

Mobilization of arachidonyl moieties from triacylglycerols into chloroplastic lipids following recovery from nitrogen starvation of the microalga *Parietochloris incisa*

Inna Khozin-Goldberg, Pushkar Shrestha, Zvi Cohen *

The Microalgal Biotechnology Laboratory, The Albert Katz Department for Desert Biotechnologies, The Jacob Blaustein Institutes for Desert Research, Ben Gurion University of the Negev, Sde-Boker Campus 84990, Israel

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Abstract

The microalga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta) was isolated from an alpine environment. It was found to accumulate unusually high amounts of arachidonic acid (AA)-rich TAG. We have hypothesized that microalgal PUFA-rich TAG might have a role as a depot of PUFA, which could be mobilized for the construction of chloroplastic membranes under sudden changes in environmental conditions. We have thus studied the changes in lipid and fatty acid composition during recovery from nitrogen starvation at 24 and 12 °C. At both temperatures, TAG was mainly consumed to support growth, however, there was a significant increase in the content of AA in the chloroplastic lipids, predominantly, monogalactosyldiacylglycerol (MGDG) at 24 °C, but much less so at 12 °C. Similar results were obtained using radiolabeled precursors. These and other findings point to the existence of three modes of operation for the construction of chloroplastic lipids that the alga can utilize to support growth under changing environmental conditions. When environmental conditions do not support growth, the prokaryotic pathway predominates. When sudden changes occur, the eukaryotic pathway is enhanced and can be even further augmented by influx of acyl moieties from TAG to maximize the exploitation of growth conditions that may possibly be transitory.

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1. Introduction

Arachidonic acid (AA, 20:4 ω 6) is a very long-chain polyunsaturated fatty acid (VLC-PUFA) that is a major component that is necessary for the development of brain cell membranes and retina in infants [1]. AA is the predominant VLC-PUFA of human breast milk and preterm babies require an external supply of AA for normal development, if they are not breast-fed. AA is also a precursor of several eicosanoids that regulate critical biological functions [2].

Abbreviations: DAG, Diacylglycerols; DGDG, digalactosyldiacylglycerol; DGTS, diacylglyceroltrimethylhomoserine; EPA, eicosapentaenoic acid; FFA, free fatty acids; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerols

* Corresponding author. Fax: +972 8 6596801.

E-mail address: cohen@bgu.ac.il (Z. Cohen).

Many microalgal species were found to contain VLC-PUFA as constituents of their polar lipids, however, the content of polar lipids is intrinsically limited [3,4]. Oleaginous algae are able to accumulate neutral lipids, mainly TAG, up to 86% of their total cell dry weight, in response to environmental stresses such as nitrogen limitation, salinity or high temperature [5–8], however, their TAG is composed mainly of saturated and monounsaturated fatty acids [9]. The oleaginous freshwater alga *Parietochloris incisa* (Chlorophyceae) has been found to accumulate an unprecedented high content of AA-rich TAG in cytoplasmic lipid bodies [10,11]. Under nitrogen starvation, over 95% of cellular AA was deposited in TAG, comprising about 60% of fatty acids [12].

Despite the potential economic importance, there have been relatively few studies on TAG-rich lipid-body accumulation in microalgae [13]. It is generally accepted that the

major role of TAG is to store carbon and energy to support growth when conditions become favorable. However, recent data indicate alternative roles of oil bodies, including various aspects of lipid trafficking [13]. Cohen et al. [14] and Bigogno et al. [11] suggested that microalgal TAG could have a role as a depot of PUFA, which can be mobilized for the construction of chloroplastic membranes under certain environmental conditions. Radiolabelling studies of the red microalga *Porphyridium cruentum* have shown that labeled AA, accumulated in TAG, was transferred to the major lipid classes constituting chloroplast membranes, predominantly monogalactosyldiacylglycerol (MGDG) [15,16]. Moreover, a mutant of *P. cruentum*, which was impaired in its ability to transfer label from TAG, showed a decrease in the proportion of the major molecular species, 20:5/20:5, of MGDG and a reduced growth at low temperatures [16]. Similarly, when *P. incisa* was transferred from optimal temperature (25 °C) to low temperatures (12 °C and 4 °C), labeled AA was transferred from TAG to polar lipids [11]. We have thus suggested that AA could be reincorporated into membranal lipids, enabling the alga to swiftly adapt to the low-temperature-induced stress [4].

In higher plants and many green algae, chloroplastic lipids are divided into two types: prokaryotic lipids are characterized by the presence of C₁₆ fatty acids at the *sn*-2 position and either C₁₆ or C₁₈ fatty acids at the *sn*-1 position of their glycerol skeletons (16/16 and 18/16, respectively), whereas eukaryotic lipids contain C₁₈ fatty acids at both positions (18/18). The C₁₈ acyl moieties of the prokaryotic lipids are entirely produced in the chloroplast while eukaryotic molecular species are produced using extrachloroplastic lipids and only the last desaturation step is chloroplastic [17]. We have suggested that the molecular species of the chloroplastic lipids of VLC-PUFA-containing microalgae can be similarly divided into prokaryotic-like, e.g., 20/16 and eukaryotic-like, e.g., 20/20. As we have found out later, these molecular species resemble those of higher plants in structure but differ in their biosynthetic origin. In every microalgal strain studied, the lipid-linked biosynthesis of VLC-PUFA, up to the stage of C₂₀ PUFA, is exclusively extra-chloroplastic [18].

In an attempt to elucidate biosynthetic pathways allowing rapid remodeling of lipid composition, we have analyzed the fatty acid composition of all the major lipid classes and the molecular species composition of MGDG. We have also followed the redistribution of label of fatty acids in both neutral and polar lipids of *P. incisa* after radiolabelling with oleic acid and recovery from nitrogen starvation at normal (24 °C) and low (12 °C) temperatures. Our findings show that when growth conditions are resumed at 24 °C, a rapid remodeling process of MGDG takes place, importing AA from TAG. At 12 °C, the significance of these processes was lower and the major adaptation mechanism consisted of almost complete ω3 desaturation of 16:2- and 18:2-containing molecular species. The findings support the role of TAG as a depot of PUFA that can be used for rapid deployment.

