Distinct Cellular and Organismic Responses to Salt Stress

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We have compared metabolic effects of high salinity between plants and cell suspension cultures from the facultative halophyte *Mesembryanthemum crystallinum* (common ice plant). This plant shows developmentally-programmed inducibility for a switch from C₄-photosynthesis to CAM (Crassulacean Acid Metabolism). The metabolic switch is enhanced by environmental factors such as drought, low temperature, and, most effectively, soil salinity. CAM induction is dependent on organized leaf tissue and cannot be elicited by salt stress in suspension culture cells. In contrast, the accumulation of proline [Thomas et al. (1992) *Plant Physiol.* 98: 626] is induced by NaCl in cultured cells as well as in plants and must be considered a cellular response to stress. We have extended our observations to include another trait of salt- and low-temperature-stress responses in the ice plant, the accumulation of putative osmoprotective sugars and sugar alcohols. In whole plants the cyclic sugar alcohol, pinitol, accumulates to amounts that approach 1 M during stress, while in suspension cells no increase in sugar alcohols is observed. The distribution of carbon to different sugars is markedly different between cells and plants under stress. Particularly obvious is the distinction between cell types in the different composition of sugars and polyols, as exemplified by the epidermal bladder cells of ice plants. Ion contents and the content of sugars and sugar alcohols of bladder cells indicate that Na⁺, Cl⁻, pinitol and an unknown carbohydrate compound provide osmotic pressure in these cells, while organic anion concentrations are low. With the ice plant, we conclude that cells in culture mimic only partly the stress response mechanisms of intact plants and we hypothesize that communication between different tissues is required to mount a complete environmental stress response.

Key words: Bladder cells — Cell culture — Environmental stress — *Mesembryanthemum crystallinum* — Polyol accumulation — Whole plant analysis.

Environmental stress is a persistent challenge for plants which significantly affects productivity. We are interested in studying molecular reactions of plants to salt stress using the ice plant (*Mesembryanthemum crystallinum*) as a model to unravel mechanisms by which some plants can tolerate severe salt stress conditions. Our approach includes the comparison of whole plant stress responses and responses that are characteristic of individual cells in culture, because of the prevalent assumption that one might learn about the underlying mechanisms by investigating cell suspensions from either stress-tolerant or stress-sensitive plants or by selecting stress-tolerant cell lines. Much has indeed been learned from the study of cells in culture. Unfortunately, however, the comparative aspect of suspension cell vs. whole plant reactivity to stress has rarely been considered in depth (Bressan et al. 1990).

Some aspects of environmental stresses appear to be perceived very similarly by cell suspensions and intact...
plants leading to similar biochemical and molecular reactions. For example, cell growth under salt stress is affected both in planta and in suspension cells, even after cells in the tissues or isolated cells have regained turgor (Binzel et al. 1985, Singh et al. 1989, Bressan et al. 1990). NaCl-mediated changes in cell wall composition are largely the same. Plants regenerated from salt-adapted cells of tobacco maintain the tolerance phenotype to some degree suggesting genetic alterations. Similarly, Winicov and coworkers have designed experiments by which photosynthetically competent, salt tolerant callus cultures were obtained (Winicov and Button 1991). Plants regenerated from such calli maintained the tolerance phenotype. These examples may indicate that salt selection in tissue culture amplifies a minority of (probably preexisting) cells tolerant to NaCl, or that tissue culture is a mutagenic procedure and a useful tool. The use of non-adapted cultures, however, in which the desired mutations have not been selected, appears to be an incomplete substitute for whole plant studies. Whole plant reactions are more complex than the reactions that can be elicited in unadapted cells (Jefferyes 1981). From our own work (Thomas et al. 1992a) and that of several other groups (Bartels et al. 1991, Bray 1990, Bressan et al. 1990) it is clear that many responses to environmental stress require the hierarchy of organized tissue to be expressed.

