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Changes in the composition of fatty acids and sterols of membrane lipids during induction and differentiation of *Brassica napus* (var. *oleifera* L.) callus

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Abstract

Changes in the membrane lipid and sterols content and composition were studied during induction and differentiation in callus cultures of *Brassica napus* var. *oleifera*. Callus induction was associated with an increase of DGDG content, significant changes in fatty acids composition of all lipid fractions and increased degree of lipid unsaturation. The membrane lipid composition of tissue at different degrees of differentiation was found to vary significantly, particularly two weeks after transfer of callus to regeneration medium. The main differences concerned the content and composition of galactolipids. Curiously in many cases, these differences declined during subsequent culture, in spite of the morphogenesis process which was in progress. Another result of differentiation was the change in free sterol composition: in shoot regenerating calli the content of stigmasterol had rose whereas the accumulation of campesterol decreased. Even though observed changes in membrane properties may not play a role in morphogenesis they are nevertheless useful as developmental markers and can be invaluable in understanding biochemical basis of morphogenesis.

List of abbreviations: 16:0, palmitic acid; 16:1, palmitooleic acid; 17:0, margaric acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; 18:3/18:2, ratio of 18:3/18:2 fatty acids; BAP, 6-benzylaminopurine; BF_3, boron trifluoride; 2,4-D, 2,4-dichlorophenoxyacetic acid; DBI, Double Bound Index = Σ (molar % fatty acid content·number of double bounds)/100; GA_3, gibberellic acid; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; MS, basal Murashige and Skoog medium; PL, phospholipids; PL/GL, ratio of phospholipids to glycolipids

Introduction

Brassica napus var. *oleifera* is an agronomically important oilseed crop. Many applications of tissue culture and genetic engineering techniques have been used to improve this crop and to facilitate studies on lipid biochemistry. Although rape is considered to be amenable to *in vitro* culture techniques, there are still significant genotype-dependent differences in regeneration ability between cultivars. The establishment of callus with a high frequency of shoot formation would be very useful especially for plant transformation and breeding. This is however difficult because plant regeneration from callus is a complex process depending on

many factors and knowledge of its inherent mechanism remains fragmentary. A better understanding of the biochemical basis of tissue differentiation could be useful in breaking the barrier of the morphogenesis initiation. Finding a biochemical marker of high morphogenic potential might be useful for early identification of responsive genotypes, for optimisation culture conditions or for monitoring the course of morphogenesis.

Genetic control is probably the most important factor controlling the ability of callus cells to differentiate, resulting in genotype-dependent variability in the regeneration abilities of particular cultivars. However, the process of differentiation is associated with changes in the tissue structure, cell size and shape which suggests that cell differentiation requires or causes the changes in physiological properties of the plasmalemma. It is well documented that, at the cellular level, membrane stability and fluidity is influenced by composition of fatty acid lipids and sterols as well as the sterol//phosholipid ratio (Moore 1982, Kuiper 1985, Duxbury *et al.*1991). Physical and chemical changes in the lipid bilayer also regulate the functions of membrane proteins and may be the part of the signal transduction mechanism (Caldwell and Whitman 1987, van Blitterswijk *et al.* 1981). Lipid changes associated with changes in the morphology of rape callus (Williams *et al.* 1991) and shoot induction in cultures of *Datura innoxia* (Manoharan *et al.* 1987) and *Aegle mermelos* (Bhardwaj *et al.* 1995) have been reported previously. The main objective of the present study was to characterise changes that occur in the membrane lipids and sterols associated with induction and differentiation in *Brassica napus* var. *oleifera* (L.) callus cultures. For the first time such studies were performed dynamically, including a culture period from callus induction to shoot regeneration. Finally, we determined the differences in membrane composition between maternal seedlings and regenerated plantlets.

