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Low temperature-induced accumulation of eicosapentaenoic acids in *Marchantia polymorpha* cells

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Abstract

The composition of fatty acids in suspension-cultured cells from the liverwort *Marchantia polymorpha* L. grown at 25 and 15°C was analyzed. The liverwort cells grown at 25°C contained approximately 18% linolenic acid (18:3ω3), 11% arachidonic acid (20:4ω6) and 3% eicosapentaenoic acid (20:5ω3) as percentages of total fatty acids. When the cells were grown at 15°C, the relative amounts of 18:3 and 20:5 showed a large increase, while those of other fatty acids were less affected. Subcellular fractionation experiments showed that the levels of 20:4 and 20:5 were increased in the chloroplast fraction but not in the extrachloroplast fraction, while the level of 18:3 was increased in both fractions. Lipid analysis revealed that galactolipids were present in chloroplasts, PE (phosphatidylethanolamine) in the extrachloroplast fraction and PC (phosphatidylcholine), PG (phosphatidylglycerol), PI (phosphatidylinositol) and DAG (diacylglycerol) in both compartments. Of lipid species bound to 20:5, only MGDG (monogalactosyldiacylglycerol) and chloroplastic PC were increased by low temperature.

Keywords: *Marchantia polymorpha*; Marchantiaceae; Liverwort; Desaturation; Eicosapentaenoic acid; Fatty acids; Lipids

1. Introduction

In higher plants and cyanobacteria, the physiological functions of polyunsaturated fatty acids (PUFAs) such as linolenic acid (18:3ω3) have been shown to include stabilization of membranes at low temperature (Harwood, 1996; Ohlrogge & Jaworski, 1997). Unlike higher plants, lower plants including bryophytes have C20 PUFAs, such as arachidonic acid (20:4ω6) and eicosapentaenoic acid (20:5ω3) (Karunen, 1990). C20 PUFAs on some eukaryotic algae (Schneider & Rossler, 1994; Khozin & Cohen, 1996; Cohen, Shiran, Khozin & Heimer, 1997) and the moss *Physcomitrella patens* (Girk, Schmidt, Zäringer, Reski & Heinz, 1998) have been indicated to be synthesized in some manner differing from that of C18 PUFAs in higher plants. Although information about the biosynthetic pathway of these acids is accumulating, their physiological function remains unknown.

In higher plants it is well known that fatty acid synthesis occurs in various subcellular compartments, such as chloroplasts, endoplasmic reticulum and mitochondrial membranes (Browse & Somerville, 1991). In lower plants including bryophytes, however, subcellular localization of fatty acids is poorly understood (Karunen, 1990). We assumed that suspension-cultured cells from the liverwort *Marchantia polymorpha* were suitable for studying the subcellular distribution of fatty acids in that they contained mature chloroplasts even in the organic medium (Ono, 1973). Although fatty acid compositions of *M. polymorpha* cells and the effect of nutritional and light conditions on these cells have been reported (Shinmen, Katoh, Shimizu, Jareonkitmongol & Yamada, 1991), their response to temperature remains unknown.

To study the physiological role of C20 PUFAs in bryophytes, we determined changes in the level of fatty acids in *M. polymorpha* cells on transfer to low tem-

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Table 1
Fatty acid composition in the lipid classes extracted from *M. polymorpha* cells grown at 25°C. Results are the average of three assays ± S.D

