Single-Cell Measurements of the Contributions of Cytosolic Na\textsuperscript{+} and K\textsuperscript{+} to Salt Tolerance\textsuperscript{1}

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Ion concentrations in the roots of two barley (\textit{Hordeum vulgare}) varieties that differed in NaCl tolerance were compared after exposure to NaCl. Triple-barreled H\textsuperscript{+}, K\textsuperscript{+}, and Na\textsuperscript{+}-selective microelectrodes were used to measure cytosolic activities of the three ions after 5 and 8 d of NaCl stress. In both varieties of barley, it was only possible to record successfully from root cortical cells because the epidermal cells appeared to be damaged. The data show that from the 1st d of full NaCl stress, there were differences in the way in which the two varieties responded. At 5 d, the tolerant variety maintained a 10-fold lower cytosolic Na\textsuperscript{+} than the more sensitive variety, although by 8 d the two varieties were not significantly different. At this time, the more tolerant variety was better at maintaining root cytosolic K\textsuperscript{+} in the high-NaCl background than was the more sensitive variety. In contrast to earlier work on K\textsuperscript{+}-starved barley (Walker et al., 1996), there was no acidification of the cytosol associated with the decreased cytosolic K\textsuperscript{+} activity during NaCl stress. These single-cell measurements of cytosolic and vacuolar ion activities allow calculation of thermodynamic gradients that can be used to reveal (or predict) the type of active transporters at both the plasma membrane and tonoplast.

In plant cells, maintaining cytosolic K\textsuperscript{+} in an environment with a high Na\textsuperscript{+} concentration is a key factor in determining the ability to tolerate salinity (Maathuis and Amtmann, 1999). In the cytosol, K\textsuperscript{+} is an essential activator for some enzymes and Na\textsuperscript{+} rarely substitutes for this biochemical function (Wyn Jones and Pollard, 1983; Flowers and Dalmond, 1992). Na\textsuperscript{+} can compete directly for K\textsuperscript{+}-binding sites on enzymes, suggesting that the cytosolic K\textsuperscript{+} to Na\textsuperscript{+} ratio, rather than the absolute Na\textsuperscript{+} concentration, is critical for tolerance. Although the relationship between the cytosolic concentrations of Na\textsuperscript{+} and K\textsuperscript{+} is of fundamental importance in understanding the response of a plant to salinity, it is difficult to obtain direct measurements of the cytosolic concentrations of these two ions in plant cells.

Most crop plants are NaCl sensitive, although cereals show a range of tolerance, with barley (\textit{Hordeum vulgare}) considered more tolerant than wheat (\textit{Triticum aestivum}) or rice (\textit{Oryza sativa}; Downton, 1984). Some barley varieties can complete their life cycle growing in 125 mM NaCl, even sustaining a 50% loss in biomass (Greenway, 1962). In a recent survey of NaCl sensitivity among barley genotypes, two varieties were identified that are representative examples from either end of the tolerance range of the species: the sensitive Triumph and the tolerant Gerbel (Flowers and Hajibagheri, 2001). The more sensitive variety accumulated more Na\textsuperscript{+} in the shoot than the tolerant variety and the authors suggested that this might reflect a more sensitive cultivar, having a higher concentration of Na\textsuperscript{+} in its cytoplasm than a more resistant variety. However, for roots growing for 15 d in 200 mM NaCl, the mean cytoplasmic Na\textsuperscript{+} concentration, estimated by x-ray microanalysis, was almost 1.4 times greater in Triumph than that in Gerbel although the differences were not significantly different (Flowers and Hajibagheri, 2001). Estimating ion activities from x-ray microanalyses requires a number of assumptions, including the approximation of cytosolic water content. The use of ion-selective microelectrodes precludes the need for such assumptions. Ion-selective microelectrode measurements in barley root cells have shown that the homeostasis of cytosolic K\textsuperscript{+} breaks down during K\textsuperscript{+} deficiency and this change is associated with an acidification of the cytosol (Walker et al., 1996, 1998).

Here, we report using ion-selective microelectrode measurements to compare Na\textsuperscript{+} and K\textsuperscript{+} homeostasis in root cells of Triumph and Gerbel during NaCl stress.

