Aspects of Salt Tolerance in a NaCl-Selected Stable Cell Line of Citrus sinensis

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ABSTRACT

A NaCl-tolerant cell line which was selected from ovular callus of ‘Shamouti’ orange (Citrus sinensis L. Osbeck) proved to be a true cell line variant. This conclusion is based on the following observations. (a) Cells which have been removed from the selection pressure for at least four passages retain the same NaCl tolerance as do cells which are kept constantly on 0.2 molar NaCl. (b) Na⁺ and Cl⁻ uptake are considerably lower in salt-tolerant cells (R-10) than in salt-sensitive cells (L-5) at a given external NaCl concentration. (c) Growth of salt-tolerant cells is markedly suppressed upon replacement of NaCl by KCl, whereas the growth of salt-sensitive cells is only slightly affected. Accumulation of K⁺ and Cl⁻ accompanies the inhibition of growth. Experiments carried out with sodium and potassium sulfate suggest that the toxic effect is due to the accumulated Cl⁻. (d) Removal of Ca²⁺ from the growth medium severely inhibits the growth of salt-tolerant cells in the presence of NaCl, whereas it has a minor effect on growth of salt-sensitive cells in the presence of NaCl. (e) Electron micrographs show that the salt-tolerant cells have very big vacuoles when exposed to salt, while the size of the vacuoles of the salt-sensitive cells does not change.

Variant cell lines have been selected from a population of cultured somatic cells of higher plants following imposition of various stresses (2, 3, 25, 28, 30). Selection has commonly been practiced following exposure to salt, drought, cold, and herbicides. Of these agriculturally useful traits, resistance or tolerance to salt is becoming increasingly important among crops of agricultural value grown in arid zones. Selection of cell lines which exhibit tolerance to salt stress has been reported before (6, 7, 11, 21) but only recently Naboras et al. (22) were able to regenerate salt-tolerant plants from a salt-tolerant selected cell line of tobacco.

Citrus plants are among the crops most sensitive to salinity (8). Therefore, it is of great importance to obtain plants capable of growth with elevated salt levels in the irrigation water. To introduce better salt tolerance, selection for salt-tolerant genetic variants in tissue culture (spontaneous or by induced mutatation) which also have regenerative capacity may provide useful starting material for conventional breeding.

A comprehensive review on the physiology of salt tolerance in plants (19) summarizes the range of known mechanisms of various plants to survive under salinity stress. Plants can either accumulate ions in response to high concentrations of salt in their environ-

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2 Deceased May 15, 1981.
RESULTS

The salt tolerance of selected (R-10) and nonselected (L-5) Citrus cell lines and the stability of the selected line are presented in Figure 1. At all NaCl concentrations tested, R-10 cell line grew much better, and its increased tolerance toward NaCl was retained even after four consecutive transfers on medium without NaCl prior to the experiment (R-10a cells). This result, further supported by data to be presented later, indicates that the R-10 cell line is a true variant.

To study the mechanism by which R-10 cells tolerate NaCl in the medium, the concentration of Na+, K+, and Cl- in L-5 and R-10 cells was determined. R-10 cells take up much less Na+ and Cl- compared with L-5 cells growing at the same external NaCl concentrations (Fig. 2). It should be noted that both cell lines contained similar concentrations of Na+ and Cl- when grown in the absence of NaCl. The percent dry weight of both L-5 and R-10 cell lines was rather constant over the entire range of NaCl concentrations examined in this study. The values were 15 to 16% and 12 to 13% for L-5 and R-10 cell lines, respectively. It is also seen in Figure 2 that the concentration of K+ in L-5 cells was not affected by the uptake of NaCl, while the concentration of K+ in R-10 cells decreased as the concentration of NaCl increased.

The data presented in Figures 1 and 2 show that L-5 cells grown in the presence of 0.1 M NaCl gain about the same fresh weight and take up similar concentrations of Na+ and Cl- as do R-10 cells grown in the presence of 0.2 M NaCl. Thus, the performance of R-10 cells grown in the presence of 0.2 M NaCl was compared throughout this paper with L-5 cells grown in the presence of 0.1 M NaCl.

Electron micrographs of L-5 and R-10 cells grown in the presence and absence of NaCl were compared. As seen in Figure 3, L-5 cells, whether grown in the presence or absence of NaCl, look very much the same, and a small vacuole, if any, can be observed. However, R-10 cells grown in the presence of NaCl have a very big vacuole, which occupies about 90% of the cell. When these cells are grown in the absence of NaCl for four consecutive transfers (R-10a cells, see Fig. 1), the big vacuoles disappear.