Materials and methods

Tissue cultures

Seeds of spring oilseed rape (*Brassica napus* L. var. *oleifera*, *cv.* Spok) were surface-sterilised with 25 % solution of commercial hypochloride ('Domestos') for 20 min, rinsed four times with sterile deionized water and aseptically germinated on MS medium (Murashige and Skoog 1962) at 25 °C with 16h/8h light/dark regime. Hypocotyls from 5-days-old seedlings were excised and cultured on MS medium supplemented with 1 $mg \cdot dm^{-3}$ BAP, 0.5 $mg \cdot dm^{-3}$ 2,4-D and 6% sucrose (MSA). 2-weeks cultured calli were isolated and transferred onto shoot induction medium i.e. MS supplemented with 3 $mg \cdot dm^{-3}$ BAP, 0.1 $mg \cdot dm^{-3}$ GA_3 and 3 % sucrose (MSB). Samples of four to five calli (aprox. 1 g) were randomly harvested at 2, 4, 6 weeks of culture (after 2 weeks induction, 2 and 4 weeks regeneration) and processed for lipid extraction. We determined also composition of membrane lipids of explant (hypocotyl of 5-day-old seedling) and regenerant (shoot of regenerated plantlet).

Lipid extraction and separation

Calli were homogenised in a Potter-Elveljem glass homogenizer in chloroform/methanol mixture (Bligh and Dyer 1959). After phase separation against 0.1 M KCl according to Flosh *et al.* (1957) and evaporation to dryness under stream of N_2 at 38 °C, lipids were stored at –50 °C under N_2. The total lipid extracts were then dissolved in chloroform and fractionated on a silica acid column (Koch-Light Laboratories Ltd, England, type 5030h, 325 mesh, activated earlier for 18 hours at 110 °C as described by Mills *et al.* (1989)). To improve the reproducibility, all separations were performed using the silica acid from the same pack.

Isolation of particular lipid fractions was performed according to Yoshida and Uemura (1984) with modifications described in Table 1.

Each lipid component was identified by co-chromatography with authentic lipid standards (Sigma) by TLC (Ryyppö *et al.* 1994). Particular fractions were then evaporated to dryness under N_2, dissolved in chloroform, and stored under N_2 at –50 °C.

Table 1. Isolation procedure of particular lipid fractions according to Yoshida and Uemura (1984) with minor modifications.

Fraction	Lipid class	Eluent	Volume [cm^3]
I	Free and esterified sterols	100 % chloroform then	9
		10 % acetone in chloroform	9
II	Acylated steryl glycoside and MGDG (if present)	50 % acetone in chloroform	10
III	Steryl glycoside and DGDG (if present)	100 % acetone	10
IV	Phospholipids and sulfolipids	100 % methanol	27

Analysis of fatty acids

The analyses of fatty acids from phospho- or glycolipids were performed according to modified procedure of Rivera and Penner (1978). Dried lipids were dissolved in 1 m^3 of 0.5 M KOH in methanol and heated for 5 minutes at 100 °C. After cooling, 100 mg of internal standard (C17:0; concentration: 4 mg per 1 cm^3 of methanol) and 1 cm^3 of 14 % BF_3 in methanol (Sigma) were added and the mixture was additionally heated at 100 °C for 2 minutes. Samples were then cooled down to room temperature and supplemented with 1 drop of saturated NaCl and 1 cm^3 of hexane. After phase separation by low-speed centrifugation, methyl esters of fatty acids were extracted to the upper hexane phase. The lower phase was washed twice by the same volume of hexane to improve extraction yield. The combined hexane phases were evaporated under N_2. Methyl esters of fatty acids were dissolved in 50 ml of hexane and then separated by gas chromatography (Hewlett Packard 5890 Series II) using capillary column GS-Aluminma (30 m length, 0.542 mm in diameter purchased from J&W Scientific). Temperature of separation: 200°C (carrier gas: He, 350 kPa). The content of particular fatty acid was defined on the basis of multilevel standard curve prepared from authentic standards purchased from Sigma.

Analysis of sterols composition

Sterol analyses were performed according to procedure of Brenac and Sauvaire (1996) with minor modifications. Sterols were acetylated (24 hours at room temperature) in 2 cm^3 of mixture: acetic anhydride - pyridine (2:1; v/v). Cholestan was used as internal standard (100 µg per sample). Samples evaporated to dryness under N_2, were dissolved in 100 µl of hexane and analysed by gas chromatography (Hewlett Packard 5890, Series II) using capillary column with liquid phase DB-1701 (15 m length, 0.254 mm in diameter) purchased from J&W Scientific. Temperature of separation: 270 °C (isotherm) and at 300 °C for inlet and outlet of detector (FID). Carrier gas: He (350 kPa). The content of particular sterols was determined on the basis of multilevel standard curve prepared from standards purchased from Sigma.

