CHAPTER 5

Transcriptional regulation of ion-transporters

CHAPTER 5: TRANSCRIPTIONAL REGULATION OF ION-TRANSPORTERS

5.1 Introduction

Salt stress disturbs cellular kinetic steady states of ion transport and the re-establishment of ion homeostasis is vital for metabolic activities and growth of the plant in saline conditions (Niu *et al*, 1995).

The hydrophobic lipid bilayer of biological membranes is almost impermeable to inorganic ions and ion flux across membranes depends on transport proteins, namely pumps, carriers and channels. Ion flux across membranes is dependent on the electrochemical gradient ($\Delta\mu_{H}^{+}$), which consists of two components, the electrical gradient (or membrane potential; $\Delta\psi$) and the chemical gradient (or proton gradient; ΔpH). Transport of ions down this gradient is passive, whereas transport against the gradient is active (Sze, 1985; Sussman and Harper, 1989). Pumps like ATPases directly utilize chemical bond energy for vectorial transport. Channels facilitate diffusion of solutes down energetically favourable gradients i.e. passive transport. Carriers couple uphill transport of one solute (against its concentration gradient) to the downhill movement of another solute, either in the same (symporter) or opposite (antiporter/exchanger) direction (Sze *et al*., 1999). Solute transport coupled to H^{+} flux via an antiport or symport mechanism is termed secondary active transport. Physiological processes that depend on secondary active transport mechanisms include mineral nutrient uptake in the roots, stomatal function, phloem loading and the response to saline growth conditions. Genotypes that are most adapted to salt tightly regulate ion uptake across the

plasma membrane at a rate that is compatible with the capacity for vacuolar compartmentation (Binzel *et al*., 1988). Thus, transport processes at the plasma membrane and tonoplast that regulate ion influx and efflux, particularly of Na^+, Cl^-, Ca^{2+}, and K^+, and maintain ion homeostasis, are of crucial importance to salinity adaptation.

The efflux of Na^+ from the cytoplasm is mediated by Na^+/H^+ exchanger/antiporter, either by SOS1 (salt overly sensitive 1) at the plasma membrane or NHX1 at the tonoplast (Apse and Blumwald, 2007). The transcript level of *SOS1* was up-regulated under salt stress in Arabidopsis and its halophytic relative *Thellungiella halophila*, indicating regulation of the transporter activity at the level of gene expression (Vera-Estrella *et al*., 2005; Chinnusamy *et al*., 2005). Halophytes accumulate Na^+ in vacuoles via a Na^+/H^+ exchanger, NHX (Apse & Blumwald, 2007). Genes for this vacuolar Na^+/H^+ antiporter have been identified and cloned from halophytes like *Suaeda salsa* (Ma *et al*., 2004) and *Atriplex gmelini* (Hamada *et al*., 2001). Expression of *SsNHX1* was increased by salt stress (Ma *et al*., 2004) whereas *AgNHX1* transcripts and AgNHX1 products were seen to increase both in roots and leaves in response to salt treatment (Hamada *et al*., 2001).

H^+-ATPases in the plasma membrane and tonoplast and the tonoplast H^+-pyrophosphatase (H^+-PPase) are primary pumps that couple the free energy of hydrolysis of ATP or PPi, respectively, to vectorial H^+ transport and generation of $\Delta\mu_H^+$. These phosphatases generate the necessary proton gradient required for activity of Na^+/H^+ and many other antiporters. NaCl-induced H^+ pumping is fundamental to Na^+/H^+ exchange and salinity tolerance (Ayala *et al*., 1996). The activity of these H^+ pumps is seen to be increased in response to salt stress and is accompanied by a concomitant increase in gene expression (Hasegawa *et al*., 2000; Vera-Estrella *et al*., 2005).

