CHAPTER 4

Identification and characterization of a plasma membrane Ca^{2+}/H^{+} antiporter

CHAPTER 4: IDENTIFICATION AND CHARACTERIZATION OF A PLASMA MEMBRANE Ca^{2+}/H^{+} ANTIPORTER

4.1 Introduction

Divalent cation calcium is an essential element in plant cells. Ca^{2+} constitutes 0.1–2.0% dry weight of plants and structurally stabilizes membranes and cell wall and acts as a counter cation for inorganic and organic ions in the vacuole and intracellular messenger in the cytosol (Marschner, 1995).

The cytoplasmic calcium ($[Ca^{2+}]_{cyt}$) is an obligate intracellular messenger in plants, regulating growth and various developmental processes. In a resting cell, $[Ca^{2+}]_{cyt}$ is strictly maintained between l00-200 nM (Bush, 1995), which is a precondition for Ca^{2+} to act as secondary messenger. Various physiological stimuli such as salinity, drought, hypo-osmotic stress, oxidative stress, cold etc. are known to transiently elevate free $[Ca^{2+}]_{cyt}$ (Bush, 1995; White and Broadley, 2003). These responses mediate mobilization of Ca^{2+} from various Ca^{2+} storage compartments and initiate a signal transduction process. However, high $[Ca^{2+}]_{cyt}$ is cytotoxic because above a critical Ca^{2+} concentration, insoluble calcium phosphate could precipitate in the cytosol, leading to a breakdown of ATP metabolism and to death (Bush, 1995; Plieth, 2005). Hence, to prevent toxic effects from elevated levels of intracellular Ca^{2+}, plants have evolved transport systems that maintain low Ca^{2+} concentrations in the cytoplasm through sequestration into apoplast or into the lumen of various intracellular organelles like vacuole, endoplasmic reticulum (ER), mitochondria, and even chloroplasts (Bush, 1995; Harper *et al*., 1998). Regulation of influx and efflux of Ca^{2+} in cytoplasm is mediated through an ensemble of transport proteins. These proteins namely, Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters/exchangers,

mediate active efflux from the cytoplasm into apoplast or intracellular organelles, while influx of Ca^{2+} into cytosol is passive through Ca^{2+} channels (Sze *et al*., 2000; Hirschi, 2001).

Vacuoles, by virtue of their size and capacity for Ca^{2+} accumulation, are the most prominent sink for Ca^{2+} in cells. But ultimately, homeostasis of $[Ca^{2+}]_{cyt}$) is achieved by export across the plasma membrane (PM) because both biochemical buffering and intracellular sequestration have finite capacities (Sanders *et al*., 1999). The steepest gradient for Ca^{2+} normally exists across the PM. Electrical potentials across the PM (ψ_{pm}) are -150 to-200 mV, yielding an electrochemical difference for Ca^{2+} ($\Delta\mu_{Ca}{}^{2+}$) of 45-60 kJ $mo1^{-1}$ (Hille, 1992; Bush, 1995). Due to this electrochemical gradient, influx of Ca^{2+} into cytosol is passive, while efflux is an active process.

Thus, Ca^{2+}-ATPases and Ca^{2+}/H^{+} antiporters regulate active Ca^{2+} efflux from cytosol and function to (1) maintain low cytoplasmic Ca^{2+} in an unstimulated cell so as to permit metabolism (2) restore $[Ca^{2+}]_{cyt}$ following perturbations (3) replenish intracellular and extracellular Ca^{2+} stores for further $[Ca^{2+}]_{cyt}$ signals (4) provide Ca^{2+} in the ER for secretory function (5) remove divalent cations such as Mg^{2+}, Mn^{2+}, Ni^{2+} or Zn^{2+}, from the cytosol, to support the specialized biochemistry of some organelles and prevent mineral toxicities (Sze *et al*., 2000; Hirschi, 2001; White and Broadley, 2003).