Statistical analysis

Results were statistically analyzed running the two-way analysis of variance (MANOVA). Post hoc comparisons were performed with the Duncan multiple range test (*Statistica 5.1*) at the 0.05 probability level.

Results and discussion

After initial small changes in the PL/GL ratio connected with callus induction, a decrease of the ratio was noted, especially in non-differentiating tissue two weeks after transfer to the regeneration medium (Table 1). This transfer was connected with changes in hormonal environment, the most important was the removal of 2,4-D, which is the factor inducing cell differentiation in the tissue, which have acquired earlier the state of the competence. However, in older tissue, the considerable increase of this parameter was observed, especially in non-differentiated calli.

First changes in the galactolipids composition were connected with callus induction (Table 1). Callus was characterised by decreased MGDG and increased DGDG components level as compared with maternal tissue. Further changes occurred after transfer to the regeneration medium: initiation of differentiation resulted in increase in the MGDG

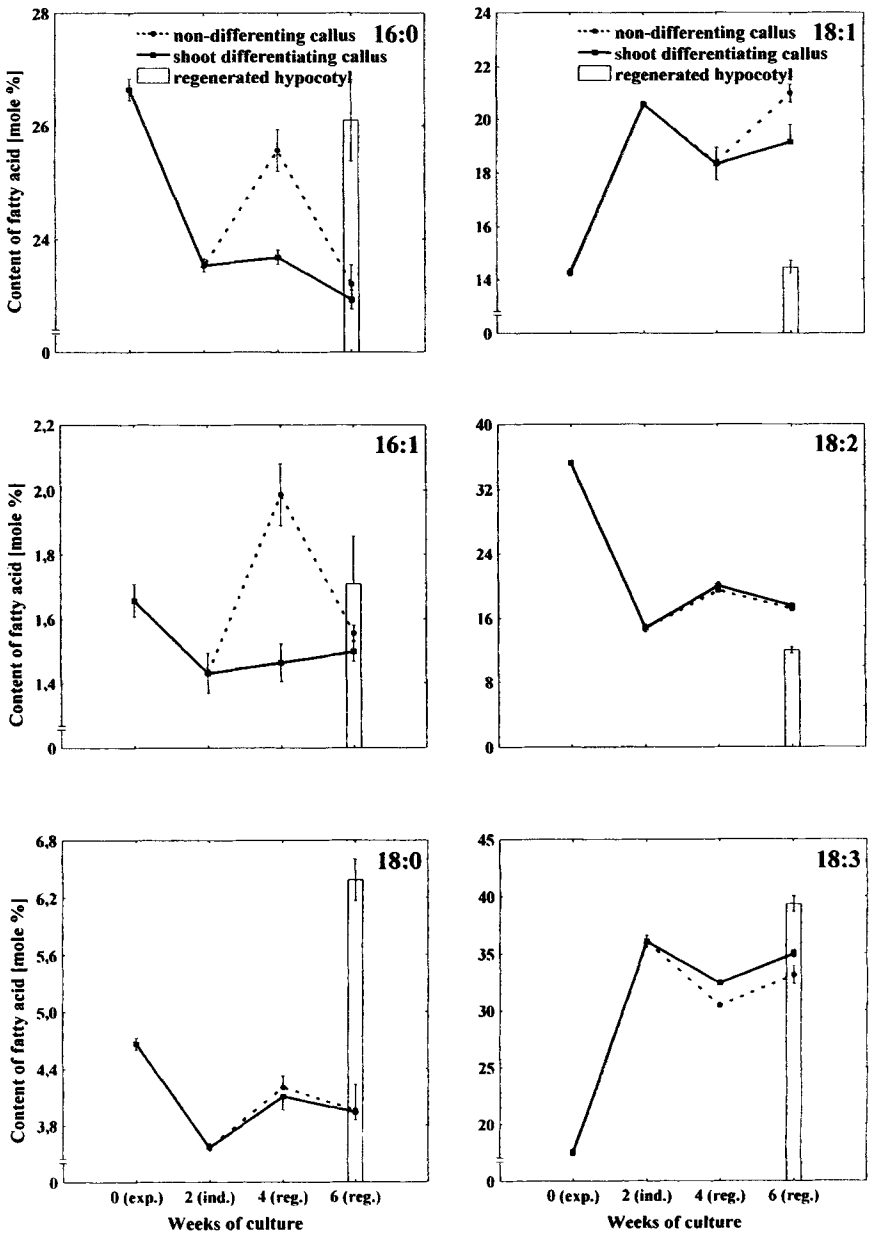

Fig. 1. The relative amounts (% of total) of fatty acids in PL fraction of *Brassica napus* cultures. Results are mean of six replicates with whiskers representing standard deviation.