Potassium is a major osmolyte in cells and plays a role in maintaining cell turgor. Potassium transport across membranes is mediated by (1) three families of potassium permeable channels - shaker-type potassium channels, 'two-pore' potassium channels and NSCCs (non-selective cation channels), and (2) three families of potassium transporters - K^+ uptake permeases (KUP/HAK/KT), high-affinity K^+ (HKT) transporters and K^+/H^+ antiporters (Shabala and Cuin, 2007). Na^+ competes with K^+ uptake (Watad *et al*., 1991; Schroeder *et al*., 1994), suggesting that the uptake mechanisms for both cations are similar. Under salinity, Na^+ gains entry mainly through NSCCs and HKT or via apoplastic pathways (Amtmann and Sanders, 1999; Chinnusamy *et al*., 2005; Shabala and Cuin, 2007). The transcript levels of several K^+ transporter genes are either down- or up-regulated by salt stress, probably reflecting the different capacities of plants to maintain K^+ uptake under salt stress.

Calcium is essential for K^+/Na^+ selectivity and membrane integrity (Cramer *et al*., 1985). NaCl causes a rapid increase in cytosolic Ca^{2+}, which acts as a general stress signal (Lynch *et al*., 1989). Re-establishment of cytosolic Ca^{2+} homeostasis is a requisite for adaptation. Severa1 transport mechanisms facilitate Ca^{2+} efflux from the cytosol, including high-affinity Ca^{2+}-ATPases in the plasma membrane and endomembranes and a high-capacity Ca^{2+}/H^+ antiporter (Hirschi, 2001; White and Broadley, 2003). Vacuolar Ca^{2+}/H^+ antiporters such as CAX1 (cation exchanger 1) play a key role in regulating cytosolic Ca^{2+} levels by transporting the ion to vacuoles. It was also shown that SOS2 (a component of the salt signaling pathway) responds to environmental cues and differentially regulates both Na^+ transporters (SOS1) and Ca^{2+} (CAX1) (Cheng *et al*.,

2004). These finding therefore provide a mechanistic link between Na^+ and Ca^{2+} homeostasis in plants

In this chapter we have compared the transcriptional expression of Na^+/ H^+ antiporter, Ca^{2+}/H^+ antiporter, PM H^+-ATPase, and K^+ transporter in the leaf tissue of FW and SW plants of *Bruguiera cylindrica*. The objective was to understand whether salt adaptation involves higher steady-state transcription levels of some genes that play a role in regulating ion transport across membranes.

5.2 Materials and Methods

5.2.1 Plant material

Mature propagules of *B. cylindrica* (L.) Blume were collected from the seacoast at Mumbai, India (19°13'45"N; 72°54'49"E). These propagules were washed under running tap water for 15 min., sown in polythene bags, containing garden soil and organic manure (1:1), and irrigated with either fresh water or 400 mM NaCl to field capacity. The propagules were maintained in the green house at 70-80% relative humidity (RH) and 25±2°C temperature. Light intensity in the greenhouse varied from 75-150 μmol m^{-2} s^{-1} during different hours of the day. Saplings of uniform size (six-leaf stage) were selected from the greenhouse-grown plants for gene expression experiment and were called fresh water (FW) plants and salt water (SW) plants, irrigated either with fresh water or 400 mM NaCl respectively.

5.2.2 Chemicals

TRI reagent was obtained from Ambion (USA). RT **(**reverse transcription**)** and PCR (polymerase chain reaction) reagents were obtained from Promega (USA) and Fermentas (India). All fine chemicals used in the experiments were obtained Sigma Aldrich (USA).

5.2.3 Total RNA isolation

Leaf tissue was ground to a fine powder in liquid N_2 in a pre-chilled mortar and pestle. The powdered tissue (100 mg) was transferred to 1 ml of TRI Reagent before it thawed and the contents mixed by inverting the microfuge tube several times. The homogenate was incubated for 5 min at room temperature to allow the complete dissociation of nucleoprotein complexes, after which chloroform (200 µl per ml TRI reagent) was added to the tube. After vortexing, the resulting mixture was incubated at room temperature for 5-10 min and centrifuged at 8228 x g (Eppendorf 5804 R, Germany) for 20 min at 4ºC. The aqueous phase was transferred to a new microcentrifuge tube and RNA was precipitated from the aqueous phase by adding 0.5 ml isopropanol (per 1 ml of TRI Reagent used for the initial homogenization). After 10 min incubation at room temperature, sample was centrifuged at 8228 x g for 20 min at 4ºC. The supernatant was discarded and the RNA pellet was washed with 1 ml of 75% ethanol. The pellet was air-dried for 5 min and dissolved in 20 µl RNase free Milli-Q water and stored at -20ºC.