Ca^{2+}/H^{+} antiporters belong to the cation/proton exchanger (CAX) family. CAX is one of the five families that form the Ca^{2+}/cation antiporter (CaCA) superfamily. CaCA proteins are integral membrane proteins that transport Ca^{2+} or other cations using H^{+} or Na^{+} gradients generated by energy-coupled primary transporters (Busch and Saier, 2002). While there are several reports on Ca^{2+}/H^{+} antiporters associated with vacuolar

membranes (Schumaker and Sze, 1985; Blumwald and Poole, 1986; Bush 1995, Sanders *et al*., 1999) their presence in PMs has been reported only in few instances. For example, a Ca^{2+}/H^{+} antiporter has been observed in isolated PM vesicles of corn leaves and roots (Kasai and Muto 1990; Vicente and Vale 1995) and also in rice PM (Qi *et al*., 2005).

Secondary active transporters like Na^{+}/H^{+} and Ca^{2+}/H^{+} antiporters, utilize the proton gradient ($\Delta\mu_{H^{+}}$) established by H^{+} pumps (H^{+}-ATPases), to transport the respective cations in the opposite direction (Schumaker and Sze, 1985; Blumwald and Poole, 1986; Rasi-Caldogno *et al*., 1987; Vicente and Vale, 1995; Kura-Hotta *et al*., 2001). For example, the plant PM H^{+}-ATPase acts as the primary transporter that pumps protons out of the cell, thus creating a pH and electrical potential gradient across the plasma membrane, which is used by the secondary transporters for ion and metabolite uptake (Michelet and Boutry 1995; Sze *et al*. 1999; Palmgren, 2001). The importance of this pump is reflected by the observation that it may consume as much as 25-50% ATP of the cellular ATP (Harper *et al*., 1990). PM H^{+}-ATPase is a P-type ATPase, forming a catalytic phosphorylated intermediate and is Mg^{2+}-dependent, K^{+}-stimulated, and vanadate inhibited (Niu *et al*., 1993b; Morsomme and Boutry, 2000).

In plants, the balance of Ca^{2+} and Na^{+} ions is an important component in salt tolerance. Ca^{2+} has at least two roles in salt tolerance, a signaling function leading to adaptation (as seen in the SOS system) and a direct inhibitory effect on Na^{+}entry into the cell (Cheng *et al*., 2004). Salinity reduces the H^{+} pumping capacity of the PM H^{+}-ATPase. Activity of this pump was found to be critical for maintaining ion homeostasis in saline environments (Niu *et al*., 1993a, 1993b; Wu and Seliskar, 1998; Muramatsu *et al*., 2002). Along with the PM H^{+}-ATPase, high affinity Ca^{2+}-ATPases and high capacity PM

Ca^{2+}/H^{+} antiporters could have crucial physiological functions when $[Ca^{2+}]_{cyt}$ concentrations are increased due to salinity.

The aim of this study was to prepare transport competent PM vesicles from the leaves of *Bruguiera cylindrica* and to study the vanadate-sensitive (P-type) ATPase and Ca^{2+}/H^{+} antiport activities in these vesicles.

4.2 Materials and Methods

4.2.1 Plant material

Saplings of *Bruguiera cylindrica* (L.) Blume were collected from the seacoast at Mumbai (19º13'45"N; 72º54'49"E), India. These saplings were of 8-12 leaf stage. The plants were brought to the laboratory in buckets along with the seabed soil and seawater in which they were growing. Plasma membrane vesicles from the leaf tissue of these saplings were prepared within 48 h of their uprooting.

4.2.2 Preparation of plasma membrane (PM) vesicles from leaves

Leaves were washed under running tap water to remove soil and dirt particles. Approximately 100 g of leaf tissue was taken to isolate plasma membrane-enriched vesicles by the procedure described by Muramatsu *et al.* (2002), with minor modifications. All procedures were conducted between 2-8ºC. Leaf tissue was homogenized with 250 ml of medium consisting of 50 mM MOPS-KOH (pH 7.6), 300 mM sucrose, 10 mM EGTA, 5 mM EDTA, 10 μg ml^{-1} BHT (butylated hydroxytoluene), 5 mM $K_2S_2O_5$ (potassium metabisulfite), 1 mM DTT, 2% (w/v) PVP, 1% (w/v) BSA, 1 mM PMSF (phenylmethylsulfonyl fluoride), 4 mM SHAM (salicylhydroxamic acid) and 1 mM ascorbic acid. Homogenate was centrifuged (Sorvall RC 5C Plus) at 8000 x g at

