3. RESULTS

3.1: Isolation of total RNA from *Salicornia brachiata*

Total RNA was isolated from young shoots as described in the materials and methods. Purity and concentration of the isolated RNA was determined spectrophotometrically. The yield of RNA ranged between 0.5-0.75 mg per gram of fresh tissue and the absorption ratio ($A_{260}/A_{280}$) was 1.98 (close to ratio for pure RNA). The integrity of the RNA was also checked on 1.0 % (w/v) agarose gel containing 2.4 M formaldehyde. Most of the RNAs were visible as distinct bands (Figure 3.1). DNA contamination was not observed. Since the integrity and quality of RNA was verified and found to be very high, hence it was used for subsequent experiments.
Figure 3.1: Analysis of total RNA isolated from *S. brachiata*. Total RNA was isolated from young shoots and separated on 1.0 % (w/v) formaldehyde agarose gel. The major RNAs bands are indicated. 10 µg of total RNA was loaded in each lane.

3.2: Isolation and cloning of full length Na\(^+\)/ H\(^+\) antiporter (*SbNHX1*) gene from *Salicornia brachiata*

3.2.1: Isolation of *SbNHX1* gene using degenerate primers

Partial length *SbNHX1* gene was obtained using degenerate forward (5’ATGTKGTCACAR TTRAGCDCTY-3’) and reverse (5’CACCAAAHACAGGCCGCATG’-3’) primers by PCR using cDNA as template. The PCR product was run on 1% agarose gel and a single predicted band was observed. The DNA from the excised band was eluted, purified and sequenced. The obtained sequence of the amplicon was aligned with known NHX1 sequences and it was confirmed that indeed the amplicon isolated by degenerated primer is part of NHX1 gene. This partial gene sequence was used to obtain the full length *SbNHX1* gene.

3.2.2: 5’-Rapid Amplification of cDNA Ends (5’-RACE)

To obtain the full-length sequence of *SbNHX1* gene, cDNA was amplified by reverse transcription PCR (RT-PCR) and 5’ and 3’-Rapid amplification of cDNA ends (RACE) were done as per details given in materials and methods. The first strand product was purified using SNAP column to remove unincorporated dNTPs and PK1 primer. TdT (Terminal deoxynucleotidyl transferase) was used to add homopolymeric tails to the 3’ ends of the cDNA. The tailed cDNA was amplified by PCR using a nested GSP primer (NHXR: 5’CACCAAHACAGGCCGCATG-3’), and
homopolymer-containing anchor primer which permit amplification from the homopolymeric tail (Invitrogen, USA). For 3’ RACE cDNA was synthesized at 42 °C for 50 min using the PK1 oligodT primer (5’-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3’). The first 3’RACE PCR reaction was carried out using adaptor primer PK2 (5’-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGCTTTTTTTTTTTTTTTTTT-3’) and GSP 1(5’ ATGTKGTCACAR TTRAGCDCTY-3’), the eluted product was gel extracted in 50 µl miliQ water and diluted to 1:50 for 2nd RACE reaction. The 2nd RACE PCR reaction was carried out using adaptor primer PK3 (5’-GAGGACTCGAGCTCAAGC-3’) and GSP-2 (5’-GAGGAGAATCGYTGGGATG-3’). The 2nd PCR RACE reaction yielded 1683 bp single band which was confirmed by sequencing.

3.2.3: Cloning of full length SbNHX1 gene

The sequences of 3’- and 5’- RACE, overlapped with the 470 bp fragment. The 3’- and 5’- RACE sequences were then assembled into full length cDNA. After conceptual In-silico translation ORF, the start codon and stop codon was defined. The full length cDNA sequence comprised of an ORF, the 3’-untranslated region (3’-UTR) of the SbNHX1 gene. The full-length SbNHX1 ORF (Figure 3.2) of 1683 bp was amplified using reverse transcriptase polymerase chain reaction (RT-PCR).

Figure 3.2: Amplification of full-length SbNHX-1 ORF. Primers were designed based on the cDNA sequence flanking the translational start and stop codons. M: 1kb DNA ladder
The primers used were designed based on the cDNA sequence flanking the start and stop codons and restriction sites sequence were also included for cloning. The amplified PCR product was cloned into pGEM-T Easy vector. For confirmation of insert into the plasmid vector, white (recombinant colony) colonies were randomly picked (Figure 3.3) and subjected to colony PCR. Plasmid was also isolated from these white colonies, purified and sequenced from both directions.

Figure 3.3: The selection of *E. coli* cells containing recombinant plasmid DNA.

