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LIPID COMPOSITION OF MICROSOMES FROM HEAT-STRESSED CELL SUSPENSION CULTURES

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Abstract—Heat-stressed (30°) cell suspension cultures of carrot (*Daucus carota* L.) attained a lower maximum cell density and showed browning earlier when compared with control cultures (22°) over a 16 day growth period. Phospholipid class profiles did not differ between cells grown at 30° and 22°. The fatty acids of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from microsomes of heat-stressed cells were less unsaturated than those of PC and PE from microsomes of control cells. In particular, there was a marked increase in the proportion of oleate [18:1(9)] at the expense of linoleate [18:2(9, 12)] at the higher growth temperature. This difference could result from inhibition or loss of the microsomal lipid-linked desaturase which inserts the double bond between carbons 12 and 13 of oleate esterified to the glycerol moiety of PC and PE.

INTRODUCTION

It is well documented that in plants as well as microorganisms a change in growth temperature often induces alteration of the fatty-acid composition of membrane glycerolipids [1–4]. The general trend observed is an increase in the extent of Fatty-acid unsaturation at lower temperatures and a decrease in the level of unsaturation at higher temperatures. This process, called 'homeoviscous adaptation' by Sinensky [4], is thought to adjust membrane fluidity, and thereby maintain membrane function, over a range of temperatures normally experienced in the natural environment. Although modification of fatty-acid unsaturation is certainly not the only change in membrane lipid composition in response to changing temperature [3, 5], its widespread occurrence supports the conclusion that it is an important parameter of temperature acclimation [6].

While there have been numerous studies of changes in plant membrane lipids induced by a decrease in growth temperature, reports on the effects of higher than normal temperatures (heat stress) are relatively few. Heat stress is a complex phenomenon that can result from either chronic exposure (days or weeks) to temperatures in the range of 30–40° or acute exposure (4–8 h) to higher temperatures (about 40–50°) [7]. Inherently, a decrease in membrane fatty-acid unsaturation induced by elevated temperature must involve turnover of existing glycerolipids with *de novo* synthesis of more saturated molecular species [6, 8].

Cell suspension cultures generated from various plant tissues or organs have proven to be useful models for studies of plant lipid metabolism because of their uniformity and the relative ease of controlling environmental conditions [3, 9, 10]. However, investigations of the effects of both high and low growth temperatures on fatty-acid composition and metabolism in cell suspension cultures have yielded confusing and sometimes contradictory results [1, 3, 9]. Substantial variation in the fatty-acid composition of cell cultures from different plant species [1, 9] is no doubt partly responsible for these discrepancies. Two additional factors are the growth phase at which sampling occurs and, in studies where only total cell fatty-acids are analysed, the extent to which storage lipids contribute to the fatty-acids pool.

In the present study, microsomal membranes were isolated from control (22°) and heat-stressed (30°) *D. carota* cells at two phases of growth, individual phospholipid classes were quantified, and the fatty-acid composition of the two major phospholipids, phosphatidylcholine and phosphatidylethanolamine, were determined. Although 30° is at the low end of the temperature range over which plants develop symptoms of heat stress [7], an increase in the incubation temperature of *D. carota* cell suspension cultures from 23° to 30° was sufficient to induce synthesis of high and low heat-shock proteins [11]. Preliminary experiments determined that 30° was the highest temperature that would allow growth

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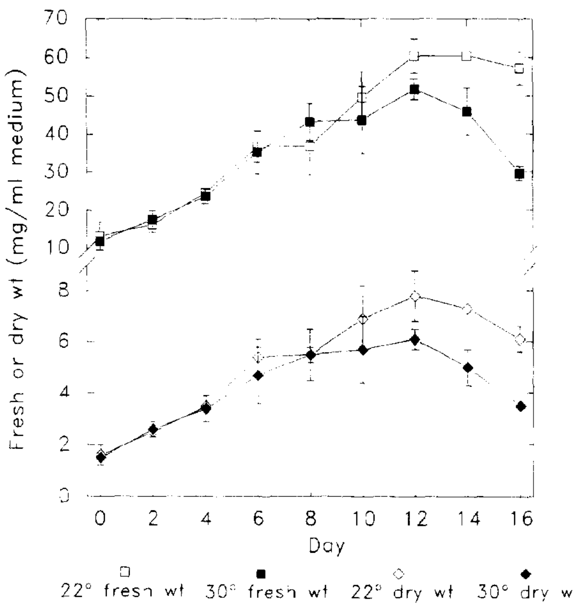

Fig. 1. Fresh and dry weight growth curves of *D. carota* cell suspension cultures at control (22°) and heat-stress (30°) temperatures over 16 days. Data points represent the mean of 3 replicate experiments. Vertical bars indicate ± 1 S.D.

of *D. carota* cell cultures for at least a week with a reasonable yield. Therefore, 30° was selected as the heat-stress temperature for this study.