	Lipid composition (mol%)	Fatty acid composition (mol%)							
		16:0	16:1	18:0	18:1	18:2 (ω 6)	18:3 (ω 3)	20:4 (ω 6)	20:5 (ω 3)
Simple lipids									
TAG	10.1 ± 0.1	26.4 ± 0.1	3.8 ± 0.1	24.9 ± 0.4	6.6 ± 0.1	15.7 ± 0.6	10.4 ± 0.1	10.1 ± 0.6	2.1 ± 0.2
DAG	4.3 ± 0.1	35.2 ± 0.4	6.6 ± 0.2	23.0 ± 0.4	14.3 ± 0.2	7.3 ± 0.2	3.5 ± 0.2	9.5 ± 0.3	0.6 ± 0.1
Galactolipids									
MGDG	25.6 ± 0.2	17.8 ± 0.2	2.2 ± 0.3	3.1 ± 0.2	3.5 ± 0.3	22.9 ± 0.1	48.5 ± 0.2	4.1 ± 0.1	0.6 ± 0.0
DGDG	5.3 ± 0.0	58.6 ± 0.7	2.9 ± 0.6	6.7 ± 0.1	4.4 ± 0.0	15.9 ± 0.2	8.2 ± 0.1	3.3 ± 0.2	–
SQDG	8.8 ± 0.0	59.4 ± 0.5	3.6 ± 0.1	5.9 ± 0.1	5.2 ± 0.0	15.0 ± 0.4	7.9 ± 0.1	3.0 ± 0.0	–
Phospholipids									
PC	14.7 ± 0.1	51.7 ± 0.1	3.9 ± 0.1	10.1 ± 0.1	3.3 ± 0.1	13.3 ± 0.0	5.1 ± 0.0	9.8 ± 0.1	1.8 ± 0.0
PE	10.4 ± 0.0	44.2 ± 0.4	1.4 ± 0.1	7.2 ± 0.9	4.2 ± 0.1	9.3 ± 0.2	5.6 ± 0.0	21.8 ± 0.1	6.3 ± 0.3
PG	4.6 ± 0.3	54.6 ± 0.7	3.4 ± 0.6	9.7 ± 0.2	7.7 ± 2.4	14.3 ± 0.8	5.4 ± 0.2	4.5 ± 0.3	0.4 ± 0.1
PI	16.2 ± 0.1	39.3 ± 0.1	1.2 ± 0.0	36.3 ± 0.2	2.3 ± 0.1	11.2 ± 0.1	4.5 ± 0.0	4.1 ± 0.0	0.7 ± 0.0
All		37.6 ± 0.3	0.9 ± 0.2	1.2 ± 0.0	3.5 ± 0.1	25.4 ± 0.1	18.3 ± 0.0	10.5 ± 0.3	2.6 ± 0.1

perature. We show here that low temperature increased the relative amounts of 20:5ω3 and 18:3ω3 in the liverwort cells and that low temperature-induced 20:5ω3 was predominantly accumulated in chloroplasts.

2. Results

The amounts and composition of fatty acids in *M. polymorpha* cells grown at 25°C (Table 1) were the same as those previously reported in *M. polymorpha* cells (Shinmen et al., 1991) and the parental plants (Gellerman, Anderson & Schlenk, 1972), except for 16:3. In our experiments the value of 16:3 was omitted because of poor separation of 16:3 with the HPLC column. Therefore, in the present experiments we focused on the changes in C18 and C20 fatty acids by low temperature. To know the response of cell growth to low temperature, the cells grown at 25°C were transferred to 15°C and subcultured. As shown in Fig. 1, the cells started to grow after the lag for 3 days on the first transfer to 15°C. After the second subculture, the cells grew without lag time (data not shown). The growth rate on the 4th subculture at 15°C was the same as that on the first and was slightly lower than that at 25°C. These facts indicate that the liverwort cells can rapidly adapt themselves to grow at 15°C. To determine the response of fatty acid composition to low temperature, their fatty acid compositions were analyzed during 10 subcultures (Table 2). Low temperature-induced changes in fatty acids were shown by the amount per cell dry weight and by the mol% of total fatty acids. The amounts of 16:0, 18:2, 18:3ω3, 20:4ω6 and 20:5ω3 were gradually increased during subculture at 15°C. However, on the basis of mol%, a large increase was found only in 18:3 and 20:5, while the levels of other fatty acids were less affected or decreased.

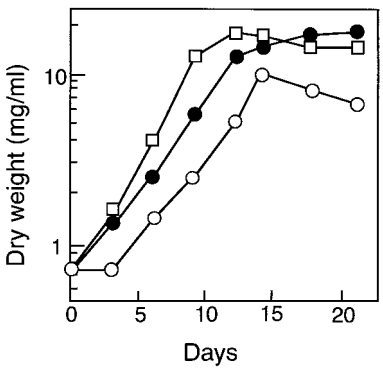

Fig. 1. Cell growth at 25 and 15°C. The cells grown at 25°C were transferred to 15°C and subcultured. The cell growth was determined on the culture at 25°C (□), on the 1st transfer to 15°C (○) and on the 4th subculture at 15°C (●). All points are means of duplicate determinations.