4ºC for 15 min. Obtained supernatant was centrifuged again at 156,000 x g at 4ºC for 45 min (Beckman Optima LE-80K, USA). The pellet was resuspended in buffer A [0.25 M sucrose, and 10 mM phosphate buffer (pH 7.8)] and centrifuged at 156,000 x g for 30 min at 4ºC. The pellets were again resuspended in buffer A and were used as crude microsomal (CM) fractions. The CM fractions were further purified by the aqueous-polymer two-phase method (Larsson *et al.,* 1994; Tanaka *et al.,* 2004) with a polymer mixture [6.2% (w/v) Dextran T-500, 6.2% (w/v) PEG (Mol Wt 3350), 30 mM NaCl, 0.25 M sucrose, 10 mM phosphate buffer (pH 7.8)]. The upper phase was diluted with buffer B [0.25 M sucrose, 5 mM MOPS-KOH (pH 7.6), 0.1 mM DTT]. An additional low speed spin at 8000 x g for 30 min at 4ºC (Sorvall RC 5C Plus) was performed to remove residual chloroplast membranes. Supernatant obtained from this step was centrifuged at 156,000 x g for 30 min at 4ºC and the plasma membrane-enriched pellets were re-suspended in buffer B and used as PM fractions. These PM fractions were stored at -80ºC for further use. Protein content was estimated by Lowry's method (1951) with BSA as a standard. The experiment was done once with three different PM vesicle preparations.

4.2.3 Measuring sensitivity of ATPase in PM vesicles to vanadate, nitrate and azide

ATP hydrolysis activity was assayed by the method of Muramatsu *et al*., (2002) with minor modifications. The 0.5 ml reaction mixture contained 10 mM Tris-MES buffer (pH 7.0), 140 mM KCl, 100 μM EGTA, 1 mM DTT, 100 μg ml^{-1} BSA, 0.5 mg ml^{-1} Brij 58 (a non-ionic surfactant), 2.5 mM Tris-ATP, and PM fraction (10 μg protein). The reaction was started by addition of 4 mM $MgSO_4$ and the assay was carried out at 25ºC for 30 min.

At the end of the assay, free phosphate (Pi) released due to ATP hydrolysis was measured according to the method of Heinonen and Lahti (1981). 4 ml of AAM (acetone: 5 N sulfuric acid: 10 mM ammonium heptamolybdate) solution was added to test tubes containing the reaction mixture (0.5 ml). The contents were mixed with a vortex mixer and 0.4 ml of 1 M citric acid was pipetted into each tube and re-mixed. Absorbance was measured at 355 nm (Ultraspec 4000, Pharmacia Biotech, USA) against a blank that had no phosphate in it. The ATP hydrolysis assays were carried out in the presence of 100 μM Na_3VO_4 (Vanadate-sensitive P-type ATPase), 50 mM KNO_3 (nitrate-sensitive V-type ATPase), or 1 mM NaN_3 (azide-sensitive F-type ATPase). The sensitivity of the ATPase to each of these inhibitors was obtained by subtracting the ATPase activity observed in presence of the specific inhibitor from the ATPase activity observed in absence of any inhibitor.

4.2.4 Measuring sensitivity of ATPase to salt

Effect of NaCl on PM H^+-ATPase (P-type) activity was studied by including 1 mM NaN_3, 50 mM KNO_3 and different concentrations of NaCl (0, 100, 200, 300, 400, and 500 mM) in the reaction mixture used for assaying ATP hydrolysis.

4.2.5 Determination of K_m for PM H^+-ATPase

Affinity of PM H^+-ATPase for its substrate ATP was determined by an ATP titration. Various concentrations of Tris-ATP (0.1, 0.2, 0.5, 1, 1.5, and 3 mM) were added to 0.5 ml reaction mixture containing 10 mM Tris-MES buffer (pH 7.0), 140 mM KCl, 100 μM EGTA, 1 mM DTT, 100 μg ml^{-1} BSA, 0.5 mg ml^{-1} Brij 58, 1 mM NaN_3, 50 mM KNO_3 and PM fraction (10 μg protein). ATP hydrolysis assay was carried out as mentioned

earlier. The K_m values for the PM H^+-ATPase was determined from Lineweaver-Burk plot. Data was analysed by using statistical software Origin 8.0.