In an attempt to understand the inhibitory effect of NaCl and the mechanism(s) by which R-10 cells reduce their accumulation of ions, cells were subjected to various salt solutions causing a comparable lowering of water potential in the medium. It has been shown (1) that R-10 cells are equally tolerant to various sodium salts, such as SO42-, NO3-, and Br-. However, replacing NaCl by KCl turned the NaCl-tolerant cell line into a much more sensitive one, while it had only a slight effect on the nonselected cells (13). As the last experiment was carried out with other salt-sensitive and salt-tolerant cell lines, it was repeated with L-5 and R-10 cell lines and extended over a wider range of K+ concentrations. The data presented in Table 1 and Figure 4 show the growth capacity of the salt-sensitive (L-5) and salt-tolerant (R-10) cell lines at various salt concentrations. It is rather clear that R-10 cells were more sensitive than L-5 cells to the addition of 0.05 and 0.1 M KCl into the medium (Fig. 4). The differential response suggests again that the two cell lines represent two variants with respect to their mechanisms of salt tolerance. The data presented in Figure 5 show that there is an increase in the amount of K+ taken up by the cells of both lines which parallels the increased K+ concentrations in the medium. At an external concentration of 0.05 M KCl, both types of cells double the amount of accumulated K+. It is also seen in Figure 5 that the amount of Cl- taken up was correlated positively with the K+ concentration in the medium, although the Cl- concentration in the medium was kept constant. The decrease in the accumulation of K+ and Cl- by R-10 cells at KCl concentrations above 0.05 M was most probably due to cell mortality. This assumption is based on the fact that translocating these cells back to a medium containing 0.2 M NaCl did not relieve the growth inhibition. The decrease in the accumulation of Na+ in these cells occurred only after the accumulation of K+ reached its maximum. Thus, replacement of NaCl by KCl in the medium increased total cation and anion uptake.

Table 1 shows that both cell lines responded similarly to the addition of 0.1 M K+ ions when added as K2SO4 salt (0.05 M K2SO4 for L-5 and 0.05 M K2SO4 + 0.05 M Na2SO4 for R-10 cells). The relative good growth of R-10 cells on this concentration of K2SO4 (0.05 M), in contrast to no growth at 0.1 M KCl, was used to determine the extent of accumulation of K+ in the medium. The experiment was carried out at varying conditions, namely, when Cl- was absent (only SO42-) and when SO42- was gradually replaced by Cl- at a constant external concentration of K+. Such an analysis should answer the question of whether the inhibitory effect of KCl on R-10 cells is due to excess accumulation of K+ or Cl-.

The data presented in Figure 6 show that K+ accumulation is high and the growth of R-10 cells is not impaired in the absence...
of Cl\(^-\) (medium containing 0.05 M Na\(_2\)SO\(_4\) and 0.05 M K\(_2\)SO\(_4\)). When KCl was introduced into the medium, the uptake of K\(^+\) remained quite constant (note that the external concentration of K\(^+\) was constant) and Cl\(^-\) was accumulated. Chloride accumulation was related linearly to its concentration in the medium and was lower at a given KCl concentration than the observed value when the rest of the salt present was NaCl (compare Figs. 4 and 6). Other anions, such as NO\(_3^-\) and Br\(^-\), behaved in a similar manner to Cl\(^-\), namely, R-10 cells were tolerant to their sodium salts but highly sensitive to their potassium salts. No other anion behaving similarly to SO\(_4^{2-}\) was found. Anions known to be impermeable, such as phosphate, succinate, and citrate, suppressed growth of both cell lines even if introduced as sodium salts.

The data presented in Figures 1 and 2 suggest that R-10 cells are more salt-tolerant because they are somehow capable of restricting the accumulation of NaCl. It was shown in several systems (18, 29) that Na\(^+\) accumulation is related to the concentration of Ca\(^{2+}\) present in the solution. High Na\(^+\)/Ca\(^{2+}\) ratios result in greater Na\(^+\) accumulation, while increasing Ca\(^{2+}\) concentrations in the medium reduced Na\(^+\) uptake. Therefore, in order to determine whether Ca\(^{2+}\) ions play any role in the mechanism of NaCl tolerance of R-10 cells, these cells as well as control L-5 cells were grown in the presence (control medium) and absence of Ca\(^{2+}\), in the presence and absence of NaCl. The data presented in Table II show that only R-10 cells grown in the presence of NaCl, for either many generations prior to the experiment or on immediate exposure to it (R-10a cells), were severely affected by the absence of Ca\(^{2+}\). L-5 cells exposed to NaCl in the absence of Ca\(^{2+}\) grew rather well. Omission of Mg\(^{2+}\) from the growth medium had only a minimal effect on R-10 cells grown in the presence of 0.2 M NaCl, as well as on L-5 cells grown in the presence or absence of NaCl and R-10 cells in the absence of NaCl (data not shown). The different response of R-10 and L-5 cells to the absence of Ca\(^{2+}\) indicates once more the genetic variability between those cell lines. The data presented in Figure 7 show the dependence of R-10 cells grown in the presence of 0.2 M NaCl, and of L-5 control cells, on the concentration of Ca\(^{2+}\) required for maximal gain in fresh weight. It is evident that Ca\(^{2+}\) ions are definitely required for growth of R-10 cells and saturation is achieved at rather low concentrations (1.5–2.0 mM).