component, whereas DGDG level remained fairly constant, which led to the increase in the MGDG/DGDG proportion (Table 1). Douce and Joyard (1980) suggest that such changes may indicate the development of chloroplasts with fully formed grana as MGDG represents up to 50 mol% of polar lipids in thylakoids, when DGDG is specifically localised in the envelope membranes. Similar changes were observed in cultures of tobacco, *Datura innoxia* (Manoharan *et al.* 1987), *Brassica napus* (Williams *et al.* 1991) and *Aegle marmelos* (Bhardwaj *et al.* 1995). Further culture totally changed lipid proportions but possibly due to significant increase of phospholipids component (Table 1).

Analyses of fatty acids composition of hypocotyl membrane revealed that phospholipids were mainly composed of linoleic (18:2) and palmitic acids (16:0), which reached a mean proportion of about 36 % and 27 %, respectively (Fig. 1). In galactolipids fraction predominated linolenic acid (18:3) although other acids like palmitic (16:0), linoleic (18:2) and oleic (18:1) were also present in significant amounts (Fig. 2, 3).

In both lipid classes, calli induction was associated with an increased degree of lipid unsaturation, mainly due to increase in 18:3 and 18:1 content (Table 2, Fig. 1-3). Nevertheless, because of concurrent reduction in linoleic acid (18:2) the double bond index (DBI) increased only in the PL fraction when in the GL fraction it remained almost unchanged (Table 2).

Subsequent culture, after calli transfer to the regeneration medium, revealed membrane involvement in induction of tissue differentiation since the response of calli varied considerably according to the differentiation degree. The most predominant effect could be seen in the DGDG fraction (Fig. 2), where significant differences between various calli types concerned almost all fatty acids. In differentiating calli considerable increase of 16:0, 16:1, and

18:0 content was observed, whereas the amounts of unsaturated C18 acids decreased (18:1 and 18:2) or remained almost unchanged (18:3) as compared with non-differentiating tissue. At the same time, in non-differentiating calli increased content of the 18:1 and 18:2 was noted. So, as a result of initiation of shoot regeneration increased degree of DGDG saturation and increase of 18:3/18:2 proportion took place. These results are comparable to those reported for *Aegle marmelos* cultures and suggest enhanced activity of enzymes desaturating dienoic to trienoic fatty acids during the development of chloroplasts (Bhardwaj *et al.* 1995).

