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Cytochemical Localization of Sodium and H⁺ATPase in the Salt Gland of Tree Mallow, *Lavatera arborea* L.

J.F. Bamidele

Department of Botany, University of Benin, Benin City, Nigeria

Abstract: Cytochemical localization of sodium ion in the salt gland of *Lavatera arborea* (L.) was carried out. Electron enzyme-cytochemical procedure was also employed to locate sites of ATPase activities in the salt gland. There was a prominent localization of electron dense precipitates in the extracytoplasmic space near the wall separating the two secretory cells in the gland. Other sites of sodium ion precipitation were in the vacuoles, dilated parts of the plasmalemma invaginations and microvacuoles. The result of the ultrastructural study demonstrated that ATPase activities were prominent in all the above sites. The close parallel in the sites of sodium localization and the areas where intense enzyme reaction were produced supports the hypothesis that the salt gland is involved in short distance intensive ion transport. X-ray microanalytical study revealed that the major ions being secreted by the salt gland are those of sodium and chloride.

Key words: Ion localization, salt gland, X-ray microanalysis, ATPase, enzyme, halophyte

INTRODUCTION

Salinity is one of the most intensely studied environmental stress factors in the ecophysiology of plants. Salinity is an increasing environmental problem throughout the world. Salt excess in soils and water has detrimental effect on plant productivity and results in substantial losses of arable soils. In Australia, for instance, one third of the agricultural area is in risk of becoming saline (Munns, 2002). Halophytes are potentially useful for ecological applications, such as landscaping, or rehabilitation of damaged ecosystems (English *et al.*, 2001; Barrett-Lennard, 2002; Marcum *et al.*, 2003). They may also present economical interest as food, forage, or for production of metabolites (Lieth, 1999; Norman *et al.*, 2002; Semple *et al.*, 2003; Truong *et al.*, 2002). Investigating salt effects on physiological and metabolic processes in halophytes is quite complex since their response depends on salt type and level, plant genotype and growth stage (Munns, 2002). The major impact of stressful NaCl loads is on functions of proteins and membranes as stabilized by water structures in the cell.

Tree mallow, *Lavatera arborea* (Malvaceae) is a perennial shrub which is frequent along coastal cliffs in the Mediterranean region, Britain and France. It is commonly used in coastal areas as a horticultural plant. In previous study, it was described that the morphology and ultrastructure of the salt glands of *L. arborea* (Bamidele

and Malloch, 2006). These earlier results suggested that salt secretion was effective in maintaining ion balance and good growth at low to moderate salinities (Okusanya, 1979). The mechanism by which ions are mobilized from the vacuole and secreted outward across the plasmalemma has been infrequently addressed and therefore, has yet to receive definite answer. Ultrastructural studies showed that the secretory cells in *L. arborea* salt glands possess a labyrinth of plasmalemma invaginations that apparently act as sites of temporary ion accumulation. The sequestered ions are passed to the cell exterior via the top cells and sub-cuticular space (Bamidele and Malloch, 2006). The abundance and proximity of mitochondria to the plasmalemma invaginations suggests that ion transport via these cells may be an active process. Earlier results showed that ion accumulation occurred within the lumina of plasmalemma invaginations and in the extra-cytoplasmic space between the two secretory cells of *L. arborea* salt gland (Bamidele and Malloch, 2006). Therefore, I tested the hypothesis that the sites of sodium ion localization and ATPase activity in *L. arborea* salt gland would correlate with the above locations. I also employed energy dispersive X-ray microanalysis to quantitatively and qualitatively analyze the ions present in the salt gland. The information gathered will shed light on the strategy by which the plant maintain its mineral ion balance and water status in the field and will contribute to an enhanced understanding of mechanism of salt tolerance in halophytes.

MATERIALS AND METHODS

Young seedlings of *Lavatera arborea* were collected from Lizard peninsula, Anglesey, Britain and raised in sand culture. Salt treatment was applied as described by Bamidele and Malloch (2006). The experiment was conducted in the laboratory at the University of Lancaster, United Kingdom.