The relative amounts of fatty acids among lipid classes in cells grown at 25°C and in cells from the 4th subculture at 15°C were determined and the ratios of mol% at 15°C to mol% at 25°C are represented in Table 3. Low temperature-induced increase in 20:5 was found in MDGD, while an increase in 18:3 was found in several lipids. This fact indicates that the synthesis of 18:3 and 20:5 may be differentially regulated by low temperature. MGDG and PE are known to be exclusively localized in chloroplast and in cytoplasm, respectively (Browse & Somerville, 1991). As shown in Table 3, the level of 20:5 in MGDG was greatly increased by low temperature, while that in PE was less affected, indicating that low temperature preferentially induced 20:5 accumulation in the chloroplast. To confirm this, fatty acid composition was determined in

Table 2
Changes in contents and compositions of fatty acids during subculture. The cells grown at 25°C (subculture 0) were transferred to 15°C and subcultured 10 times. The amounts of fatty acids are shown as μmol/g dry weight and as mol% of the total fatty acids. All values are means of triplicate determinations. The value in the parenthesis means the ratio of the value of the subculture at 15°C to that of subculture 0

Subcultures at 15°C	16:0	16:1	18:0	18:1	18:2 (ω 6)	18:3 (ω 3)	20:4 (ω 6)	20:5 (ω 3)
μmol/g dry weight								
0	24.4	0.1	2.1	0.9	14.8	11.5	7.5	2.1
1	39.5 (1.6)	0.1	3.2 (1.5)	5.1 (5.7)	20.5 (1.4)	21.2 (1.8)	14.3 (1.9)	4.2 (2.0)
2	31.2 (1.3)	0.2	3.1 (1.5)	3.5 (3.9)	14.5 (1.0)	18.5 (1.6)	10.2 (1.4)	9.2 (4.4)
3	43.5 (1.8)	0.1	2.6 (1.2)	0.9 (1.0)	17.0 (1.2)	35.3 (3.1)	18.6 (2.5)	12.3 (5.9)
10	48.3 (2.0)	0.1	2.5 (1.2)	0.8 (0.9)	30.5 (2.1)	42.3 (3.7)	18.3 (2.4)	18.6 (8.9)
mol%								
0	37.5	0.0	2.4	2.3	24.5	17.3	13.5	2.5
1	38.5 (1.0)	0.1	2.5 (1.0)	3.1 (1.3)	19.5 (0.8)	19.2 (1.1)	10.5 (0.8)	6.6 (2.6)
2	33.3 (0.9)	0.3	1.8 (0.8)	3.3 (1.4)	17.2 (0.7)	24.1 (1.4)	10.8 (0.8)	9.2 (3.7)
3	38.0 (1.0)	0.1	0.4 (0.2)	0.2 (0.1)	13.3 (0.5)	30.5 (1.8)	9.0 (0.7)	8.5 (3.4)
10	34.0 (0.9)	0.2	0.0 (0.0)	0.0 (0.0)	14.3 (0.6)	33.0 (1.9)	9.2 (0.7)	9.3 (3.7)

chloroplast and extrachloroplast fractions. Intact chloroplasts were isolated from the cells at the 4th subculture at 15°C and the cells at 25°C, osmotically ruptured and washed with centrifugation. The resultant chloroplast fraction included thylakoid membranes and, presumably, chloroplast envelopes. The lipids in both fractions were isolated and mol% of fatty acids in each lipid was determined. Typical results of the ratio of mol% at 15°C to mol% at 25°C in chloroplast and extrachloroplast fractions were represented on the basis of mol% of total C18 fatty acids (18:0, 18:1, 18:2 and 18:3) and C20 fatty acids (20:4 and 20:5) (Fig. 2A) and on the basis of mol% of each fatty acid (Fig. 2B). The steady state levels of 18:3 and 20:5 are considered to be dependent not only on the activity of desaturase but also on the rate of synthesis of C18 and C20 fatty acids, respectively. With respect to C18 fatty acids, the activities of fatty acid synthesis and desaturation can be estimated from Fig. 2A and B, respectively. In both fractions the amount of total C18 fatty acids was not affected by low temperature in total lipids or any lipid class (Fig. 2A; total). However, 18:3 in total lipids was increased in both fractions (Fig. 2B; total). The increase in chloroplast 18:3 in total lipids is due to increase in 18:3 in PC and DAG, while the increase in the extrachloroplast fraction is due to an increase in extrachloroplast PG. Unlike C18 fatty acids, an increase of total C20 fatty acids in total fatty acids was found only in the chloroplast (Fig. 2A). Among lipid classes, the increase in 20:4 and 20:5 was found only in MGDG and chloroplastic PC.