2. Materials and methods

2.1. Growth condition

Cultures of *P. incisa* were batch cultivated on BG-11 nutrient medium in glass columns under controlled temperature (24 °C) and light conditions (170 μmol quanta m⁻² s⁻¹), as previously described [10]. Continuous bubbling of an air-CO₂ mixture (99:1, v/v) was provided from the bottom of the column. For nitrogen starvation experiments, cultures in the early stationary phase (6 days) were resuspended and maintained in nitrogen-free BG11 medium for 14 days at 24 °C and 12 °C. NaNO₃ was omitted from the medium and ferric ammonium citrate was substituted by ferric citrate. Recovery was initiated by resuspending the cells in four volumes of complete medium. For radiolabelling studies, cultures were grown in 150 mL Erlenmeyer flasks under an air/CO₂ (99:1) atmosphere, in an incubator-shaker, at a speed of 170 rpm, and illuminated at a light intensity of 115 μmol quanta m⁻² s⁻¹ [10].

2.2. Growth parameters

Growth of the culture was estimated on the basis of chlorophyll content and dry weight measurements. Chlorophyll concentration was measured after extraction with dimethyl sulfoxide (DMSO) at 70 °C for 5 min [10]. The biomass concentration was estimated by dry weight determination on pre-weighed 25 mm glass fiber paper filters (Whatman GF/C, Schleicher and Schuell Co., Dassel, Germany) [9].

2.3. Lipid extraction

Lyophilized biomass was extracted as previously described [18] by DMSO at 70 °C for 5 min, followed by continuous mixing with methanol at 4 °C for 30 min. The methanol-DMSO extract was mixed with peroxide-free diethyl ether, hexane and water to form a ratio of 1:1:1:1 (v/v/v/v). The upper phase was collected and the water phase was re-extracted twice with a diethyl ether-hexane mixture. The upper lipid-containing phases were combined, evaporated to dryness and stored in a small volume of chloroform under argon at -20 °C.

2.4. Fatty acid and lipid analysis

The total lipid extract was separated into neutral and polar lipids on silica gel cartridges (Bond-Elut, JR-SI, Varian), using 0.5% methanol in chloroform (v/v) and methanol to elute neutral and polar lipids, respectively [19]. Polar lipids were further separated into individual lipids by two-dimensional TLC (Silica Gel 60, 10 × 10 cm, 0.25 mm thickness, Merck, Darmstadt, Germany) using a solvent system of chloroform/methanol/water (65:25:4, v/v/v) for the first direction, and of chloroform/methanol/1-ethylpropylamine/25% ammonia (65:35:0.5:5, v/v/v/v) for the second direction [15]. Neutral lipids were resolved with petroleum ether/diethyl ether/acetic acid (70:30:1, v/v/v). Lipids on TLC plates were visualized by brief exposure to iodine vapors, scraped from the plates and transmethylated (see below) or extracted from the silica using a mixture of chloroform/methanol (1:1, v/v). Biomass, individual lipids or lipid-containing silica gel scraping were transmethylated using sulfuric acid in methanol, in the presence of an internal standard (17:0), as previously described [10,15].

2.5. Molecular species analysis

MGDG was resolved into its component molecular species by reverse-phase HPLC (LiChrospher 100, RP-18, 5 μM, Merck) utilizing a mobile phase of methanol/water (93:7, v/v) for 40 min and 94:6 (v/v) for another 20 min with a flow rate of 1.5 mL/min. The HPLC system was equipped with a variable-wavelength detector (Lamda-Max 481, Waters, Milford, MA) and an evaporative light scattering detector (ELSD IIA, Varex, Md), or a radioactivity detector (Flo-One β series A-100, Radiomatic Instruments and Chemical Co., Inc., Tampa, FL).

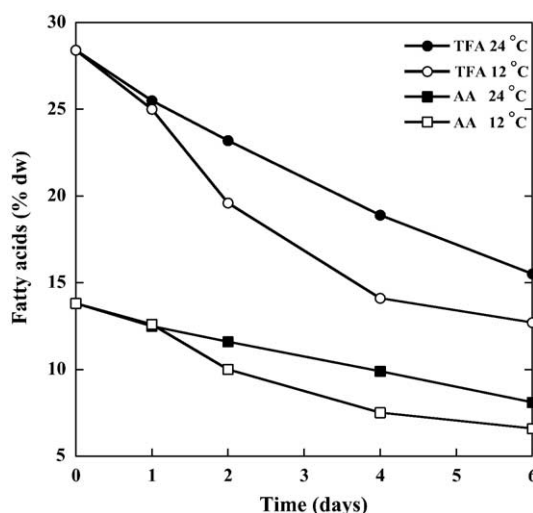


Fig. 1. Changes in total fatty acids (□) and arachidonic acid (■) during recovery from N-starvation at 24 °C and 12 °C.

2.6. Radiolabelling experiments

Cultures maintained under nitrogen starvation for 14 days were concentrated to half of the original volume by centrifugation and incubated with 10 μCi [$1\text{-}^{14}\text{C}$] oleic acid (Sp. act. 55 mCi/mmol, Amersham, Little Chalfont, UK) for 24 h. Labeled oleic acid was added as its ammonium salt, which was obtained by neutralization of the free fatty acid with an equimolar amount of 2 M NH_4OH . After incubation, cells were centrifuged, washed

repeatedly with the label-free nutrient medium, followed by resuspension in four times the original volume of the complete growth medium and cultivated in flasks for 48 h as detailed above. HPLC analysis of TAG fatty acids was performed as previously described [20].

3. Results

3.1. Nitrogen starvation

Cultures of *P. incisa* were maintained in nitrogen (N)-free medium for 14 days at 25 °C to maximize the cellular content of AA-rich TAG [12]. Fatty acid analysis of the N-starved cultures showed that net synthesis of fatty acids and particularly AA proceeded during starvation, reaching after 14 days, a content of 29.2% (of dry weight) and 14.4%, respectively (Fig. 1). TAG accounted for 86% of total lipids, comprising 25.1% (of dry weight) of the cell biomass. AA was the major fatty acid of TAG constituting 50% of its fatty acid content (Table 1). More than 80% of total cellular AA was accumulated in TAG, during this period.

3.2. Recovery from nitrogen starvation

Recovery from N-starvation was induced by resuspension of the cells in complete nutrient medium. Recovery of growth in terms of increase in chlorophyll and biomass contents of the cultures was followed for 6 days. Samples for lipid

Table 1
Fatty acids composition of major lipid classes of *P. incisa* following recovery (Rec.) from N-starvation (Starv.)