Recently, a new type of Na\textsuperscript{+}-selective microelectrode was reported (Carden et al., 2001) and we have used this tool to investigate the hypothesis that toxicity occurs as Na\textsuperscript{+} replaces K\textsuperscript{+} in the cytosol, and
that there may be similarities to cellular responses to 
K$^+$ deficiency. This new information has also enabled 
the thermodynamic feasibility of various cellular 
transport mechanisms for Na$^+$ and K$^+$ to be cal-
culated. These cell measurements show how this 
method has broader application for the analysis of 
plants, including Arabidopsis mutants with altered 
expression or regulation of transporters.

RESULTS

Triple-barreled microelectrode measurements of 
root cortical cells were made in young barley seed-
lings treated with 200 mM NaCl. For both varieties of 
barley, growth in 200 mM NaCl and 0.1 mM K$^+$ 
eventually ceased, showing that NaCl tolerance un-
der these conditions was a matter of differing sur-
vival times. During the first 28 d of NaCl treatment 
(over the first 4 d of which the concentration was 
increased by 50 mM per day), both varieties contin-
tued to grow, the biomass of both roots and shoots 
increased if measured as fresh or dry weight (data 
not shown). All the seedlings of Triumph were, how-
ever, dead after 44 d in NaCl, whereas 80% of the 
variety Gerbel had survived, although by 64 d all the 
plants were dead.

Cortical Cell Electrode Measurements of pH, Membrane 
Potential Difference ($E_m$), Na$^+$ Activity ($a_{Na}$), and K$^+$ 
Activity ($a_{K}$)

The Na$^+$ and K$^+$ measurements were separated 
to two populations by assigning each value to ei-
ther the cytosol (Fig. 1) or the vacuole (Fig. 2) using 
measured pH values (Walker et al., 1995). Two nor-
mal distributions having means of pH 5.6 and 7.4 (see Table I) described the combined pH 
measurements obtained using both Na$^{+}$- and K$^{+}$-
selective microelectrodes. Figures 1 and 2 show the 
mean intracellular pH, $E_m$, $a_{Na}$, and $a_{K}$ measured in the cytosol. Each value was obtained from between four and 28 samples.

Figure 1. Histograms showing the results of triple-barreled Na$^+$ and 
K$^+$ measurements in the cytosol of Triumph (white) and Gerbel 
(shaded) cortical root cells of seedlings treated with NaCl for 5 and 
8 d. The values are means ± se of $E_m$, $a_{Na}$, $a_{K}$, and pH and each value 
was obtained from between five and 28 samples.

Figure 2. Histograms showing the results of triple-barreled Na$^+$ and 
K$^+$ measurements in the vacuoles of Triumph (white) and Gerbel 
(shaded) cortical root cells of seedlings treated with NaCl for 5 and 
8 d. From the top to the bottom, Mean ± se values of $E_m$, $a_{Na}$, $a_{K}$, and 
pH measured in the cytosol. Each value was obtained from between 
four and 28 samples.
19.5 mm, but these varietal differences had gone by 8 d (3 d at 200 mm NaCl; Fig. 1). At 5 d, cytosolic aK was very similar in Triumph and Gerbel, with both varieties having values of about 60 mm, but by 8 d, only in Triumph, the NaCl-sensitive variety, was there a significant decrease to 39 mm (Fig. 1). The cytosolic pH was not significantly different between varieties on either day.

Figure 3 shows a summary of the triple-barreled microelectrode data for the cortical cell compartments. At 5 d, both varieties had higher aNa in the vacuole than in the cytosol, and this difference was greater in the tolerant Gerbel than in Triumph (Fig. 3). By 8 d, these varietal differences had gone and in the tolerant Gerbel the vacuolar aNa had decreased when compared with 5 d (Fig. 2), but these changes were not significant (Fig. 3; ANOVA P < 0.05). The measurements of vacuolar aK showed similar values for both varieties around 18 mm at 5 d, although in Gerbel vacuolar K accumulation had increased by 8 d; this change was not statistically significant (Fig. 3; ANOVA P = 0.21). For both varieties, aK was larger in the cytosolic than in vacuolar compartment (Fig. 3).

