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Changes in the composition of membrane lipids in relation to differentiation in *Aegle marmelos* callus cultures

L. Bhardwaj[1], J.-M. Mérillon[2,*] & K.G. Ramawat[1]

[1]*Department of Botany, M. -L. Sukhadia University, Udaipur, 313001, India;* [2]*Laboratoire de Biologie Cellulaire et Biochimie Végétale (EA 1370), Faculté des Sciences Pharmaceutiques, Université de Tours, France (*present address and requests for offprints: Laboratoire de Mycologie et de Biologie Végétale JE 095, Faculté des Sciences Pharmaceutiques, 3 Place de la Victoire, 33000 Bordeaux, France)*

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Abstract

Changes in fatty acid, phospholipid and galactolipid contents during cellular and organ differentiation in *Aegle marmelos* have been described. Decrease in phosphatidylinositol content and presence of 3-*trans*-hexadecenoic acid in phosphatidylglycerol were related to greening and shoot buds differentiation. The galactolipids level, the monogalactosyl diglyceride/digalactosyl diglyceride ratio and the linolenic acid level (mainly in monogalactosyl diglyceride) increased with the degree of differentiation, indicating the possible biogenesis of functional chloroplasts.

Abbreviations: 2,4-D – 2,4 dichlorophenoxyacetic acid, BA – benzylaminopurine, DW – dry weight, FW – fresh weight, PC – phosphatidylcholine, PE – phosphatidylethanolamine, PI – phosphatidylinositol, PG – phosphatidylglycerol, PS – phosphatidyl serine, MGDG – monogalactosyl diglyceride, DGDG – digalactosyl diglyceride, 16:0 – palmatic acid, 18:0 stearic acid, 18:1 – oleic acid, 18:2 – linoleic acid, 18:3 – linolenic acid, trans-16:1 – 3-trans-hexadecenoic acid

Introduction

A balanced ratio of plant growth regulators (cytokinin/auxin) plays an important role in differentiation (Wilson et al. 1991). But from incorporation of growth regulators in the medium to the appearance of visible organs, a plethora of events occurs, which are not clearly understood (Tran Thanh Van 1981; Manoharan et al. 1987; Wilson et al. 1991).

Studies have been made to understand the complex process of differentiation by monitoring different events separately, i.e. carbohydrate metabolism (Thorpe & Laihsley 1973), protein and nucleic acid synthesis (Sengupta & Raghavan 1980), ultrastructural changes (Ramawat et al. 1989) and lipid composition (Manoharan et al. 1987).

Plant lipids play an important role in growth and development of plants (Kader & Laurent 1991). We know that plant growth factors such as auxins and cytokinins alter membrane lipid composition. For example, cytokinins decrease the free sterol/phospholipid ratio and modulate fatty acid composition of phospholipids in *Catharanthus roseus* cells (Mérillon et al. 1993). Some phospholipids, particulary phosphatidyl inositol derivatives are involved in the transduction of signals such as auxins and light (Ettlinger & Lehle 1988; Morse et al. 1989). Also light-induced changes in the membrane lipids of green tissues have been shown to take place mainly in the galactolipids, which are major lipids of chloroplasts (Douce & Joyard 1980; Kader & Laurent 1991).

Therefore, there are some assumptions of interrelations between the effects of external stimulus and the membrane lipid composition in several physiological processes.

Aegle marmelos is a familiar woody fruit tree grown in India and a plant of economic importance (Arya et al. 1981). Some studies showed *in vitro* regeneration from hypocotyl explants (Arya et al. 1981) or nucellar tissues (Hossain et al. 1993) of *A. marmelos* through organogenesis. We have established different strains of *A. marmelos* differing in their levels of differentiation and in their growth hormone requirement. These strains have shown differential auxin oxidation and presence of auxin protectors, varied secondary metabolite content and subcellular structures (Bhardwaj 1991). We therefore studied the changes in lipid composition during greening and shoot bud initiation, in non-differentiated and differentiated strains.

