Intracellular Compartmentation of Ions in Salt Adapted Tobacco Cells

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ABSTRACT

Na\(^{+}\) and Cl\(^{-}\) are the principal solutes utilized for osmotic adjustment in cells of *Nicotiana tabacum* L. var Wisconsin 38 (tobacco) adapted to NaCl, accumulating to levels of 472 and 386 millimolar, respectively, in cells adapted to 428 millimolar NaCl. X-ray microanalysis of unetched frozen-hydrated cells adapted to salt indicated that Na\(^{+}\) and Cl\(^{-}\) were compartmentalized in the vacuole, at concentrations of 780 and 624 millimolar, respectively, while cytoplasmic concentrations of the ions were maintained at 96 millimolar. The morphometric differences which existed between unadapted and salt adapted cells, (cytoplasmic volume of 22 and 45% of the cell, respectively), facilitated containment of the excited volume of the x-ray signal in the cytoplasm of the adapted cells. Confirmation of ion compartmentation in salt adapted cells was obtained based on kinetic analyses of \(^{22}\)Na and \(^{36}\)Cl efflux from cells in steady state. These data provide evidence that ion compartmentation is a component of salt adaptation of glycophyte cells.

A typical response of many plants to saline environments, particularly halophytes, is to accumulate high intracellular concentrations of Na\(^{+}\) and Cl\(^{-}\) (9, 11, 23, 26, 28). Since the *in vitro* activities of enzymes isolated from glycophytes or halophytes are inhibited equally by NaCl (9, 11), it has been generally accepted that the accumulated ions are sequestered in the vacuole and 'compatible solutes,' such as sugars, proline, and glycinebetaine, function to balance the osmotic pressure of the cytoplasm (9, 11, 26). This ability to compartmentalize Na\(^{+}\) and Cl\(^{-}\) has been considered to be a mechanism of tolerance of halophytes (26).

Although salt tolerance of glycophytes, particularly agronomic species, is usually attributed to the ability to exclude Na\(^{+}\) and Cl\(^{-}\), especially from the shoot (8, 11), intracellular ion compartmentation also may occur in these species. Despite indications of negative correlations between ion accumulation and salt tolerance in glycophytes, such as tomato (22), rice (29), wheat (27), and maize (12), examination of the data reveals that, even in tolerant genotypes, the accumulated levels of Na\(^{+}\) and Cl\(^{-}\) are substantial. In some instances whole cell Na\(^{+}\) and Cl\(^{-}\) accumulation was similar for both tolerant and sensitive genotypes (22, 27, 29). Thus, the ability to compartmentalize Na\(^{+}\) and Cl\(^{-}\) may be an underlying determinant of the tolerance not only of halophytes but also of many crop species.

Verification of ion compartmentation has been restricted by the difficulty in obtaining reliable measurements of subcellular ion concentrations (7, 15, 23, 30). Despite this, cytoplasmic Na\(^{+}\) concentration in leaf cells of *Suaeda maritima* was determined to be 165 mM by efflux analysis when the whole cell concentration was about 600 mM (28). Data obtained by x-ray microanalysis of frozen hydrated leaves of *Atriplex spongiosa* (23) revealed that cytoplasmic ion concentrations were considerably lower than those in the vacuole.

Results from investigations of glycophytes exposed to salinity have been less conclusive. Analytical transmission electron microscopic analysis of freeze substituted root cells of *Zea mays* treated with 100 mM NaCl indicated the majority of Na\(^{+}\) and Cl\(^{-}\) was compartmentalized in the vacuole (13), while the opposite result was reported for leaf mesophyll cells from wheat treated with the same concentration of NaCl (14). Using steady state efflux analysis, slightly higher concentrations of Na\(^{+}\) were observed in the cytoplasm than in the vacuole of root cells of oat plants grown in 50 mM NaCl, although the concentrations in both compartments were less than 50 mM (15).