Preliminary experiments in the search for possible candidates for osmoregulation in R-10 cells gave negative results. No excess of malic acid, soluble sugars, or free amino acids was accumulated in R-10 cells grown in 0.2 M NaCl over L-5 cells grown in the

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**Table 1. Effect of Various Na\(^+\) and K\(^+\) Salts on Shamouti Salt-Sensitive (L-5) and Salt-Tolerant (R-10) Cell Lines**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Salt</th>
<th>Growth (% control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-5</td>
<td>NaCl, 0.1</td>
<td>100</td>
</tr>
<tr>
<td>L-5</td>
<td>NaCl, 0.05 + KCl, 0.05</td>
<td>95</td>
</tr>
<tr>
<td>L-5</td>
<td>KCl, 0.1</td>
<td>38</td>
</tr>
<tr>
<td>L-5</td>
<td>Na(_2)SO(_4), 0.05</td>
<td>94</td>
</tr>
<tr>
<td>L-5</td>
<td>K(_2)SO(_4), 0.05</td>
<td>57</td>
</tr>
<tr>
<td>L-5</td>
<td>Na(_2)SO(_4), 0.025 + K(_2)SO(_4), 0.025</td>
<td>80</td>
</tr>
<tr>
<td>R-10</td>
<td>NaCl, 0.2</td>
<td>100</td>
</tr>
<tr>
<td>R-10</td>
<td>NaCl, 0.15 + KCl, 0.05</td>
<td>60</td>
</tr>
<tr>
<td>R-10</td>
<td>NaCl, 0.1 + KCl, 0.1</td>
<td>0</td>
</tr>
<tr>
<td>R-10</td>
<td>Na(_2)SO(_4), 0.1</td>
<td>80</td>
</tr>
<tr>
<td>R-10</td>
<td>K(_2)SO(_4), 0.1</td>
<td>0</td>
</tr>
<tr>
<td>R-10</td>
<td>Na(_2)SO(_4), 0.05 + K(_2)SO(_4), 0.05</td>
<td>64</td>
</tr>
</tbody>
</table>
absence of NaCl. Research into other possible organic solutes is now underway.

**DISCUSSION**

The purpose of this study was first to establish that a NaCl-tolerant selected cell line of Shamouti orange is indeed a variant cell line, and second, to understand the mechanism by which these cells survive at elevated salt levels.

From the data presented in this paper, there are good indications that the R-10 cell line which was selected on a medium containing 0.2 mM NaCl is a true genetic variant. First, the acquired tolerance to NaCl which was achieved through a prolonged procedure of passages on medium containing NaCl is a stable trait, namely, it is not lost when the cells are transferred through numerous generations in the absence of selection pressure (Fig. 1). Second, the extent of Na\(^+\) and Cl\(^-\) uptake at a given external NaCl concentration is lower in the selected cells than that observed for the nonselected cells (Fig. 2). Third, R-10 cells are very sensitive to replacement of NaCl by KCl, while the nonselected cells (L-5) are relatively indifferent to this replacement (Fig. 4). This finding suggests that R-10 and L-5 cells may differ in either the mechanism by which Na\(^+\), K\(^+\), and Cl\(^-\) are accumulated, or in compartmentation of these ions within the cells. Additional evidence for the assumption that L-5 and R-10 cell lines differ in their mechanism(s) of ion accumulation is provided by the marked effect which the lack of Ca\(^{2+}\) in the medium has on the sensitivity of R-10 cells toward NaCl. This characteristic is not shared by L-5 cells grown in the presence of NaCl (Table II). The morphological differences found between L-5 and R-10 cells also suggest that these cells belong to distinct variant lines. Confirmation of the R-10 cell line as a true variant is important because Shamouti orange

![Image](https://example.com/image.png)

**Fig. 5.** Accumulation of Na\(^+\), K\(^+\), and Cl\(^-\) in L-5 and R-10 cells as a function of KCl concentrations. Cells harvested in the experiment described in Figure 4 were analyzed as described in "Materials and Methods." (O), Na\(^+\); (C), K\(^+\); (A), Cl\(^-\).