Table 3
Low temperature-induced changes in fatty acid composition. Lipids were extracted from cells grown at 15 and 25°C. Fatty acid contents in each lipid were determined as mol%. Results are the average of three assays ± S.D

	Increase in total fatty acids (mol% at 15°C/mol% at 25°C)	Increase in fatty acids (mol% at 15°C/mol% at 25°C)							
		16:0	16:1	18:0	18:1	18:2 (ω 6)	18:3 (ω 3)	20:4 (ω 6)	20:5 (ω 3)
Simple lipids									
TAG	0.5 ± 0.0	1.1 ± 0.0	1.5 ± 0.0	0.7 ± 0.0	1.6 ± 0.0	0.9 ± 0.0	1.0 ± 0.0	0.9 ± 0.1	1.0 ± 0.1
DAG	0.7 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	0.7 ± 0.0	0.9 ± 0.0	2.2 ± 0.1	1.0 ± 0.1	0.7 ± 0.1
Galactolipids									
MGDG	1.6 ± 0.0	1.1 ± 0.0	1.2 ± 0.2	1.2 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	1.3 ± 0.0	2.4 ± 0.0	4.0 ± 0.3
DGDG	1.5 ± 0.0	0.8 ± 0.0	1.3 ± 0.2	1.5 ± 0.0	0.9 ± 0.1	0.9 ± 0.0	1.7 ± 0.1	1.8 ± 0.1	–
SQDG	1.5 ± 0.0	0.7 ± 0.0	1.0 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	1.2 ± 0.0	2.4 ± 0.0	1.8 ± 0.1	–
Phospholipids									
PC	0.6 ± 0.0	1.0 ± 0.0	0.6 ± 0.0	1.0 ± 0.0	1.6 ± 0.0	0.9 ± 0.0	1.5 ± 0.0	1.1 ± 0.0	1.3 ± 0.1
PE	0.7 ± 0.0	1.1 ± 0.0	2.5 ± 0.2	1.7 ± 0.2	0.9 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	1.0 ± 0.0	0.8 ± 0.0
PG	1.8 ± 0.1	1.0 ± 0.0	1.5 ± 0.2	1.1 ± 0.0	0.6 ± 0.1	0.9 ± 0.0	2.2 ± 0.0	0.9 ± 0.1	–
PI	0.4 ± 0.0	1.1 ± 0.0	2.7 ± 0.1	0.3 ± 0.0	1.9 ± 0.1	1.2 ± 0.0	3.2 ± 0.0	1.8 ± 0.1	1.9 ± 0.4
All		0.9 ± 0.0	1.0 ± 0.3	0.9 ± 0.0	0.5 ± 0.0	0.8 ± 0.0	1.5 ± 0.0	1.1 ± 0.0	1.2 ± 0.0