RESULTS

Control cultures inoculated to an average cell density of 13.2 mg ml^{-1} fr. wt and incubated at 22 ± 1° reached a maximum density of 60.5 mg ml^{-1} by day 12 (Fig. 1). Slight browning of the cultures was noted at this time. The stationary phase extended to day 16 when cell density had decreased to 57.3 mg ml^{-1}. Cultures incubated at 30 ± 1° were inoculated to an average cell density of 11.8 mg/ml fr. wt with cells previously grown at 22°. This (relatively low) heat-stress temperature allowed maintenance over the same interval (16 days) as that established for the 22° controls (Fig. 1). Although browning of heat-stressed cultures was observed by day 8, the log phase of growth continued to day 12 when a maximum cell density of 51.9 mg ml^{-1} was attained. In contrast with cultures held at 22°, cell density fell rapidly after day 12 and had decreased to 29.7 mg ml^{-1} by day 16. The curves of changing dry weight of cells per ml of medium showed similar trends to those of the fresh weight curves for both control and heat-stressed cultures (Fig. 1). Cultures held at 22° increased from 1.6 mg ml^{-1} to a maximum of 7.8 mg ml^{-1} on day 12, then declined to 6.1 mg ml^{-1} by day 16. Cultures held at 30° increased from 1.5 mg ml^{-1} to a maximum of 6.1 mg ml^{-1} on day 12, then fell sharply to 3.5 mg ml^{-1} by day 16.

There was considerable variation between replicates in the amount of phospholipid recovered from microsomal preparations (per gram fresh weight of cells), but the same trend was observed with advancing age in control and heat-stressed cultures (Table 1). There was little difference in the amount of microsomal phospholipid obtained from cells used as the day 0 inoculum (grown 7 days at 22°), cells held 8 days at 22°, and cells held 8 days at 30°. As shown in Fig. 1, each of these cultures was harvested in the mid-log phase of growth when phospholipid synthesis and membrane proliferation should be quite active. For both control and heat-stressed cultures, the amount of microsomal phospholipid recovered at day 14 was only about half of that obtained at day 8 (Table 1). This decrease in microsomal phospholipid per gram fresh weight of cells was somewhat greater in heat-stressed cultures. In accord with these data, both sets of cultures were in the stationary phase by day 14, and cell density in heat-stressed cultures had begun to decline (Fig. 1).

Table 1. Total phospholipid content of microsomal membrane fractions from *D. carota* cell suspension cultures grown at control (22°) or heat-stress (30°) temperature and harvested 0, 8, or 14 days after inoculation.

	Day of Harvest		
Growth Temp.	0	8	14
22°	0.43 ± 0.11	0.49 ± 0.09	0.26 ± 0.09
30°	—	0.49 ± 0.12	0.21 ± 0.04

Values are expressed as μmol PL per g fr. wt of cells and represent the mean ± s.d. ($n = 4$).

The two predominant phospholipids in microsomes from control and heat-stressed cells were phosphatidylcholine and phosphatidylethanolamine, which constituted about 50 and 30 mol%, respectively, of the total phospholipid (Table 2). Phosphatidic acid, phosphatidylinositol and phosphatidylglycerol comprised most of the remaining phospholipid, but a few minor components were not identified or quantified. There was no significant change in the phosphatidylcholine:phosphatidylethanolamine ratio (range 1.67–1.85) or in the overall phospholipid composition with either growth phase or growth temperature (Table 2).