Lipid	Conditions	Temp (°C)	% TFA	TFA ($\mu\text{g mL}^{-1}$)	Fatty acid composition (% of total)											
					16:0	16:1 ω 7	16:2 ω 6	16:3 ω 3	18:0	18:1 ω 9	18:1 ω 7	18:2 ω 6	18:3 ω 6	18:3 ω 3	20:3 ω 6	20:4 ω 6
TAG	Starv.		86.0	779.2	8.0	0.2	0.4	0.3	3.5	16.1	3.6	13.1	1.0	0.9	1.0	50.0
	Rec. 24	24	77.6	546.6	9.0	0.2	0.3	0.7	3.1	12.6	3.6	13.2	0.9	2.1	0.9	51.1
	Rec. 12	12	78.2	532.4	8.6	0.2	tr	0.8	3.2	11.9	3.7	10.8	1.0	3.6	1.0	52.4
MGDG	Starv.		1.8	16.0	3.6	0.3	23.6	14.6	0.3	3.2	0.4	28.9	0.5	20.4	–	2.7
	Rec. 24	24	4.3	30.5	2.1	0.3	4.4	13.3	0.7	6.2	2.6	22.5	1.2	21.9	0.4	22.9
	Rec. 12	12	2.7	18.3	2.1	0.5	1.7	23.2	0.6	2.2	2.2	6.7	1.1	42.0	–	16.1
DGDG	Starv.		1.6	14.1	16.7	0.3	6.3	1.2	0.5	5.8	0.9	42.7	1.1	10.5	0.3	11.3
	Rec. 24	24	3.8	26.6	7.1	0.4	1.8	0.9	2.3	8.8	3.9	37.0	1.1	13.9	0.4	20.9
	Rec. 12	12	3.2	21.5	7.3	0.5	2.4	3.0	1.2	2.4	2.9	12.6	1.2	44.5	0.5	20.2
SQDG	Starv.		3.2	28.8	62.3	0.2	–	–	0.7	2.7	8.5	21.1	0.3	3.3	–	0.4
	Rec. 24	24	4.9	34.8	54.2	0.3	–	–	2.5	5.7	9.6	17.4	0.5	7.9	–	1.8
	Rec. 12	12	5.5	37.5	56.8	0.2	–	–	1.6	1.2	16.8	4.7	0.4	16.2	–	1.4
DGTS	Starv.		2.1	19.1	35.6	1.5	tr	0.2	5.5	3.7	3.8	22.1	6.2	0.6	0.6	13.1
	Rec. 24	24	2.2	15.7	36.2	1.2	–	–	6.9	1.9	3.3	22.7	2.8	1.3	0.3	17.4
	Rec. 12	12	3.4	23.4	43.8	1.1	–	0.2	7.5	1.0	5.7	13.1	4.3	2.3	0.7	16.4
PE	Starv.		0.5	4.8	12.0	0.4	–	–	7.3	1.5	32.9	5.5	2.6	0.2	4.6	27.2
	Rec. 24	24	0.7	5.1	14.9	–	–	–	9.8	1.1	38.7	5.1	0.8	0.2	3.1	25.6
	Rec. 12	12	0.8	5.6	18.1	0.3	–	–	10.0	1.1	34.3	3.5	0.9	0.4	4.3	24.4
PC	Starv.		0.9	8.5	23.6	1.3	tr	0.2	6.5	5.0	15.7	16.8	4.9	0.3	1.5	18.6
	Rec. 24	24	1.9	13.1	18.7	0.8	0.2	–	6.0	3.4	11.6	18.9	2.8	1.0	1.1	31.8
	Rec. 12	12	1.9	13.3	33.0	0.7	–	–	6.8	2.3	14.8	9.9	3.4	2.3	1.5	21.9
DAG	Starv.		1.1	9.9	15.6	1.1	–	0.5	8.4	30.9	6.3	12.0	1.1	0.3	0.5	20.3
	Rec. 24	24	1.0	6.9	21.4	–	–	–	9.3	25.7	5.3	12.2	0.8	0.8	–	24.5
	Rec. 12	12	0.8	5.6	17.5	0.5	–	0.6	8.8	22.1	5.4	11.9	1.1	1.3	0.6	27.0
FFA	Starv.		0.8	6.8	22.2	2.5	–	1.0	11.1	25.0	6.2	12.3	3.6	0.5	3.1	3.8
	Rec. 24	24	0.5	3.4	19.9	–	–	–	11.1	15.6	3.5	10.8	1.3	0.5	2.0	31.1
	Rec. 12	12	0.4	2.5	27.3	1.4	–	–	14.8	15.3	3.6	6.3	5.9	1.2	6.2	12.9

Cultures were resuspended in full medium and grown at 24 °C (2 days) or 12 °C (4 days). tr—traces.

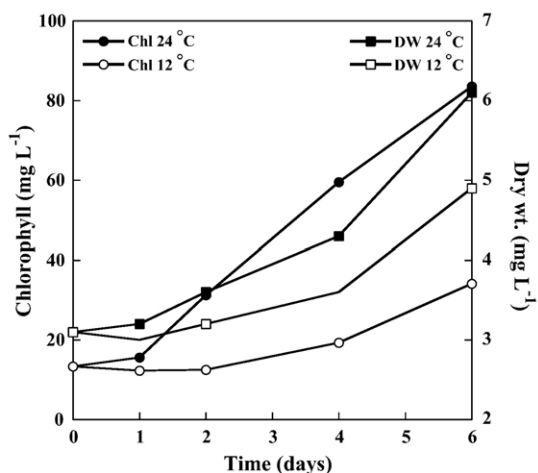


Fig. 2. Changes in chlorophyll (●) and dry weight (■) during recovery from N-starvation at 24 °C and 12 °C.

analysis were taken after 2 days at room temperature (24 °C) and after 4 days at low temperature (12 °C). These time points were chosen on the basis of similar increases in dry weight. After a 1-day lag, growth was resumed and significant chlorophyll and biomass synthesis commenced at room temperature (Fig. 2). At low temperature, however, chlorophyll accumulation and biomass production were much slower (Fig. 2).

3.3. Alterations in lipid and fatty acid content and composition

After 4 days of recovery the total fatty acid content decreased by 33.5 and 50.4% at 24 °C and 12 °C, respectively (Fig. 1). The decrease in AA content was lower, amounting to only 28.3 and 45.7%, respectively. At both temperatures, recovery was accompanied by a decrease in the proportion of TAG (from 86 to 78% of total lipids) and by an increase in the relative proportions of the chloroplastic lipids, MGDG (from 1.8 to 4.3 and 2.7%, respectively), DGDG (from 1.6 to 3.8 and 3.2%, respec-

tively) and SQDG (from 3.2 to 4.9 and 5.5%, respectively) (Table 1).