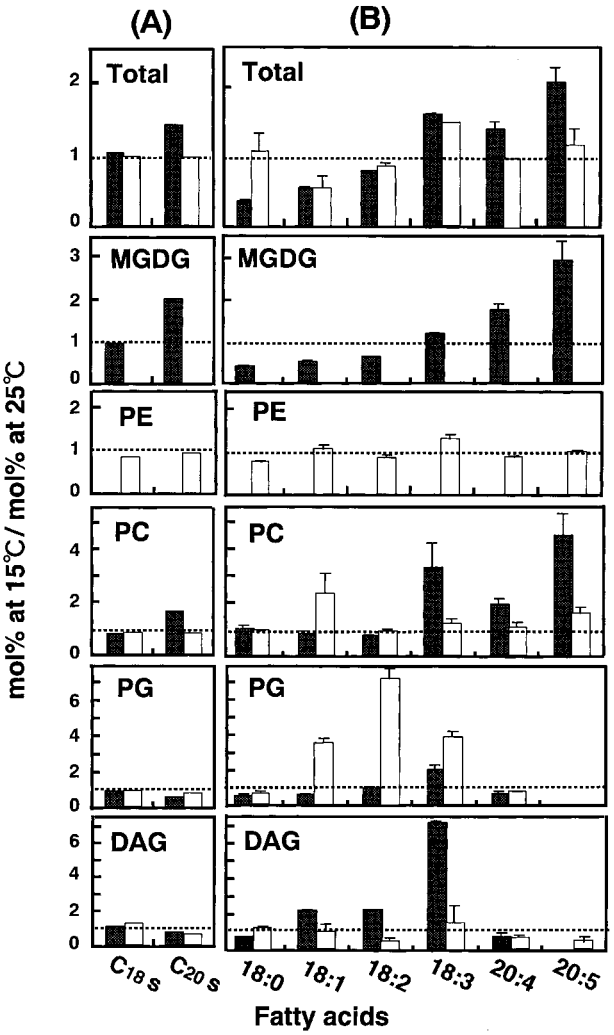

Fig. 2. Low temperature-induced changes in the relative amount of fatty acids in chloroplast and extrachloroplast fractions. Chloroplast (black bar) and extrachloroplast (white bar) fractions were prepared from cells grown at 15°C at the 4th subculture and grown at 25°C. The lipids were isolated from each fraction and their fatty acid contents were determined as mol%. (A) The ratio of the sum of mol% of 18:0, 18:1, 18:2 and 18:3 (C18s) and the sum of 20:4 and 20:5 (C20s) at 15°C to those at 25°C. (B) The ratio of mol% of each fatty acid at 15°C to that at 25°C. Legend: MGDG: monogalactosyldiacylglycerol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PG: phosphatidylglycerol; DAG: diacylglycerol.

3. Discussion

The present experiments demonstrated that in liverwort cells grown at low temperature, accumulation of C20 PUFAs was stimulated only in chloroplast lipids, while that of C18 PUFAs was stimulated in both chloroplast and extrachloroplast lipids. The former result is inconsistent with the results from the protonema culture of the moss *Ceratodon purpureus*, in which an increase in 20:5ω3 by a shift of growth temperature from 25 to 4°C was detected in PE and MGDG, which are a cytosolic lipid and the major lipid of chloroplasts, respectively (Aro, Somersalo & Karunen, 1987). In the moss *Leptobryum pyriforme*, furthermore, the amount of 20:5ω3 was considerably increased in PE after transferring the protonema from normal growth conditions to a temperature of 1°C (Hartmann, Beutelmann, Vandekerkhove, Euler & Kohn, 1986). In the present experiments, the growth temperature was shifted from 25 to 15°C. Further experiments are required if an additional desaturation may occur at a temperature lower than 15°C and for a prolonged subculture.

As shown in Table 3 and Fig. 2B, low temperature-induced accumulation of 20:5ω3 bound MGDG exceeded that of other thylakoid lipids, suggesting that low temperature-induced accumulation of 20:5ω3 is involved in the adaptation of thylakoid membranes to low temperature. As shown in Table 2, the mol% of 18:3ω3 was gradually increased through subcultures at 15°C while that of 20:5ω3 maintained the same level after the second subculture. These facts indicate that 20:5ω3 has a specific role at an early stage during adaptation of liverwort cells to low temperature.

In relation to the biosynthetic pathway of C20 PUFAs, feeding external fatty acids to red algae, *Porphyridium cruentum* and eustigmatophyte, *Monodus subterraneus*, indicated the existence of two possible pathways (Khozin & Cohen, 1996):

1. 18:2ω6 → 18:3ω6 → 20:3ω6 → 20:4ω6 → 20:5ω3,
2. 18:2ω6 → 18:3ω3 → 18:4ω3 → 20:4ω3 → 20:5ω3.

In the major ω6 pathway (1), 18:2ω6-PC is sequentially converted to 20:4ω6-PC by a sequence including a Δ6 desaturase, an elongation system and a Δ5 desaturase. In the minor ω3 pathway (2), 18:2ω6-PC is presumably desaturated to 18:3ω3 which is sequentially converted by the enzymatic sequence of the ω6 pathway to 20:5ω3-PC. Destruction of the Δ6 desaturase gene in the moss, *Physcomitrella patens*, also supported this scheme, although species of lipids were not determined (Girk et al., 1998). Unlike these algae, however, the moss was unable to convert 20:4ω6 to 20:5ω3 in that the moss mutant recovered 20:4ω6 production but did not 20:5ω3 by supplementation with 20:3ω6 (Girk et al., 1998). According to this scheme, the biosynthetic pathway of 20:4ω6 and 20:5ω3-MGDG in the liverwort, *M. polymorpha*, can be suggested as follows:

1. cytosol (18:2ω6-PC → → → 20:4ω6-PC)rarr(20:4ω6-PC → 20:4ω6-DAG → 20:4ω6-MGDG) chloroplast,
2. cytosol (18:2ω6-PC → 18:3ω3-PC → → → 20:5ω3-PC) → (**20:5ω3-PC** → 20:5ω3-DAG → **20:5ω3-MGDG**) chloroplast.

