⁺ as well as K⁺/H⁺ antiporter activities (Venema et al., 2002). Thus it is plausible that under normal conditions, the Na⁺/H⁺ antiporter maybe involved in sequestering K⁺ into the vacuoles (Scttosanto et al., 2004).

The leaf and root K⁺ contents in transgenic plants grown in 300 mM NaCl was lower than those of plants grown in absence of salt. Potassium concentrations are maintained at 100-200 mM. Following NaCl stress Na⁺ ions may displace K⁺ from its carrier binding sites and this competition could lead to impaired K⁺ uptake and lower K⁺ cytosolic concentrations. However the growth of transgenic plants was not significantly affected by high salinity, suggesting that K⁺ nutrition was not compromised. Further, under conditions of potassium deficiency, cytosolic K⁺ is maintained by mobilization of vacuolar K⁺.
significant difference was observed in $\text{Ca}^{2+}$ contents of the plants. Upon NaCl stress, the leaves and stems showed high levels of $\text{Na}^+$ accumulation. A 1.5-2.8-fold increase in the $\text{Na}^+$ content was observed in leaves of transgenic plants watered with salt. Similarly a 1.2-2.5-fold increase in $\text{Na}^+$ content was observed in the stems and a similar increase was observed in the roots. Seeds show 0.04 mg of $\text{Na}^+$ per 100 mg dry weight. Sequestration of $\text{Na}^+$ into the vacuole of hair cells decreases the cellular water potential and facilitates water uptake through root hairs under NaCl stress. This suggests that PgNHXI is an ideal candidate gene for transferring NaCl tolerance capabilities, without the use of tissue specific promoters. It is possible that the targeting signals for tissue localization may reside within the gene itself.

The present study demonstrates the ability of plants to tolerate salt at all stages of the life cycle, from seed germination to the seed set stage. Also PgNHX was found to be better in its ability to tolerate stress compared to OsNHX (Ohta et al., 2002) and AtNHX (Apse et al., 1999) genes. It should be noted that the stems and seeds show an increase in the $\text{K}^+$ content. However, the roots had no alterations in $\text{K}^+$ levels. Interestingly, no significant changes in the $\text{Ca}^{2+}$ levels were observed in transgenic plants before and after stress treatments on providing exogenous calcium although salt tolerance was ability was enhanced. However, an increase in stem $\text{Ca}^{2+}$ levels after stress treatment was observed in control plants. These observations point out that over-expression of PgNHX does not alter the $\text{H}^+$ balance and thus does not adversely affect activities of other transporters.

Proline contributes to osmotic adjustment and protection of macromolecules during dehydration and as a hydroxyl radical scavenger (Kishor et al., 1995). Proline levels were measured in control and transgenic plants after subjecting 10-day-old seedlings to sub-lethal NaCl stress. Plants over-expressing PgNHXI showed lower proline induction in comparison to control plants. This is consistent with the ability of transgenic plants to overcome osmotic stress. However, the proline content was increased by 2-3-fold in NaCl treated versus
Untreated transgenic lines. An increase in proline levels has been shown previously in tomato over expressing AtNHXI (Zhang et al., 2001). These observations suggest that proline helps in maintenance of osmotic stress.

Thus the present study demonstrates the use of PgNHXI as an efficient candidate to impart salt tolerance in plants.