After 5 d growing in NaCl, the E m values reported from both intracellular compartments differed between the varieties with Triumph having more negative values (comparing Figs. 1 and 2). By 8 d, both varieties showed significantly more negative vacuolar E m values than at 5 d (Fig. 3), but at 8 d the only varietal differences were in cytosolic E m, which was more negative for Triumph (−94 ± 6 mV) compared with Gerbel (−72 ± 5 mV). After 8 d of salinization, the trans-tonoplast potential was −9 mV for Triumph and +9 mV for Gerbel (using the convention of Bertl et al., 1992). The microelectrode measurements of aK, aNa, and E m (Figs. 1 and 2) were used to investigate the energetic feasibility of likely transport mechanisms for Na + and K + across the plasma membrane and tonoplast.

**Thermodynamics of Na + Transport across the Plasma Membrane and Tonoplast**

The Na + electrochemical potential differences (ΔµNa) across both the plasma membrane and the tonoplast were calculated to determine whether active or passive transport was required to maintain these gradients (Table II). Extracellular aNa was measured by the Na + microelectrodes at 150 mm, both in the nutrient solution containing 200 mm NaCl and in the apoplast between epidermal and cortical cells (data not shown). This value of aNa is similar to the calculated value of 142 mm for the nutrient solution obtained using an activity coefficient of 0.71, determined using the Debye-Hückel equation (Robinson and Stokes, 1970). The values in Table II show that for

*Table I. Statistical separation of the pH measurements in barley root cortical cells a,b,c,d*

<table>
<thead>
<tr>
<th>Distribution Pattern</th>
<th>Mean pH: Population 1</th>
<th>Mean pH: Population 2</th>
<th>χ²</th>
<th>d.f.</th>
<th>P</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Single normal</td>
<td>6.9 ± 0.1</td>
<td>−</td>
<td>60.3</td>
<td>16</td>
<td>1.00</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Double normal, equal variance</td>
<td>5.6 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>13.5</td>
<td>14</td>
<td>0.27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Double normal, unequal variance</td>
<td>5.6 ± 0.1</td>
<td>7.4 ± 0.1</td>
<td>13.3</td>
<td>13</td>
<td>0.27</td>
<td>&lt;0.05</td>
</tr>
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</table>

*aUsing the data pooled from both varieties on both days (n = 137–140). **Using three different biologically relevant distribution patterns, the success of distinguishing the subcellular compartment was assessed. The goodness of fit of each distribution was tested for by a chi-square analysis (χ²) at 95% limit of confidence with associated degrees of freedom (d.f.). The proportion of values (P) falling into the first population was also estimated. *Means are presented with se. **The confidence of a good fit of the data to the distribution is also expressed (P).
the cytosol, regardless of variety or time in NaCl, there was a large inwardly directed driving force for Na\(^+\) across the plasma membrane of between \(-113\) and \(-182\) mV. The vacuolar accumulation of Na\(^+\) required active transport at the tonoplast, for Gerbel on 5 d, but this requirement had gone by 8 d. These results indicate that energy is needed to maintain this ion gradient, with efflux mechanisms removing Na\(^+\) from the cytosol into either the extracellular solution or the vacuole, or both. The feasibility of several active Na\(^+\) transport mechanisms was assessed by calculating the associated free energy change (\(\Delta G' / F\)) for each mechanism (Table III). In most of the conditions for the intracellular electrode measurements, an Na\(^+\)/H\(^+\) antiport operating at the plasma membrane, transporting Na\(^+\) out of the cell, would be energetically feasible for maintaining the measured ion gradients. An exception is Gerbel on 5 d; for these conditions, the \(\Delta G' / F\) at 5 d is +27 mV, showing that operating alone an antiport mechanism is not feasible for the measured ion gradients. The energy provided by the hydrolysis of ATP for Na\(^+\) efflux via either an Na\(^+\) or Na\(^+\)/K\(^+\) ATPase was also assessed, despite a lack of evidence for such a mechanism in higher plant cells. This calculation reveals the efflux of Na\(^+\) across the plasma membrane via an ATPase to be energetically feasible (Table III).

In most cases for both varieties, a tonoplast H\(^+\)/Na\(^+\) antiport was energetically feasible to explain the measured ionic gradients; the one exception was for Gerbel on d 5 (Table III).

