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Variation in polar-group content in lipids of cowpea (*Vigna unguiculata*) Cell cultures as a mechanism of haloadaptation

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Abstract

Callus and cell suspension cultures of cowpea (*Vigna unguiculata*) were induced with 2,4-dichlorophenoxyacetic acid and grown at different NaCl concentrations. The cell biomass yield and its total lipid content decreased with increasing salinity. However, while the hexose content in lipids was higher, the amount of lipid phosphorus was significantly lower in both agar and cell suspension cultures. Ion-transport rates with artificial membranes prepared with different lipid fractions showed that lipids from cells grown in a saline medium were less permeable to Na^+ and to Cl^- than those grown in a non-saline medium. Also the permeability of membranes prepared with glycolipids was lower than those prepared with phospholipids and whole lipids. Apparently, the increase of hexose/phosphorus ratio in membrane lipids is induced in response to the halo-adaptation process.

Introduction

A number of halo-tolerance mechanisms have been described for non-halophyte plants, for example: the high ion uptake for turgor maintenance and the accumulation of compatible organic solutes as osmoregulants (Greenway & Munns 1980). Two critical aspects of cellular metabolism have been analyzed with particular attention: trans-membrane movements and cellular compartmentalization of sodium, and carbon acquisition and allocation (Cheeseman 1988). The membrane lipids could participate in these metabolic aspects of halo-tolerance.

Apparently, the lipid composition of roots of grape root-stock, and the accumulation and transport of ions in the leaves, are correlated with the degree of halotolerance (Kuiper 1968a, 1968b). The lipid composition of salt-sensitive bean, the less salt-sensitive barley and the salt-tolerant sugar beet have also shown different content of sterols and sterol esters in the roots according to their salt resistence (Stuiver et al. 1978). In the root lipid composition of six plant species, the proportion of glycolipids with respect to phospholipid was correlated with the salt-tolerance level of species in question; in contrast, no correlation of this kind was found in leaf lipid composition (Hirayama & Mihara 1987). Thus, root lipid composition of plants may be indicative of their salt-tolerance level.

In roots particularly, the level of glycolipids in *Plantago* species has been increased at high mineral nutrition, while other lipid constituents were affected in a different manner (Kuiper & Kuiper 1978); however, in this study the salinity has not been involved. Little information is available concerning the synthesis and degradation of lipids in plants under saline stress (Kuiper 1980). In orach for example, with increasing external

NaCl concentration the glycolipid proportion was largely increased, while the phospholipid one was decreased. In cucumber, the glycolipids were increased slightly, whereas the phospholipids showed no change. Thus, the ratio glycolipid/phospholipid was increased in both species in response to salinity (Hirayama & Mihara 1987).

In contrast, the plasma membrane and endomembranes from barley roots, grown at different NaCl concentrations, showed a lipid composition that was not altered by salinity (Brown & Du Pont 1989). The lipid modification in response to salinity by sugar beet roots was related to their genotypes (Stuiver et al. 1981). On the contrary, other tissue, e.g. the chloroplast membranes of barley, had a considerable decrease in the glycolipid content with the increase in salinity. The accumulated NaCl in the leaf may affect the galactosyl-and acyl-transferases, which are lipid-synthesizing enzymes (Müller & Santarius 1978).

Plant tissue culture techniques possess several advantages for assessing physiological effects of salts at the cellular level, because the organ-organ and plant-environment interactions are removed or controlled. With this technique the production of halo-tolerant callus tissues has been feasible (Nabors et al. 1975, 1980; Stavarek & Rains 1984; Kavi-Kishor & Reddy 1985; Pandey & Ganapathy 1985; Chandler & Thorpe 1986) and the salt effect on callus growth and the osmoregulation process has been studied (Kavi-Kishor 1988; Paek et al. 1988). Yet no information is available concerning the effect of the salt concentration on the lipid composition of cells in tissue cultures under saline stress.

In this work the lipid composition of callus and cell suspension cultures of halo-tolerant cowpea was analyzed in order to determine the role of lipids in the halo-adaptation process.