The volumetric content of TAG decreased from 779 to 547 and 533 $\mu\text{g mL}^{-1}$. At 24 °C, the net buildup of DGDG and especially MGDG was more intense than at 12 °C (Table 1). MGDG increased from 16.0 to 30.5 and 18.3 $\mu\text{g mL}^{-1}$, DGDG from 14.1 to 26.6 and 21.5 $\mu\text{g mL}^{-1}$ and SQDG from 28.8 to 34.8 and 37.5 $\mu\text{g mL}^{-1}$ at 24 °C and 12 °C, respectively (Fig. 3). During the recovery, there was also a decrease in the content of DAG, from 9.9 to 6.9 and 5.6 $\mu\text{g mL}^{-1}$, and of FFA, from 6.8 to 3.4 and 2.5 $\mu\text{g mL}^{-1}$, respectively.

During recovery at 12 °C, but much less so at 24 °C, the proportion of 18:3 ω 3 in the three major chloroplastic lipids and of 16:3 ω 3 (in MGDG and DGDG), increased at the expense of their respective ω 6 precursors, 18:2 and 16:2 (Table 1). The proportion of AA in MGDG increased sharply from 2.7 to 22.9 and 16.1% and in DGDG from 11.3 to 20.9 and 20.2%, at 24 °C and 12 °C, respectively. The content (% dw) of these lipids also increased, especially at 24 °C. Consequently, in MGDG AA increased from 0.4 to 7.6 and 1.7 $\mu\text{g mL}^{-1}$ and in DGDG from 1.3 to 7.9 and 2.7 $\mu\text{g mL}^{-1}$, at 24 °C and 12 °C, respectively. The proportion of AA increased also in the extraplastidial polar lipids, PC and DGTS. The proportion of AA in TAG did not change but the content of AA in TAG decreased from 285 to 139 and 203 $\mu\text{g mL}^{-1}$, respectively. These findings suggest a transfer of AA from TAG to polar lipids at 24 °C, but much less so at 12 °C (Table 1, Fig. 3). There was also a sharp increase in the proportion of AA in FFA (from 3.8 to 31.1 and 12.9%, respectively), indicating an enhanced activity of TAG lipase. At 12 °C, but not at 24 °C, the proportion of 18:2 in the extrachloroplastic lipids DGTS, and PC decreased from 22.1 and 16.8% to 13.1 and 9.9%, respectively. Lower decreases were noted in the proportion of 18:1. Correspondingly, the proportion of 16:0 increased. These findings indicate DGTS and PC as likely sources of acyl moieties that can be remodeled into chloroplastic lipids.

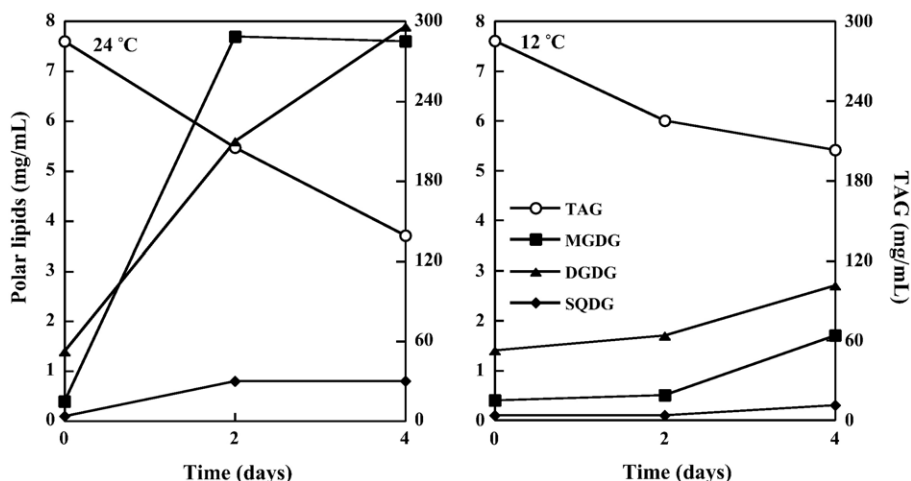


Fig. 3. Changes in the volumetric content of arachidonic acid in TAG (O), MGDG (■), DGDG (▲) and SQDG (◆) during recovery from N-starvation at 24 °C (left panel) and 12 °C (right panel).

Table 2

Changes in the molecular species distribution (% of TFA) and content ($\mu\text{g mL}^{-1}$) in MGDG of *P. incisa*, following recovery (Rec.) (2 days at 24 °C, or 4 days at 12 °C) from N-starvation

Molecular species	Molecular species distribution and content					
	N-starvation 14 days		Rec. 24 °C 2 days		Rec. 12 °C 4 days	
	% of TFA	$\mu\text{g mL}^{-1}$	% of TFA	$\mu\text{g mL}^{-1}$	% of TFA	$\mu\text{g mL}^{-1}$
18:2/16:2	31.3	5.0	2.6	0.8	tr	tr
18:3/16:2	16.0	2.6	4.9	1.5	1.2	0.2
18:2/16:3 ω 3	12.5	2.0	4.3	1.3	0.6	tr
18:3 ω 3/16:3 ω 3	24.2	3.9	25.4	7.7	53.1	9.7
Total 18/16	83.9	13.4	37.1	11.3	54.9	10.0
18:1/18:2	tr	tr	3.8	1.2	tr	tr
18:2/18:2	2.0	0.3	6.4	1.9	tr	tr
18:1/18:3 ω 3	0.8	tr	tr	tr	tr	tr
18:2/18:3 ω 3	2.1	0.3	2.8	0.8	1.6	0.3
18:3 ω 3/18:3 ω 3	1.3	0.2	2.4	0.7	8.0	1.5
Total 18/18	6.1	1.0	15.3	4.7	9.6	1.7
20:4/18:1	2.4	0.4	10.1	3.1	1.8	0.3
20:4/18:2	4.4	0.7	21.2	6.5	6.7	1.2
20:4/18:3 ω 3	2.1	0.3	7.2	2.2	20.2	3.7
Total 20/18	8.9	1.4	38.5	11.8	28.6	5.2
20:4/20:4	1.0	0.2	9.0	2.8	6.9	1.3

Positional analysis was not performed. TFA—total fatty acids; tr—traces; unless otherwise indicated, PUFA are of the ω 6 family.