The fatty-acid moieties of microsomal phosphatidylcholine and phosphatidylethanolamine were almost all 16 or 18 carbon fatty acids, predominantly palmitate (16:0), stearate (18:0), oleate (18:1), and linoleate (18:2) (Table 3). The percentage of 16:0 was smaller and that of 18:1 greater in phosphatidylcholine compared with phosphatidylethanolamine, regardless of growth phase or temperature. Not surprisingly, the fatty acid compositions of both phosphatidylcholine and phosphatidylethanolamine were quite similar in microsomes from the day 0 inoculum cells (grown 7 days at 22°) and in microsomes from the 8 day/22° cells. In contrast, phosphatidylcholine and phosphatidylethanolamine from microsomes of cells grown 8 days at 30° had a much greater percentage of 18:1, mostly at the expense of 18:2. The ratio of 18:1 to 18:2 in microsomal phosphatidylcholine increased from 0.35 in the 0-day inoculum to 1.36 in the 8-day heat-stressed cells. Similarly, the 18:1 to

Table 2. Phospholipid composition of microsomal membrane fractions from *D. carota* cell suspension cultures grown at control (22°) or heat-stress (30°) temperature and harvested 0, 8, or 14 days after inoculation. Values indicate mol % total PL and represent the mean of four experiments. Values within a row followed by the same letter do not differ significantly at the 5% level (LSD test).

	Day of harvest				
	0	8		14	
Phospholipid	—	GT 22°	GT 30°	GT 22°	GT 30°
PC	50.8a	53.0a	53.9a	51.7a	51.8a
PE	29.8a	29.2a	29.1a	31.0a	28.8a
PI	7.5a	7.8a	5.2a	7.3a	7.4a
PG	5.9a	4.1a	4.9a	4.1a	5.3a
PA	6.0a	5.9a	6.8a	5.9a	6.6a

Table 3. Fatty-acid composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from microsomal membranes of *D. carota* suspension culture cells grown at control (22°) or heat-stress (30°) temperature and harvested 0, 8, or 14 days after inoculation. Values indicate wt.% of total PC or PE fatty acids and represent the mean of four experiments. Values within a row followed by the same letter do not differ significantly at the 5% level (LSD test).

	Day of harvest				
	0	8		14	
	—	GT 22°	GT 30°	GT 22°	GT 30°
PC fatty acid					
16:0	18.4a	18.3a	17.2a	15.5a	14.7a
18:0	6.7c	6.7ca	8.0b	8.7b	11.2a
18:1	18.8c	19.7c	11.5d	43.4a	28.9b
18:2	54.5ab	53.6b	61.4a	32.0d	42.7c
18:3	1.7b	1.7b	1.9b	1.5b	2.6a
PE fatty acid					
16:0	24.8a	21.9a	20.0a	22.4a	21.6a
18:0	7.2c	6.8c	9.8b	10.8ab	13.3a
18:1	9.6c	11.4c	6.1d	27.2a	17.6b
18:2	56.4a	57.7a	61.5a	37.6b	44.6b
18:3	2.0a	2.2a	2.6a	2.3a	2.9a

18:2 ratio in microsomal phosphatidylethanolamine increased from 0.17 to 0.72. At both 22° and 30°, as cultures passed from mid-log phase (8 days) to stationary phase (14 days) increases in 18:2 and (to a lesser extent) 18:0 offset a decrease in 18:1 in microsomal phosphatidylcholine and phosphatidylethanolamine. Despite this, the 18:1 to 18:2 ratio in microsomal phosphatidylcholine and phosphatidylethanolamine remained much higher in heat-stressed compared with control cell cultures.

The changes in fatty-acid composition that occurred with incubation of cultures at heat-stress temperature (30°) resulted in a significant decrease in the unsaturation index of microsomal PC and PE (Table 4). There was also evidence of a slight overall increase in fatty-acid chain length (shown as a decline in the 16C:18C ratio), but this was not statistically significant. A modest increase in the unsaturation index of microsomal phosphatidylcholine and phosphatidylethanolamine occurred at both growth temperatures as cultures progressed from log to stationary phase.

DISCUSSION

Two previous studies of lipid and fatty-acid composition in suspension culture cells of *D. carota* found significant amounts of mono-, di-, and tri-acylglycerols, as well as free fatty acids, in total lipid extracts [9, 10]. In light of this, the present study focused on phospholipid composition and fatty-acid unsaturation in isolated microsomes to ensure that lipid changes observed following transfer from 22° to 30° are relevant to the process of high temperature acclimation [2]. The proportions of phosphatidylcholine and phosphatidylethanolamine found in the microsomal fraction agree well with those previously reported for whole cells by Kleinig and Kopp [10] (about 51% phosphatidylcholine and 29%