Materials and methods

Source of explants and culture conditions

Cowpea seeds from the 'Universidad Autonoma de Baja California Sur' (*Vigna unguiculata* H002) were surface-disinfested by soaking in 0.5% sodium hypochlorite for 15 min, followed by five rinses in sterile distilled water. The seeds were inoculated in glass vessels (150 ml), capped with translucent autoclavable lids, containing 20 ml of Murashige & Skoog (1962) medium (MS) without plant growth regulators. After ten days, 0.5 cm^2 leaf explants from germinated seeds were transferred to basal agar medium (MS) supplemented with 10 μM of 2,4-dichlorophenoxyacetic acid. The calluses were induced at 26 ± 2°C under continuous fluorescent light (100 μmol m^{-2} s^{-1}).

Salinity treatments of callus were carried out by transplanting 0.4 g of cell aggregates obtained after 120 days of culture to basal agar medium containing 0, 150 or 300 mM of NaCl. After 30 days culture, the calluses were weighed and one portion dried at 70°C for 24 h, cooled and weighed again to determine dry weight; the other part was used for lipid analysis. The experiments in solid medium were carried out in triplicate.

Cell suspension was produced by dissociation of 5 g of fresh callus in 250 ml Erlenmeyer flasks containing 50 ml of MS liquid basal medium and cultured for 30 days at 26 ± 2°C and shaken at 150 rpm. Then the cultures were filtered through gauze and 10 ml aliquots of filtrate were transferred to 40 ml of fresh medium. After 30 days culture, 25 ml were transferred to 500 ml Erlenmeyer flasks containing 250 ml of MS liquid basal medium with 0 or 300 mM of NaCl. The cultures were carried out in five independent experiments.

Lipid analysis and artificial membrane preparation

Calluses and cells from suspension cultures were suspended in methanol and heated for 5 min at 100°C in a glass-stoppered tube to inactivate degradative enzymes. Then, the suspensions were cooled and chloroform was added to obtain 5 ml of mixture methanol-chloroform (2:1). With this mixture the biomass was extracted for 24 h at 4°C. The extract was filtered and the residue was washed two times with 5 ml of methanol-chloroform (2:1). Ten ml of water were added to the combined filtrates in a glass-centrifuge tube and the phases allowed to separate by centrifugation. The chloroform layer was

withdrawn and the aqueous phase washed two times with 3 ml of chloroform. The chloroform solution, containing the lipids, was evaporated under vacuum at 45°C. The dry residue was weighed and immediately dissolved in 1 ml of chloroform containing 0.02% of 2,6-di-t-butyl-4-methyl phenol (BHT). Aliquots were dried under N_2 and assayed to determine the total phosphorus with Amidol reagent (Bartlett 1959) and the total sugar according to the phenol-sulfuric acid procedure (Dubois et al. 1956).

The lipid material for artificial membrane preparation was obtained from cell suspension cultures carried out in 1000 ml flat-bottom flasks containing 800 ml of MS liquid basal medium, aerated, magnetically stirred and kept under sterile conditions. Ten flasks were prepared with 200 mM of NaCl and ten without NaCl. Cell viability in cell suspension cultures was determined by the fluorescein diacetate technique described by Widholm (1972). The neutral lipids were obtained by fractionation in alumina column chromatography according to Vazquez-Duhalt & Greppin (1987). Then, the glycolipids and phospholipids were eluted successively with 10 volumes of acetone and 10 volumes of methanol respectively. The different fractions were dried under vacuum at 45°C and stored with 1 ml of chloroform containing 0.2% of BHT. The polar lipid analysis was carried out by two-dimensional thin-layer chromatography developed in a solvent system containing:

- Chloroform - methanol - 28% ammonia (65:25:5), and
- Chloroform - acetone - methanol - acetic acid - water (6:8:2:2:1).

The spots were developed according to Kates (1972):

- general detection with 5% H_2SO_4 in ethanol at 180°C;
- glycolipids with alpha-naphthol;
- phospholipids with ammonium molybdate, and
- amino-lipids with ninhydrin.

The measurements of ion-transport rates across artificial lipid membranes were carried out according Hirayama et al. (1987). Artificial membranes were prepared with the help of teflon Millipore filters (LCWPO1300) immersed into 10 mg ml^{-1} lipid solution. The impregnated filters were placed in a 1 cm^2 partition-hole between two 200 ml aqueous compartments. Ion flow through lipid membrane was measured with ion-electrodes under a concentration gradient of 0.6 M of NaCl in a 5 mM potassium phosphate buffer (pH 7.0) at 20°C.