We are interested in distinguishing between cellular responses and tissue-specific, or whole-plant, reactions to environmental stress, and salt stress in particular. Many organisms, from different kingdoms (Yancey et al. 1982), and probably all plant cells, will recruit a number of specific mechanisms when challenged by high salinity. The accumulation of proline is one example of such an interkingdom reaction (Rhodes 1987, Csonka and Hanson 1991). A plant-specific response to many osmotic stress conditions is the accumulation of the plant growth regulator ABA (Zeevar and Creelman 1988, Bray 1990, Thomas et al. 1992b). This does not necessarily mean that the cells exhibiting the specific reaction are salt-tolerant. It appears that we must distinguish between cellular responses to salinity which may be ubiquitous to all cells and tissue-specific reactions which require organized tissue to be displayed.

An important consideration must be the plant chosen to study the effects of salt stress. We have selected the ice plant (Mesembryanthemum crystallinum), a facultative halophyte. This plant provides a well-understood model for the physiological, biochemical and molecular analysis of the effect of environmental stress on plants (Cushman et al. 1990a, b, Winter and Gademann 1991). We have previously shown that in cell suspension cultures from the ice plant the cellular stress indicator, proline, accumulates when the cells are challenged by 400 mM NaCl (Thomas et al. 1992a). Increases in proline are also observed in whole plants (Demming and Winter 1986). In contrast, the gene Ppc1, encoding an isoform of the enzyme PEPCase, which is transcriptionally induced in salt-stressed plants (Cushman et al. 1989) does not respond correspondingly in suspension cells (Thomas et al. 1992a). One might argue that this failure is because the cells are non-photosynthetic and that a photosynthetically active cell would respond by inducing PEPCase. This remains to be seen, although we think it unlikely, based on other experiments.

We have recently studied another aspect of whole plant reactions, the accumulation of the cyclic polyol, pinitol, in salt-stressed plants. Pinitol is derived from methylation of myo-inositol to the intermediary product ononitol (Vernon and Bohnert 1992a) and ononitol appears to be epimerized (Dittrich and Brandl 1987) to pinitol. Pinitol accumulates to over 9% of dry matter in long-term stressed ice plants (Paul and Cockburn 1989). Pinitol accumulation, like the accumulation of other sugar alcohols, is correlated with drought tolerance found in a number of plant species from different families (Loewus and Loewus 1983, Ford 1984, Dittrich and Korak 1984).

We hypothesized that the biosynthesis of pinitol might be, like the biosynthesis of proline, an induced cellular character. The substrate, myo-inositol, is present in all cells and it is supplied as one component of the tissue culture medium. Pinitol does not derive from a pathway that is connected with photosynthetic activity, as is the establishment of the CAM pathway for which PEPCase is a key enzyme. Surprisingly, however, cell suspension cultures, which were established in the absence of salt stress, did not synthesize pinitol when the cells were subjected to high concentrations of salt. Thus, we assume that proline accumulation in cells and plants may provide a general stress-mediating effect, while the induction of polyol accumulation is dependent on organized tissues. Further experiments indicated that within organized leaf and stem tissue of the ice plant polyol accumulation is different in different specialized cells. After long-term stress, the epidermal bladder cells of the ice plant accumulate pinitol and another, yet unidentified, carbon compound to a different extent than in leaf mesophyll cells. These two compounds accumulate such that they appear to provide a substantial osmotic pressure in the cytosol.

Materials and Methods

Plants of Mesembryanthemum crystallinum were grown, tissue material was processed, and proline and protein determinations were performed as described (Ostrem et al. 1987, 1990, Thomas et al. 1992b, Vernon and Bohnert 1992a). Cell suspension cultures were established and analyzed as described (Thomas et al. 1992a). For the isolation of soluble carbohydrates, ground frozen cell, leaf, or root material was extracted with MeOH/Chloroform/H2O (12:5:3) and two phases form-
ed by adding H$_2$O. The MeOH/H$_2$O phase was desalted (BioRad AG50W-X4, H$^+$-form, Amberlite IRA-68, OH$^-$-form; followed by a MeOH/H$_2$O wash of the columns). Desalted extracts were vacuum-dried, resuspended in H$_2$O and filtered through a nylon filter (0.2 μm, Gelman) before injection onto the HPLC column. The analysis of soluble sugars and sugar alcohols was performed using a Dionex (Sunnyvale, CA) HPLC system in conjunction with a Dionex Carpack PA1 column with 150 mM NaOH and/or a BioRad (Richmond, CA) HPX-87C Aminex column with H$_2$O at 85°C equipped with a Gilson (Middleton, WI) model 302 pump. Carbohydrate detection was by pulsed amperometric detection (Dionex Advanced PAD) at 35°C. Detection limits for each sugar were at least 10 pmol/50 μl sample. For calibration standards, various sugars from different sources were used (Tarczynski et al. 1992a, b, Vernon and Bohnert 1992a).