### Thermodynamics of K\(^+\) Transport across the Plasma Membrane and Tonoplast

Using the data for intracellular compartmentation of K\(^+\) (Figs. 1 and 2), the feasibility of K\(^+\) cotransport mechanisms was also assessed. Under these experimental conditions, K\(^+\) uptake into the cytoplasm across the plasma membrane cannot occur by a channel mechanism. The positive electrochemical potential values for K\(^+\), with \(\Delta \mu_{K}/F\) between +68 and +106 mV, show that active transport was required for K\(^+\) uptake from the external solution into the cytosol across the plasma membrane (Table II). Transport of K\(^+\) across the tonoplast into the cytosol would also require energy input with the \(\Delta \mu_{K}/F\) of +17 to +22 mV (Table II). Testing the thermodynamics of K\(^+\) uptake at the plasma membrane by both Na\(^+\)- and H\(^+\)-coupled symport mechanisms showed that both are feasible for maintaining the measured cytosolic a\(_K\) in both varieties at both 5 and 8 d (data not shown).

## DISCUSSION

One unavoidable consequence for plants growing in high concentrations of NaCl is that some of these
ions will enter root cells and the ability of plants to survive depends on the balance between the entry and efflux from the cytoplasm. In this paper, we report directly measured root cellular activities of Na\(^+\), K\(^+\) and H\(^+\) under extreme conditions of NaCl stress. These measurements have then been used to compare the possible mechanisms that the cell may have for removing Na\(^+\) from the cytoplasm.

**Varietal Differences in Cellular Cation Activities**

The microelectrode measurements of \(a_{Na}\) and \(a_K\) in the root cortex show that there are large cellular differences between the varieties after just 5 d of treatment with NaCl (summarized in Fig. 3). After 5 d, the tolerant variety Gerbel seemed to be more effective at cytosolic Na\(^+\) exclusion and vacuolar sequestration than the more sensitive variety Triumph (Fig. 3), despite the large cytosol-directed electro-chemical potentials for Na\(^+\) (Table II). However, statistical comparison of the mean values of \(a_{Na}\) for 5 d for the two varieties were not significantly different (ANOVA \(P = 0.08\)). After 8 d, Gerbel was also better at maintaining cytosolic \(a_K\) in the high background of 200 mM NaCl (ANOVA \(P < 0.05\)). Expressing the cytosolic K\(^+\):Na\(^+\) ratios, 34.7 for Gerbel and 3.2 for Triumph, illustrates clearly the large differences in the response of the two varieties after 5 d in NaCl. However, after 8 d of growing in 200 mM NaCl, the cytosolic K\(^+\):Na\(^+\) ratios of the two varieties were very similar (Gerbel, 2.1; and Triumph, 2.9). These changes in cytosolic cation activities agree with the whole-plant responses of the two varieties showing that NaCl tolerance under these conditions was a matter of differing survival times.

**K Deficiency, Compartmental pH, and NaCl Stress**

Undoubtedly, the application of NaCl to plants alters the intracellular pools of K\(^+\), but is this cellular response similar to that brought about by a lack of K\(^+\) in the nutrient solution? Subcellular compartmentation of K\(^+\) in barley is known to change in response to changes in external K supply (Memon et al., 1985). Under K\(^+\)-replete conditions, there is cytosolic \(a_K\) homeostasis at around 70 mM, whereas vacuolar \(a_K\) changes in response to changes in external K\(^+\) (Walker et al., 1996). Vacuolar activities decreased from 100 mM in replete plants to less than 25 mM for deficient plants, when the external K\(^+\) supply was decreased (Walker et al., 1996, 1998). During extreme K\(^+\) starvation, barley root epidermal cells showed a decrease in cytosolic \(a_K\) to 40 mM accompanied by an acidification of the cytosol to pH 6.7 (Walker et al., 1996). In the present study, treatment of plants with 200 mM NaCl, combined with low external K\(^+\) of 0.1 mM, produced cortical cell cytosolic \(a_K\) as low as 39 mM in the variety Triumph by 8 d (Figs. 1 and 3). At this cytosolic \(a_K\), decreases in the rates of K-dependent biochemical processes and, hence, growth would result (Leigh and Wyn Jones, 1984; Walker et al., 1998). Cytoplasmic K\(^+\) concentrations (rather than activities) of 60 to 80 mM, for plants growing at the same sodium concentration but 60 times the potassium concentration for 15 d and calculated from x-ray microanalyses (Flowers and Hajibagheri, 2001), were remarkably similar to our microelectrode measurements.