3.4. Molecular species analysis

In order to further elucidate the transfer of AA from TAG to chloroplastic lipids, we have analyzed the molecular species composition of MGDG, which was found to be the major lipid sink of AA during recovery. Under nitrogen starvation, the molecular species of MGDG were mostly of the 18/16 type (Table 2), constituting about 84% of total MGDG. During recovery at 24 °C, these molecular species were diluted by the production of 18/18, 20/18 and 20/20 molecular species, whose content increased from 1 to 4.7,

1.4 to 11.8 and 0.2 to 2.8 $\mu\text{g mL}^{-1}$, respectively. Within the 18/16 group, there was a decrease in the content of those containing ω 6 fatty acids (16:2 and 18:2), in favor of the fully desaturated species, 18:3 ω 3/16:3 ω 3. The share of the latter increased from 28.8 to 68.5% of total 18/16 molecular species, indicating an intensive ω 3 desaturation. At 12 °C too, the content of the 18/16 species decreased (from 13.4 to 10.0 $\mu\text{g mL}^{-1}$), whereas that of the 18/18, and especially the 20/18 and the 20/20 increased, but much less than at 24 °C (from a total of 2.6 to 8.2 $\mu\text{g mL}^{-1}$). Molecular species containing ω 3 fatty acids (16:3 and 18:3) dominated at the expense of their less unsaturated ω 6 precursors. In each of the first 3 groups, the share of 18:3 ω 3-containing molecular species constituted 96.7, 83.3 and 70.6% of their respective groups.

3.5. Radiolabelling

A radiolabelling study was carried out to monitor the transfer of AA from TAG to the chloroplastic lipids following recovery. Cells of *P. incisa* were labeled with $[1-^{14}\text{C}]18:1$ for 24 h under N-starvation conditions. During this period most of the label was converted to $[1-^{14}\text{C}]20:4$ and deposited in TAG (data not shown). The redistribution of label was followed during the recovery period at room and low temperatures. The label of TAG continuously decreased and was partially transferred to the chloroplastic lipids, MGDG, DGDG and SQDG during recovery at 24 °C, but much less so at 12 °C (Fig. 4). At 12 °C, the decrease of label of TAG was observed only in the first 4 h and the transfer to chloroplastic lipids was much lower. Decreases in label were noted also in DGTS (at both temperatures) and in PC (only at 12 °C) (Fig. 5). An early transitory rise was noted in the label of AA in PC and even more in DGTS. At 12 °C, it was less pronounced, mostly in DGTS. The relatively high label of DGTS and its subsequent decrease may indicate this lipid

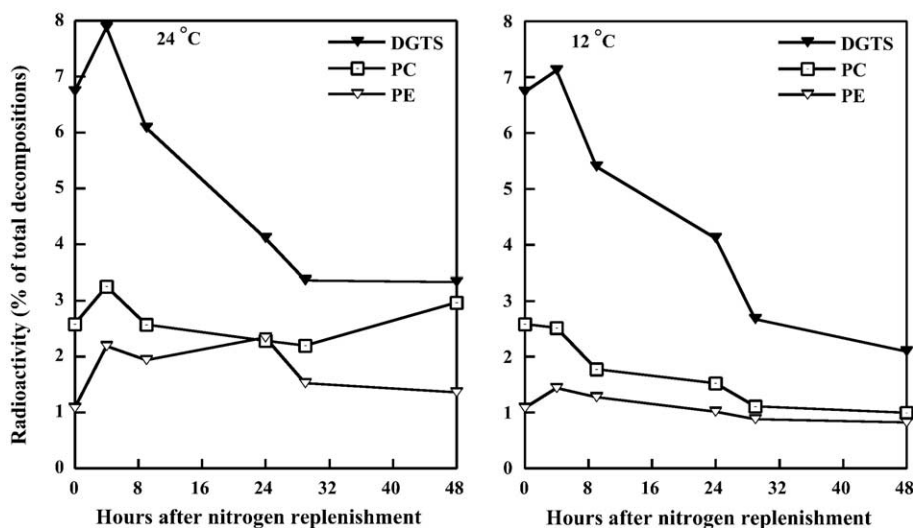


Fig. 4. Redistribution of radioactivity in chloroplastic lipids (left scale) and TAG (right scale) of *P. incisa* in the first 48 h following nitrogen starvation for 14 days, labelling with $[1-^{14}\text{C}]18:1$ for 24 h and nitrogen replenishment at 24 °C (left panel) or 12 °C (right panel); TAG (○), MGDG (■), DGDG (▲) and SQDG (◆), respectively.

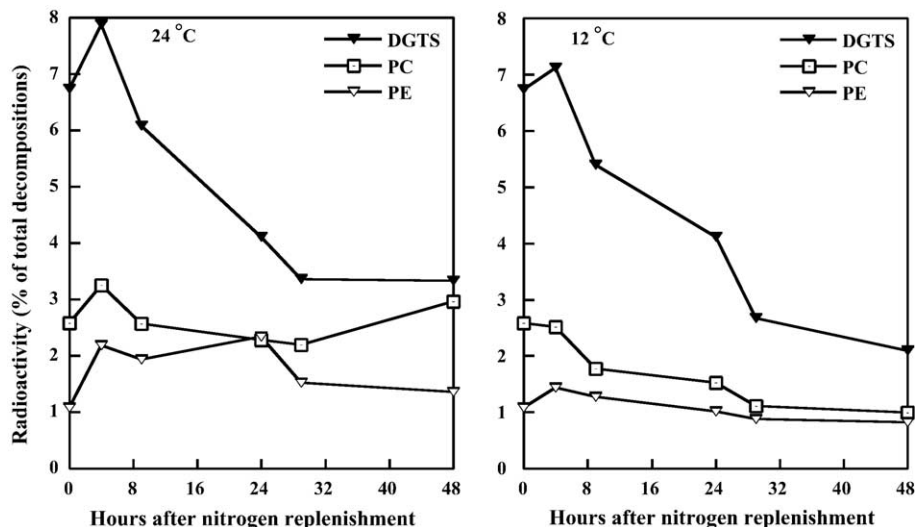


Fig. 5. Redistribution of radioactivity in the extrachloroplastic lipids of *P. incisa* in the first 48 h following nitrogen starvation for 14 days, labelling with [1-¹⁴C]18:1 for 24 h and nitrogen replenishment at 24 °C (left panel) or 12 °C (right panel); DGTS (▼), PC (□), PE (▽).

together with PC as the primary intermediates for both TAG and MGDG.

4. Discussion

The unique accumulation of AA-rich TAG in oil bodies, especially under N-starvation [10,12] lead us to hypothesize that in *P. incisa*, TAG may have an additional role, other than being a storage of carbon and energy [11]. While most microalgae reside in large water bodies where temperature and nutritional changes are relatively slow, quite a few microalgae grow in ecological niches that may be subject to rapid short-term fluctuations, e.g., temperature, salinity or nitrogen availability. Indeed, *P. incisa* was isolated from an alpine environment characterized by sudden changes in environmental conditions [10]. Adaptation to these changes may require significant and rapid alterations in the fatty acid and molecular species composition of chloroplast membrane lipids. However, under such conditions, the de novo synthesis of PUFA would be too slow. We have thus hypothesized that in algae growing in such habitats, PUFA-rich TAG can be metabolically active serving as a buffering capacity for PUFA, providing specific acyl groups, in order to enable rapid adaptation of the membranes [4].