The concentration of RNA was determined spectrophotometrically. Electrophoresis of the RNA on a 1% agarose gel was used to check the quality of the isolated RNA.

5.2.4 Semi-quantitative RT-PCR (reverse transcription-polymerase chain reaction)

5.2.4.1 cDNA synthesis

The following components were combined in a sterile 200 µl microcentrifuge tube:

1 µg of total RNA
0.5 µg OligodT_{15} primer

Total volume of above mixture was made up to 5 µl. This mixture was then heated at 70ºC for 10 min and stored on ice.

In the second step, following components for first strand cDNA synthesis were added:

4 µl 5X Reaction Buffer
2.4 µl 25 mM $MgCl_2$
4 µl 10 mM dNTP mix
0.5 µl (40U/ µl) RNasin
1 µl /20 µl reaction Reverse Transcriptase (Im-PromII, Promega)

Then the 5µl RNA template was added to the reverse transcriptase mix and the tubes were incubated at room temperature for 5 min. Reverse transcription was performed at 42ºC for 60 min followed by inactivation of reverse transcriptase at 70ºC for 15 min. An additional 5 min of final heat inactivation at 95ºC was done for storage purpose. The reaction was stored at -20ºC until it was used for PCR. The cDNA synthesis was performed in a thermocycler (Eppendorf, Germany).

5.2.4.2 Primer Design for Semi-quantitative RT-PCR

Gene-specific oligonucleotide primers for Na^+/H^+ antiporter, $Ca^{2+}H^+$ antiporter, PM H^+-ATPase and K^+ transporter were designed from available expressed sequence tag sequences (ESTs) of burma mangrove, *B. gymnorrhiza* (***Box 5.1***; Miyama *et al*., 2006). The constitutively expressed 25S rRNA sequence (from Arabidopsis) was used as a loading control. These primers were designed using Primer 3 software (***Table 5.1***;http://fokker.wi.mit.edu/primer3/).

Table 5.1 Primers used for amplification of the ion transporters and H^+-ATPase from *B. cylindrica*.

Gene	**Primer**	**Tm** (^oC)	**Sequence**
Na^+/H^+ Antiporter	FP	63.8	*5'-AGATGTGAAGGGATCGTTGG-3'*
	RP	64.0	*5'-CAAAAGATGGCCAATCACG-3'*
Ca^{2+}/H^+ Antiporter	FP	67.5	*5'-GGCAACCTATGGACTTGAACTTCC-3'*
	RP	69.6	*5'-GGCATTTGGAATGTGTAACCTACGC-3'*
PM H^+-ATPase	FP	64.4	*5'-GATGGGCTGGTGTCATCTG-3'*
	RP	63.5	*5'-AAGCCTTGCAACTTCAGCTC-3'*
K^+ Transporter	FP	75.2	*5'-TTTCCGGTTGCAGCCATACAGATTGC-3 '*
	RP	71.1	*5'-GATCCTTGGGAAACAACCATGTGC-3'*
25s rRNA	FP	55.3	*5'-GCCGACCCTGATCTTCTG-3'*
	RP	*53.7*	*5'-GATGGTTCGATTAGTCTTTCGC-3'*

5.2.4.3 PCR conditions

PCR reaction mixture was prepared by adding 1.6 µl 10 X Buffer, 1.2 µl 25 mM $MgCl_2$, 0.2 µl 10 mM dNTP, and 0.2 µl 5 U/µl Taq Polymerase. Gene-specific primers (forward (FP) and reverse (RP)) were added in a concentration of 40 pmol per 20 µl reaction. 2 µl of cDNA template was used in the PCR reaction mixture.