The pH electrode measurements of both K\(^+\) and Na\(^+\) triple-barreled electrodes separated into two distinct populations, and the more alkaline population of measurements was assumed to be from the cytosol. These values for the cytosolic pH are very similar to those measured previously for unstressed barley roots using microelectrodes (7.3 ± 0.1; Walker et al., 1996, 1998). In the salinized cells described here, there was no evidence for an associated acidification with depletion of cytosolic \(a_K\) (Figs. 1 and 3). This result suggests that within cells, the depletion of cytosolic \(a_K\) resulting from NaCl treatment is physiologically different from that associated with K starvation, although another possible explanation is that epidermal and cortical cells respond differently to depletion of cytosolic \(a_K\). In support of this idea, we know that the expression pattern of a high-K\(^+\)/low-Na\(^+\) affinity transporter (HKT1) in wheat is stronger in root cortical cells (Schachtman and Schroeder, 1994) and during K\(^+\) withdrawal (Wang et al., 1998).

The pH values for both the cytosol and the vacuole broadly agree with values published for barley roots obtained using \(^{31}\)P-NMR (Martinez and Läuchli, 1993; Katsuahara et al., 1997). These NMR measurements for unsalinized barley roots gave mean pH values of 7.5 to 7.7 and 5.5 to 5.7 in the cytosol and vacuole, respectively. In both reports, the addition of NaCl resulted in an alkalization of the vacuole (to over pH 6), but almost no change in the pH of the cytosol (Martinez and Läuchli, 1993; Katsuahara et al., 1997). Although these values obtained using NMR are similar to the results reported here, where the mean cytosolic pH was 7.5 for both varieties, there was no significant alkalization of the vacuolar pH from 5 to 8 d (Fig. 2). In wheat, NaCl stimulation of proton pumping at the plasma membrane has been measured (Ayala et al., 1997), and this is consistent with the measured more negative membrane potential in Triumph compared with Gerbel at 8 d (Figs. 1 and 3), possibly indicating a greater level of NaCl stress in this variety.

**Transport Mechanisms That Are Important in NaCl Tolerance**

Na\(^+\) might enter cells through nonselective cation channels (Schachtman et al., 1991; Davenport and Tester, 2000; Maathuis and Sanders, 2001), and HAK1- (Santa-Maria et al., 1997) and HKT1-type transporters (Rubio et al., 1995; Rus et al., 2001). The ability of the plant cell to prevent, at least in the short
brane potential was also measured in root cells of the plant. Katsuhara et al., 1997). An increased activity of the plasma membrane H/K+ ATPase is implicated in the NaCl tolerance of marine algae (Shono et al., 2001), and although energetically feasible (see Table III), a similar direct role for a primary pump removing Na+ from the cytosol in higher plants has yet to be shown. The role and molecular identities of H+/Na+ exchangers, at both the plasma membrane (Qiu et al., 2002) and tonoplast (Apse et al., 1999; Zhang and Blumwald, 2001), in the NaCl tolerance of higher plants is now well established. For most of the conditions tested in this paper, a plasma membrane H+/Na+ antiporter with a stoichiometry of 1:1 could operate to achieve the measured ion gradients; only for Gerbel on d 5 was this mechanism not energetically feasible (Table III). This result for Gerbel at 5 d may indicate that the antiporter has a variable H+/Na+ stoichiometry ratio enabling the maintenance of such a low cytosolic aNa+. The activity of a plasma membrane H+/Na+ antiporter should lead to an acidification of the cytosol between 5 and 8 d, but no such change was measured (Figs. 1 and 3), a result that agrees with measurements obtained using 31P-NMR (Martinez and Läuchli, 1993; Katsuhara et al., 1997). An increased activity of the plasma membrane H+ pump may function to regulate cytosolic pH, compensating for any acidification caused by an antiporter removing Na+ from the cytosol. Our microelectrode measurements show that there was a hyperpolarization of the plasma membrane potential in Triumph root cells between 5 and 8 d, becoming more negative by between 10 and 20 mV (Figs. 1 and 3). However, this change in membrane potential was not statistically significant (ANOVA P = 0.2), but a similar change in the membrane potential was also measured in root cells of Triumph treated directly with 200 mm NaCl (Carden et al., 2001).