Materials and methods

Tissue cultures

Callus cultures were raised from surface sterilized stem explants (1.5 cm) from a wild tree of *Aegle marmelos* Correa (*Rutaceae*) (Bhardwaj 1991). Cultures were maintained on MS medium (Murashige & Skoog 1962) supplemented with 11.2 μM 2,4-D, 2.2 μM BA and 0.46 μM kinetin (medium A). Suppression of plant growth regulators (medium B) for four passages caused greening and further incorporation of azaindole (25.4 μM) produced shoot buds (medium C). All the cultures were maintained separately under light for 16 h (36 μmol $m^{-2}s^{-1}$) at 28 ± 1 °C. Experiment was carried out after 6 passages and 3-week-old tissues were used.

Chlorophyll analysis

Chlorophyll content was estimated according to Arnon (1949).

Lipid analysis

Cells were fixed in boiling water for 10 min or in liquid nitrogen and were lyophilized. The following operations were performed as described earlier (Mérillon et al. 1993). Briefly, lipids were extracted four times with chloroform-methanol (1/1, v/v) and twice with chloroform. Phospholipids and galactolipids were separated by two-dimensional thin layer chromatography on silica gel 60 plates with a first development in chloroform/methanol/water (65/25/4, v/v/v) and a second development in chloroform/acetone/methanol/acetic acid/water (50/20/10/10/5, v/v/v/v/v). After visualization with 2′,7′-dichlorofluorescein under UV light, the separated lipids were scraped off, saponified and esterifed with boron trifluoride-methanol.

Heptadecanoic acid was added as internal standard. Analysis of methyl esters of fatty acids was performed by FID-GLC, with a DB-23 (J & W Scientific) column (30 m × 0,32 mm). Peak areas were recorded by integration. Each analysis was run at least twice. Lipids were quantified on the basis of their fatty acid content.

Data presentation

Results are the mean of two separate experiments; trends were consistent. Standard deviations calculated for 3 replicates (one experiment) were less than 5%.

Results and discussion

Tissues turned green when 2,4-D was removed from the culture medium and a 27-fold increase in chlorophyll content was observed (Table 1). Chlorophyll content of the tissues subsequently increased (37 fold) with the appearance of shoot buds. Shoot buds themselves contained 89-fold higher chlorophyll content than undifferentiated creamish callus, but only about half of that of *in vivo* grown leaves. Induction of the process of differentiation might be due to 2,4-D depletion (Ramawat et al. 1989; Wilson et al. 1991).

Total lipid content (phospholipids + galactolipids) reached approximatively the same yield in undifferentiated non-green (17.8 mg/g DW) and green callus (17.2 mg/g DW) while a higher content was recorded in callus producing shoot buds (19.4 mg/g DW) (Table 1). But shoots themselves showed a low lipid content. This may be due to development of thicker walled cells in shoot buds. This is supported by results obtained with stem tissue (not presented) and leaf tissue (*in vivo*) analysed separately.

Both processes of differentiation (greening or shoot buds differentiation) were associated with a decrease in the level of phospholipids and an increase in the level of galactolipids (Table 1). The galactolipid amount in these callus cultures was twice as high as in creamish callus.

Minor changes were recorded in amounts of phospholipids during greening of tissues, the prominent being the decrease in the relative as well as the abso-

Table 1. Chlorophyll content and lipid composition of cultures at various stages of development.

Tissues	Medium*	Chlorophyll mg/g FW	Total lipid mg/g DW	Amount (%)	
				Phospholipid	Galactolipid
Non-differentiated cream	A	0.01	17.8	90.8	9.1
Non-differentiated green	B	0.37	17.2	84.2	15.7
Differentiated green with shoot buds	C	0.52	19.4	82.8	17.1
Shoot buds only	C	1.25	15.4	–	–
Leaves (*in vivo*)	–	2.57	16.5	–	–

*See Materials and Methods for media composition.

Table 2. Percentages of phospholipids and galactolipids at various stages of cellular differentiation.