Indicated in the figure are blue colonies (containing non-recombinant plasmid) and white colonies (containing recombinant plasmid).
3.3: *In silico* characterization of *Salicornia brachiata* Na\(^+\)/H\(^+\) antiporter (*SbNHX1*) gene

The cDNA of Na\(^+\)/H\(^+\) antiporter gene from *Salicornia brachiata* named *SbNHX1* (accession number: EU448383) is 2110 bp, consisting of a 3’–noncoding stretch of 427 bp. The open reading frame of *SbNHX1* gene is 1683 bp and encoded a polypeptide of 560 amino acid residues (Figure 3.4). The estimated molecular mass and isoelectric points of the Na\(^+\)/H\(^+\) antiporter protein were 62.44 kD and 6.83, respectively. Blast result and the phylogenetic tree analysis showed that *SbNHX1* was Na\(^+\)/H\(^+\) antiporter located on vacuolar membrane. A conserved NHE (Na\(^+\)/H\(^+\) exchange) domain was found by BLASTp. An N-terminal transmembrane region and a C-terminal cytoplasmic tail exist along the peptide chain of *SbNHX1*.

*Salicornia brachiata* *SbNHX1* is closely similar to *Salicornia europaea* *SeNHX1*, *Salicornia bigelovii* *NHX1*, *Kalidium foliatum* *KfNHX1*, *Salsola soda* *SsNHX1*, *Atriplex qmelini* *AqNHX1* and *Suaeda japonica* *SjNHX1* by 99%, 98%, 92%, 87%, 86% and 85% identity respectively (Figure 3.5 and Figure 3.6). The *SbNHX1* gene showed higher similarity with Class-I type NHX genes of halophytes. Bioinformatics analysis predicts that *SbNHX1* protein has a common structure with other reported Na\(^+\)/H\(^+\) antiporters, which composed of a hydrophobic N-terminus, 11 strong transmembrane helix region and a regulatory hydrophilic C-region, in which an amiloride-sensitive motif and a CaM binding domain are conserved. A tandem of negatively charged amino acid between helix 4 and 5 showed regularly arranged pattern, conservatively presenting in Na+/H+ transporters among organisms, where a Na+/H+ transport pore structure was proposed combined with its hydropathy profile. Additionally, the glycosylation, N-myristoylation and phosphorylation sites were also examined.
Figure 3.4: The nucleotide and deduced amino acid sequences of SbNHX1.

The amino acid residues are indicated by a single letter code; A potential translation initiation codon (ATG) is boxed; termination codon is marked with red asterisk; The untranslated region (3'-UTR) is underlined; Gray shading with dark letters reflects the binding site of amiloride binding site.
3.3.1: Alignment of deduced amino acid sequence of SbNHX-1 with other Na⁺/H⁺ antiporters

The BLAST hits were manually screened for the sequence alignment of SbNHX1 with other SbNHX1 antiporters. The comparison of Na⁺/H⁺ antiporters across plant species revealed a high degree of conservation in protein structure (Figure 3.6). The membrane-spanning regions are well conserved in eukaryotic Na+/H+ antiporters. Within these regions, SbNHX1 shares high degree of similarity with other vacuolar Na+/H+ antiporters such as SeNHX1, SbiNHX1 and KfNHX1 (Figure 3.6). The protein sequence of 85-LFFIYLLPPI-94 in SbNHX-1 is highly conserved. In mammals, this region was identified as the binding site of amiloride, which inhibits the eukaryotic Na+/H+ exchanger. These features confirm that the SbNHX1 is a vacuolar-type Na+/H+ antiporter. Sequences of NHX1 proteins from different plant species were obtained from the GenBank. Phylogenetic analysis showed higher degree of conservation of sequence throughout the length. The alignment of SbNHX1 with NHX1 proteins from other plant species shows that it bears close identity: 99% to NHX1 of Salicornia europaea and Salicornia bigelovi, 94% to KfNHX1 of Kalidium foliatum, 88% to SjNHX1 of Suaeda japonica and 87% to AdNHX1 of Atriplex dimorphostegia and AgNHX1 of Atriplex gmelini (Figure 3.6), suggesting SbNHX1 is a homolog gene of NHX1 type antiporters.
Figure 3.5: Graphic display of protein blast (blastp) results for SbNHX1 antiporter against NCBI database. 43 protein hits were found and some of them are shown as red bars depending on their percentage of identity to SbNHX1 antiporter. This graphic display provided an idea about the number of protein hits for SbNHX1 protein screened for further phylogenetic analysis.
Figure 3.6: Homology analysis of amino acid sequence of vacuolar Na+/H+ antiporters from different plant species.


3.3.2: Phylogenetic analysis

To investigate the phylogenetic relationships of different NHXI genes in plants, closely related sequences were aligned and analysed (Figure 3.7) using the CLC sequence viewer version 5.1.1 software. The phylogenetic tree was generated based on minimal evolution criterion using the neighbor-joining method with 1000 times of bootstrap. The following Na+/H+ antiporter genes were analyzed to assess their phylogenetic relationship: *Salicornia europaea* (AY131235), *Salicornia bigelovii* (DQ157454), *Kalidium foliatum* (AY825250), *Suaeda japonica* (AB198178), *Atriplex gmelini* (AB038492), *Atriplex dimorphostegia* (AY211397), *Suaeda salsa* (AF370358), *Salsola soda* (EU073422), *Suaeda maritima* subsp. Salsa (AY261806) *Chenopodium glaucum* (AY371319), *Mesembryanthemum crystallinum* (AM746985), *Tetragonia tetragonoides* (AF527625), *Ipomoea nil* (AB033989).
Figure 3.7: Phylogenetic tree of Na+/H+ antiporters. A multiple sequence alignment was generated using CLC sequence viewer version 5.1.1 software and evolutionary distances were calculated by the neighbor joining method.