**FIG. 6.** Growth and accumulation of Na\(^+\), K\(^+\), and Cl\(^-\) in R-10 cells as a function of external Cl\(^-\) concentrations. Growth was determined after 30 d. 0 KCl means 0.05 M Na\(_2\)SO\(_4\) plus 0.05 M K\(_2\)SO\(_4\). Any concentration of added KCl was deducted from the K\(_2\)SO\(_4\) to maintain the concentration of K\(^+\) constant. Growth, □—□, Na\(^+\); ○—○, K\(^+\); ——, Cl\(^-\).

**Table II.** Effect of Ca\(^{2+}\) and Mg\(^{2+}\) on Growth of Selected and Nonselected Cell Lines in the Presence and Absence of NaCl

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>NaCl</th>
<th>CaCl(_2)</th>
<th>MgCl(_2)</th>
<th>Growth (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-5</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.880 ± 0.095</td>
</tr>
<tr>
<td>L-5</td>
<td>0.1</td>
<td>0</td>
<td>3</td>
<td>0.495 ± 0.090</td>
</tr>
<tr>
<td>R-10</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0.610 ± 0.050</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>0</td>
<td>3</td>
<td>1.453 ± 0.140</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>0</td>
<td>3</td>
<td>1.563 ± 0.280</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.120 ± 0.010</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.640 ± 0.035</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.120 ± 0.010</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.792 ± 0.080</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.465 ± 0.060</td>
</tr>
<tr>
<td>R-10</td>
<td>0.2</td>
<td>3</td>
<td>3</td>
<td>0.530 ± 0.050</td>
</tr>
</tbody>
</table>

* See Figure 1.

**FIG. 7.** Growth of R-10 cells in the presence of salt, and of L-5 cells in the absence of salt, as a function of Ca\(^{2+}\). Growth was determined after 28 d. (O), L-5 cells; (C), R-10 cells in 0.2 mM NaCl.
callus cells (L-5) are capable of embryogenesis and, subsequently, plant regeneration (14–17). Only a stable genetic variant can have the potential eventually to regenerate plants possessing the desired trait selected for at the callus level. It is an absolute requirement but not necessarily a sufficient one. It was reported that a stable cold-resistant carrot cell line did not regenerate cold-resistant plants (27). However, NaCl-resistant tobacco plants were regenerated from NaCl-resistant callus (22). As preliminary results indicate that Shamouit orange embryos regenerated from R-10 cells survive in a medium containing NaCl much better than do embryos regenerated from nonselected cells (13), it is hoped that this will also be characteristic of plants following regeneration.

There are several systems described in the literature in which ion uptake, especially Na⁺, K⁺, and Cl⁻, was analyzed for nonadapted and NaCl-adapted cultures. Heyser and Naboros (12) describe the ion uptake of tobacco cells selected in the presence of 0.13 M NaCl, and Croughan et al. (6) describe the ion uptake of alfalfa cell cultures selected in 10 g L⁻¹ NaCl. Tal et al. (26) describe the ion uptake of cell cultures derived from either salt-sensitive or salt-tolerant tomato plants. In all these studies, as well as in Citrus cell cultures, Na⁺ and Cl⁻ uptake increases as a function of external concentrations of NaCl. However, a comparison between the extent of uptake of these ions in nonselected and selected line of each crop shows varied response. Whereas in the case of alfalfa there is essentially no difference between the extent of uptake of Na⁺ and Cl⁻ by NaCl-selected and nonselected cell lines, the response of tomato callus derived from NaCl-resistant plants is rather different from that of the callus derived from NaCl-sensitive plants (26). At a given external concentration of NaCl, the callus derived from salt-resistant plants accumulates larger amounts of both Na⁺ and Cl⁻ than does the callus derived from salt-sensitive plants. A comparison between NaCl-sensitive and NaCl-tolerant Citrus cell lines reveals an opposite pattern of Na⁺ and Cl⁻ uptake. At a given external concentration of NaCl, the salt-tolerant (R-10) cells accumulate less Na⁺ and less Cl⁻ than do the salt-sensitive (L-5) cells (Fig. 2). This result suggests that while the salt-selected alfalfa cell line survives salinity by a general shift toward a halophytic mode of salt tolerance, the salt-selected Citrus cell line most probably survives the elevated levels of salinity by partial avoidance of NaCl. Tal et al. (26) show a direct relationship between ion accumulation under saline conditions in tomato plants and the callus derived from them. Therefore, it is reasonable to hypothesize that such a relationship may also hold in the opposite direction, namely, in going from a salt-tolerant callus line to the plants regenerated from it.