We have previously characterized the growth and osmotic adjustment of *Nicotiana tabacum* L. var Wisconsin 38 cells (1) adapted to high levels of salinity (428 mM NaCl). Na\(^{+}\) and Cl\(^{-}\), the principal components of the extensive osmotic adjustment exhibited by these cells, accumulated to an average whole cell concentration of 500 and 400 mM, respectively (2). Presented in this paper are results of an investigation utilizing steady state efflux kinetic analysis and scanning electron energy dispersive x-ray microanalysis to identify the intracellular localization of Na\(^{+}\) and Cl\(^{-}\) accumulated by cells adapted to 428 mM NaCl. The majority of Na\(^{+}\) and Cl\(^{-}\) was sequestered in the vacuole, such that the concentrations of Na\(^{+}\) and Cl\(^{-}\) in the cytoplasm were below 100 mM. These findings verify the occurrence of ion compartmentation and illustrate that it is a mechanism whereby glycophytes as well as halophytes may tolerate the inhibitory quantities of ions which are accumulated in response to salinity.

MATERIALS AND METHODS

Cell Culture. Cell suspensions of *Nicotiana tabacum* L. var Wisconsin 38, adapted to different concentrations of NaCl (up to 600 mM), were maintained according to procedures described previously (1). Unadapted cells (grown in medium without NaCl) and cells adapted to 428 mM NaCl were used in the experiments. Determinations of intracellular ion compartmentation were made on cells in the early stationary phase of growth since this population of cells still exhibited maximal viability and their Na\(^{+}\) and Cl\(^{-}\) contents most closely approximated steady state.

X-Ray Microanalysis. Unetched frozen-hydrated cells were bulk
analyzed with a system consisting of a CT1000 Cryotrans System (Hexland/Oxford, Bedford, MA), Jeol JSM 840 Scanning Electron Microscope (Cambridge, MA) and Kevex Analyst 8000 Microanalyzer (Foster City, CA). Cells adapted to 428 mM NaCl are characterized by numerous cytoplasmic strands which traverse the vacuole (1). Prior to analysis, cells were centrifuged in an Eppendorf microcentrifuge at 15,000 rpm for 5 min. This process resulted in the vacuole becoming a spherical compartment with discrete peripheral boundaries (Fig. 1). On the basis of visualization using a vital stain (Evans’s blue), this treatment did not reduce cell viability. Cells began to reform vacuolar strands within 15 min, and after 1 h it was not possible to distinguish between centrifuged and uncentrifuged cells.

Immediately after centrifugation, cells were placed into wells of an aluminum sample holder and frozen in liquid N$_2$ slush. The sample holder was transferred to a cold (−170°C) prechamber where the cell mass was fractured with a liquid N$_2$ cooled knife. Prior to coating, cells were observed at 1 kV to inspect the fracture face of the sample. Samples were then evaporative coated with a thin layer of carbon before analysis.

The operating parameters for the x-ray microanalysis were as follows: accelerating voltage, 10 kV (tungsten filament); aperture, 70 μm; probe current (calculated) 1.2 × 10$^{-4}$ amp; working distance, 39 mm; specimen tilt, 5° (creating a 45° take-off angle); magnification, ×2500. An accelerating voltage of 10 kV was used since it represented a satisfactory compromise between better efficiency of x-ray generation and better spatial resolution.

Point analyses were conducted for 200 s in at least four locations within the cytoplasm and vacuole of each cell analyzed. Criteria used to choose cells for analysis were: readily distinguishable cytoplasm and vacuole, sufficient cytoplasmic area to contain the excited volume (minimum of 7 μm in diameter, at an accelerating voltage of 10 kV the limits of resolution are estimated to be 2–3 μm [18]), and a relatively even surface across the cell so that the topography at the various points of analysis would be consistent.

The x-ray data were quantitated using the peak to background ratio (P/B) method (10). Standard element windows of 14 eV were used to integrate the area under the elemental peaks. The background was estimated by integrating the area in two windows (width of 7 eV) flanking each peak, but offset by 12 eV so that spillover from the peak would not artificially elevate the estimate of the background. The concentrations of Na$^+$ and Cl$^-$ in the cytoplasm and vacuole were calculated on the basis of the P/B, the relative volumes of the two compartments and the whole cell Na$^+$ and Cl$^-$ concentrations.