Procedure for antimonate precipitation: The rapid method described by Harvey *et al.* (1979) was adopted. Potassium antimonate reagent (2.5%, w/v) was prepared by boiling 5% (w/v) potassium antimonate gently until all the solid dissolved. The solution was allowed to cool down to room temperature and was then mixed with equal volume of 2% (w/v) osmium tetroxide in deionized water. The pH was adjusted to 7.4 with 50 mM acetic acid and the volume readjusted with deionized water to give a 2.5% potassium antimonate solution. Small pieces of leaf tissue (1×5 mm) were carefully cut with a grease-free razor and fixed for 1 h at 4°C in 2.5% potassium antimonate/1% osmium tetroxide reagents. The tissue were rinsed for 10 min in 50 mM sodium cacodylate/acetate buffer (pH 7.2), dehydrated with graded ethanol series and embedded in Spurr's resin (Spurr, 1969). Ultrathin sections were prepared using a Reichert microtome and examined for antimonate precipitation using the electron microscope.

Cytochemical localization of ATPase activity: ATPase activity was localized in *L. arborea* salt gland using the lead precipitation procedure modified from Hall (1971). Small leaf tissue (1 mm²) obtained as described above were cut from fully mature *L. arborea* leaves, fixed for 2 h in cold 2% (v/v) glutaraldehyde buffered with 50 mM sodium cacodylate at pH 7.2. The tissue segments were subsequently washed for a minimum of 3 h with 6 changes of cold buffer. This was followed by 20 min rinses in 0.1 M Tris maleate buffer (pH 7.2). The leaf pieces were incubated for 3 h at 37°C in a medium containing 2 mM ATP, 2 mM Mg(NO₃), 3.6 mM Pb(NO₃)₂, 48 mM Tris-maleate buffer (pH 7.0). After incubation, the tissue was rinsed and stained with 2% (w/v) osmium tetroxide buffered with 0.05 M sodium cacodylate (pH 7.2) overnight at 4°C. Tissue was dehydrated through a graded ethanol series and embedded in Spurr's resin (Spurr, 1969). Control consisted of tissue incubated in a medium lacking ATP (Hall, 1971). Sections were not post-stained unless otherwise stated. Ultrathin sections were cut a Reichert microtome. Sections were observed and photographed with a Philips 301 TEM at 60 Kv.

X-ray microanalysis: Leaf segments were washed with distilled water three times. The leaves were dipped in 5% agar, inserted to a depth of 1.0 cm in a copper holder and immediately sliced free-hand with a razor blade to get transverse sections and then frozen in liquid nitrogen. The samples were freeze-dried, carbon coated in a high vacuum sputter coater and stored in a desiccator (Van Steveninck and Van Steveninck, 1991). The coated specimens were analysed in a Leo 420 stereoscope (Oxford INCA EDX Systems) fitted with an energy dispersive X-ray detector and a Link AN10000 X-ray microanalyser (Oxford Instruments Microanalysis Group, High Wycombe, Bucks, UK). Spectra from 0 to 10 keV were recorded at an accelerating voltage of 20 kV, using a beam diameter of about 1 nA. Spectra were analysed by the proprietary ZAF-P/B program. The P-B/B (peak/background/background) ratios represent only semiquantitative measurements of ion concentration. Elemental maps were collected in real-time mode. A secondary electron image of the specimen was first stored direct to disk at a resolution of 512×512 pixels. Multiple scans were then made of the same region of the specimen to collect elemental distribution maps. Specimen drift was minimized by keeping the specimen/stage temperature at a constant -145°C.

RESULTS

The salt gland is globose and multicellular (Fig. 1). Figure 1d shows electron- opaque precipitates localized in the extracellular space close to the cell wall separating the two secretory cells of *L. arborea* salt gland. Another site of sodium ion precipitate deposition is in the lumen of the dilated paired plasmalemma invaginations. Precipitates of ions also occur in vacuoles and minivacuoles (Fig. 1e). Electron dense black deposits of lead phosphate were found chiefly in the dilated regions of the plasmalemma invaginations (Fig. 2e). Some enzyme activities were also observed on vacuolar membranes (Fig. 2d). The result indicates a close parallel in the sites of sodium ion deposition and areas where ATPase activity was observed (e.g., Fig. 1d and 2e). In both experiments, the plasmalemma invaginations in the control tissue lacked precipitate. The results of the energy dispersive analysis show that the main ions being secreted from the salt gland are those of sodium and chloride. The result also revealed the presence of a little amount of magnesium ions (Fig. 1b and c and 3b-e).