The concentrations of cations in the collected sap of bladder cells were determined (Desert Analytics, Tucson, AZ) by flame spectroscopy (Na$^+$, K$^+$ and Ca$^{2+}$). Ion chromatography HPLC was used for anion detection (Dionex OmniPac PAX-100 column with isocratic 40 mM NaOH/5% MeOH with the conductivity cell of the Dionex pulsed electrochemical detector using suppressed conductivity). Reproducibility in repeated analyses was approximately 3%.

### Results and Discussion

When whole plants of *M. crystallinum* grown in hydroponic tanks or in soil are stressed by addition of high amounts of NaCl (e.g. 400 mM), the plants wilt, but regain turgor within one to two days and continue to grow. In the stressed plants the expression of novel genes important for the CAM pathway (Bohnert et al. 1992) is induced as well as for other events more directly related to salt tolerance. Among short-term reactions, the accumulation of sugar alcohols appears to be one mechanism used by the plant for protection against salt stress and drought (Tarczynski et al. 1992b, Vernon and Bohnert 1992a, b). The sugars and alcohols were identified by UV absorption or electrochemical detection, and quantitated by HPLC (Dionex OmniPac column with isocratic 40 mM NaOH or 50% piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) and 5% MeOH with the conductivity cell of the Dionex pulsed electrochemical detector using suppressed conductivity). Recognizable quantities of sugars were calculated by isocratic elution of standard solutions and comparison with retention times of the HPLC-UV and HPLC-PAD profiles of the samples.

### Table 1  Concentrations of sugars and sugar alcohols in plants and suspension culture cells of *Mesembryanthemum crystallinum* in the absence and presence of 400 mM NaCl

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Treatment</th>
<th>Duration of stress</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Fructose</th>
<th>Inositol</th>
<th>Pinitol/Ononitol</th>
<th>Unknown polyol</th>
<th>Proline (μmol PCV$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>−NaCl</td>
<td></td>
<td>1.2</td>
<td>5.7</td>
<td>3.9</td>
<td>0.2</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.5±0.2</td>
</tr>
<tr>
<td></td>
<td>−NaCl</td>
<td>5 days later</td>
<td>1.6</td>
<td>10.0</td>
<td>7.4</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+NaCl</td>
<td>1 day</td>
<td>1.9</td>
<td>4.5</td>
<td>2.0</td>
<td>1.3</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3 days</td>
<td>3.5</td>
<td>1.3</td>
<td>1.1</td>
<td>3.8</td>
<td>1.2</td>
<td>trace</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 days</td>
<td>2.5</td>
<td>0.7</td>
<td>0.7</td>
<td>2.8</td>
<td>3.3</td>
<td>trace</td>
<td>7±1</td>
</tr>
<tr>
<td>Roots</td>
<td>−NaCl</td>
<td></td>
<td>1.2</td>
<td>0.3</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>&lt;0.1</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>+NaCl</td>
<td>5 days</td>
<td>1.9</td>
<td>0.5</td>
<td>0.9</td>
<td>0.1</td>
<td>0.6</td>
<td>trace</td>
<td>—</td>
</tr>
<tr>
<td>Bladder cells</td>
<td>+NaCl</td>
<td>Long term</td>
<td>&lt;0.1</td>
<td>3.9</td>
<td>3.0</td>
<td>0.3</td>
<td>20.2</td>
<td>21.2</td>
<td>—</td>
</tr>
</tbody>
</table>

| Suspension cells | NaCl | 0 (a) | 3.5 | 1.7 | 0.7 | 0.7 | <0.1 | <0.1 | 0.1±0.02 |
|                 |      | 0 (b) | 7.1 | 5.3 | 1.4 | 0.7 | <0.1 | <0.1 | —       |
|                 | NaCl | 7 days (a) | 3.7 | 0.8 | 0.5 | 0.2 | <0.1 | <0.1 | 1.0±0.2 |
|                 |      | 7 days (b) | 6.9 | 3.4 | 0.4 | 0.1 | <0.1 | <0.1 | —       |

*PEPC levels in plants and cells have been reported (Ostrem et al. 1987, Thomas et al. 1992a).