Results and discussion

Callus induction was obtained in MS basal agar medium supplemented with 1 μM of 2,4-D. The calluses were transferred to saline medium and cultured under different NaCl concentrations. After 30 days (growth decreased with the increase of NaCl concentration) as shown by both fresh and dry weight (Table 1). In this way, the biomass production in 150 mM of NaCl was 65% of that without salt and it was only 15% in a medium containing 300 mM of NaCl, on dry weight basis. However, the growth obtained in saline media is indicative that the cells achieved the adaptation. The water content in the calluses was not affected by the NaCl concentration, but the lipid content decreased (Table 1). The most important effect of the salinity on the callus cells was the drastic change in the proportions of polar lipids. Thus, the hexose/phosphorus ratio in the lipids was five times higher at 300 mM of NaCl (Table 1). Our results are in agreement

Table 1. Biomass yield, moisture and lipid characteristics of callus of cowpea grown for 30 days under different salinities.[a]

	NaCl concentration (mM)		
	0	150	300
Fresh weight (mg)[a]	1129 ± 2[b]	737 ± 290	458 ± 19
Dry weight (mg)	80 ± 4	62 ± 13	36 ± 6
Moisture (%)	92.9 ± 0.4	91.9 ± 1.4	92.2 ± 0.9
Total lipids (% d.w.)	2.4 ± 0.2	1.9 ± 0.1	1.6 ± 0.2
Hexose in lipids (μmol g^{-1} lipid)	1354 ± 49	1296 ± 3	2794 ± 414
Phosphorus in lipids (μmol g^{-1} of lipid)	400 ± 39	210 ± 48	155 ± 24
Hexose/phosphorus (molar ratio)	3.4 ± 0.2	6.2 ± 2.8	18.1 ± 0.1

[a]400 mg of fresh cell aggregates were transfered to basal agar medium containing different NaCl concentrations.
[b]The standard deviation was calculated form values obtained in three replicate experiments.

with those obtained with *Plantago* roots (Kuiper & Kuiper 1978) and orach and cucumber roots (Hirayama & Mihara 1987), but differ from those found with membranes from barley roots where no significant changes were found (Brown & Du Pont 1989).

Sometimes, the cell aggregates or calluses could present heterogeneous cellular composition and the cells could be under different levels of the stress condition. In order to eliminate these problems and to determine the proportion of viable cells in the culture, cowpea cells were cultivated in cell suspension cultures with 300 mM of NaCl and without NaCl. The cultures were carried out for one week in saline medium to determine the changes in the synthesis and degradation of lipids during the early halo-adaptation process, minimizing growth or new cell production. Table 2 shows the total lipid content and the amount of polar lipids in the cell biomass after one week of culture under saline stress. In both, saline culture and controls, no significant growth was detected in an one-week period. The total lipid content of the cells was lower than in callus; however, the overall lipid level was also decreased by salinity as in the calluses. The polar lipid content in cells from suspension cultures was lower than in callus cells; however, in both culture systems the glycolipid content increased and the phosphorus decreased (Table 2). Moreover, the molar ratio of polar groups was the same in either system and changed in proportion with salinity.

As mentioned, the increase of glycolipid content in response to salinity has been detected in whole plants, especially in root lipid composition (Kuiper & Kuiper 1978; Hirayama & Mihara 1987). All these studies have suggested that the lipid modification may induce a change in membrane permeability. The method for determining the ion-transport through artificial lipid membranes described by Hirayama et al. (1987) may help to explain the lipid role in the haloadaptation process. Therefore, artificial lipid membranes were prepared with the lipid fractions of cowpea cells grown at different NaCl concentrations. As shown in Table 3, artificial membranes prepared with total lipids of cells grown in saline medium, transported both Na^+ and Cl^- ions at a rate nearly 24 times lower than those prepared with total lipids of control cells (grown without NaCl). These results suggest that the cowpea cells can easily change their membrane permeability in response to external NaCl concentration.

Different lipid fractions employed to prepare artificial membranes showed different ion-transport properties. Ion transport across membranes prepared with glycolipids was significantly lower than those prepared with phospholipids from cowpea cells (Table 3). The neutral lipids (mainly sterols and sterol esters) showed no ion permeability, as occurred with the control experiment, without lipid impregnation, because such lipids are not capable of forming bilayers. The differences in permeability of the lipid frac-

Table 2. Lipid content and polar group ratio of lipids from cell suspension cultures of cowpea grown during 7 days at different NaCl concentrations.