4.2.6 Ca^{2+}/H^+ antiport assay and determination of its K_m

ATP-dependent proton transport into PM vesicles was assayed by observing the quenching of Acridine orange fluorescence. Recovery of the fluorescence by addition of Ca^{2+} was used to determine the Ca^{2+}/H^+ antiport activity. Fluorescence was monitored at excitation and emission wavelengths of 492 (slit width 0.45 nm) and 525 nm (slit width 3 nm) respectively with a Fluoromax-4 spectroflourometer (Horiba Jobin Yvon, Edison, NJ, USA). PM vesicles (140 µg protein) were added to 1.5 ml of reaction medium containing 10 mM Tris-MES (pH 7), 140 mM KCl, 100 µM EGTA , 0.5 mg ml^{-1} Brij 58, 1 mM NaN_3, 50 mM KNO_3 , 2 mM Tris-ATP, 0.1 mg ml^{-1} BSA, and 1 mM DTT. Proton accumulation inside the vesicles was initiated by adding $MgSO_4$ (4 mM) to activate the ATPase and fluorescence quenching was measured till it reached a minimal level. At this stage, various concentrations of $CaCl_2$ were added. Free calcium concentrations [Ca^{2+}] were calculated using a web-based EGTA calculator (http://brneurosci.org/egta.html). Recovery of fluorescence was measured for about 10 min, till no further increase of fluorescence was observed. Finally addition of $(NH_4)_2SO_4$ completely abolished the pH gradient and led to the original fluorescence levels. The samples were continuously stirred during the measurements. The Ca^{2+}/H^+ exchange activity was expressed as the initial rate of recovery of fluorescence intensity observed after the addition of Ca^{2+} and was expressed as rate of change in fluorescence counts per second (cps). The K_m values for the Ca^{2+}/H^+ antiporter was determined from Hanes-Woolf plot. Data was analysed by using statistical software Origin 8.0.

4.3 Results

PM vesicles were used to measure the rate of ATP hydrolysis by PM H^+- translocating ATPases. Assays were performed in the presence of inhibitors that differentiate types of H^+-ATPases (from PM, tonoplasts and mitochondria) to determine the purity of PM fraction. Vanadate-sensitive (P-type) ATPase activity was inhibited by more than 70% in the isolated membrane fractions (***Fig 4.1***). In contrast, activities of nitrate-sensitive (V-type) ATPase (tonoplast) and azide-sensitive (F-type) ATPase for (mitochondria) were inhibited by approximately 23%. Hence the membrane fractions were predominantly of PM origin. Performing the ATPase assays in presence of increasing concentrations of ATP showed typical Michaelis-Menten type kinetics as seen from the Lineweaver-Burk plot. The mean K_m (ATP) value of the PM H^+-ATPase determined from the three PM preparations was 1.437 mM (***Fig. 4.2***).

On studying the effect of different salt concentrations on the activity of P-type ATPase, it was seen that the activity remained unaltered at 100 mM NaCl treatment (***Fig.4.3***). NaCl concentrations above 100 mM, showed inhibition of ATPase activity.

Fluorescence of acridine orange was used to study the proton pumping competence and vesicular sidedness of the PM vesicles. Fluorescence quenching due to the transport of H^+ into the PM vesicles was observed on $MgSO_4$ addition to the reaction medium containing ATP (***Fig 4.4***), indicating that inverted vesicles with cytoplasmic-side-out orientation were obtained (***Fig 4.5***).