At the tonoplast, there was also a cytosol-directed ΔµNa+/F indicating that an active transport process was operating on the tonoplast transporting Na+ into the vacuole (Table II). Na+/H+ antiports have been found on the tonoplast of barley, with energy provided by the proton pumps (Garbarino and DuPont, 1989; Martinez and Läuchli, 1993). Moreover, the tonoplast ATPase of barley is stimulated by the presence of NaCl (Garbarino and DuPont, 1988; Matsumoto and Chung, 1988). The operation of a H+/Na+ antiport might be expected to result in an alkalization of the vacuole, but this was not observed between 5 and 8 d (Fig. 2). NMR measurements showed barley vacuolar alkalization upon exposure to NaCl and these measurements used excised root tips measured for up to 6 h after NaCl treatment (Martinez and Läuchli, 1993; Katsuhara et al., 1997). The microelectrode measurements show significant vacuole alkalization when the values in Figures 2 and 3 are compared with values for root cells not treated with NaCl (pH 5.1–5.3; Walker et al., 1995, 1996). Therefore, there is evidence to support a tonoplast-located Na+/H+ antiport as the main mechanism for vacuolar sequestration of Na+. The microelectrode measurements show that in the longer term response to NaCl stress, the increased activity of tonoplast H+ pumps can maintain vacuolar pH. This result agrees with the observation that transgenic plants overexpressing a vacuolar H+/Na+ antiporter have improved NaCl tolerance, accumulating NaCl in the vacuole but also able to maintain normal pH regulation within intracellular compartments (Apse et al., 1999; Zhang and Blumwald, 2001). In addition, the removal of protons from the cytosol by the tonoplast pumps would offset an acidification of the cytosol by the actions of a plasma membrane Na+/H+ antiporter. This may also explain the lack of cytosolic acidification observed between 5 and 8 d as the cytosolic aK is depleted and that occurs during K+ deficiency (Walker et al., 1996, 1998).

Clearly salinity tolerance depends on sustaining the cytosolic environment, limiting Na+ accumulation and maintaining K+ concentration. Microelectrode measurements of root cell responses to salinity suggest that for the first 5 d, the tolerant variety Gerbel is better able to exclude Na+ and then by 8 d better at maintaining K+ when compared with Triumph (see Fig. 3). In root cells, these varietal differences only seem to occur early on in the salinity response; by 15 d, no significant differences were measured (Flowers and Hajibagheri, 2001). The differences between Gerbel and Triumph at 5 d cannot be explained by the activity of a plasma membrane H+/Na+ antiporter because this mechanism is not energetically feasible to explain the measured electrochemical gradients in the tolerant plant. Possible differences in the Na+ exclusion mechanisms for uptake...
may be important for explaining these early differences between the two varieties. Both this result and the differences after 8 d between Gerbel and Triumph in the ability to maintain cytosolic K⁺ could be explained by subtle changes in the properties of either a root HKT1-type transporter (Rubio et al., 1995; Rus et al., 2001) or HAK1-type transporter (Santa-Maria et al., 1997).

The microelectrode data have provided direct intracellular measurements of three cations during the early onset of NaCl stress well before any visual stress symptoms have appeared in the plants. This nondestructive technique can be used for the analysis of transport mechanisms in single cells of mutant plants with altered expression of transporters; for example, Arabidopsis mutants with altered expression and activity of H⁺/Na⁺ exchangers (Qiu et al., 2002). The subsequent data analysis can show the feasibility of transport mechanisms at both the plasma membrane and tonoplast in single cells.

**MATERIALS AND METHODS**

**Plant Material**

Barley seeds (Hordeum vulgare L. cv Triumph and cv Gerbel) were germinated in darkness for 3 d on filter paper moistened with 0.2 mM CaSO₄. Five seedlings of each variety were transferred to 1.5-L pots containing a solution containing the following nutrients: Ca(NO₃)₂ (5 mM), KH₂PO₄ (0.1 mM), MgSO₄ (2 mM), plus micronutrients (Hoagland and Arnon, 1938), and a buffer, MES/Tris (5 mM), pH 6.0. The final K⁺ concentration in the solution was only 0.1 mM compared with the 6 mM used in the experiments conducted by Flowers and Hajibagheri (2001). The seedlings were grown in a cabinet at 20°C (day/night), 16 h d⁻¹ photon flux density of 300 µmol m⁻² s⁻¹, and 75% relative humidity. The nutrient solution was vigorously aerated with compressed air and was replaced every 2 d. After 4 d, the NaCl concentration in the solution was increased in steps of 50 mM to a final value of 200 mM NaCl. The microelectrode measurements were made on seedlings that had been growing for either 5 or 8 d in NaCl (1 or 4 d, respectively, in 200 mM NaCl). During this 8 d in NaCl, no significant differences in growth, measured as root fresh or dry weight, were found between the two varieties (data not shown).