When N-starved cells of *P. incisa* are transferred to optimal growth conditions, resumption of photosynthesis requires swift construction of the various photosynthetic membrane components, among them the chloroplastic lipids. This requirement could be crucial since the window of opportunity for exponential growth could be rather narrow. During recovery, the AA content decreased but somewhat less than the fatty acid content (Fig. 1). There was a significant reduction in the content of TAG and a smaller increase in that of chloroplastic lipids (Fig. 3). The simultaneous decrease of AA-rich TAG and increase of AA in chloroplastic lipids (Table 1, Fig. 4) strongly supports our hypothesis that TAG are not only a source of carbon and energy but can export stored PUFA for

the construction of chloroplastic lipids. Similarly, when the growth temperature of an exponentially growing culture of *P. incisa* labeled with [1-¹⁴C]arachidonic acid was suddenly dropped to 4 °C, the label in TAG, which was mostly associated with AA, was turned over to polar lipids [11]. Likewise, it was found that AA-rich TAG of *P. cruentum* contributed AA moieties for the production of eukaryotic-like molecular species of MGDG, containing EPA at the *sn*-1 and *sn*-2 positions of the glycerol skeleton following N-replenishment [16]. Also in higher plants, lipid bodies were recently shown to be metabolically active in seeds and other plant organs [13]. Furthermore, Stobart et al. [21] produced evidence that supports a transacylation mechanism that can account for TAG turnover in microsomal membranes of developing safflower seeds.

Under optimal conditions the major PUFA of MGDG in *P. incisa*, as in many other green algae, are 16:3 ω 3 and 18:3 ω 3. However, it contains also 13.9% AA (of total fatty acids) [10]. Under N-starvation, the share of AA decreased to 2.4% (Table 1) and the ω 3 fatty acids were mostly replaced by their ω 6 precursors, 16:2 and 18:2. Attenuation of ω 3 desaturation and accumulation of the ω 6 precursors, under conditions that do not support optimal growth, is a well-documented phenomenon in both higher plants [22] and algae [23,24]. Similarly, in *P. cruentum*, under suboptimal growth conditions, chloroplastic lipids, predominantly MGDG, accumulate AA (20:4 ω 6) rather than EPA (20:5 ω 3). The increase in membrane unsaturation and consequently of its fluidity, as a means of cold adaptation, is a well-studied phenomenon in both lower and higher plants [25,26]. Indeed, upon recovery at 12 °C, the need for high level of unsaturation in MGDG is provided by an intensive ω 3 desaturation of the C16- and C18-containing molecular species (Table 2).

Our findings indicate that in *P. incisa*, there are two pathways leading to the production of molecular species of chloroplastic lipids (Fig. 6). The first pathway is the prokaryotic pathway that in similarity to 16:3-plants such as

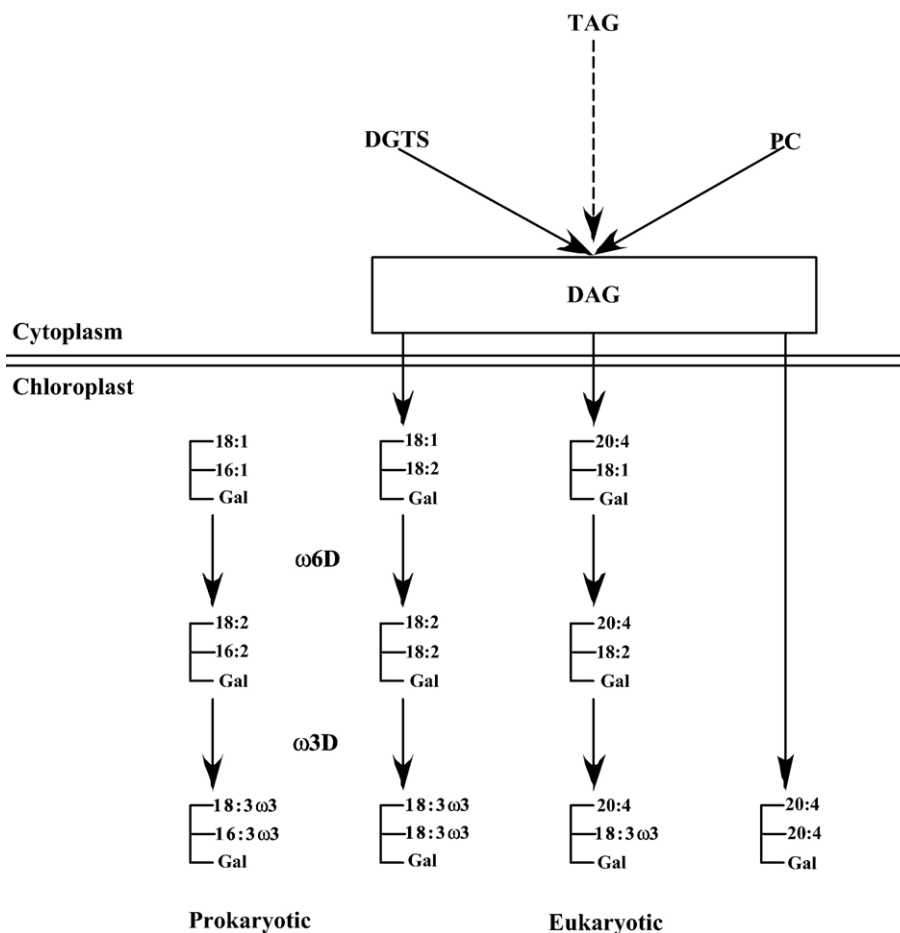


Fig. 6. Outline of suggested pathways in the biosynthesis of MGDG in *P. incisa*. Dashed arrow—rapid deployment pathway. Gal—galactose moiety.

Arabidopsis thaliana [17] and several green algae, e.g., *Chlorella* [27,28] and *Chlamydomonas* [29], gives rise to the 18/16 type molecular species of MGDG (and presumably other chloroplastic lipids) in *P. incisa*. The second pathway is the eukaryotic pathway, also common in higher plants and green algae. In these organisms, this pathway most likely import DAG from extrachloroplastic lipids and provides membranal lipids with C18-containing molecular species that can be further desaturated by the chloroplastic $\omega 3$ desaturase [17,28]. In *P. incisa*, 3 types of molecular species, 18/18, 20/18 and 20/20 are produced via this pathway. These molecular species appears to derive from DGTS and PC but also from TAG (Figs. 4, 5).