PCR was carried out for 20, 25, and 30 amplification cycles at the following cycling conditions:

1. 95ºC – 15 min
2. 94ºC – 30 s
3. 50ºC – 30 s
4. 72ºC – 1 min
5. Repeat step 2-4 for 20/25/30 cycles
6. 72ºC – 10 min

5.2.4.4 Analysis of PCR products

Agarose gel electrophoresis was used to study comparative expression of amplified gene products. PCR products were separated on 1.8% agarose (Sigma, USA) gel. 20 µl of amplified cDNA product was mixed with 5 µl of 6X loading dye (0.4% bromophenol blue, 50% glycerol). 1X TAE buffer was used as running buffer. The electrophoresis was performed at 45 mA for an hour after which the gel was stained with ethidium bromide (0.2nM). The gel images were captured using a gel documentation system (Alpha Innotech, USA). The band sizes were noted by comparing their positions with those of a molecular size marker (φx 174 DNA Hae-III digest, MB Fermentas, Canada). Changes in expression of the genes were calculated from the gel images by measuring band intensities. These intensities were normalized against the 25S rRNA band intensities to determine –fold expression. Densitometric analysis was performed using AlphaEaseFC 4.0 software. The experiments were carried out at least thrice.

5.2.4.5 Sequencing of PCR amplified products

Specificity of the PCR amplification products was confirmed by sequencing. The PCR products were purified using QIAquick PCR amplification kit (Quiagen Gmbh, Germany) and given for sequencing to a commercial firm (GeneOmbio Technologies, Pune, India) along with the respective forward and reverse primers.

5.3 Results

Blastx (translated query with protein database) analysis of the EST sequences from *Bruguiera gymnorhiza* showed similarity to (a) tonoplast Na^+/H^+ antiporter (TIGR 00840), (b) PM H^+-ATPase (TIGR 01647), (c) Ca^{2+}/H^+ antiporter (TIGR 00846) and (d)

KT (TIGR 00794). Primers designed from the *B. gymnorhiza* ESTs were used to study differential expression of these genes in FW and SW plants using semi-quantitative RT-PCR. All the primer sets gave single amplified products at the annealing temperature of 50^{o}C even though their T_ms differed, so amplifications were carried out at this temperature for all the genes studied.

FW and SW cDNA band intensities were normalized against the respective 25S rRNA band intensities at 25 and 30 cycles of cDNA amplification. Only the readings at 25 cycles were considered for determining -fold expression, since saturation of the amplification reaction was seen for some genes at 30 cycles. SW plants showed over 2-2.5 fold higher expression of all the genes investigated as compared to the expression in FW plants (***Fig 5.1a, b***). The results of only one experiment have been presented, since all experiments showed the same trend.

5.4 Discussion

Increases in ambient salt concentrations can lead to toxic accumulation of ions such as Na^+ in the cytosol. The disproportionate presence of Na^+ in both cellular and extracellular compartments has a negative impact on the acquisition and homeostasis of essential nutrients such as K^+ and Ca^{2+}. The plant vacuole plays a major role in the intracellular sequestration of Na^+ ions (Marschner, 1995). Compartmentation of Na^+ ions into vacuoles has been attributed to tonoplast localized NHX-like antiporters (Apse *et al*., 1999; Apse and Blumwald, 2007). Over-expression of *AtNHX1* conferred salt tolerance to transgenic *Arabidopsis* plants, thus confirming its role in salt sequestration (Apse *et al*. 1999). In the halophyte *Suaeda salsa*, expression of *SsNHX1* increased in response to

NaCl treatment and the increase was proportional to the concentration of salt (Ma *et al*., 2004). Higher Na^+/H^+ antiporter expression in SW plants as compared to FW plants in our experiments is suggestive of its role in salt adaptation/tolerance in *B. cylindrica* in saline conditions.