To further deduce the phylogenetic relationships among Na+/H+ antiporters in plants, Na+/H+ antiporters protein sequences from both glycophytes and halophytes were aligned and phylogenetic tree was constructed. Relationship of SbNHX1 protein is illustrated by tree view (Figure 3.8). The following Na+/H+ antiporter genes were analyzed to assess their phylogenetic relationship: Salicornia europaea (SeNHX1, AY131235), Kalidium foliatum (KfNHX, AY825250), Suaeda japonica (SjNHX1, AB198178), Chenopodium glaucum (CgNHX, AY371319), Mesembryanthemum crystallinum (McNHX1, AM746985), Arabidopsis thaliana (AtNHX1, NM_122597; AtNHX4, NM_111512), Lycopersicon esculentum (LeNHX1, CAC84522.1), Oryza sativa (OsNHX2, AY360145.1), Zea mays (ZmNHX1, AY270036.1), Arabidopsis thaliana (AtNHX5, NM_104315; AtNHX6, NM_106609). SbNHX1 gets clustered with halophytes within the Class-I type of NHX proteins (Figure 3.8).
Figure 3.8: Tree showing relationship of SbNHX1 protein with other NHX1 proteins. Scale indicates branch length. A multiple sequence alignment was generated using the software MEGA 4 and evolutionary distances were calculated by the neighbor joining method.

3.3.3: Prediction of hydrophobicity, transmembrane domains and subcellular localization

Hydrophobicity plot of SbNHX1 was created by the method of Kyte and Doolittle (1982). The hydropathy plot analysis was carried out using the program of Protoscale (http://us.expasy.org/tools/protscale.html). The membrane-spanning regions and their orientation were predicted by the method of Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Figure 3.9A).

The hydropathy plot shows SbNHX1 has 12 hydrophobic peak regions (Figure 3.9A), indicating its potential helix regions. The output of ConPred II suggests that
SbNHX1 contains 11 strong transmembrane (TM) domains (Table 3.1) which are fallen into the 1-5 and 7-12 hydrophobic regions (Figure 3.9B). The 6th hydrophobic region is not enough long to span membrane. Its N-amphipathic portion is located in the outside of the vacuole, whereas the hydrophilic C-terminal portion is thought to be vacuolar luminal. In order to predict the number of transmembrane domains (TMD) of the cloned Na+/H+ antiporter a consensus prediction for transmembrane alpha helices was carried out. The results for NHX1 antiporter is plotted in Figure 3.9C. The comparison of Na⁺/H⁺ antiporters across plant species revealed a high degree of conservation in protein structure.
Figure 3.9: Hydrophobicity plot of SbNHX1 gene product. The hydrophobicity values were calculated by the program TMpred available at http://www.ch.embnet.org/software/TMPRED-form.html. Hydrophobicity/hydrophilic analysis indicated that the SbNHX1 had 12 hydrophobic segments (A) containing 11 strong potential transmembrane segments (B) as confirmed by ConPred II. Cut off of 0.6 in the TMD probability was applied in order to define a certain region as transmembrane domain (C) as confirmed by THMHH.

Table 3.1: The amino acid sequence of SbNHX1 is a MEMBRANE PROTEIN which has 11 strong transmembrane helices.

<table>
<thead>
<tr>
<th>No.</th>
<th>N terminal</th>
<th>transmembrane region</th>
<th>C terminal</th>
<th>type</th>
<th>length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>VVSMNLFVALLCGCIVIGHLL</td>
<td>44</td>
<td>PRIMARY</td>
<td>21</td>
</tr>
<tr>
<td>2</td>
<td>54</td>
<td>ITALLIGLCTGVVILLIS</td>
<td>71</td>
<td>PRIMARY</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>78</td>
<td>LLVFSEDLFFIYLLPIIFNAGF</td>
<td>100</td>
<td>SECONDARY</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>111</td>
<td>FITIIMFGAIGTLVSVISLGAAT</td>
<td>137</td>
<td>PRIMARY</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>142</td>
<td>IGSLELGDYLAICAIFAATDSVCTL</td>
<td>166</td>
<td>SECONDARY</td>
<td>25</td>
</tr>
<tr>
<td>6</td>
<td>221</td>
<td>YLFFASTMLGAMTGLLSAYVI</td>
<td>241</td>
<td>SECONDARY</td>
<td>21</td>
</tr>
<tr>
<td>7</td>
<td>271</td>
<td>LFYLSGILTVFFCGIVMSHYTW</td>
<td>292</td>
<td>PRIMARY</td>
<td>22</td>
</tr>
<tr>
<td>8</td>
<td>306</td>
<td>AFATLPFVAEIFLFLYVGMDAL</td>
<td>327</td>
<td>SECONDARY</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>345</td>
<td>VSSILLGLMVGRAAFVF</td>
<td>362</td>
<td>SECONDARY</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>385</td>
<td>IVIWWAGLMRGAVSMALAY</td>
<td>403</td>
<td>SECONDARY</td>
<td>19</td>
</tr>
<tr>
<td>11</td>
<td>420</td>
<td>MITSTITIVALFSTMVGLLT</td>
<td>439</td>
<td>SECONDARY</td>
<td>20</td>
</tr>
</tbody>
</table>
The analysis of SbNHX1 showed potential four N-glycosylation sites, nine N-myristoylation sites were predicted by PPsearch. Further, there are fifteen protein kinase phosphorylation sites of casein kinase II (8 sites) and protein kinase C (7 sites) and a leucine zipper pattern were also found in the SbNHX1 (Table 3.2).