22Na$^+$ and 36Cl$^-$ Efflux. Isotopic exchange washout experiments were conducted under steady state conditions (15–17, 25) on cells preloaded with either 22Na$^+$ (as NaCl in H$_2$O, Amersham No. SKS-1, carrier free) or 36Cl$^-$ (as NaCl in H$_2$O, ICN No. 63006, 12.8 mCi [g Cl$^-$]$^{-1}$). In order to allow cells to incubate

![Fig. 1. Photomicrographs showing reduced cell size and vacuole to cytoplasm ratio of cells adapted to 428 mM NaCl. Nomarski interference-contrast optics were used to view unadapted (A, B) and adapted (C, D) cells which had been centrifuged as described in “Materials and Methods;” bars represent 25 μm. At this stage of the growth cycle, unadapted cells are predominantly of the form shown in A.](image-url)
for at least one \( t_{2e/2} \), prior to reaching early stationary phase (approximate fresh weight density of 0.32 g ml\(^{-1}\)) for unadapted cells and 0.14 g ml\(^{-1}\) for adapted cells) label was added at least 72 and 120 h prior to this point for unadapted cells and adapted cells, respectively. Six ml of culture were transferred to a sterile 25 ml Erlenmeyer flask containing the radiolabeled ion. Adapted cells were incubated with either 50 \( \mu Ci\) \(^{22}\)Na\(^{+}\) or 40 \( \mu Ci\) \(^{36}\)Cl\(^{-}\) and unadapted cells with 20 \( \mu Ci\) \(^{22}\)Na\(^{+}\) or 15 \( \mu Ci\) \(^{36}\)Cl\(^{-}\). The cells were maintained on a gyratory shaker at 25°C during the incubation period.

An efflux procedure was developed to minimize disturbance of the metabolic state of the cells, particularly with respect to Na\(^{+}\) and Cl\(^{-}\) transport. A 10 ml disposable syringe barrel was used to hold the cells during efflux. Tygon tubing was placed over the end of the barrel and the flow regulated with a pinch clamp. The base of the syringe barrel was lined with a disk of Miracloth (Calbiochem, No. 475855) which was held in place by an o-ring. A capillary tube connected to a peristaltic pump was positioned along the inner wall of the syringe so that a stream of air was directed at the bottom of the vessel to gently suspend the cells and to aerate the medium. Cells effluxed in this manner remained viable (as determined by microscopic examination with Evan’s blue viability stain) for the duration of the washout period. The Na\(^{+}\) and Cl\(^{-}\) contents of the cells prior to and following the efflux were equivalent, indicating that the cells remained in steady state with respect to these two ions for the duration of the efflux period.

Efflux was conducted on duplicate samples from three separate cultures for salt adapted cells and two separate cultures for unadapted cells. At the start of the efflux, 0.25 g (fresh weight) of cells were gently pipetted into the syringe barrel and the incubation medium drained by gravity and collected. Two ml of nonlabeled medium were added to the cells and replaced with fresh medium at successive sampling points. The fresh nonlabeled medium (pH adjusted to that of the culture medium) contained 0 or 428 mm NaCl for unadapted cells or for adapted cells, respectively. At the end of the washout period the radioactivity remaining in the cells was determined. Cell samples were frozen and lyophilized prior to analysis. Duplicate samples were used for all determinations. Cell samples for analysis of Na\(^{+}\) content were acid digested (2); radioactivity and Cl\(^{-}\) content were determined on water extracts of the cell samples. One ml of H\(_2\)O was added to each 2.5 mg (dry weight) of cells. The samples were immersed in a boiling water bath for 30 min, then centrifuged in a microfuge for 3 min at 15,000 rpm and the supernatant collected. This procedure was repeated three times and the supernatants pooled. The extracts were frozen, lyophilized, and resuspended in 1 ml of H\(_2\)O. Na\(^{+}\) and Cl\(^{-}\) were measured as described previously (2). The radioactivity in the media samples and cell extracts was determined with a Beckman LS6800 liquid scintillation counter in 4 ml of aqueous counting cocktail (Aquasol, Amer sham).