We hypothesize that *P. incisa* have three modes of operation with respect to the production of chloroplastic lipids. When environmental conditions do not support growth, e.g., in the stationary phase [10] or under N starvation, the prokaryotic pathway predominates, comprising over 70% of total MGDG. Upon recovery from nitrogen deficiency that is compounded by a low temperature shift, maximal growth cannot be immediately supported, as the organism has to cope with both recovery and low temperature. Under such circumstances, the eukaryotic pathway is invoked, importing acyl (mostly 18:1, 18:2 and AA) moieties from DGTS and PC (presumably as DAG). This mode is also contributing under exponential

conditions. The third mode is utilized when there is a sudden requirement for enhanced desaturation of chloroplastic lipids under conditions that support exponential growth, e.g., upon recovery from N-starvation at room temperature or when an exponentially growing culture is undergoing a drastic temperature down shift [11]. In such cases, the acyl content available from DGTS and PC would not suffice and TAG are rapidly deployed to enhance the flux in the eukaryotic pathway, releasing AA-rich acyl moieties that can be exported into the chloroplast. The use of this mode is only transitory and after 9 days AA returned to its normal level in chloroplastic lipids (data not shown). We presume that AA is imported as DAG or an acyl moiety that is released from a phospholipid, DGTS, or TAG (Fig. 4). A TAG lipase is likely the key enzymatic activity involved in the release of AA from TAG as evidenced by the increase in AA in FFA. Indeed, the presence of a highly active TAG lipase was recently detected in cell-free homogenates of *P. incisa* [30].

In higher plants, the ability to change the ratio of eukaryotic to prokaryotic molecular species, under different environmental conditions, is rather limited. However, in the red alga, *P. cruentum*, the share of eukaryotic-like molecular species of MGDG increases from 42% (of total acyl MGDG) at 30 °C to 58% at 20 °C [31]. Indeed, this alga was isolated from wet saline soil, another ecological niche characterized by rapid

fluctuations in environmental conditions. Recently, Falcone et al. [32] have shown that in higher plants too, the ratio of eukaryotic to prokaryotic molecular species is affected by temperature.

The data we have shown suggest that upon transfer to growth conditions at low temperatures, the major adaptation processes used by cells of *P. incisa* are the construction of new molecular species, produced via the eukaryotic pathway and the enhancement of ω 3 desaturation of preexisting, as well as newly formed, molecular species of MGDG (and apparently also of DGDG). At 24 °C, however, ω 3 desaturation is lesser than at 12 °C. Instead, the eukaryotic mechanism is significantly intensified. The share of molecular species produced in the eukaryotic pathway increased from 16.0% to 62.8% of total MGDG (Table 2). However, the reason why two diverse types of PUFA, 16:3 and 18:3 of the ω 3 family, and AA of the ω 6 family, are preferentially produced under different conditions is still unclear. Possibly, the role of the AA-containing molecular species is to provide increased chain length, as well as enhanced desaturation.

While the ratio of the different groups of molecular species was significantly different between 12 °C and 24 °C, the ratio of the eukaryotic molecular species of the 18/18, 20/18 and 20/20 types was only slightly different, being 24:61:14 at 24 °C and 21:63:15 at 12 °C, respectively. This finding indicates that the rapid deployment of acyl groups does not change the ratio of these groups in the sink although under N-starvation the AA content of TAG is 50% in comparison to only 13.1 and 18.6% in DGTS and PC, respectively. Indeed, the enrichment of the latter lipids with 16:0 during recovery at 12 °C suggests that only certain molecular species are selected for export.

Plant desaturases are classified according to their specificities. Several desaturases introduce a double bond at a certain distance from the carboxylic end, e.g., Δ 6 or Δ 9, while other desaturate at a position that is relative to the methyl end of the fatty acid, e.g., ω 3. There is a general agreement in the literature that the ultimate chloroplastic desaturase is an ω 3, rather than a Δ 15, desaturase, since it is able to desaturate both 16:2 and 18:2 [17]. Surprisingly, it appears that in *P. incisa*, this enzyme can desaturate both fatty acids, but not 20:4 ω 6 to 20:5 ω 3. In *P. cruentum*, the chloroplastic ω 3 desaturase can desaturate AA to EPA, however, the 18:2/16:0 molecular species of MGDG is not further desaturated [15]. In contrast, Sakuradani et al. recently showed [33] that the ω 3 desaturase of the AA-producing fungus *Mortierella alpina* 1S-4 can desaturate both C18 and C20 PUFA. This is, to the best of our knowledge, the first reported case of an ω type enzyme, i.e., an enzyme that “counts” from the methyl end of the fatty acid chain that is specific both to the carbon atom position and to the chain length.