Table 3.2: The PROSITE patterns for SbNHX1: positions of important sites

<table>
<thead>
<tr>
<th>Position</th>
<th>Predicted sites/patterns</th>
</tr>
</thead>
<tbody>
<tr>
<td>51 - 54</td>
<td>ASN_GLYCOSYLATION N (N-glycosylation site)</td>
</tr>
<tr>
<td>294 - 297</td>
<td></td>
</tr>
<tr>
<td>369 - 372</td>
<td></td>
</tr>
<tr>
<td>499 - 502</td>
<td></td>
</tr>
<tr>
<td>11 - 14</td>
<td>CK2_PHOSPHO_SITE (Casein kinase II phosphorylation site)</td>
</tr>
<tr>
<td>17 - 20</td>
<td></td>
</tr>
<tr>
<td>205 - 208</td>
<td></td>
</tr>
<tr>
<td>251 - 254</td>
<td></td>
</tr>
<tr>
<td>374 - 377</td>
<td></td>
</tr>
<tr>
<td>458 - 461</td>
<td></td>
</tr>
<tr>
<td>479 - 482</td>
<td></td>
</tr>
<tr>
<td>547 - 550</td>
<td></td>
</tr>
<tr>
<td>257 - 278</td>
<td>LEUCINE ZIPPER (Leucine zipper pattern)</td>
</tr>
<tr>
<td>298 - 300</td>
<td></td>
</tr>
<tr>
<td>302 - 304</td>
<td></td>
</tr>
<tr>
<td>371 - 373</td>
<td></td>
</tr>
<tr>
<td>376 - 378</td>
<td></td>
</tr>
<tr>
<td>464 - 466</td>
<td></td>
</tr>
<tr>
<td>507 - 509</td>
<td></td>
</tr>
</tbody>
</table>
3.3.4: Predicted Secondary structure of SbNHX1

The secondary structure was predicted using the PSIPRED Protein Structure Prediction Server (http://www.combio.dundee.ac.uk/) and it reveals a structure of 24 coils, 22 alpha-helices and 5 beta-strands (Figure 3.10).

Figure 3.10: Predicted secondary structure for Na+/H+ antiporter from *Salicornia brachiata.*
3.4: Transcript profiling of *SbNHX1* gene by real-time PCR

Total RNA was isolated from NaCl treated (0.10, 0.25, 0.5, 1.0, 1.5, 2.0 M for 48 h; 0.5 M for 6, 12, 24, 48, 72h) *S. brachiata* plant. The cDNA was prepared using 5 μg total RNA by Superscript RT II first-strand cDNA synthesis kit (Invitrogen, San Diego, CA). Real Time qPCR was performed on a Bio-Rad IQ5 detection system (Bio-Rad, U.S.A.) for transcript profiling under different salt treatments as described. At the end of the PCR cycles, the products were put through a melt curve analysis to check the specificity of PCR amplification. The amplified product was run on the 1% agarose to confirm expected size. The experiments were repeated twice independently. Fold changes were calculated using the CT method (Livak and Schmittgen 2001). CT values of different NaCl treatment are given below in table 3 and 4. CT values for individual variants were compared to CT values for a reference control (β-tubulin) for all treated samples (Figure 3.11A & B).

Table 3.3: CT values and Relative fold of different concentration treatment.