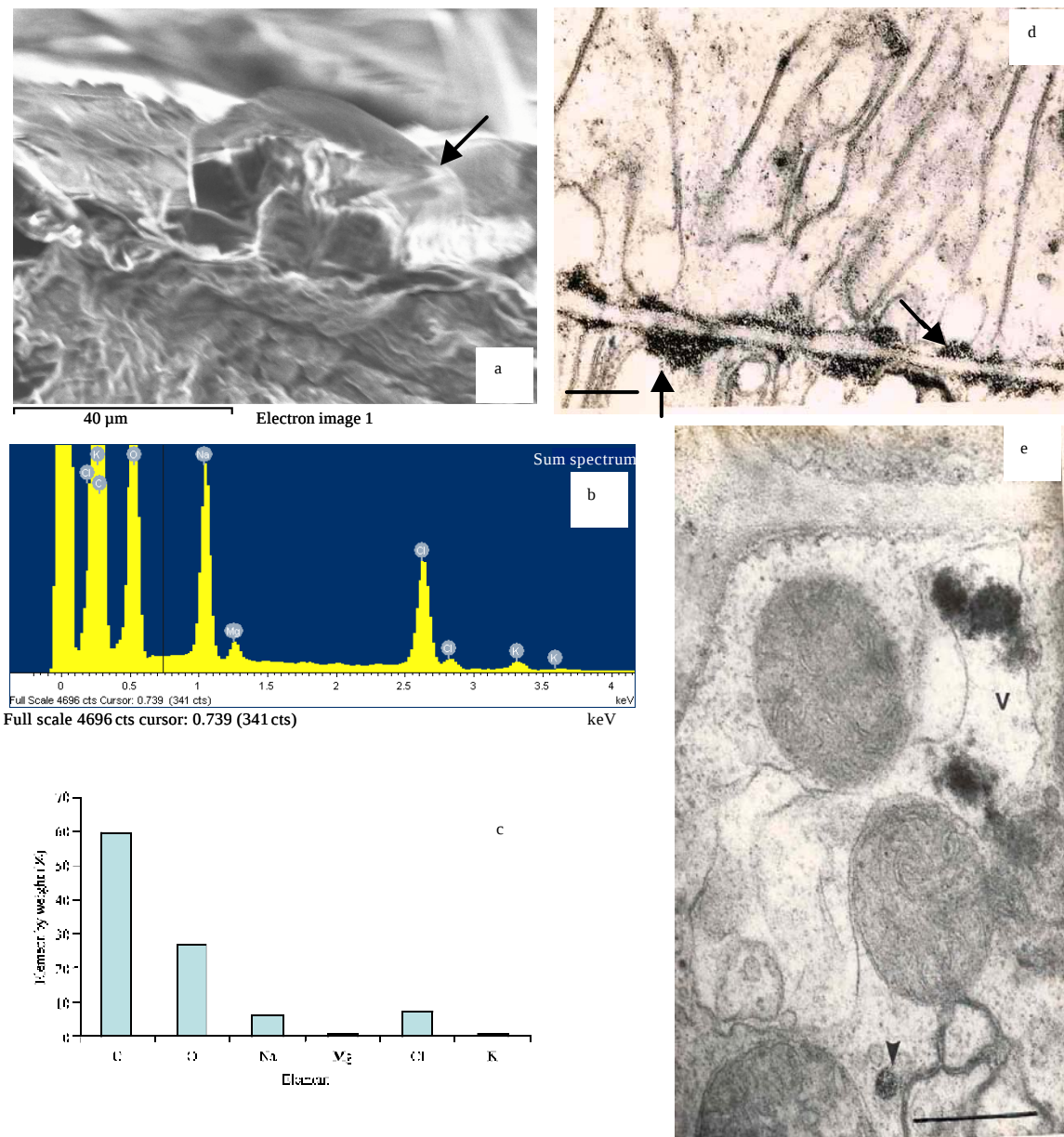


Fig. 1: a): Scanning electron micrograph of *L. arborea* salt gland; b): X-ray emission spectrum of salt secretion in the salt gland of *Lavatera arborea*. Major peaks were Na and Cl; c): Percentage element by weight in *L. arborea* salt gland; d): Transmission electron micrograph showing electron dense precipitates (arrow) near the wall separating the two secretory cells of *L. arborea* salt gland. Scale marker = 0.5 μm and e): Electron micrograph of portion of *L. arborea* salt gland. Note the electron opaque precipitates in the vacuole (v) and minivacuole (lower arrow). Scale marker is 0.5 μm