The content of the bladder cells can only be removed from the cells after stress, in unstressed plants these cells are not filled, but are pressed to the epidermis. Amount of the unknown polyol is estimated using pinitol units.

* Plants were five weeks old at the start of the experiment; a single experiment is shown. Variations in the relative amounts of sugars between experiments are considerable. Upon stress in the leaves, glucose and fructose invariably declined, pinitol/ononitol amounts increased. Variations in repeat determinations from the same sample were ±3%. (a) established from hypocotyl; (b) established from roots. Amounts in cells are expressed as μmoles ml$^{-1}$ PCV (packed cell volume); for bladder cells it was estimated that the vacuole contributed ~98% of cell weight.

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sugar alcohols listed (Table 1, Figure 1a, b) were determined by HPLC analysis and pulsed amperometric detection. This analysis indicates the stress-related changes in the allocation of carbon in the leaves. In leaf extracts from unstressed plants the most prevalent sugars are glucose, fructose and sucrose (Table 1). Minor amounts of myo-inositol and several other sugars are present at levels of less than one percent of all soluble carbohydrates. Similar compositions are found in roots; however, the amount of sucrose is typically higher than that of glucose and fructose in the roots than in the leaves and stems.

Upon salt stress the levels of sucrose increased or, at least, were maintained, while the absolute amounts of glucose and fructose decreased dramatically. The amount of myo-inositol increased, although during longer stress periods myo-inositol reached a plateau and later decreased again. Continuously increasing are the amounts of ononitol and pinitol. They migrate close to the injection peak on the Dionex Carbopac PA1 column, but can be resolved on the BioRad HPX-87C column, as shown in Figure 1. Fructose and myo-inositol co-migrated on the Bio-Rad column. They were resolved by also separating each extract on a Dionex column where fructose eluted as a separate peak. The difference between the two peaks gave the amount of myo-inositol.

The composition of sugars and sugar alcohols in suspension cells is different from sugar and polyol content in intact plants. These cells are growing with 30 g liter\(^{-1}\) of sucrose (87.7 mM) and 100 mg liter\(^{-1}\) of myo-inositol (0.56 mM) supplemented in the medium. Assuming a packing of cells as rigid spheres, the volume of the centrifuged cell samples would result in 74\% internal volume. This leads to estimates of the content of sucrose, fructose and glucose of the well-washed cell pellet of 4.7–9.6 mM, 1.1–7.2 mM, and 0.5–1.9 mM, respectively. Inositol was present at 0.14–0.95 mM. Under conditions of salt stress (Table 1; Figure 2a, b), sucrose levels did not change significantly, while glucose and fructose declined. Most importantly after seven days of salt stress in the suspension culture cells, the amounts of each of the three cyclic sugar alcohols, inositol, pinitol and ononitol, did not increase. The cells do not appear to use these carbohydrates for osmotic protection.

That inositol and its methylated forms did not accumulate in suspension cells is in stark contrast to the behaviour of whole plants. In previous work (Thomas et al. 1992a) in which we measured the accumulation of proline under salt stress, we observed much slower growth of the cells in suspension under salt stress, although the putative osmoprotectant proline accumulated (see Table 1). In these cultured cells the CAM-indicator PEPCase did not increase (Thomas et al. 1992a) and here we show that pinitol does not increase either. Our interpretation is that the suspension culture cells established from this halophytic plant are actually salt-sensitive, because the