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>Mean CT value of β-tubulin</th>
<th>Mean CT value of SbNHX1</th>
<th>Relative fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0 M</td>
<td>42.02</td>
<td>28.69</td>
<td>1</td>
</tr>
<tr>
<td>0.1 M</td>
<td>42.26</td>
<td>26.85</td>
<td>4.11</td>
</tr>
<tr>
<td>0.25 M</td>
<td>41.95</td>
<td>25.93</td>
<td>5.55</td>
</tr>
<tr>
<td>0.5 M</td>
<td>42.48</td>
<td>25.32</td>
<td>9.95</td>
</tr>
<tr>
<td>1.0 M</td>
<td>41.09</td>
<td>26.97</td>
<td>1.85</td>
</tr>
<tr>
<td>1.5 M</td>
<td>41.98</td>
<td>27.56</td>
<td>1.93</td>
</tr>
<tr>
<td>2.0 M</td>
<td>42.14</td>
<td>27.97</td>
<td>1.89</td>
</tr>
</tbody>
</table>
Figure 3.11 A: Amplification chart for concentration treatment

Figure 3.11 B: Amplification chart for time treatment
### Table 3.4: CT values and Relative fold of different time point treatment at 0.5 M NaCl.

<table>
<thead>
<tr>
<th>Time point (hours)</th>
<th>Mean CT value of ( \beta )-tubulin</th>
<th>Mean CT value of ( SbNHX1 )</th>
<th>Relative fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41.35</td>
<td>31.99</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>41.73</td>
<td>31.32</td>
<td>2.07</td>
</tr>
<tr>
<td>12</td>
<td>41.68</td>
<td>30.85</td>
<td>2.77</td>
</tr>
<tr>
<td>24</td>
<td>41.59</td>
<td>29.11</td>
<td>8.69</td>
</tr>
<tr>
<td>48</td>
<td>41.88</td>
<td>29.05</td>
<td>11.08</td>
</tr>
<tr>
<td>72</td>
<td>41.55</td>
<td>29.42</td>
<td>6.82</td>
</tr>
</tbody>
</table>

#### 3.4.1: Expression analysis of \( SbNHX1 \)

For studying the expression analysis of \( SbNHX1 \), real time PCR was carried out using cDNA from different NaCl concentrations (0.1, 0.25, 0.5, 1.0, 1.5 and 2.0 M) treated plants (Figure 3.12). The relative fold expression of \( SbNHX1 \) gene was analyzed against the control plant. \( SbNHX1 \) transcripts increased gradually from 4-fold to 12-fold by increasing the NaCl concentration up to 500 mM NaCl and thereafter reduced. Plants were also treated at 0.5 M NaCl for different time period to see the sequential pattern of transcript. The transcript was increased from 2-10 folds concomitantly by increasing the duration treatment from 6 h to 48 h, however at 72 h it reduced further.
Figure 3.12: Real time PCR analysis of the SbNHX1 gene under NaCl stress condition. a: Different concentration of the NaCl was given for 48 h. b: 500 mM NaCl for different time periods (h)

3.5: Construction of overexpressing SbNHX1 transformation vector

The full-length coding sequence of SbNHX1 gene was amplified from Salicornia brachiata cDNA as described in materials and methods. PCR product of 1683 bp, which was consistent with the theoretical length of SbNHX1 ORF and adapter sites on both ends for restriction enzymes, was cloned in pGEM-T Easy Vector (Figure 3.13 A). It was confirmed by colony PCR of independent transformed DH5α colonies that carries pGEM-T-SbNHX1 construct (Figure 3.13 B). The recombinant plasmid pGEM-T-SbNHX1 was digested with KpnI (Figure 3.13 C) and XbaI (Figure 3.13 D; lane-1) restriction enzymes for excision of SbNHX1 ORF with over-hanged restriction sites. Agarose gel electrophoresis of digested product revealed a DNA band of approx 1683 bp (Figure 3.13 D; lane-2) and then this band was excised from gel and ligated into the intermediate plant expression vector pRT101 (Figure 3.14), resulting in pRT101-SbNHX1 construct (Figure 3.15). pRT101 was used in cloning to provide CaMV 35S promoter to overexpress SbNHX1 gene and 35S terminator.
Subsequently, the pRT101-\textit{SbNHX1} construct was digested with \textit{PstI}. After restriction digestion of pRT101-\textit{SbNHX1} construct with \textit{PstI}, fallout of approx 2400 bp was obtained and that was ligated into the \textit{PstI} site of the binary vector pCAMBIA1301. To confirm the ligation of above fallout of pRT101-\textit{SbNHX1} construct in pCAMBIA1301, pCAMBIA1301-\textit{SbNHX1} construct (Figure 3.16) was digested with \textit{PstI} which yielded an approximately 2400 bp fragment (Figure 3.16 E, Lane 2).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3.png}
\caption{Cloning of \textit{SbNHX-1}gene into the pGEM-T Easy Vector. (A) Recombinant plasmid pGEM-T-\textit{SbNHX-1}. (B) PCR confirmation of independent transformed DH5\textalpha{} cell lines that carrying \textit{SbNHX-1} gene. (C) Single restriction enzyme digestion analysis of \textit{SbNHX1} gene in pGEM-T Easy Vector and pRT101 with \textit{KpnI}. (D) pGEM-T-\textit{SbNHX 1} construct and pRT101 digested with \textit{KpnI} and \textit{XbaI} (double digested).}
\end{figure}
Figure 3.14: Cloning of *SbNHX-1* ORF into the plant expression vector pRT101. (A) Gel extracted product of pGEM-T-*SbNHX1* construct that was digested with *KpnI* and *XbaI*. (B) Purified plant expression vector pRT101. (C) pRT101-*SbNHX1* construct. (D) PCR confirmation of recombinant DH5α cell lines that carries *SbNHX1* gene in pRT101. E: Digestion analysis of pRT101-*SbNHX1* construct; lane 1: pRT101-*SbNHX* construct digested with *XhoI* and *KpnI*, lane 2: pRT101-*SbpAPX* construct digested with *SdaI*. 
Figure 3.15: The recombinant pRT101-\textit{SbNHX-1} construct.