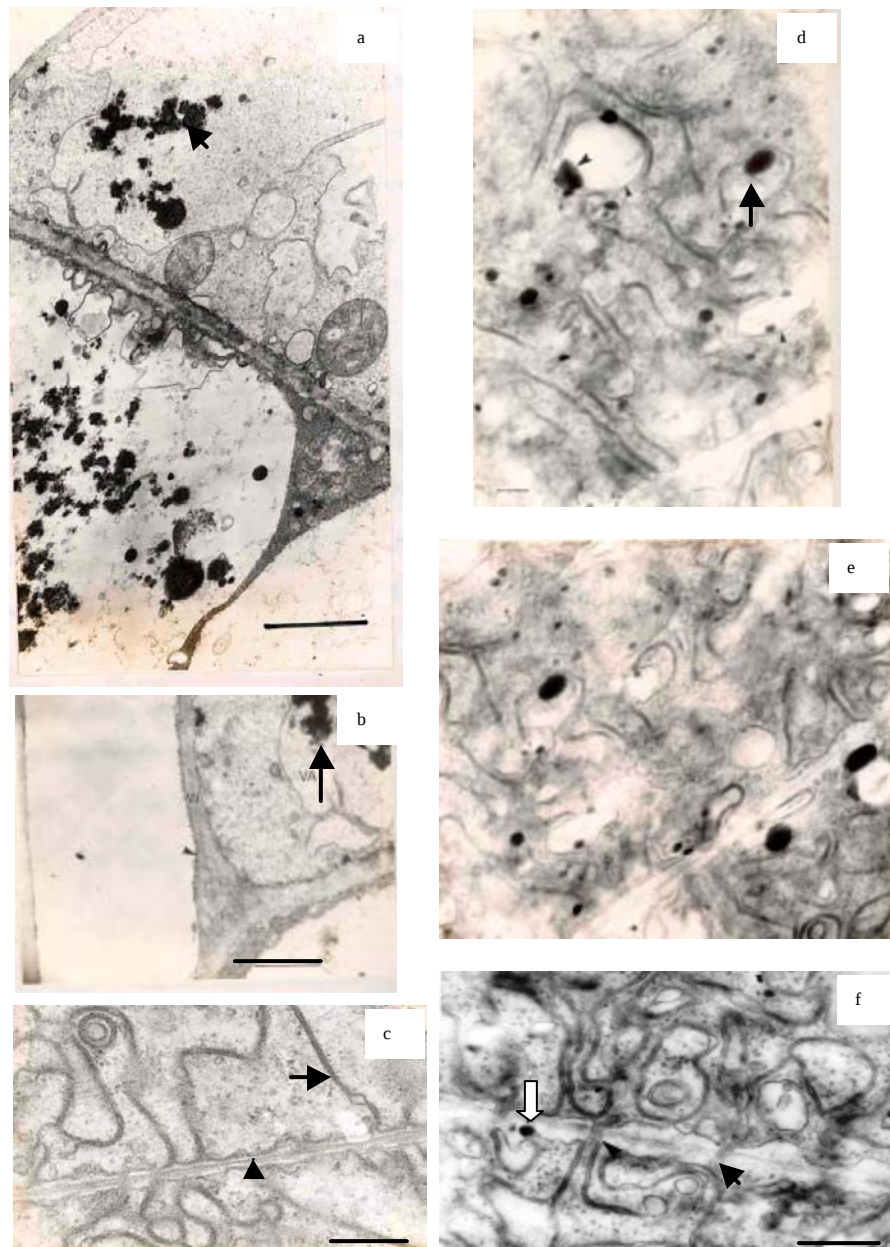


Fig. 2: a): Transmission electron micrograph of parts of the top cells of *L. arborea* salt gland showing electron dense precipitates within the vacuoles (arrows). Scale marker = 5 μ m; b): Micrograph of part of *L. arborea* salt gland. Note dark precipitates largely within the vacuole (arrow) and the cell wall next to the subcuticular space (arrowhead). Scale marker = 0.5 μ m; c): Electron micrograph showing lack of dark precipitates in the control specimen (lacking ATP or potassium pyroantimonate treatment). Scale marker = 0.25 μ m; d): Micrograph in *L. arborea* salt gland showing sites of ATPase activity. Electron dense precipitates are prominent on the tonoplast (arrowhead) and areas where the plasmalemma invaginations dilate (arrow). Scale marker = 0.2 μ m; e): Micrograph of *L. arborea* salt gland. Note dark precipitates near the cell wall separating the two secretory cells (small arrows) and on the plasmalemma (long arrow). Scale marker = 0.2 μ m and f): Micrograph showing part of the cell wall separating the two secretory cells of *L. arborea* salt gland. Note the plasmodesmata (short arrow) and the precipitate near the cell wall (white arrow). Scale marker = 0.5 μ m