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**Fig. 1** Analysis of sugar and sugar alcohol content by HPLC-PAD in extracts from ice plant leaves after separation on a Bio-Rad HPX-87C column. (a) no stress; (b) five days of salt stress. 1— injection peak; 2— sucrose; 3— glucose; 4— ononitol/pinitol; 5— fructose and inositol; 6— methanol peak (from extraction medium).
cells in suspension do not mimic stress responses that are observed with organized leaf or root tissue. The gene Imt1, encoding the methyl transferase enzyme that eventually leads to ononitol and pinitol production (Vernon and Bohnert 1992a), is apparently not induced in these suspension cultures, judged by the lack of ononitol/pinitol accumulation. From many previous experiments we know that increases in enzyme protein, followed by product increases from the enzyme reaction, usually follow increased expression of a gene. Assuming that Imt1 is no exception, we suggest that the lack of pinitol in suspension culture cells is due to a lack of expression of, at least, the methyl transferase encoded by this gene, and probably also other enzymes in this pathway. The reasons for the differences in gene expression and metabolic reactions between cells and intact plants are not known, but several explanations are possible. Based on ongoing experiments we think that the altered hormone composition in cells is, in part, responsible. It appears that the ratio between ABA and cytokinin in stressed plants may be a signal that results in changes in gene expression (Thomas et al. 1992b). Suspension cells in culture have different hormone levels. In addition, we think that cells in different tissues are crucial for the salt stress response chain. For example, as the stress is perceived by the root system, different cells in the root cortex and/or central cylinder will react and, perhaps, will be responsible for the generation of signals the generation of which depends on organized tissue. We are investigating such mechanisms at present. A lucid example for the importance of specialized cells within an organized tissue has come from the analysis of the ion, sugar and polyol content of epidermal bladder cells.

A prominent structure of the ice plant are epidermal bladder cells. These cells differentiate during development of the unstressed plant, but they remain appressed to the leaf or stem surface. Following water stress, turgor is generated in these cells—they may contain up to five μl of solution per cell—and the cells conspicuously protrude from the epidermis. Bladder cells are found in a number of plant species which experience drought or salt stress. Their function may be in generating a "second epidermis" by which evaporation of water from the leaves is further reduced (Steudle et al. 1977). In addition, storage of water in these cells may represent a buffer under conditions of temporarily or diurnally changing water availability. For CAM plants the vacuolar space of the bladder cells, which in Mesembryanthemum was estimated to be approximately 20% of the leaf water volume (Steudle et al. 1977), could be a storage compartment for organic ions. The epidermal bladder cells of Mesembryanthemum crystallinum have been investigated before with respect to ion content and conductivity (Lütte et al. 1978). From these studies it was deduced that the cuticle of the cells is extremely impermeable, that the cells exchange water (and presumably
ions) with the underlying mesophyll cells, and that the vacuolar Na\(^+\) and Cl\(^-\) content followed approximately the concentration of these ions in the solution used for watering the plants. Lüttge et al. (1978) found a discrepancy in charge by measuring inorganic ions and suggested that the difference is due to the organic anion, oxalate. Studies which measured other compounds, for example sugars and polyols, contained in bladder cells have not been conducted.

Bladder cells are particularly large at the tips of the side shoots and on the surface of the fruits and seed pods. We have collected the vacuolar sap of bladder cells from the largest cells at the surface of fruits and have analyzed their ion content (Table 2). We observed relatively low amounts of organic ions, malate (1 to 4 mM) and oxalate (23 to 42 mM), in different collections of bladder cell contents. The low concentration of malate probably indicates that the bladder cells are not active in CAM. The higher amount of oxalate (particularly in plants grown in the field) is in equilibrium with Ca\(^{2+}\) and Mg\(^{2+}\) and their oxalate salts. The pH of the cell sap was 6. HPLC analysis of sugars and sugar alcohols indicated the prevalence of ononitol/pinitol, sucrose was very low, but fructose and glucose were 3–4 mM (Table 1).

Analysis of the bladder cells from long-term stressed plants (Table 2) showed that the major inorganic ions are Na\(^+\) (~600–800 mM) and Cl\(^-\) (~700 mM), as reported previously (Steudle et al. 1978), as well as K\(^+\) (~50 mM) in plants that were stressed long term by watering with 500 mM NaCl solutions. Ca\(^{2+}\)-levels did vary; they should be dependent on the amount of oxalate present. Oxalate was indeed highly variable, as seen, for example, in the comparison of growth chamber-grown vs. field-grown ice plants.