Fatty acid desaturases are suggested to operate in a PC-bound form in cytosol and PG- and a galactolipid-bound form in chloroplasts (Browse & Somerville, 1991; Harwood, 1996). At low temperature, chloro-

plastic 20:5ω3-PC was greatly increased compared to that in chloroplastic 20:4ω6-PC (Fig. 2). In addition, chloroplastic 18:3ω3-MGDG was not affected by a low temperature, while chloroplastic 18:3ω3-PC and 18:3ω3-DAG were increased. These facts indicate that low temperature activated the cytosolic ω3-desaturase resulting in an increase in 20:5ω5-PC and 20:5ω3-MGDG, but did not affect chloroplastic ω3-desaturases. In eukaryotic algae, the PC-bound fatty acids in cytosol were indicated to be exported to chloroplasts in the form of DAG, then galactosylated (Khozin & Cohen, 1996). However, in *M. polymorpha* cells considerable amounts of PC-bound 20:4ω6 and 20:5ω3 were found in both cytosolic and chloroplast fractions (Fig. 2). The contamination of cytosolic membranes in the chloroplast fraction is improbable, because PE was absent from the chloroplast fraction and a low temperature-induced increase in 20:5ω3-PC was found only in the chloroplast fraction. At present, the reason is unknown, although detection of 20:5ω3 and 18:3ω3-PC in the chloroplast fraction was also reported in the moss *C. purpureus* (Aro et al., 1987).

The ability to desaturate C20 fatty acids is considered to have been lost during the evolution of plants, since C20 PUFAs are generally absent in higher plants (Karunen, 1990). The present experiments indicate that in liverwort cells 20:5 plays a specific role in the adaptation of chloroplast membranes at low temperature. Further characterization of the biosynthetic mechanism of 20:5ω3 will provide additional information in the physiological role of C20 PUFAs in bryophytes.

4. Experimental

4.1. Plant material

Cells of *Marchantia polymorpha* L. (A-18) (Ono, 1973) were propagated in MS medium by shaking on a gyratory shaker at 110 rpm at 25 ± 1°C in the light and subcultured at biweekly intervals in 20 ml of fresh MS medium as described previously (Harashima & Ono, 1991). For growth at low temperature, cells at the stationary phase were transferred to fresh medium and incubated at 15°C under the same conditions as described above.

4.2. Subcellular fractionation

The chloroplast and extrachloroplast fractions were prepared at 4°C by the method of Murata, Sato, Takahashi & Hamazaki (1982). 20 g of fresh cells were ground in a mortar with 40 ml of the grinding buffer (50 mM Na/K–phosphate buffer, pH 7.2 and 0.4 M sucrose). The homogenate was filtrated through miracloth and the filtrate was centrifuged at $2000 \times g$ for 8 min. The supernatant was used as the extrachloroplast fraction. The precipitate was resuspended with the same grinding buffer and centrifuged at $200 \times g$ for 30 s. The resultant supernatant was recentrifuged at $2500 \times g$ for 5 min. Chloroplasts in the precipitate were resuspended in 5 mM Na/K–phosphate buffer (pH 7.2) and the suspension was centrifuged at $20{,}000 \times g$ for 30 min. The pellet was used as the chloroplast fraction.

4.3. Lipid isolation

Lipids were extracted with methanol/chloroform (2:1) at 1°C (Bligh & Dyer, 1959). For analysis of lipid classes, lipids were separated by TLC on silica gel plates (Merck 5715) with chloroform/methanol/28% ammonia (65:35:5, v/v) in the first dimension and with chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v) in the second dimension. Spots were detected with primuline under UV (366 nm) and eluted with methanol. For identification, phospholipids were stained with molybdenum blue (Dittmer & Lester, 1964), glycolipids with anthrone (Heinz, 1967), amino groups with ninhydrin (Fahmy, Niederwieser, Pataki & Brenner, 1961) and quaternary amino groups with Dragendorff reagent (Munier & Macheboeuf, 1951). TAG (triacylglycerol) and DAG were separated from polar lipids and pigments by TLC with hexane/diethyl ether/acetic acid (80:30:1, v/v).

4.4. Fatty acid analysis

The fatty acid solution (30 μl) was mixed with an equal volume of 5% (w/v) 9-anthryldiazomethane solution and incubated at room temperature for 30 min. Then, the solution was applied to an HPLC column of TSK-GEL OCTYL-80TS (25 cm × 4.6 mm) (Shimadzu Seisakusho Ltd., Kyoto) and eluted with acetonitrile/water (9:1, v/v) at 40°C at the flow rate of 1 ml/min (Nimura & Kinoshita, 1980). Fluorescence from fatty acids was measured at 412 nm, with excitation at 365 nm.

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