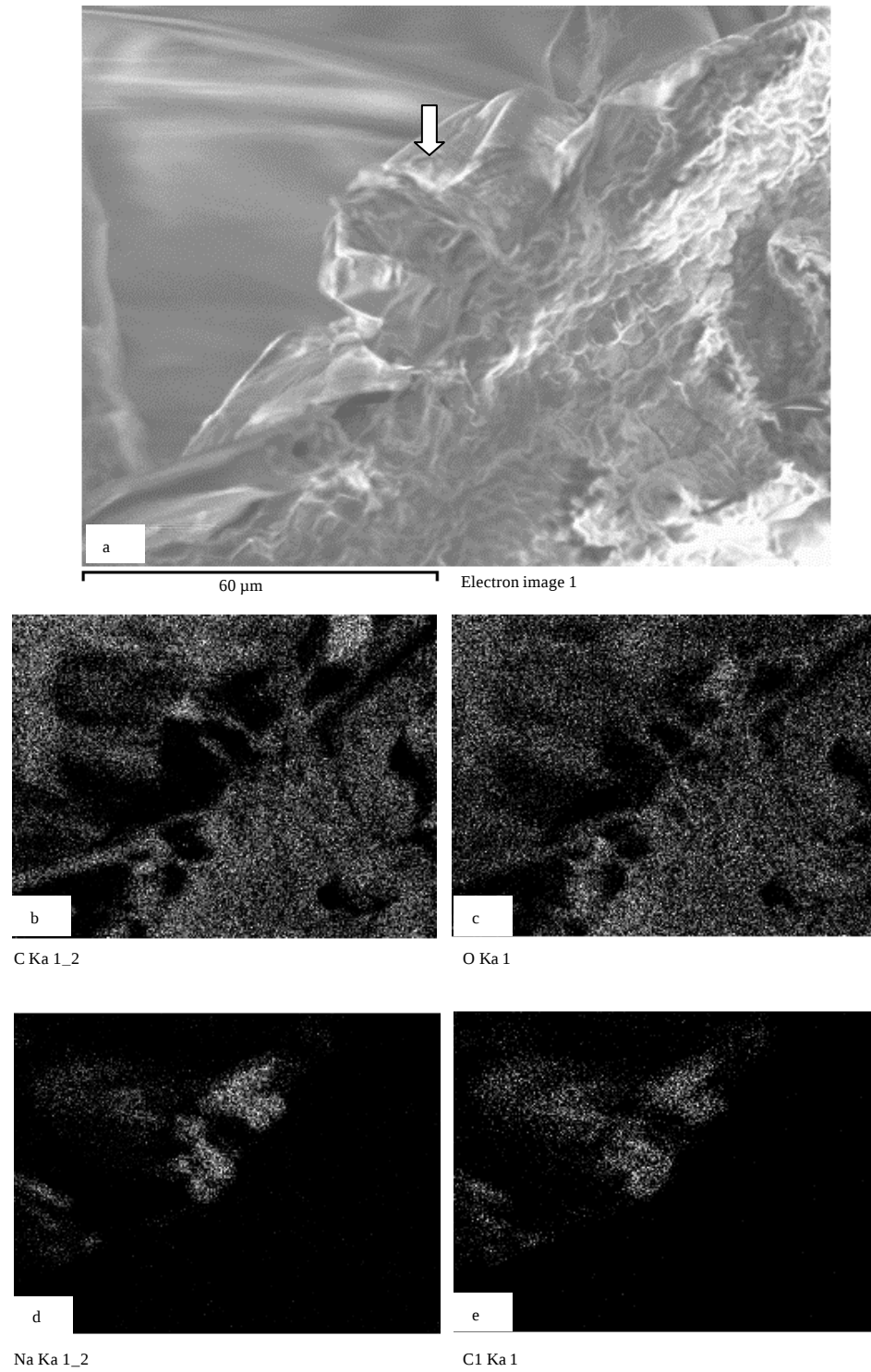


Fig. 3: a) Scanning electron micrograph of *L. arborea* showing the salt gland (arrow) and b-e): Distribution of C, O, Na and Cl in *L. arborea* salt gland