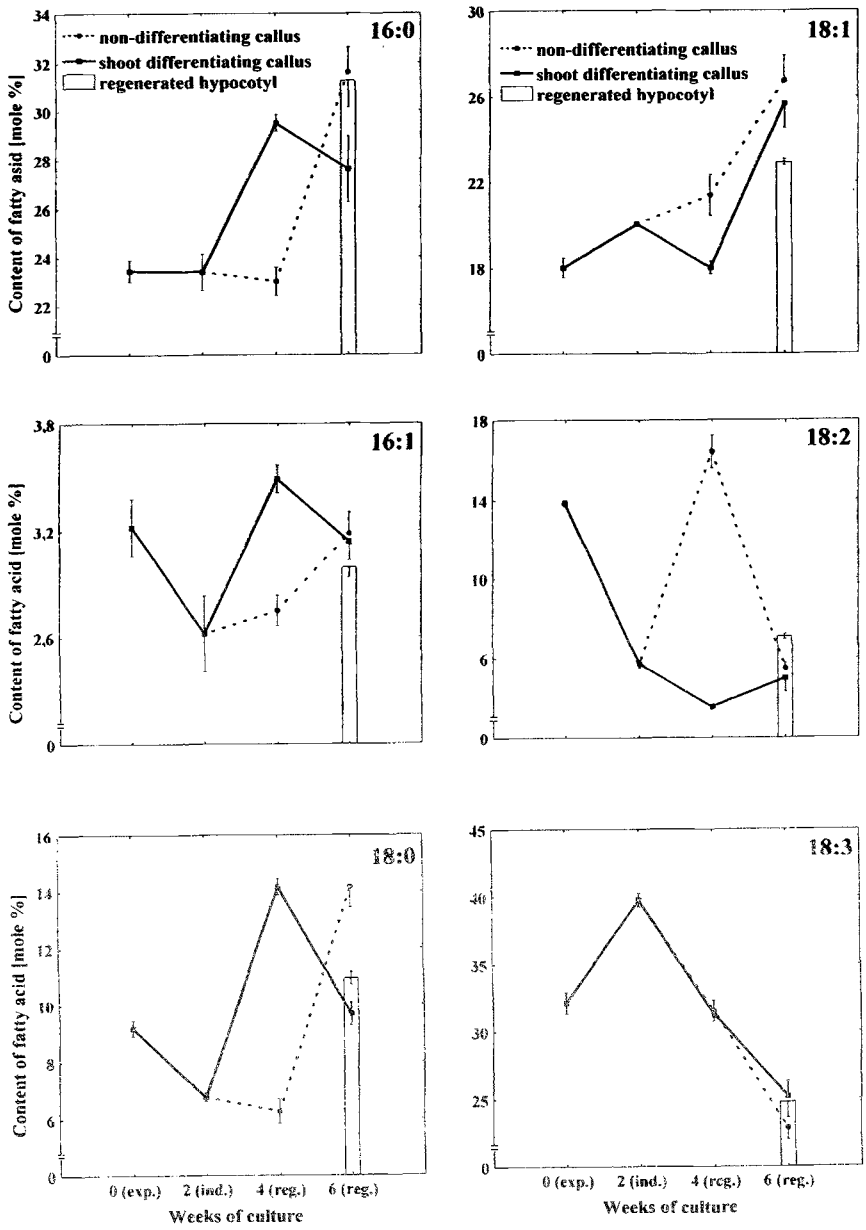

Fig. 2. The relative amounts (% of total) of fatty acids in DGDG fraction of *Brassica napus* cultures. Results are mean of six replicates with whiskers representing standard deviation.

As a result of transfer onto regeneration medium, non-differentiating calli increased their 16:1 and 16:0 amount in PL or 16:1 and 18:2 amount in MGDG fractions. In differentiating calli the amount of these acids remained almost constant (Fig. 1, 3). In the MGDG more differences, assumed to be associated with callus differentiation (reduced levels of 16:0 and 18:1 and increased amount of 18:3), appeared two weeks later, after four weeks culture on regeneration medium. As a result of these changes, proportion of 18:3/18:2 in the MGDG fraction increased in differentiating calli 2 weeks after passage to MSB medium. In phospholipids the proportion of saturated fatty acid predominated in non-differentiating tissues as compared with differentiating ones, but no differences were observed in values of DBI and 18:3/18:2.

Curiously in many cases, the differences in membrane fatty acids composition and saturation level between calli on various differentiation degrees were declined during subsequent culture, although the morphogenesis process was in progress.

The contents of the main phytosterols produced by *Brassica napus* callus on various differentiation degrees are given in Table 3. The analyses of the lipid fraction revealed an overwhelming predominance of stigmasterol (86 per cent of total free sterols).