Table 4. Unsaturation Index (UI) and 16 carbon: 18 carbon fatty-acid ratio (16C: 18C) of PC and PE from microsomal membranes of *D. carota* suspension culture cells grown at 22° or 30° and harvested 0. 8, or 14 days after inoculation. $\mathrm{UI} = [\%\ 18{:}1 + 2(\%\ 18{:}2) + 3(\%\ 18{:}3)]/100$. Values represent the mean of four experiments. Values within a row followed by the same letter do not differ significantly at the 5% level (LSD test)

	Day of harvest				
	0	8		14	
	—	GT 22°	GT 30°	GT 22°	GT 30°
Unsat. Index					
PC	1.33ab	1.32ab	1.40a	1.11c	1.22bc
PE	1.29ab	1.33a	1.37a	1.09c	1.15bc
16C:18C					
PC	0.23a	0.22a	0.21a	0.18a	0.17a
PE	0.33a	0.28a	0.25a	0.29a	0.28a

phosphatidylethanolamine, with PC:PE = 1.80). Phosphatidic acid constituted a considerably larger percentage of microsomal than whole cell phospholipids, possibly due to the action of phospholipase D during membrane isolation [10]. The unsaturation indexes of phosphatidylcholine and phosphatidylethanolamine from whole cells reported by Gregor [12] are appreciably higher than those determined for microsomal phosphatidylcholine and phosphatidylethanolamine. The cultivar used, the growth conditions, and the specific membrane fraction are all factors that could be responsible for this difference.

The only comparable report in the literature on the effects of a shift up in growth temperature on unsaturation of phosphatidylcholine and phosphatidylethanolamine in plant cell suspension cultures is that by Toivonen *et al.* [3]. They found that transfer of *Catharanthus roseus* cultures from 23° to 32° resulted in an increased proportion of 16:0, a general decrease in unsaturation of 18-carbon fatty acids, and (consequently) a decrease in the unsaturation index in (whole cell) phosphatidylcholine and phosphatidylethanolamine. These results differed from those for *D. carota* cultures with respect to the change in the level of 16:0 in phosphatidylcholine and the type and magnitude of changes in unsaturation of 18-carbon fatty-acids. The level of 18:3 was much higher in phospholipid from *C. roseus* cells. This was perhaps related to the intense greening of the cells that occurred at each growth temperature [3]. It is interesting to note that in a companion study of temperature effects on membrane lipid composition in hairy root cultures of *C. roseus* [13], the proportions of 18:1 and 18:2 in phosphatidylcholine and phosphatidylethanolamine, as well as the magnitude of the change in these two fatty-acids with a shift from 24° to 32°, much more closely resembled our results with (root) cell suspension cultures of *D. carota*.

Most of the research on the correlation between lipid unsaturation and high temperature tolerance/acclimation has focused on chloroplasts, because photosynthesis is impaired at high temperature and chloroplast membranes are rich in polyunsaturated fatty acids [6, 14]. The consensus from this work is that decreased unsaturation of chloroplast lipids can be important in thermal tolerance [6, 14, 15], but this mechanism is not always involved in acclimation of plants to high temperature [14, 16]. The same generalization appears to apply when considering the role of changes in fatty-acid unsaturation in the ER and other 'eukaryotic' membranes in response to heat stress. A recent investigation of resistance to extreme temperatures and changes in fatty-acid composition in cotton seedlings showed that whereas acclimation to low temperature was associated with an increase in 18:2 in phospholipids, a decrease in phospholipid unsaturation did not accompany the increase in thermal tolerance induced by a 6-hr heat shock at 40° [16]. On the other hand, ten days after shifting from a 30°/20° to a 50°/40° day/night temperature regime, thermal tolerance in the desert succulent *Ferocactus acanthodes* was increased by about 8°, and the unsaturation index of polar lipid fatty-acids from both chloroplast and microsomal fractions was significantly decreased [14].

Clearly, many years after establishing that adjustment of membrane fatty-acid unsaturation often accompanies a change in growth temperature, there is still little known about the mechanism or physiological significance of this phenomenon. It appears that cell suspension cultures of *D. carota* would serve as a relatively simple and controllable model for further studies. Because the level of 18:3 in microsomal phospholipids is quite low, and the increase in the 18:1 to 18:2 ratio at high temperature is dramatic, future work could focus on the lipid-linked desaturase which introduces the double bond between carbons 12 and 13 of 18:1 esterified to phosphatidylcholine and phosphatidylethanolamine [6, 8]. This microsomal desaturase, designated as *fad* 2 in studies of *Arabidopsis* mutants [6], has recently been cloned [17]. Thus, it should now be possible to establish whether the decrease in desaturase activity in response to heat stress is the result of gene regulation, changes in the physical properties or types of lipids specifically associated with the enzyme (affecting its kinetics) [5, 18, 19], or perhaps merely the reduced availability of O_2 (a required cofactor) [3].