Efflux data were analyzed as described by Pallaghy and Scott (16). The values for \( k_{\text{vac}} \) and \( Y_{\text{vac}} \), as well as for \( k_{\text{cyt}} \) and \( Y_{\text{cyt}} \), were determined from a reverse stepwise linear regression of a semilog transformation of the data, similar to the method of Rygiewicz et al. (21). Data points were sequentially included in the linear regression starting with points from the last sample until three successive points caused the value of \( r^2 \) to decline. Typically, at least eight points were included in the regression and the \( r^2 \) values were >0.95. This type of analysis yields results similar to those obtained via nonlinear regression routines, particularly when individual efflux components are characterized by rate constants which differ by at least an order of magnitude (21).

**RESULTS**

Cell Morphometry. X-ray microanalysis of bulk frozen hydrated samples offers excellent potential for in situ ion localization. However, in order to resolve accurately the ion content within an intracellular compartment, it is imperative that the generation of x-rays be restricted to a volume contained exclusively within that compartment. This volume is a function of the electron beam accelerating voltage, the average atomic number of the elements in the sample, and the atomic number of the element analyzed. At an accelerating voltage of 10 kV, the excitation volume in frozen hydrated tissue for Na\(^{+}\) and K\(^{+}\) (as well as other elements of similar atomic number) has been estimated to be 2 to 3 \( \mu m \) in diameter (18).

Cells adapted to 428 mm NaCl were approximately one-eighth the size of unadapted cells (Fig. 1; Table I) at the growth stage used for these experiments. The cytoplasm occupied 22 and 45% of the total volume of unadapted and adapted cells, respectively. The altered morphology of salt adapted cells facilitated the determination of ion compartmentation since the excited volume of the x-ray signal could be contained exclusively within the cytoplasm (Fig. 2, A–D).

**X-ray Microanalysis.** X-ray microanalysis was conducted with the electron beam in a static position (point analysis) in order to maximize resolution of cytoplasmic ion contents. Vascular and cytoplasmic spectra contained similar types and amounts of background (Bremsstrahlung) radiation and a predominant O peak (salt adapted cells contained approximately 85% H\(_2\)O). Spectra collected from random locations within a compartment of a given cell were very similar and comparisons made between vacuolar spectra collected by point analysis and scanning analysis of small areas did not reveal any appreciable differences (data not shown).

Unprocessed ion spectra collected from a NaCl adapted cell by point analysis illustrate the large differences in the vascular and cytoplasmic Na and Cl concentrations (Fig. 3, B and D). The ratios of Na and Cl in the vacuole to those in the cytoplasm (V/C) (calculated as the average ratio of the element P/B from spectra of the vacuole to the element P/B from spectra of the cytoplasm for each cell analyzed) was 8.2 and 6.5, respectively (Table II). Although these ratios actually reflect the elemental...
Table I. Morphometry of Unadapted Cells and Cells Adapted to 428 mM NaCl

Volume of the cytoplasm was calculated as the difference between the whole cell and vacuolar volumes.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>( V_{\text{total}} ) (( \mu m^3 \times 10^{-5} ))</th>
<th>( V_{\text{vac}} )</th>
<th>( V_{\text{cyt}} )</th>
<th>( \frac{V_{\text{vac}}}{V_{\text{total}}} ) ratio</th>
<th>( \frac{V_{\text{cyt}}}{V_{\text{total}}} ) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadapted(^a)</td>
<td>5.95 ± 0.71(^c)</td>
<td>4.77 ± 0.60</td>
<td>1.18 ± 0.12</td>
<td>0.78 ± 0.01</td>
<td>0.22 ± 0.01</td>
</tr>
<tr>
<td>Adapted(^b)</td>
<td>0.68 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>0.30 ± 0.01</td>
<td>0.55 ± 0.01</td>
<td>0.45 ± 0.01</td>
</tr>
</tbody>
</table>

\(^a\) n = 40. \(^b\) n = 120. \(^c\) ±SE.