To study the Ca^{2+}/H^+ antiporter activity, aliquots of Ca^{2+} were added (10.2 µM–153.16 µM) to the assay medium after the pH gradient reached a steady state and the rate of fluorescence recovery was recorded. Addition of $CaCl_2$ to PM vesicles caused the

dissipation of a preformed pH gradient, which was measured as the recovery of acridine orange fluorescence (***Fig 4.4***). The rate of the Ca^{2+}/H^{+} antiporter activity was seen to increase with increasing Ca^{2+} concentrations (***Table 4.1***).

The Ca^{2+}/H^{+} antiport activity displayed Michaelis- Menten kinetics with respect to Ca^{2+} concentration (***Fig 4.6***). The mean K_m (Ca^{2+}) value of the three PM fractions as obtained from Hanes-Woolf plot was 12.8 μM.

4.4 Discussion

A typical feature of biological membranes is the asymmetric arrangement of constituents across the lipid bilayer. The most useful approach for characterizing the asymmetric properties of a membrane, including its vectorial activities, is to prepare sealed membrane vesicles of either orientation. Two-phase partitioning separates particles according to their surface properties and vesicles of opposite orientations can be obtained (Palmgren *et al*., 1990). With such preparations, each membrane surface can be probed selectively using impermeable agents, and transport in either direction can be measured. The vesicles are devoid of cytoplasmic constituents of the intact cell and their metabolic activities are limited to those enzymes associated with the membrane itself. Using such vesicles it is therefore possible to determine whether solute transport is coupled to proton gradients generated by ATPase activity.

In our experiments, inside-out PM vesicles were prepared from leaves of mangrove *B. cylindrica* using the non-ionic detergent Brij 58, a polyoxyethylene acyl ether. *B. cylindrica* PM vesicles were shown to be transport-competent and formed proton gradients due to the activity of the plasma membrane-localized H^{+}-ATPase as detected by

quenching of acridine orange fluorescence (Palmgren, 1991; Clerc and Barenholz, 1998). In neutral and acidic solutions, acridine orange behaves as a weak base (pK_a = 10.45) and exists predominantly as the protonated cation in equilibrium with minute amounts of the neutral free base. Acridine orange monomers show maximal fluorescence emission at 525 nm when excited at 492 nm. When this dye is added to a suspension of vesicles whose internal compartments are made acidic due to proton pumping activity of the PM H^+-ATPase, the free base diffuses across the vesicle membrane and is protonated in the vesicle interior. Increasing concentration of acridine orange in the vesicles causes the aggregation of the dye molecules (Clerc and Barenholz, 1998). This process causes a decrease of the monomer concentration in the external medium and hence a decrease of fluorescence. The vesicles also create a localized filter effect that diminishes the intensity of light emitted by the trapped fluorophores (Lakowicz, 1983). The net result is a reduction in fluorescence emission at 525 nm when the dye penetrates into the vesicle internal compartment. That the H^+-ATPase activity was of the PM and not the vacuolar or mitochondrial enzyme, was determined by addition of specific inhibitors of for each of these ATPases. Addition of 0.1 mM Na_3VO_4 (specific inhibitor of PM H^+-ATPase) led to 70% inhibition of the ATPase acitivity indicting that the vesicles were predominantly of PM origin. In plants, K_m of PM H^+-ATPase has been shown to vary between 0.3 mM to 1.4 mM for its substrate Mg^{2+}-ATP (Michelet and Boutry, 1995). We observed a K_m (ATP) value of 1.437 mM for PM H^+-ATPase in *B. cylindrica*, which lies in the reported range.

Since the vesicles were isolated from *B. cylindrica* plants growing in their natural habitat on the sea-coast, we studied the salt sensitivity of the PM H^+-ATPase by including salt in