The pGEM-T-\textit{SbNHX1} construct was \textit{KpnI/XbaI} restriction digested and ligated into \textit{KpnI/XbaI} restricted sites of the intermediate plant expression vector pRT101.

The pCAMBIA1301-\textit{SbNHX1} construct was then introduced into the \textit{Agrobacterium tumefaciens} strain LBA4404 by freeze-thaw method according to the procedure described in materials and methods section. \textit{A. tumefaciens} colonies carrying pCAMBIA1301- \textit{SbNHX1} construct were identified by PCR analysis. Plasmid
DNA of LBA4404 cell lines were extracted using the alkaline lysis method and PCR was performed using plasmid DNA with gene specific primers. PCR results confirmed the presence of $SbNHX_1$ in the LBA4404 cell lines as shown in Figure 3.16 E. These recombinant cells were used for Agrobacterium-mediated transformation of tobacco. Schematic map of pCAMBIA1301- $SbNHX_1$ plant expression construct is shown in Figure 3.17.
Figure 3.16 Cloning of pRT101-SbNHX-1excised product into the binary expression vector pCAMBIA1301.

(A) Purified plasmid pCAMBIA1301. (B) Colony PCR after cloning of excised product of pRT101-SbNHX1 construct in pCAMBIA1301. (C) pCAMBIA 1301-SbNHX 1 constructs. (D) pCAMBIA1301-SbNHX1 construct digested with PstI. (E) Colony PCR to confirm insert in A. tumefaciens. Marker: molecular Marker.
Figure 3.17: Schematic map of pCAMBIA 1301-SbNHX-1 plant expression construct.

Construct used to transform tobacco. Expression of SbNHX1 is driven by the cauliflower mosaic virus 35S promoter.
3.6: Transformation and production of transgenic tobacco plants

Transformation studies were carried out in *Nicotiana tabacum* cultivar Xanthi to generate *SbNHX1* expressing plants. *A. tumefaciens* LBA4404 strain containing the recombinant binary plasmid pCAMBIA1301-*SbNHX1* was used as vector for transformation. This plasmid specifies the kanamycin resistance in the host bacteria while the *A. tumefaciens* LBA4404 strain has resistance to rifampicin and streptomycin. Thus, the strains were maintained on LB agar plates containing 50 mg/l kanamycin, 25 mg/l rifampicin and 25 mg/l streptomycin. Single colonies of the strain were grown overnight at 28°C and 200 rpm in 50 ml of LB medium supplemented with appropriate antibiotics. Agrobacterium grown LB medium was centrifuged at 5000 rpm and 4°C for 10 minutes and bacterial pellet was dissolved in ½ strength MS medium. This bacterial suspension was poured in a sterile petriplate so as to make a thin film (2–3 mm) at the base of the petriplate. The leaf discs explants were used for genetic transformation as described in materials and methods.

A large number of putatively transformed lines were obtained. The transgenic nature of these plants was tested using molecular approaches. Over 100 independently transformed putative transgenic plants were maintained in the greenhouse for seed production. Transgenic plants appeared phenotypically normal. No apparent undesirable genetic change due to tissue culture or transformation process has been observed in the greenhouse grown plants. Plants grew normally and flowering occurred after two months of transfer in greenhouse. After 3–4 months, seeds (*T₁*) were harvested (Figure 3.18, 3.19 & 3.20).
Figure 3.18: Representative transformation in tobacco and regeneration of putative transgenic line. (A) wild type tobacco cv. Xanthi growing in vitro (B) regeneration of shoots from leaf explants after transformation with A. tumefaciens harbouring pCAMBIA1301-SbNHX1 plasmid (C) putative tobacco transgenic lines growing in selection medium (D) tobacco transgenic line in plastic pot after hardening.

Figure 3.19: Representative independently transformed transgenic (T₀) plants maintained in pots for seed production in greenhouse.
3.7: Gus analysis of transgenic tobacco lines

The leaves of putative T₀ transgenic lines were analyzed for GUS expression. The lines tested for GUS assay were found to be GUS-positive (blue colour) and no blue colour was observed from control or non-transformed tissue (NT). These GUS-positive transgenic lines were also found positive for PCR using gus gene specific primers (Figure 3.21).