Fig. 2. Scanning electron micrographs of tobacco cells adapted to 428 mM NaCl. Portions of the cytoplasm can be readily distinguished from the vacuole (A and C). B is an inset of A, showing an enlarged view of the cytoplasm. The area of the box superimposed over the cytoplasm (B) is approximately 42 \( \mu m^2 \). At an accelerating voltage of 10 kV the limits of resolution for x-ray microanalysis of Na\(^+\) and Cl\(^-\) should be 2 to 3 \( \mu m \); therefore, reliable measurements of ion contents within the cytoplasm should be possible without contaminating spill-over from the vacuole. Although unsuitable for x-ray microanalysis, deeply etched cells reveal more intracellular detail (D) and lend support to the identification of cytoplasmic and vacuolar compartments in unetched samples. The fracture plane across the cell in D was uneven, leaving a section of what appears to be tonoplast exposed in the lower right portion of the cell. Cytoplasm (C), vacuole (V), tonoplast (T).

Another notable difference indicated in the x-ray spectra of salt adapted cells was the higher levels of K and P observed in the cytoplasm as compared to the vacuole (Fig. 3, B and D). P was not detected in the vacuole of salt adapted cells, while a small P peak was routinely observed in the cytoplasm. Despite the fact that K concentrations were consistently higher in the cytoplasm than in the vacuole of salt adapted cells, the levels of K in the vacuole were near the limits of detection which resulted in low P/B and apparently contributed to a high degree of variability in the data for this element.

At the early stationary phase of the growth cycle, only a low

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Content on a per volume basis rather than a cell water basis, they provide an estimate of the elemental concentration difference between the two compartments (23).

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The inability to detect K in unadapted cells in the early stationary phase of growth (when whole cell concentrations of K were approximately 80 mM) suggests that under the operating conditions of these analyses the threshold of detection for K was above 80 mM. Since detection of K in the cytoplasm was possible with salt adapted cells (Fig. 3B), despite whole cell K concentrations of about 60 mM, it is likely that K is being accumulated in this compartment at concentrations beyond those which exist in either the cytoplasm or the vacuole of unadapted cells.

**22Na** and **36Cl** Efflux Kinetics. **22Na** and **36Cl** steady state efflux kinetics of unadapted cells (Fig. 4, A and B) and adapted cells (Fig. 4, C and D) could be resolved into isotherms which appeared to represent the loss of radiotrace from the exponential compartments arranged in series (15, 16, 25). We have associated this most rapidly exchanging component with the free space, intermediate component with the cytoplasm, and slowest component with the vacuole. The **Kcyt** and **t1/2cyt**, **Kvac** and **t1/2vac** (Table IV) differed by more than an order of magnitude and were similar to values commonly reported in the literature (15, 17, 25). Comparisons of plots of log **Q**, (tracer content) versus time (t) and log **dQ/dt** versus t confirmed that the observed kinetics of efflux of the second and third components were following first order approximations (25). Whole cell **Na** and **Cl** contents were constant prior to and following the efflux period indicating that the cells were in steady state with respect to these ions during the course of the experiment.

Exchange of both **22Na** and **36Cl** was much slower across the tonoplast for salt adapted cells than for unadapted cells (Table IV). Based on the calculations of ion contents of the vacuole and cytoplasm (**Qvac** and **Qcyt**, respectively, Table IV) and meas-

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**Table II. X-Ray Data from the Analysis of Cells Adapted to 428 mM NaCl**

Data for **P/B** and **V/C** are the average ± se from the analysis of 20 cells. Within each cell, at least 4 locations in each compartment were analyzed. **P/B** ratios were calculated as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Ion</th>
<th><strong>[P/B]_vac</strong></th>
<th><strong>[P/B]_cyt</strong></th>
<th><strong>V/C</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>2.18 ± 0.10</td>
<td>0.32 ± 0.03</td>
<td>8.16 ± 0.94</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>1.49 ± 0.09</td>
<td>0.28 ± 0.04</td>
<td>6.53 ± 0.81</td>
</tr>
</tbody>
</table>

\[ \frac{V}{C} = (\frac{[P/B]_{vac}}{[P/B]_{cyt}}) \]

percentage of unadapted cells possessed sufficient cytoplasmic area (3 \( \mu m \)) to permit x-ray microanalysis (Fig. 1). In these cells oxygen was the only element that was routinely detected in either the vacuole or the cytoplasm (Fig. 3, A and C), although a small K peak was observed occasionally. Other elements which were detectable in salt adapted cells, i.e., **Na**, **Cl**, and **P** were apparently distributed throughout the cells at concentrations below the threshold of detection.