To what extent sugars and polyols are contained in the vacuolar sap or whether they are restricted to the cytoplasm of the epidermal bladder cells has not been investigated. A second polyol with a retention time of 21.65 minutes was identified (BioRad HPX-87C column) (Figure 3a). It was a minor component among the sugars/sugar alcohols of extracts from whole leaves (Figure 3b) and was absent from suspension cell extracts (Figure 3c). However, it was present at approximately 30% of all polyol and sugar compounds in the bladder cells (Figure 3a) and slightly higher than the amount of pinitol. Such high amounts most likely indicate that these two compounds serve as osmotically active solutes. If pinitol and the unknown polyol (mass of 186 by mass spectroscopy; unpublished) were located in the cytoplasm and partially counterbalancing the osmotic pressure of the salts in the vacuole, their concentration would be between 0.7 and 1.4 M. In that case the cytoplasmic volume containing the polyols would be 3 to 5% of the cell volume, an estimation in agreement with previous measurements and electron microscopic examination of mature bladder cells (Kramer 1979). The sum of the ion contents from bladder cells of growth chamber-grown plants (samples 1–4, Table 2) indicates an excess of anions over cations. While our ion chromatography separation system easily indicated all major anions, the same was not the case for cations. Undoubtedly Mg\(^{2+}\) is present. An in-depth, complete analysis, which is not the topic of this study, would be needed to resolve this discrepancy.

We have compared the pattern of the sugars and their accumulation in plants with the pattern obtained for sugar compounds in stressed and unstressed suspension cells. The suspension cells were not salt-adapted cells, but they were established and maintained in the absence of NaCl and then stressed by the addition of NaCl (400 mM) similar to

### Table 2 Ion content of epidermal bladder cells from salt-stressed ice plants

<table>
<thead>
<tr>
<th>Ion</th>
<th>Concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5*</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>616 726 730 777 n.d.</td>
</tr>
<tr>
<td>K(^+)</td>
<td>48 50 43 38 n.d.</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>0.06 0.2 0.9 0.05 n.d.</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>712 715 725 760 333</td>
</tr>
<tr>
<td>NO(_3)(^-)</td>
<td>38 48.5 52.8 42.0 181.1</td>
</tr>
<tr>
<td>SO(_4)(^2-)</td>
<td>2.6 2.1 1.9 2.4 3.9</td>
</tr>
<tr>
<td>Malate</td>
<td>&lt;0.1 1.4 1.0 4.4 3.5</td>
</tr>
<tr>
<td>Oxalate</td>
<td>&lt;0.1 24.2 23.7 42.0 106.1</td>
</tr>
<tr>
<td>Phosphate</td>
<td>&lt;0.1 1.2 1.2 1.4 2.0</td>
</tr>
</tbody>
</table>

The content of bladder cells was removed from a large number of cells located in the epidermis of the leaves, the fruits and along the stems by a Hamilton syringe. Average amount of fluid per cell was 2–3 \(\mu\)l, containing the vacuolar sap and the cytoplasm of the bladder cells. The sample contained less than 4 \(\mu\)g of chlorophyll ml\(^{-1}\). Chloroplasts were removed by centrifugation. The values reported here were determined for the collected sap (600 to 1,400 \(\mu\)l) from three sets of between 8 and 12 plants each which had been salt-stressed by watering with Hoagland's solution containing 500 mM NaCl for 25 to 38 days. The data are biased for ions found in large bladder cells which could easily be emptied.

Among the organic ions detected, the low content of malate probably indicates that these cells are not performing CAM and it also indicates that the vacuole of the epidermal bladder cells, estimated to contribute more than 97% of the volume, performs osmoregulation using inorganic ions.

Numbers 1 to 4—different sets of plants that were used for extraction of solution from bladder cells.

* Analysis of bladder cells from non-salt stressed, four months old plant (weight approximately 9 kg) covering an area of ca. 1 m\(^2\) and growing at a Tucson, Arizona, location in full sunlight with occasional watering. n.d.—not determined.