Figure 3.21: Example of expression of gus gene. (A) Blue colour appearing in GUS-positive transformed leaf of tobacco (B) Control (non-transformed) leaf of tobacco.
3.8: Characterization of SbNHX1 transgenic T₀ plants

Following plant transformation and hygromycin selection, fifty five independent transformants were obtained. Although selective media containing hygromycin was used at different stages of the regeneration and propagation experiments and GUS assay results also show the confirmation of integration of SbNHX1 gene in putative transgenic plants, further analysis was performed to verify the presence of T-DNA in T₀ putative transgenic plants. For this purpose DNA was extracted for each putative T₀ transgenic line using CTAB method (Figure 3.22). PCR analysis with SbNHX1, hpt and gus specific primers was done to check the presence of SbNHX1 gene construct, hygromycin resistant gene and gus marker gene.

![Figure 3.22: Total genomic DNA isolated from transgenic lines.](image)

3.8.1: Confirmation of transgenes in transgenic lines

PCR analysis confirmed the presence of stable SbNHX1 transgene of 1683 bp in independent transgenic lines, where total genomic DNA was used as the template and end sequences of SbNHX1 were used as the primers in the PCR analysis. Further, the presence of hptII and gus genes was also confirmed by using respective gene specific primers (Figure3.23).
3.8.2: Southern blotting

Five PCR positive transgenic lines 45, 61, 75, 77 and 88 and one PCR negative wild type as control were analysed for copy insertion of *SbNHX1* gene with Southern blot analysis and all the five transgenic lines showed single copy insertion of the gene while wild type control plant didn’t show any signal of gene insertion (Figure 3.24).

**Figure 3.23: Confirmation of transgenes in transgenic lines by PCR amplification.**

(A) *SbNHX1* gene (B) *hptII* gene (C) *gus* gene. M: molecular marker; PC: DNA from plasmid pCAMBIA1301-*SbNHX1* (positive control), NT: Non-transformed controls (negative control) and L- carry samples from putative transformants.

**Figure 3.24: Southern analysis of Wt and transgenic plants**
3.9: Characterization of *SbNHX1* transgenic T$_1$ plants

55 independent T$_0$ plants were grown in greenhouse out of which 22 plants produced seeds. PCR analysis of T$_1$ lines confirmed the successful integration of T-DNA into the plant genome. PCR analysis for *hptII* and *gus* genes was performed using genomic DNA extracted from 10 independent T$_1$ lines. Eight T$_1$ lines produced the desired band of 963 bp for *hptII* and 1286 bp for *gus* as shown in Figure (Figure 3.25).

![Figure 3.25: Confirmation of transgenes in T$_1$ transgenic lines by PCR amplification.](image)

(A) *hptII* gene (B) *gus* gene M: molecular marker; NT: Non-transformed controls (negative control) and L1–L10 carry samples from T$_1$transgenic lines.

3.9.1: Analysis of T$_1$ transgenic lines for salinity tolerance

Wild type (WT) seeds were germinated on MS medium and kept for 8 days. T$_1$ seeds of two transgenic lines L77 and L88 were germinated on MS medium containing hygromycin. These seedlings (both WT and T$_1$) were transferred to MS medium with 0, 100, 200 and 300 mM NaCl. In the presence of NaCl both the Wt and transgenics showed growth retardation in a dose-dependent manner, but the retardation was more in case of WT plants. At 100 mM NaCl both Wt and transgenics survived but growth
of Wt was less as compared to the transgenic lines. However, at 200 mM and 300 mM NaCl Wt seedlings were greatly stunted, but the transgenics showed slow growth. The growth parameters such as number and length of roots, leaf area and fresh weight of seedlings were determined. The growth was more in case of transgenics as compared to WT after 30 days (Figure 3.26)
Figure 3.26: Analysis of SbNHX1 transgenics (T1).

Growth comparison of WT, L77 and L88 transgenic lines on A. 0 mM, B. 100 mM, C. 200 mM and D. 300 mM NaCl. E-H. Comparison of shoot and root length of WT, L77 and L88 transgenic lines on 0, 100, 200 and 300 mM NaCl.

3.9.2: Chlorophyll content measurement

Tolerance of T1 transgenic lines L77 and L88 to salt stress was studied by leaf chlorophyll estimation. Chlorophyll content was measured after 30 days in both Wt and transgenic plants treated with different NaCl concentration as described in materials and methods. The chlorophyll content in Wt plant reduced significantly with increasing salt concentration. Transgenic lines showed higher chlorophyll amount, and its chlorophyll content in 200 and 300 mM salt did not get affected much as compared with Wt plants. Out of two transgenic lines L77 showed higher chlorophyll content (Table 5 & Figure 3.27).