the reaction medium. Salt concentrations above 100 mM had inhibitory effects on its activity. The effect of salinity on transport proteins may occur through: (a) changes in lipid-protein interactions since salt-induced alterations in plasma membrane lipid composition and structure have been reported in various species (Brown and DuPont, 1989; Wu *et al*., 1998). ATPase activity could therefore be altered by changes in the membrane lipids (Carruthers and Melchior, 1986; Yu *et al*., 1999) (b) increased transcription activity of ATPase (Niu *et al*., 1993a, 1993b; Binzel, 1995) (c) changes in kinetic properties due to changes in protein structure (Svennelid *et al*., 1999; Kerkeb *et al*., 2001) (d) alteration in intracellular calcium which affects calmodulin, as a second messenger, and thus affects the functions of ATPase (Palmgren and Harper, 1999) and (e) direct denaturation of membrane transport proteins. The PM H^+-ATPase from both control and salt-adapted callus from the halophyte S*partina patens* was inhibited in the presence of salt in the assay solution (Wu and Seliskar, 1998). *In vitro* NaCl inhibition kinetic data revealed a pattern of non-competitive inhibition of PM H^+-ATPase by NaCl, in which NaCl could either bind to the enzyme or the enzyme-substrate complex, (Wu and Seliskar, 1998).

Salt treatment has been reported to decrease PM H^+-ATPase activity in tomato roots (Sanchez-Aguayo *et al*., 1991), stimulate its activity was in cell cultures of *B. sexangula* (Kura-Hotta *et al*., 2001) or have no effect on the enzyme activity in *Acer pesudoplatanus* cells (Zingarelli *et al*., 1994). The importance of this enzyme in salt tolerance has been suggested from biochemical analyses of PM fractions isolated from halophytes such as *Salicornia bigelovii*, *S. patens*, and in seagrass *Zostera marina* (Ayala *et al*., 1996; Wu and Seliskar 1998; Muramatsu *et al*., 2002). The lack of PM H^+-ATPase activity in

presence of salt was interpreted as one of the physiological factors involved in inhibiting normal plant development in saline conditions. However, inhibition of PM H^+-ATPase activity was also suggested to have a distinct advantage with respect to salt tolerance, through depolarization of the plasma membrane, which, in turn, decreased Na^+ absorption under salinity (Suhayda *et al*., 1990; Serrano *et al*., 1999). Whether salt leads to inhibition or activation of the H^+-ATPase under *in vivo* conditions remains to be studied in *B. cylindrica*.

An important role of the PM H^+-ATPase is to provide the proton gradient that is used for energizing secondary active transporters for solute transport. Ca^{2+} transport by Ca^{2+}/H^+ antiporter has been shown to be driven by H^+-ATPase mediated proton gradient. This antiporter is known to function in sequestering Ca^{2+} into vacuoles (Blumwald and Poole, 1986; Schumaker and Sze, 1990) Two parallel systems for Ca^{2+} efflux, a Ca^{2+}ATPase and Ca^{2+}/H^+ antiporter, were reported in the plasma membrane of maize leaves and roots (Kasai and Muto, 1990; Vicente and Vale, 1995). However the role of Ca^{2+}/H^+ antiporter in Ca^{2+} extrusion through PM is not yet well established.

Inside-out membrane vesicles are a good system for studying the mechanisms by which Ca^{2+} is transported out of the cytoplasm against a large concentration gradient that exists across the PM, since it is possible to vary the Ca^{2+} concentration outside the vesicles and follow its entry into the vesicle. Transport competent *B. cylindrica* PM inside-out vesicles were used for identification of Ca^{2+}/H^+ antiport activity in the PM and a prominent Ca^{2+}/H^+ antiporter activity in these PM vesicles was characterized. This Ca^{2+}/H^+ antiporter showed a K_m for Ca^{2+} of 12.8 μM. Ca^{2+}/H^+ antiport of corn root PM had a K_m of ~ 75 μM (Vicente and Vale, 1995), while the tonoplast Ca^{2+}/H^+ antiporters

showed K_m values ranging from 10-40 μM (Blumwald and Poole, 1986; Schumaker and Sze, 1990; Vicente and Vale, 1995). A major function for the Ca^{2+}/H^+ antiporter is thought to be to lower the concentration of cytosolic Ca^{2+} to 1-2 μM after an increase in its concentration due to external and/or internal stimuli (Maeshima, 2001). The Ca^{2+}-ATPase may then act to lower the concentration of Ca^{2+} still further. The Ca^{2+}/H^+ antiporter in *B. cylindrica* leaves is a moderately high affinity and high capacity antiporter might have an important role in maintaining cytoplasmic Ca^{+2} concentrations under the saline environment in which this mangrove grows.