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References

- [1] M.A. Crawford, K. Costeloe, K. Ghebremeskel, A. Phylactos, L. Skirvin, F. Stacey, Are deficits of arachidonic and docosahexaenoic acids responsible for the neural and vascular complications of preterm babies? *Am. J. Clin. Nutr.* 66 (1997) 1032–1041.
- [2] C.D. Funk, Prostaglandins and leukotrienes: advances in eicosanoid biology, *Science* 294 (2001) 1871–1875.
- [3] W. Eichenberger, C. Gribi, Lipids of *Pavlova lutheri*: cellular site and metabolic role of DGCC, *Phytochemistry* 45 (1997) 1561–1567.
- [4] Z. Cohen, I. Khozin-Goldberg, Searching for PUFA-rich microalgae, in: Z. Cohen, C. Ratledge (Eds.), *Single Cell Oils*, American Oil Chemists' Society, Champaign, IL, 2005, pp. 53–72.
- [5] Z. Dubinsky, T. Berner, S. Aaronson, Potential of large-scale algal culture for biomass and lipid production in arid lands, *Biotech. Bioeng. Symp.* 8 (1978) 51–68.
- [6] A.H. Scragg, R.R. Leather, in: R.S. Moreton (Ed.), *Single Cell Oil*, Longman, UK, 1988, pp. 71–98.
- [7] P.G. Roessler, Environmental control of glycerolipid metabolism in microalgae: commercial implications and future research directions, *J. Phycol.* 26 (1990) 393–399.
- [8] Z. Cohen, Production of polyunsaturated fatty acids by the microalga *Porphyridium cruentum*, in: Z. Cohen (Ed.), *Production of Chemicals by Microalgae*, Taylor and Francis, London, 1999, pp. 1–24.
- [9] R.J. Henderson, E.E. Mackinaly, P. Hodgson, J.L. Harwood, Differential effects of the substituted pyridazinone herbicide Sandoz 9785 on lipid composition and biosynthesis in photosynthetic and non-photosynthetic marine microalgae: II. Fatty acid composition, *J. Exp. Bot.* 41 (1990) 729–736.
- [10] C. Bigogno, I. Khozin-Goldberg, S. Boussiba, A. Vonshak, Z. Cohen, Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid, *Phytochemistry* 60 (2002) 497–503.
- [11] C. Bigogno, I. Khozin-Goldberg, Z. Cohen, Accumulation of arachidonic acid-rich triacylglycerols in the microalga *Parietochloris incisa* (Trebouxiophyceae, Chlorophyta), *Phytochemistry* 60 (2002) 135–143.
- [12] I. Khozin-Goldberg, C. Bigogno, P. Shrestha, Z. Cohen, Nitrogen starvation induces the accumulation of arachidonic acid in the freshwater green alga *Parietochloris incisa* (Trebouxiophyceae), *J. Phycol.* 38 (2002) 991–994.
- [13] D.J. Murphy, The biogenesis and functions of lipid bodies in animal, plants and microorganisms, *Prog. Lipid Res.* 40 (2001) 325–328.
- [14] Z. Cohen, I. Khozin-Goldberg, D. Adlerstein, C. Bigogno, The role of triacylglycerol as a reservoir of polyunsaturated fatty acids for the rapid production of chloroplastic lipids in certain microalgae, *Biochem. Soc. Trans.* 28 (2000) 740–743.
- [15] I. Khozin, D. Adlerstein, C. Bigogno, Y.M. Heimer, Z. Cohen, Elucidation of the biosynthesis of eicosapentaenoic acid in the microalga *Porphyridium cruentum*: II. Studies with radiolabeled precursors, *Plant Physiol.* 114 (1997) 223–230.
- [16] I. Khozin-Goldberg, Y. Hu Zheng, D. Adlerstein, S. Didi-Cohen, Y.M. Heimer, Z. Cohen, Triacylglycerols of the red microalga *Porphyridium cruentum* can contribute to the biosynthesis of eukaryotic galactolipids, *Lipids* 35 (2000) 881–889.
- [17] J. Browse, C. Somerville, Glycerolipid synthesis: biochemistry and regulation, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 42 (1991) 467–506.
- [18] I. Khozin-Goldberg, S. Didi-Cohen, I. Shayakhmetova, Z. Cohen, Elucidation of the biosynthesis of eicosapentaenoic acid (EPA) in the freshwater eustigmatophyte *Monodus subterraneus*, *J. Phycol.* 38 (2002) 745–756.
- [19] Z. Cohen, S. Didi, Y.M. Heimer, Over-Production of γ -linolenic and eicosapentaenoic acids by algae, *Plant Physiol.* 98 (1992) 569–572.

- [20] C. Bigogno, I. Khozin-Goldberg, D. Adlerstein, Z. Cohen, Biosynthesis of arachidonic acid in the oleaginous microalga *Parietochloris incisa* (Chlorophyceae): radiolabeling studies, *Lipids* 37 (2002) 209–216.
- [21] K. Stobart, M. Mancha, M. Lenman, A. Dahlqvist, S. Stymne, Triacylglycerols are synthesized and utilized by transacylation reactions in microsomal preparations of developing safflower (*Carthamus tinctoris* L.) seeds, *Planta* 203 (1997) 58–66.
- [22] C. Somerville, J. Browse, Dissecting desaturation: plants prove advantageous, *Trends Cell Biol.* 6 (1996) 148–153.
- [23] G.L. Klyachko-Gurvich, L.N. Tsoglin, J. Doucha, J. Kopetskii, I.B. Ryabykh, V.E. Semenenko, Desaturation of fatty acids as an adaptive response to shifts in light intensity, *Physiol. Plant.* 107 (1999) 240–249.
- [24] J.-M. Routaboul, S.F. Fischer, J. Browse, Trienoic fatty acids are required to maintain chloroplast function at low temperatures, *Plant Physiol.* 124 (2000) 1697–1705.
- [25] N. Murata, H. Wada, Acyl-lipid desaturases and their importance in the tolerance and acclimatization to cold of cyanobacteria, *Biochem. J.* 308 (1995) 1–8.
- [26] M. Miquel, D. James Jr., H. Dooner, J. Browse, *Arabidopsis* requires polyunsaturated lipids for low temperature survival, *Proc. Natl. Acad. Sci. U. S. A.* 90 (1993) 6208–6212.
- [27] G.A. Thompson, Lipids and membrane function in green algae, *Biochim. Biophys. Acta* 1302 (1996) 17–45.
- [28] N. Sato, M. Tsuzuki, A. Kawaguchi, Glycerolipid synthesis in *Chlorella kessleri* 11 h I. Existence of a eukaryotic pathway, *Biochim. Biophys. Acta* 1633 (2003) 27–34.
- [29] C. Giroud, A. Geber, W. Eichenberger, Lipids of *Chlamydomonas reinhardtii*. Analysis of molecular species and intracellular site(s) of biosynthesis, *Plant Cell Physiol.* 29 (1988) 587–595.
- [30] P. Shrestha, D. Cohen, I. Khalilov, I. Khozin-Goldberg, Z. Cohen, Triacylglycerol biosynthesis in microsomes and oil bodies of the oleaginous green alga *Parietochloris incisa*, Proceedings of 16th International Plant Lipid Symposium, 1–4 June, 2004 (Budapest, Hungary, <http://www.mete.mtesz.hu/pls/proceedings>).
- [31] D. Adlerstein, I. Khozin, C. Bigogno, Z. Cohen, Effect of environmental conditions on the molecular species composition of galactolipids in the alga *Porphyridium cruentum*, *J. Phycol.* 33 (1997) 975–979.
- [32] D.L. Falcone, J.P. Ogas, C. Somerville, Regulation of membrane fatty acid composition by temperature in mutants of *Arabidopsis* with alterations in membrane lipid composition, *BMC Plant Biol.* 4 (2004) 17, (<http://www.biomedcentral.com/1471-2229/4/17>).
- [33] E. Sakuradani, A. Takahiro, I. Keita, S. Shimizu, A novel fungal ω 3-desaturase with wide substrate specificity from arachidonic acid-producing *Mortierella alpina* 1S-4, *Appl. Microbiol. Biotechnol.* 66 (2005) 648–654.