	NaCl concentration (mM)	
	0	300
Dry biomass ($mg\,l^{-1}$)[a]	214 ± 24	233 ± 14
Total lipids (% d.w.)	1.5 ± 0.1[b]	0.8 ± 0.2
Hexose in lipids ($\mu mol\,g^{-1}$ of lipid)	549 ± 92	754 ± 113
Phosphorus in lipids ($\mu mol\,g^{-1}$ of lipid)	172 ± 35	55 ± 14
Hexose/phosphorus (molar ratio)	3.2 ± 0.3	11.6 ± 2.8

[a]The dry biomass concentration after inoculation (time = 0) was $219\,mg\,l^{-1}$.
[b]The standard deviation was calculated from values obtained in five independent experiments.

Table 3. Ion transport of artificial lipid membranes prepared from lipids of cowpea cells grown at different NaCl concentrations.

	Ion-transport rates ($\mu eq\,cm^{-2}\,h^{-1}$)	
	Na^+	Cl^-
Dioleyl phosphatidyl choline (Sigma reagent standard)	42.2 ± 14.8[a]	78.0 ± 15.1
Total lipids from cultures without NaCl	166.9 ± 25.1	188.9 ± 36.2
Total lipids from cultures with 200 mM of NaCl	6.9 ± 3.3	8.2 ± 0.8
Glycolipids	0.8 ± 0.1	2.3 ± 1.9
Phospholipids	10.1 ± 4.4	8.5 ± 5.4
Neutral lipid	0.0	0.0

[a]The standard deviation was obtained from values of three membrane preparations.

tions is in agreement with the relationship found between the increase of the glycolipid/phospholipid ratio and the decrease of ion-transport rate with the increase in salinity. That is, high salinity caused an increase in the glycolipid content, which in turn repressed ion-flow. Obviously, the artificial membranes from total lipids are not representative of the function of whole plasma membranes. This is a destructive technique and does not consider the membrane proteins and active ion-transport. Nevertheless, the artificial membrane technique seems good in order to evaluate, easily and quickly, the pasive ion-transport as a consequence of changes in the lipid composition of cells.

It has been previously reported that artificial membranes prepared with mono- and di-galactosyl diglycerides (Kuiper 1968b; Hirayama et al. 1987), and with glycolipids from samphire and from cucumber (Hirayama & Mihara 1987) are more permeable to Na^+ and Cl^- ions than the respective phospholipids. In plants, the surface-active mono- and di-galactosyl diglycerides increased the transport of chloride and sodium, while cerebrosides with saturated fatty acids seemed to be less permeable to ions than the phospholipids (Kuiper 1969).

The TLC analysis of polar lipids from cowpea showed that the major glycolipids are the glycosylceramides or cerebrosides, followed by the monogalactosyl diglycerides and digalactosyl diglycerides. On the other hand, the proportion of phospholipids found in cowpea was as follows: phosphatidyl choline > phosphatidyl serine > phosphatidyl ethanolamine. Also phosphatidic acid, phosphatidyl glycerol, diphosphatidyl glycerol, free fatty acids and neutral lipids were detected.

The glycosylceramides have also been reported as the most abundant glycolipid in different membranes fractions of barley roots (Brown & Du Pont 1989). In animal cells, glycosylceramides have been found in the myelin membrane of neural axons and in the brush border membranes of the intestine and kidney, where they have been associated with permeability reduction to ions (Curatolo 1987) as occurred in artificial membranes from lipids of cowpea cells.

In conclusion the results obtained so far show that the lipid composition of cultured cells of the halo-tolerant cowpea is modified by the salinity of the medium. Its hexose content is increased, while its phosphorus content is decreased. The artificial membranes prepared with lipids from cells grown under saline stress are less permeable to Na^+ and Cl^- ions, than those prepared with the lipids from cells grown in a medium without NaCl. This difference in permeability may be attributed to an increase in the amount of glycolipid content. Thus, the change in the polar lipid composition in cowpea cell cultures could be considered as an important halo-adaptation response. This property could be used to detect halo-adapted plant cell-lines precociously.

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