<table>
<thead>
<tr>
<th>Lines</th>
<th>Wt</th>
<th>L77</th>
<th>L88</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>0.19±0.017</td>
<td>0.19±0.007</td>
<td>0.35±0.05</td>
</tr>
<tr>
<td>100mM</td>
<td>0.56±0.089</td>
<td>0.69±0.005</td>
<td>0.61±0.011</td>
</tr>
<tr>
<td>200mM</td>
<td>0.46±0.013</td>
<td>0.61±0.001</td>
<td>0.5±0.009</td>
</tr>
<tr>
<td>300mM</td>
<td>0.22±0</td>
<td>0.5±0.014</td>
<td>0.385±0.007</td>
</tr>
</tbody>
</table>

3.9.3: Fresh weight measurement

Fresh weight for transgenic lines and wild type seedlings were measured after 30 days of growth on MS media containing different NaCl concentration. The
transgenic plants showed better fresh weight compared to Wt plants at different salt concentration. Fresh weight for wild type seedlings at 100 mM, 200 mM and 300 mM NaCl concentration was remarkably reduced as compared to the fresh weight for transgenic line at same NaCl concentrations. For transgenic line L77, fresh weight was constantly decreased with increasing concentration of NaCl. For transgenic line L88, fresh weight for seedling at 200 mM NaCl was higher than fresh weight at 100 mM, but fresh weight at 300 mM NaCl concentration was remarkably reduced (Table 6 & Figure 3.28).

**Table 3.6: Fresh weight in mg**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Wt</th>
<th>L77</th>
<th>L88</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>136±81.79</td>
<td>194.5±47.31</td>
<td>241.75±70.01</td>
</tr>
<tr>
<td>100mM</td>
<td>39.75±5.9</td>
<td>175.25±44.82</td>
<td>105.25±27.24</td>
</tr>
<tr>
<td>200mM</td>
<td>37.75±9.21</td>
<td>115.75±66.07</td>
<td>121.5±21.2</td>
</tr>
<tr>
<td>300mM</td>
<td>22±2.7</td>
<td>39.75±12.23</td>
<td>55.75±19.05</td>
</tr>
</tbody>
</table>

**3.9.4: Root length Measurement**

Root lengths for transgenic lines and wild type seedlings were obtained after 30 days of growth on 0, 100, 200 and 300 mM NaCl concentration. Root length for transgenic lines and wild type seedlings at 0 mM NaCl concentration had no significant difference. Whereas root length for wild type plants at 100mM, 200 mM and 300 mM NaCl concentration was retarded. Root length for transgenic lines at 100mM and 200 mM NaCl concentration was increased when compare to the root length of same lines on 0mM NaCl medium and was much higher than the wild type
seedling while on 300 mM NaCl concentration, root length of all the lines was drastically retarded (Table 7 & Figure 3.29).

**Table 3.7: Root length in cm**

<table>
<thead>
<tr>
<th>Lines</th>
<th>Wt</th>
<th>L77</th>
<th>L88</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM</td>
<td>5.05±0.288</td>
<td>5.62±0.478</td>
<td>5.55±0.31</td>
</tr>
<tr>
<td>100mM</td>
<td>5.12±0.853</td>
<td>7.8±0.391</td>
<td>7.15±0.251</td>
</tr>
<tr>
<td>200mM</td>
<td>4.3±0.244</td>
<td>6.32±0.537</td>
<td>6.57±0.505</td>
</tr>
<tr>
<td>300mM</td>
<td>1.65±0.238</td>
<td>2.46±0.792</td>
<td>2±0.559</td>
</tr>
</tbody>
</table>

**Figure 3.27: Chlorophyll assay of Wt, L77 and L88 plants in 0, 100, 200 and 300 mM NaCl.** Graph represents, mean and SD of chlorophyll content in leaves of Wt, L77 and L88 at 0, 100, 200 and 300 mM NaCl.

**Figure 3.28: Comparison of fresh weight of 30-day-old seedlings (Wt, L77 and L88) on 0, 100, 200 and 300 mM NaCl.** The graphs represent the mean and SD over three replicates for fresh weight of seedlings.
Three months old transgenic plants (L77) were used to study their salt tolerance. The T1 transgenic and WT plants were watered every third day with 200 ml of NaCl solution (200 mM) for 30 days.

3.9.5: Salt Tolerance of Transgenic Lines under Greenhouse Conditions:

Three months old transgenic plants (L77) were used to study their salt tolerance. The T1 transgenic and WT plants were watered every third day with 200 ml of NaCl solution (200 mM) for 30 days.

Figure 3.29: Comparison of root length of 30-day-old seedlings (Wt, L77 and L88) on 0, 100, 200 and 300 mM NaCl. The graphs represent the mean and SD over three replicates for root length of seedlings.

Figure 3.30: Comparison of salt tolerance of T1 transgenic and Wt.
Noticeable difference was not observed in transgenic and WT plants when irrigated with tap water. When both the plants were watered with 200 mM NaCl solutions, WT plants showed severe wilting, yellowing of lower mature leaves and stunted growth whereas transgenic line (L77) was able to tolerate this salt treatment and performed better (shown in Figure 3.39).