The intracellular Ca^{2+} concentrations fluctuate in response to environmental and internal cues (Sanders *et al*., 1999). Stress conditions, such as salinity, also induce cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) accumulation (Knight *et al*., 1997). During these biological responses, Ca^{2+} is mobilized from the plant vacuole, the most important storage compartment for Ca^{2+}, to act as an intracellular signaling ion. Since high cytosolic Ca is toxic to cell, it must be removed and steady levels of ($[Ca^{2+}]_{cyt}$) has to be re-established. Along with Ca^{2+}-ATPase, Ca^{2+}/H^+ antiporters or calcium exchangers (*CAX*) mediate Ca^{2+} homeostasis by transporting Ca^{2+} from the cytosol into vacuoles or out of the cell (Hirschi, 1999; Sze *et al*., 2000). A putative tonoplast Ca^{2+}/H^+ antiporter from halophyte *Suaeda salsa*, SsCAX1 showed higher expression levels in the presence of 100 mM $CaCl_2$ and also 100 mM NaCl. This suggests that SsCAX1 has an important role in lowering the cytosolic Ca^{2+} burst induced by salt stress (Han *et al*., 2010). Increased transcript levels of Ca^{2+}/H^+ antiporter in SW leaves of mangrove *B. cylindrica* observed by us suggests the role of this antiporter for maintaining Ca^{2+} homeostasis in saline conditions. In addition we also observed Ca^{2+}/H^+ antiporter activity in plasma membrane vesicles prepared from *B.cylindrica* leaves (see Chapter 4). Both these observations are suggestive of the importance of Ca^{2+} transport in adaptation to salinity.

Potassium is the major osmolyte in cells and perhaps the one that occurs at highest concentrations (100-200 mM). Members of the HAK/KUP/KT family are involved in

both low- and high-affinity K^+ transport, which maintain K^+ homeostasis (Shabala and Cuin, 2007). They are ubiquitously expressed in plant tissues, functioning both in the plasma membrane and the tonoplast. These transporters mediate some low-affinity Na^+ influx at high external Na^+ concentration, besides K^+ influx. The transcript levels and activity of a number of these transporters are modified under saline conditions (Shabala and Cuin, 2007). A decrease in transcription of KUP2, which is predominantly expressed in rapidly growing tissues, has been observed in shoots of NaCl-treated *Arabidopsis* plants (Elumalai *et al*., 2002). Its down-regulation resulted in a general reduction in growth rate. In contrast, KUP6 and KUP11 were seen to be up-regulated during salt stress. Transcript abundance of KUP1 and KUP4 homologues was also found to increase in the ice plant *Mesembryanthemum* during K^+ starvation and salt exposure (Su *et al*., 2001). Additional transcription activity of the K^+ transporters may be required under saline conditions since external Na^+ is known to reduce K^+ uptake and replace K^+ within cells (Shabala and Cuin, 2007). Increased expression of K^+ Transporter in the salt acclimated *B. cylindrica* plants might play a role in maintaining K^+ homeostasis and/or turgor regulation in the saline habitat of this plant.

For most of these ion transporters, an electrochemical gradient generated by H^+-ATPase and / or H^+-PPase provides the driving force for the movement of ions across membranes. Plasma membrane H^+-ATPases are encoded by a multigene family and their expression is regulated in spatial and temporal contexts as well as by chemical inducers like salt (Niu *et al*., 1993a; 1993b; Binzel, 1995; Niu *et al*., 1995). Accumulation of PM H^+-ATPase mRNA was induced by NaCl in expanded leaves and in roots of the glycophyte tobacco and the halophyte *Atriplex nummularia*, however in roots of halophyte the increase in

transcript abundance was substantially greater than that in the roots of glycophytes (Niu *et al*., 1993a). A rice PM H^+-ATPase isoform *OSA3* was also seen to be specifically induced by salt stress (Zhang *et al*., 1999). A tomato PM H^+-ATPase isoform showed higher transcription upon salt exposure, but not on exposure to iso-osmotic levels of PEG, indicating that *LHA8* expression is triggered specifically by ionic effects of NaCl rather than by its osmotic effects (Kalampanyil and Wimmers, 2001). Expression of PM H^+-ATPase was markedly decreased in salt-sensitive *Populus popularis* leaves in response to salt stress, while salt-resistant *P. euphratica* maintained a high H^+-pumping activity (Ma *et al*., 2010). The higher PM H^+-ATPase expression in leaves of SW plants of *B. cylindrica* as compared to its expression in FW plants indicated that this pump plays an important role in salt adaptation. It could provide the proton gradient required for operating the Na^+/H^+ as well as the Ca^{2+}/H^+ antiporters that play a role in Na^+ and Ca^{2+} exclusion from the cytosol respectively. Whether the differences in transcript levels of these transporters in FW and SW plants of *B. cylindrica* translates into differences in their functional activity remains to be studied.