The cytoplasmic and vacuolar Na\(^+\) and Cl\(^-\) concentrations were calculated on the basis of the **V/C** ratio of these two elements (Table II), the whole cell ion concentrations (Table III), and the relative volumes of the vacuolar and cytoplasmic compartments (Table I). In the adapted cells the cytoplasmic concentrations of both Na\(^+\) and Cl\(^-\) were 96 mM, while the vacuolar concentrations were calculated to be 780 and 624 mM, respectively (Table III).
Table III. Summary of Compartmentation Data from X-ray Microanalysis and Efflux Kinetic Analysis of Cells Adapted to 428 mM NaCl

The concentration of Na\(^+\) and Cl\(^-\) in the cytoplasm, \(C_{\text{cyt}}\), and the vacuole \(C_{\text{vac}}\) were calculated from the morphometry data of Table I \((V_{\text{vac}}/V_{\text{cyt}})\), the whole cell ion concentration, \(C_{\text{total}}\), and either the vacuole:cytoplasm ion concentration ratio, \(V/C\), (x-ray Table II) or the vacuole:cytoplasm ion content ratio, \(Q_{\text{vac}}/Q_{\text{cyt}}\), (efflux, Table IV).

<table>
<thead>
<tr>
<th>Ion</th>
<th>(V_{\text{vac}}/V_{\text{cyt}}) ratio</th>
<th>(C_{\text{total}}) mm</th>
<th>EDS X-ray Microanalysis</th>
<th>Efflux Kinetics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(V/C) mm</td>
<td>(C_{\text{vac}}) mm</td>
<td>(C_{\text{cyt}}) mm</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>55</td>
<td>472</td>
<td>8.2</td>
<td>780</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>55</td>
<td>386</td>
<td>6.5</td>
<td>624</td>
</tr>
</tbody>
</table>

Fig. 4. Efflux of \(^{22}\)Na\(^+\) (A, C) and \(^{36}\)Cl\(^-\) (B, D) from unadapted cells (A, B) and cells adapted to 428 mM NaCl (C, D). Inset shows the loss of radiolabel from the fast (free space) and medium (cytoplasmic) components after subtracting the slow component from the primary curve.

Table IV. \(^{22}\)Na\(^+\) and \(^{36}\)Cl\(^-\) Efflux Kinetics of Unadapted Cells and Cells Adapted to 428 mM NaCl

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Radiolabel</th>
<th>(k_{\text{cyt}}) ((\text{min} \times 10^5)^{-1})</th>
<th>(t_{1/2\text{cyt}}) (min)</th>
<th>(k_{\text{vac}}) ((\text{min} \times 10^5)^{-1})</th>
<th>(t_{1/2\text{vac}}) (min)</th>
<th>(Q_{\text{vac}}/Q_{\text{cyt}}) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadapted</td>
<td>(^{22})Na(^+)</td>
<td>7.6 ± 1.6</td>
<td>9.9 ± 2.7</td>
<td>3.8 ± 0.3</td>
<td>31.1 ± 3.0</td>
<td>20.9 ± 2.9</td>
</tr>
<tr>
<td>Adapted</td>
<td>(^{22})Na(^+)</td>
<td>10.5 ± 2.5</td>
<td>7.9 ± 1.0</td>
<td>1.7 ± 0.7</td>
<td>70.1 ± 2.8</td>
<td>18.4 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>(^{36})Cl(^-)</td>
<td>4.3 ± 0.5</td>
<td>17.1 ± 2.0</td>
<td>2.7 ± 0.3</td>
<td>46.9 ± 5.8</td>
<td>9.0 ± 0.1</td>
</tr>
</tbody>
</table>

\(n = 4\). \(^b\) \(n = 6\). \(^c\) ± SE.