DISCUSSION

The present results confirm the initial hypothesis that the salt glands on the leaves of *Lavatera arborea* are involved in ion regulation. The results a correlation between the sites of sodium ion localization and sites ATPase activity in *L. arborea* salt gland (Fig. 1d-e, 2a-b and 2d-f). Salt glands occur on leaf surfaces of some halophytes. Salt gland excretion is typically highly selective for sodium and chloride although small amounts of other ions (e.g., calcium and magnesium) may also be excreted. The available evidence is that excretion is an active, metabolically driven process. In marine couch, for example, the localization of the energy consuming (ATPase) activity within salt gland basal cells is indicative of active ion loading at these sites (Naidoo and Naidoo, 1999). The large number of mitochondria associated with the secretory cells of *L. arborea* salt gland apparently provides the necessary energy to drive the secretion process. It has been asserted that cell vacuoles carry out numerous metabolic functions (Fitzgerald *et al.*, 1982; Battey *et al.*, 1999). Under some physiological conditions, they participate in the export of a range of solutes ranging from simple sugars and organic acids to amino acids and mineral ions (MacRobbie, 1999). Echeverria (2000) pointed out that vacuole also appears to serve as an interim location in the process of sugar secretion in nectaries and ion secretion by salt glands. The latter role is demonstrated in the present study on *L. arborea* salt gland. Numerous minivacuoles were observed in the secretory cells of *L. arborea* salt gland (Bamidele and Malloch, 2006). This earlier result also showed that ion accumulation occurred within the lumina of plasmalemma invaginations and in the extra-cytoplasmic space between the two secretory cells of the salt gland. The present results confirm that sodium ions are sequestered in the vacuoles and minivacuoles of the secretory cells of *L. arborea* salt gland. Sodium ions were also localized in the extra-cytoplasmic space between the two secretory cells. These occur as darkly stained deposits (Fig. 1d) in the micrographs. The close parallelism between the sites of ion accumulation and sites of antimonite precipitation in *L. arborea* salt gland suggests that the electron opaque precipitates are not mere artefacts (Fig. 1d and 2e). Lack of random precipitates within the cytoplasm further suggests that the method is reliable. X-ray microanalysis result further demonstrated that the dominant cation being secreted by *Lavatera arborea* is sodium. Recent evidence has demonstrated the vacuole as the site of salt accumulation and the existence of ion antiports, pumps and channels at the tonoplasts of plant cells (Blumwald and Gelli, 1997). It is expected that there will be intensive

enzyme activities at sites where ion are being pumped. This assertion is corroborated in the result of enzyme localization. The sites of ATPase activity and sodium ion localization mirror the sites where ions were observed in earlier studies. Intense ATPase activities were observed on vacuolar membranes, in areas where the plasmalemma invaginations are dilated and along the cell wall separating the two secretory cells. The numerous mitochondria previously shown to be abundant in the secretory cells (Bamidele and Malloch, 2006) might be the source of ATP for the active transport process. Naidoo and Naidoo (1999) had demonstrated similar enzyme localization in the grass *Sporobolus virginicus*. X-ray microanalysis is a method for mapping the spatial distribution of ions throughout the gland and in this study it provides new information about ion accumulation in the salt gland of *L. arborea*. Figure 3a is a scanning micrograph showing a transverse section of *Lavatera arborea* leaf. One of the salt glands on which ion distribution was carried out is shown in Fig. 3a. The x-ray ion distribution maps (Fig. 3b-e) further confirm that sodium and chloride ions are the principal minerals being secreted by *L. arborea* salt gland.

In summary, these results indicate that *L. arborea* is a moderately salt tolerant halophyte, which accumulates ions to achieve a negative water potential gradient. Salt glands in the leaves are involved in the regulation of ions within the plant. Cytochemical studies revealed the presence of sodium in extracytoplasmic spaces as well as (ATPase) enzyme activities on the plasmalemma invaginations and tonoplasts. These findings support the postulate that the secretory cells and micro-vacuoles are associated with ion transport and regulation via the salt gland.

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