Tissue	Phospholipids and galactolipids (% total)							
	PC	PE	PI	PG	PS	MGDG	DGDG	MGDG/DGDG
Non-differentiated creamish	50.9	21.2	13.7	2.7	2.3	4.6	4.5	1.02
Non-differentiated green	51.3	19.5	9.8	2.3	1.3	7.7	8.0	0.96
Differentiated green with shoot buds	46.7	23.3	8.9	2.9	1.0	9.8	7.3	1.34

lute level of PI during greening and shoot buds formation i.e. 9.8% (1.7 mg/g DW) and 8.9% (1.7mg/g DW) respectively instead of 13.7% (2.4 mg/g DW) in creamish callus. This is in accordance with studies employing cultures of *Datura innoxia* (Manoharan et al. 1987). These results point to an involvement of the PI system in the process of differentiation. This process resulted in an increase of MGDG and DGDG amounts (Table 2). There was a higher MGDG/DGDG ratio in differentiated callus with shoot buds (1.34), compared with undifferentiated callus (1.02 and 0.96) (Table 2); this may indicate the development of chloroplasts with formed grana (Douce & Joyard 1980; Manoharan et al. 1987). Such changes were recorded in herbaceous plant species tobacco and *Datura innoxia* (Manoharan et al. 1987). However, in *A. marmelos*, a tree species, increase was limited. This may be due to the fact that shoot buds possess little leaves and were not as chlorophyllous as herbaceous plants. Perhaps, this is the reason why woody species required much care and are difficult to establish after plantlet formation as compared to herbaceous plants. Moreover, a low MGDG/DGDG ratio may be a characteristic of plants adapted to arid or semi-arid climates (Guillot-Salomon et al. 1991).

The total lipid fatty acid composition showed a significant decrease in 16:0 with concurrent increase in 18:3 in the green and shoot-differentiating callus tissues (Table 3). In these tissues we observed the presence of trans-16:1 in PG (result not shown). This fatty acid is found only in PG molecules in photosynthetic structures and is probably implicated in the molecular organization of the light-harvesting protein chlorophyll a/b complex (Dubacq & Trémolières 1983). The ratio of 18:2 to 18:3 in total lipids was consistently greater in non-differentiated creamish callus compared with green and differentiated callus (Table 3). Analysis of fatty acids in phospholipid and galactolipid classes showed that this change was the most prominent in MGDG and DGDG; only the corresponding data are given in Table 4. These results suggested that the enzyme activity which desaturates dienoic to trienoic fatty acids, was greatly enhanced during the development of chloroplasts. The decrease of the ratio 18:2/18:3 and the increase of 18:3 were more marked in MGDG than in DGDG. This observation suggested the accumulation of MGDG (18:3/18:3) molecules playing an important role in photosystem activity (Guillot-Salomon et al. 1991). There was a lower proportion of 16:0 in galactolipids in the green and shoot-differentiating callus cultures, compared with non-differentiated creamish callus. Moreover, MGDG was always poorer in 16:0 than DGDG, as shown in jojoba and spinach by Guillot-Salomon et al. (1991).

Table 3. The relative amounts of fatty acids in total lipids of *A marmelos* tissues.

Tissues	% fatty acid composition					18:2/18:3
	16:0	18:0	18:1	18:2	18:3	
Non-differentiated cream	37.8	4.9	14.1	32.7	10.4	2.76
Non-differentiated green	26.8	2.7	11.6	38.2	20.7	1.84
Differentiated green with shoot buds	28.0	3.1	12.5	33.3	23.1	1.44

Table 4. The relative amounts of fatty acids in galactolipids of *A marmelos* tissues.

Tissues	Galactolipid	% fatty acid composition					18:2/18:3
		16:0	18:0	18:1	18:2	18:3	
Non-differentiated cream	MGDG	16.1	5.4	8.7	28.6	41.1	0.696
	DGDG	29.9	8.8	8.2	25.7	27.3	0.941
Non-differentiated green	MGDG	6.0	2.3	6.4	24.3	60.9	0.399
	DGDG	15.8	4.6	5.9	21.7	52.0	0.417
Differentiated green with	MGDG	6.2	2.2	5.9	18.1	67.5	0.268
shoot buds	DGDG	21.1	7.4	7.6	17.3	46.5	0.372

To conclude, we show that there are marked changes in lipids during greening and shoot buds differentiation of *A. marmelos* callus differing in their growth hormone requirement, mainly that in the level of PI suggested the involvement of the PI system in the process of differentiation. Also we found an increase of galactolipid amount, MGDG/DGDG ratio and 18:3 level indicating the possible development of functional chloroplasts with formed grana.

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