S.D. = ±6% in repeated analysis of the same sample. The pH of the bladder cell extract (mid-morning) was 6.0.
Responses to salt stress by plants and cells

the procedure used for whole plants. The sugar composition in the cells was altered by salt stress (Table 1, Figure 3). Myo-inositol inside the suspension culture cells approximately equaled the amount supplied in the medium. In contrast to leaves, the methylated inositol-derivatives, ononitol and pinitol, were not accumulating when the cells were suspended in 400 mM NaCl. The most prevalent sugars, glucose, fructose and sucrose, were present at concentrations of 0.5 to about 10 mM. For comparison, the combined concentration of these sugars in the medium was approximately 80 mM.

Generalization is not possible without several caveats. Proline accumulated ten-fold following treatment with 400 mM NaCl. This increase in the suspension culture cells was due to either an endogenous cell-specific or ubiquitously perceived signal, the perception of either higher osmotic pressure in the medium, or the influx of NaCl. This "scenario" had no effect on sugar (pinitol) metabolism in the cells. In contrast, the whole plants increased both proline and pinitol/ononitol levels. It appears tempting to speculate that the perception of stress leads to several signals and to their transduction, and that the various signals must be integrated to elicit a whole plant response. If this is so, only one or few of these signals appear to be expressed or recognized by cells in culture.

After several years of molecular analysis of the ice plant model we begin to see mechanisms that allow this plant to survive severe salt stress. The entire response must be seen in the context of whole plant development (Cushman et al. 1990a), likely a reflection of the ecological niche that the plant occupies (Winter et al. 1978, Bloom and Troughton 1979). Concerning specific responses, CAM induction (Cushman et al. 1989, Bohnert et al. 1992) appears to provide long-term survival of the energy-producing leaves and, especially, of the reproductive structures. The latter aspect is documented by the specialized accumulation of pinitol and of the unknown carbohydrate in the bladder cells of the fruits. Short-term, the capacity to photosynthesize is protected during a salt challenge by a largely undisturbed gene expression program of photosynthesis-related proteins (Michalowski et al. 1992). Leaf polyol production is both a short-term and a long-term means to conserve water and, likely, for protection of cellular structures (Vernon and Bohnert 1992a, b). How these reactions are influenced by changes in the concentration of growth regulators, particularly the levels and the interplay between the levels of ABA and cytokinin (Thomas et al. 1992b, McElwain et al. 1992), remains to be studied in detail. What is clear, however, is that the entire spectrum of responses appears to be recognized only in organized tissues.

From published comparisons and from the comparison between cells and whole plants reported here it appears obvious that the cellular response leading to proline

Fig. 3 Comparison of polyol and sugar profiles from epidermal bladder cells, leaves and cells in suspension culture. (a) epidermal bladder cell extract; (b) extracts from stressed leaves; (c) extracts from stressed cells from suspension cultures. S—sucrose; G—glucose; P—pinitol and ononitol; F—fructose and inositol; U—unknown carbohydrate of mass 186. Column separation as in figure 1.
accumulation in the ice plant (Demmig and Winter, 1986, Treichel, 1986, Thomas et al. 1992a) is ubiquitous (Yancey et al. 1982), but probably not sufficient for protection against severe stress. Proline accumulation might be a reaction sufficient for survival under moderate salinity or water stress conditions. From the studies reported here it appears that this halophyte, when encountering severe salt stress, can utilize unique and specialized mechanisms that allow the ice plant to provide more protection than that obtained by the general proline accumulation mechanism. The additional accumulation of glycine-betaine (McCue and Hanson 1990) and of polyols, such as pinitol (Ford 1984, Paul and Cockburn 1989, Vernon and Bohnert 1992a) has apparently been utilized for similar purposes in a number of different organisms. In the ice plant, pinitol accumulation does not occur in cultured cells, but appears to be, like CAM induction, a response that requires organized tissues, leaves and roots. Obvious future experiments will have to focus on providing the genetic basis for polyol production, cellular localization of the polyols, and whether polyol producing transgenic plants are salt tolerant as a consequence of this accumulation. To understand the mechanisms by which some plants perform better than others is a research topic which should eventually lead to engineering strategies by which transgenic plants expressing stress-alleviating polyol- or cyclitol-producing traits can be investigated (Tarczynski et al. 1992b).

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