Measurements of cytoplasmic and vacuolar volumes (Table I), the concentrations of Na\(^+\) and Cl\(^-\) in each compartment were determined (Table III) and the data confirmed that these ions are compartmentalized in the vacuole of the salt adapted cells. However, the results from steady state efflux kinetics indicated a lower cytoplasmic Na\(^+\) concentration (55 mM) than did the x-ray microprobe analysis (96 mM). This discrepancy could be due to a problem in the quantitation of the efflux parameters, especially since the determination of \(k_{\text{cyt}}\) and \(Y_{\text{cyt}}\) are inherently prone to a larger degree of error than the vacuolar parameters.
(7, 21). Also, it is possible that in some instances the excited volume for Na extended into the vacuole resulting in a larger x-ray signal and the perception of greater Na contents in the cytoplasm. Since the excited volume for Cl would be smaller than for Na (18), cytoplasmic Cl content should be resolved more accurately than the Na content. However, if this had been the case, it is likely that problems with excited volume overlap would have resulted in much greater variability from analysis to analysis than was observed. Regardless, the conclusion that these cells compartmentalize Na$^+$ and Cl$^-$ in the vacuole remains unaltered.

**DISCUSSION**

Na$^+$ and Cl$^-$ exclusion, particularly by cells of the growing regions of the shoot, has been implicated as a primary mechanism of salt tolerance in glycophytes (8, 14). Ion compartmentation is not generally considered to be a mechanism inherent to glycophytes; in fact, the inability to compartmentalize ions is often considered a fundamental basis for the salt sensitivity of glycophytes (9, 26). However, whether or not glycophytes compartmentalize ions as a mechanism of salt tolerance has not been resolved definitively. Despite the fact that glycophytes are not thought to utilize Na$^+$ and Cl$^-$ as principal osmotics, salt tolerant glycophytes accumulate substantial levels of ions even at moderate levels of salinity (12, 22, 27, 29). When different glycophyte genotypes are compared for salt tolerance (22, 29) the more tolerant genotypes generally accumulate less salt. This has been taken as evidence that glycophytes exclude Na$^+$ and Cl$^-$ as a tolerance mechanism. However, even the most tolerant glycophytes absorb substantial amounts of Na$^+$ and Cl$^-$ (amounts often considered toxic to cytoplasmic functions) over a limited exposure time and it may be more appropriate to consider these tolerant genotypes as slow absorbers rather than excluders. To survive indefinitely in the presence of high levels of NaCl, even slowly absorbing glycophyte genotypes would eventually have to deal with substantial amount of absorbed Na$^+$ and Cl$^-$. Therefore, mechanisms to deal with absorbed Na$^+$ and Cl$^-$ should exist within glycophyte species. In fact, since it appears that glycophyte genotypes differ in NaCl-tolerant genotypes have not been exposed experimentally to NaCl for long time periods (several sexual or vegetative reproductive cycles), whether or not tolerant genotypes eventually accumulate as much Na$^+$ and Cl$^-$ as less tolerant genotypes remains unknown.

Tobacco cells which are adapted to 428 mm NaCl accumulate 489 and 396 mm Na$^+$ and Cl$^-$, respectively. Results from x-ray microprobe and steady state efflux kinetic analyses (Table III) have demonstrated that Na$^+$ and Cl$^-$ are compartmentalized in the vacuole of adapted cells. Cytoplasmic levels of both ions are kept below 100 mm, while vacuolar concentrations are in excess of 700 mm for Na$^+$ and 600 mm, for Cl$^-$. These results not only indicate that ion compartmentation is an essential mechanism for salt tolerance (26), but also support the concept that intracellular compartmentation is a mechanism which is inducible in glycophytes and is not restricted solely to salt tolerant species endemic to saline environments.

It appears from our work (5, 6) that a reduced growth rate is a primary response of glycophytes to salt exposure. Coincident with the reduced rate of cell expansion is also a reduced rate of vascular expansion (6; Table I). Since Na$^+$ and Cl$^-$ are accumulated in the vacuole, the reduced expansion rate would force the maintenance of higher ion gradients across the tonoplast. This would require increased control of ion compartmentation to prevent accumulation of Na$^+$ and Cl$^-$ in the cytoplasm and avoid toxicity. We have suggested that growth inhibition in response to NaCl may result from the inability of glycophytes to respond differently to salt and desiccation (1).