EXPERIMENTAL

Initiation and maintenance of cell suspension cultures. Seeds of *Daucus carota* L. 'Royal Chantenay' were surface-sterilized in a soln containing 20% household bleach (1% NaClO) and 0.1% (v/v) Tween 20 for 30 min, then placed on moist, presterilized filter paper in 10 cm Petri dishes. The dishes were sealed with parafilm and stored in the dark. Seeds were allowed to germinate for one week at 22 ± 1°. Cell suspension cultures were initiated by transfer of root explants to Murashige and Skoog basal salts plus vitamins liquid medium supplemented with 2,4-dichlorophenoxyacetic acid at 1 mg l^{-1} and 2% (w/v) sucrose (MS medium) [20]. Cell density (fr. wt per ml) was determined by collecting cells from 15 ml of suspension culture on a tared Whatman No. 4 filter paper and filtering to dryness prior to weighting. Dry wt was determined after heating filtered cell samples in an oven at 73 ± 1° for 48 hr. Cells were subcultured into fresh MS medium every 7 days by harvesting on sterile 145 μm mesh screening and adding an inoculum to obtain an initial density of 0.2 g fr. wt per 25 ml. Control cultures were maintained at 22 ± 1° on a gyratory shaker at 120 rpm under continuous light provided by cool white fluorescent lamps at 21 μmol m^{-2} s^{-1}. Heat-stressed cultures were inoculated with cells grown at 22° and then incubated at 30 ± 1°. Conditions were the same as those for control cultures, with the exception that the light intensity was lower (15 μmol m^{-2} s^{-1}).

Isolation of microsomes. Cell suspension cultures were harvested by vacuum filtration on days 0, 8, and 14 after inoculation. Harvests at day 8 and day 14 were chosen to represent the mid-log and early stationary phases of growth, respectively, for both control and heat-stressed cultures (Fig. 1). Filtered cells were resuspended at 1 g per ml in cold homogenization medium (250 mM sucrose, 70 mM Hepes, 3 mM EDTA, 0.5% PVP-40, pH 8.0) and disrupted in a blender. Cell homogenates were centrifuged at 13 000 *g* for 15 min, followed by centrifugation of the supernatant at 80 000 *g* for 30 min to recover the microsomes. Microsomal pellets were washed once by resuspending in cold 1 mM Hepes buffer plus 250 mM sucrose, pH 7.2, and recentrifugation at 80 000 *g* for 30 min. The washed microsomes were resuspended in a few ml of 1 mM Hepes, pH 7.8, and stored at −60° prior to lipid analysis.

Lipid extraction, fractionation and analysis. After thawing and recentrifugation of microsomal suspensions, the membranes were resuspended in 0.8% aq. NaCl and extracted with 3 vols of $CHCl_3$–MeOH, 2:1. The $CHCl_3$ phase containing total lipids was washed with MeOH–H_2O, 1:1 then evaporated under a stream of N_2. The total lipid extracts (TLEs) were dissolved in 4 ml of $CHCl_3$ and two 25 μl aliquots were used for assay of total PL by the method of ref. [21]. A portion of the TLE containing 1 μmol of PL was dissolved in 1 ml of $CHCl_3$ and sepd by silicic acid CC into neutral lipid, glycolipid, and PL fractions as described in ref. [22]. Equal portions of the PL column fractions were used for analysis of PL class composition by TLC and PL fatty-acid composition by GC. Individual PL classes were sepd by 1–D TLC on silica gel, 60 plates developed in $CHCl_3$–MeOH–CH_3COOH–H_2O, 170:32:25:7, then quantified by the method of results [21] after scraping and eluting with $CHCl_3$–MeOH, 2:1. Fatty acids of total PL were converted to their Me esters (FAMEs) with 14% (w/v) BF_3 in MeOH, and FAMEs were analysed by FID GC using a 0.25 mm × 15 m SP-2330 fused silica capillary column [23].

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