**Electrode Manufacture and Calibration**

Triple-barreled microelectrodes were prepared as described previously (Walker et al., 1995) and filled using pH (Miller and Smith, 1992), K⁺ (Walker et al., 1995), and Na⁺ (Carden et al., 2001) sensor cocktails. The ion-selective barrels were backfilled from the blunt end with the sensors using a 29-gauge metal needle and a 1-mL glass syringe, leaving 48 to 72 h between backfilling neighboring barrels.

All ion-selective barrels were calibrated before and after an intracellular impalement. The pH-selective barrel was calibrated using 1 mM BisTris/ MES buffers in the range 5.0 to 8.0. These calibration solutions also contained 50 mM NaCl to maintain the function of the Na⁺-selective barrel as the performance of an ion-selective microelectrode deteriorates when not immersed in the primary sensing ion (Miller, 1995). No interference to the response of the pH-selective barrel was detected over a range of 0.1 to 100 mM NaCl (data not shown). The Na⁺-selective barrel was calibrated as described previously (Carden et al., 2001) and all calibration lines were obtained by fitting a Nicolsky-Eisenman equation to the calibration data.

**Microelectrode Measurements**

For intracellular measurements, intact barley seedlings were mounted on a microscope stage with the root fixed to the base of a perfusion chamber using small Plexiglas blocks and grease (Walker et al., 1995). The root was perfused with nutrient solution containing 200 mM NaCl. Microelectrode recordings were made using a high-impedance differential electrometer and recorded as described previously (Walker et al., 1995). Measurements were made on root epidermal and cortical cells, 10 to 20 µm from the root tip. The initial impalement of an epidermal cell could be confirmed visually, after which it was not possible to see the location of the tip. Electrode impalements in epidermal cells reported poor membrane potentials, suggesting that these cells were damaged by the NaCl treatment (Carden et al., 2001).

**Electrochemical Gradients and the Energetic Feasibility of Transport Mechanisms**

The electrochemical difference for Na⁺ (ΔGNa) across a membrane was calculated by subtracting the calculated Nerst potential for specified measured conditions from the Eᵢ measured in identical conditions (Table II). A negative result indicates that ion uptake was passive, whereas a positive result indicates that active uptake was required. The energetic feasibility of a particular transport mechanism for an ion moving down its electrical gradient was assessed by calculating the ΔG/F given by the general equation in which the free energy is in terms of the electrochemical potential of the ion (mV). The individual equations used for these calculations are shown below for Table III.

For the plasma membrane H⁺/Na⁺ antiport, the equation ΔG/F = −92.4 log((caH)/[Na⁺][aNa],out)/(caNa⁺)[aH] in with a 1:1 stoichiometry was used. A similar equation for the tonoplast-located antiport was used. For a plasma membrane–located Na⁺/ATPase, the equation ΔG/F = −(Eᵢ − 92.4 log(([(ATP)][aNa]₁/[(ADP][Pi][aNa]₀)) + ΔG_ATP/F was used. For a plasma membrane–located Na⁺/K⁺-ATPase mechanism, transporting Na⁺ from, and K⁺ into, the cytosol across the plasma membrane, the equation ΔG/F = −92.4 log(([(ATP)][aNa]₁/[aK]₀)/[(ADP][Pi][aNa]₀[caK]₀) + ΔG_ATP/F was used. The energetic calculations requiring ATP hydrolysis are based on a Na⁺K stoichiometry of 1:1 and using ΔG_ATP/F = −283 mV (Rosing and Slater, 1972), 0.4 mM ATP, 150 µM ADP, and 2.5 mM Pi free concentrations (Roberts et al., 1985).

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**LITERATURE CITED**


Cellular Measurements of Salt Tolerance