By application of the Ussing-Teorell flux ratio equation (15, 17), it can be demonstrated that Na$^+$ flux is active from the cytoplasm across the plasma membrane to the outside (J$_{Na}$) and across the tonoplast to the vacuole (J$_v$) in salt adapted cells. If an ion is distributed passively across a membrane, then the predicted flux ratio, a$^{Na}$/a$^v$, should be equivalent to the experimental flux ratio (15, 17): a$^{Na}$/a$^v$ = C$^v$/C$_{cyt}$ exp [zFERT)] = J$_{Na}$/J$_v$ If J$_{Na}$/J$_v$ is greater or less than a$^{Na}$/a$^v$, then transport is considered active inward or outward, respectively (15). Based on the constant whole cell Na$^+$ and Cl$^-$ concentrations during the course of the efflux experiments, the cells were assumed to be in steady state and under these conditions the experimental flux ratio is equal to one. For Na$^+$, a$^{Na}$/a$^v$ across the plasma membrane (outside to the cytoplasm) was 46 when a membrane potential difference of -60 mV was assumed and was 0.12 across the tonoplast (cytoplasm to the vacuole) if the tonoplast membrane potential difference was assumed to be zero. Varying the value assumed for the electrical potential difference across the plasma membrane from -60 to -120 mV, and across the tonoplast from 0 to +15 mV (ranges of membrane potential differences commonly reported; 15, 17) would not alter the predicted direction of transport since such changes would only steepen the electrochemical gradients. Only if the membrane potential difference was +38 mV across the plasma membrane or -53 mV across the tonoplast would a$^{Na}$/a$^v$ = 1. Similar calculations using the subcellular concentrations of Cl$^-$ yielded an a$^{Na}$/a$^v$ of 0.40 for the plasma membrane (outside to the cytoplasm) and 0.30 for the tonoplast (cytoplasm to the vacuole) using membrane potential differences of -60 and 0 mV, respectively, which suggests that Cl$^-$ is transported against the electrochemical gradient into the cytoplasm and vacuole. With Cl$^-$, a$^{Na}$/a$^v$ = 1 for the plasma membrane and the tonoplast if the membrane potential differences were -38 and +47 mV, respectively.

It has been suggested that glycophytes may not be as capable of maintaining ionic gradients (26) as halophytes and this inability may account for their susceptibility to salinity. However, the ability of salt adapted tobacco cells to maintain low cytoplasmic levels of Na$^+$ and Cl$^-$ despite steep concentration gradients across the plasma membrane and tonoplast is indicative that membrane properties required to achieve and maintain such gradients are inherent to glycophytes. The rate at which glycophytes can establish the steep gradients which are necessary, especially after the vascular expansion rate is reduced, compared to halophytes, may explain substantial differences in salt tolerance between glycophytes and halophytes. The ability to establish and maintain ion gradients after adaptation could be facilitated by membrane transport adaptations which result in increased proton motive force generating capacity across the plasma membrane and the tonoplast (19, 20, 24) which facilitates the transport of ions. The activity of the tonoplast Na$^+$/H$^+$ antiport of red beet cells is induced by NaCl (4). A similar antiport driven by proton motive force likely exists on the plasma membrane and would function to evacuate Na$^+$ from the cytoplasm to the external environment.

Reduced passive permeability of the plasma membrane and the tonoplast to Na$^+$ and Cl$^-$ could facilitate the maintenance of high ion gradients. The increased t$_{1/2}$ vac observed for $^{22}$Na$^+$ and $^{36}$Cl$^-$ in salt adapted cells (Table IV) may be indicative of such a change in the tonoplast as a result of adaptation to salinity. Further examination of the ion transport and membrane properties of these salt adapted cells as well as the mechanisms of growth reduction will be necessary to evaluate the role of these processes in Na$^+$ and Cl$^-$ compartmentation and salt tolerance.

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