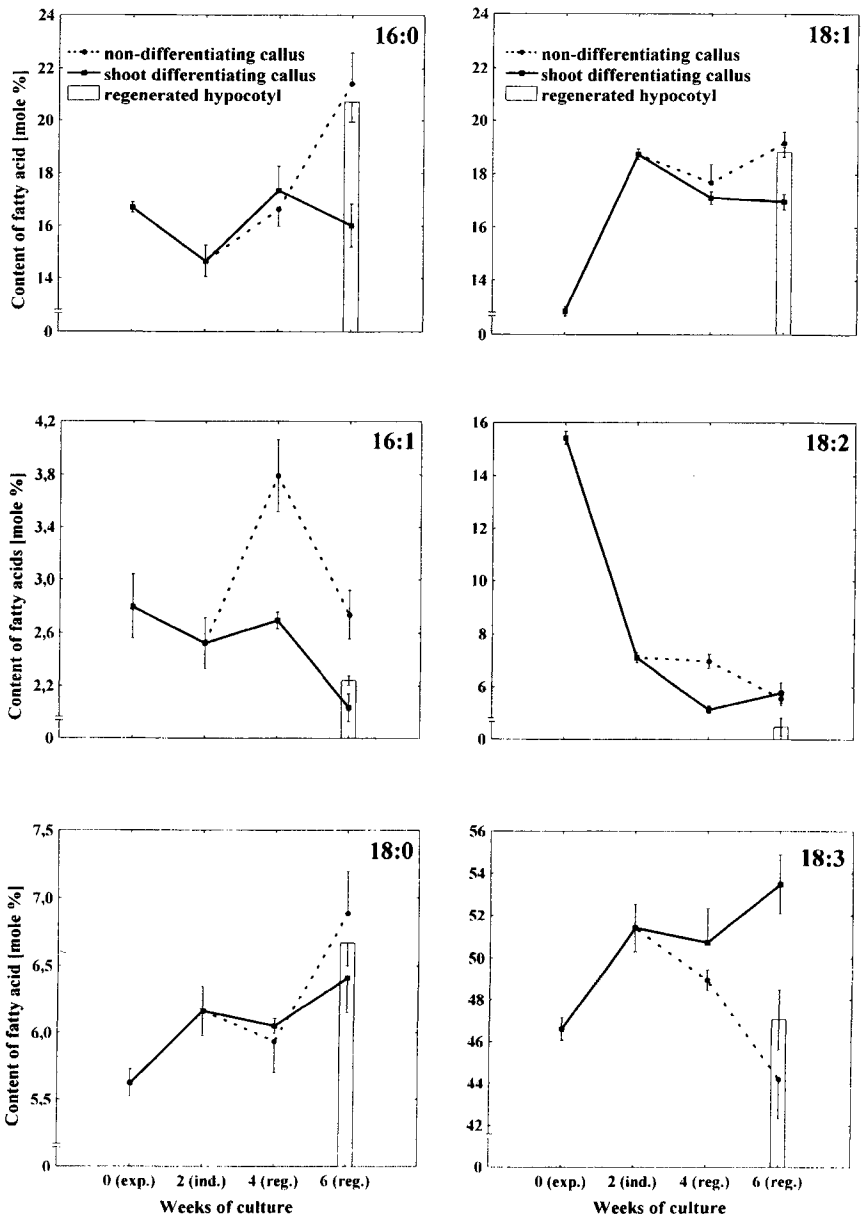

Fig. 3. The relative amounts (% of total) of fatty acids in MGDG fraction of *Brassica napus* cultures. Results are mean of six replicates with whiskers representing standard deviation.

Stigmasterol and campesterol together accounted for about 97% of total free sterols. Sitosterol was only a minor component, moreover synthesised in older tissue – so its presence seems to be connected rather with the age of tissue than with the differentiation degree. No traces of cholesterol were found. In shoot regenerating callus the synthesis and accumulation of campesterol decreased whereas content of stigmasterol rose. As a result the ratio of stigmasterol/sitosterol increased. Such effect was also observed in regenerated shoots and somatic embryos of flax (Cunha and Ferreira 1997). As membrane permeability increases in the order: cholesterol < campesterol < sitosterol < stigmasterol (Grunwald 1975) observed changes suggest enhancement of membrane fluidity. These changes might concern more specifically the plasmalemma than other cell membranes, since it contains most of free sterols of the cell (Hartmann *et al.* 1975).

These results imply the involvement of plant cell membranes in initiation and/or first stages of morphogenesis process. The mechanism for this involvement is still unknown and only speculations on its nature can be put forward. The observed differences in terms of membrane composition may eventually determine differences in membrane fluidity, permeability and affect activities of some membrane-bound enzymes.

As we know changes in membrane fluidity may be a part of the signal transduction system, may profoundly affect the binding of developmental regulatory proteins and/or action of membrane receptors (Wilson *et al.* 1991). Membrane permeability could affect the transport of metabolically important substances, and that this, in turn influence on course of tissue development.