The extent of accumulated K⁺ as a function of external concentrations of NaCl is also different among the various cell cultures. While the K⁺ concentration of the nonselected alfalfa cells was lower than that found in the NaCl-selected cells upon addition of NaCl into the growth medium, the reverse pattern was obtained with both selected and nonselected Citrus cell lines, as well as calli derived from salt-sensitive and salt-tolerant tomato plants. In the latter systems, salt-tolerant calli had lower K⁺ concentration upon addition of NaCl than did the salt-sensitive ones. It seems possible to explain this result in the Citrus cell culture if one assumes that K⁺ is accumulated mostly in the cytoplasm. While there is no difference in the relative contribution of cytoplasm to the cell volume of L-5 cells in the presence or absence of salt, such a difference is observed in R-10 cells. Upon addition of NaCl to R-10 cells, the size of vacuoles increases so that the cytoplasmic volume decreases. Thus, the observed decrease of accumulated K⁺ may arise from a shrinkage of cytoplasmic volume rather than from a decrease in its internal concentration. In that case, there is no difference, in respect to internal concentration of K⁺, between L-5 and R-10 cells and tobacco cells grown in the absence or presence of salt (12).

The accumulation of Na⁺ and K⁺ by Citrus cell lines is not dependent on the nature of the anion, but is only a function of the external concentrations of these ions. However, the accumulation of Cl⁻, which is also a function of external concentrations of Cl⁻, is markedly enhanced by the presence of K⁺ (Fig. 5). Since increased accumulation of K⁺ by itself does not affect the growth of either L-5 or R-10 cells (Table I and Fig. 6), it may be concluded that Cl⁻ is the toxic element for Citrus callus cells grown in the presence of NaCl. However, this conclusion is oversimplified. Calculations of internal Cl⁻ content show that L-5 cells grown at 0.1 M NaCl (50% inhibition of growth; see Fig. 1) have 1.5% Cl⁻ by dry weight (Fig. 2), while the same cells grown at 0.05 M NaCl + 0.05 M KCl have about 3% Cl⁻ by dry weight (Fig. 5), and no further inhibition of growth is observed (Fig. 4). When the same calculations are made for R-10 cells at concentrations of 0.2 M NaCl or 0.15 M NaCl + 0.05 M KCl, one finds a correlation between higher levels of Cl⁻ accumulation and further inhibition of growth (Figs. 4 and 5). At present, we do not have any explanation for the difference between the performance of L-5 and R-10 cells in the presence of KCl. Thus, the above conclusion, concerning the toxicity of accumulated Cl⁻, seems valid at least in respect to R-10 cells. Similar results were described by Cooper and Gorton (4) and Furr and Ream (9), where a correlation was found between salt tolerance of various Citrus trees and their accumulation of Cl⁻. This similarity implies that Citrus callus may serve as a reliable model and obviously is a much easier system to study the mechanism of salt tolerance in Citrus plants.

R-10 cells seem to survive in elevated levels of NaCl by avoiding its accumulation. The enhanced sensitivity of these cells to the lack of Ca²⁺ may suggest that extrusion of Na⁺ from the cells depends on uptake of Ca²⁺. It was shown by Layhaye and Epstein (18) that in Phaseolus vulgaris the accumulation of Na⁺ decreased upon addition of Ca²⁺. Such a decrease was also found in soybean (29). These data may explain the preliminary results described in this study, but more thorough analysis of ion content at various Ca²⁺ concentrations is required. The main reason for presenting these results at such an early stage is that they lend support to the evidence for R-10 cells being a variant cell line rather than evaluation of any mechanism concerning salt tolerance.

In conclusion, the Citrus NaCl-tolerant cell line selected from ovular callus of Shamouiti orange is a stable and true variant isolated during a prolonged procedure of selection. This cell line tolerates NaCl by its partial avoidance and the accumulated Cl⁻ seems to be the toxic ion, as suggested previously for the effect of NaCl on Citrus trees. Thus, callus may serve as a reliable and easy model system for studying the mechanism of salt tolerance in Citrus.

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