However, even though particular biochemical changes in membrane properties may not play a role in morphogenesis they are still worthwhile studying as they could be useful as developmental

Table 2. Lipid composition [% total] of *in vitro* grown cultures of *Brassica napus* at various stages of differentiation. Amount of particular lipid class was determined on the basis of its fatty acid content. Means ± S_D for 5 replicates.
PL, phospholipids; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; PL/GL, ratio of phospholipids to glycolipids;

Plant material	PL	MGDG	DGDG	MGDG/DGDG	PL/GL
Seedling hypocotyl (explant)	85.0 ± 0.6	6.5 ± 0.1	8.5 ± 0.6	0.76	5.67
2 weeks old callus (non-differentiating)	84. 0 ± 2.0	5.2 ± 0.2	10.8 ± 1.9	0.48	5.25
4 weeks old callus (non-differentiating)	71.5 ± 2.5	6.4 ± 0.6	22.1 ± 1.9	0.29	2.51
4 weeks old callus (shoot differentiating)	80.1 ± 0.7	10.8 ± 0.3	9.1 ± 0.4	1.19	4.03
6 weeks old callus (non-differentiating)	94.5 ± 0.4	3.6 ± 0.2	1.9 ± 0.2	1.89	17.18
6 weeks old callus (shoot differentiating)	87.9 ± 0.7	3.8 ± 0.1	8.3 ± 0.8	0.46	7.26
shoot of regenerant	81.2 ± 0.1	5.5 ± 0.0	13.3 ± 0.1	0.41	4.32

markers and could be invaluable in understanding morphogenesis.

Regenerating shoot showed a lipid composition that resembled callus tissue rather then typical seedling hypocotyl (Table 2, Fig. 1-3). This result is in disagreement with data reported by Williams *et al.* (1991), where regenerating leaf tissue showed a lipid composition resembling typical plant leaves. On the other hand, Tattrie and Veliky (1973) concluded that lipids found in tissue culture were not necessarily comparable to those of the part of the intact plant from which the cell culture was been established. Lipid composition could be similar in different tissues and organella of plants growing in the same environmental condition, because plants can regulate their lipid composition to optimise the membrane structure and function.

The unsaturation degree of the PL fraction was similar in explants and regenerants, when in GL fraction significant differences were noted (Table 3). This effect possibly reflects the difference in chloroplasts development between autotrophic plant and plantlet regenerated from photomixotrophic callus culture.

Table 3. Changes in the saturation degree of membrane lipids of *Brasssica napus in vitro* cultures due to differentiation stages. Means ± S_D for 5 replicates. PL, phospholipids; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; DBI, Double Bound Index = Σ (molar % fatty acid content·number of double bounds)/100; U/S, ratio of unsaturated to saturated fatty acids

Plant material	U/S			DBI			18:3/18:2		
	PL	MGDG	DGDG	PL	MGDG	DGDG	PL	MGDG	DGDG
Seedling hypocotyl (explant)	2.19±0.04	3.48±0.14	2.1±0.1	1.39±0.04	1.86±0.06	1.44±0.18	0.50±0.03	3.03±0.16	1.99±0.53
2 weeks old callus (non-differentiating)	2.69±0.05	4.02±0.65	2.1±0.6	1.60±0.06	1.90±0.10	1.36±0.55	2.45±0.25	7.26±0.57	4.34±2.19
4 weeks old callus (non-differentiating)	2.36±0.14	3.46±0.42	2.0±0.7	1.51±0.04	1.82±0.07	1.33±0.36	1.57±0.07	7.07±0.74	2.52±0.91
4 weeks old callus (shoot differentiating)	2.60±0.07	3.19±0.58	1.3±0.1	1.57±.05	1.82±0.13	1.23±0.04	1.63±0.09	9.82±0.61	8.84±0.79
6 weeks old callus (non-differentiating)	2.69±0.20	2.57±0.41	1.2±0.2	1.56±0.08	1.66±0.17	0.97±0.24	1.93±0.14	8.01±1.27	3.51±1.44
6 weeks old callus (shoot differentiating)	2.72±0.06	3.68±0.67	1.7±0.2	1.61±0.05	1.91±0.13	1.26±0.25	2.0±0.05	9.45±1.59	6.47±1.44

Table 4. Free sterol composition of *Brassica napus* callus at various stages of differentiation. Means of three replicates ± S_D

Plant material	[% of total free sterols]		
	Campesterol	Stigmasterol	Sitosterol
4 weeks old callus, non-differentiating	11.46 ± 1.30	88.54 ± 1.30	0.0
4 weeks old callus, shoot differentiating	11.01 ± 0.40	88.99 ± 0.40	0.0
6 weeks old callus, non-differentiating	11.93 ± 0.66	84.68 ± 1.61	3.39 ± 1.26
6 weeks old callus, shoot differentiating	10.73 ± 0.79	86.34 ± 0.64	2.93 ± 0.78

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