

Triacylglycerols of the Red Microalga *Porphyridium cruentum* Can Contribute to the Biosynthesis of Eukaryotic Galactolipids

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ABSTRACT: A mutant of the red microalga *Porphyridium cruentum* was selected on the basis of impaired growth at suboptimal temperatures (15 vs. 25°C). Fatty acid and lipid analyses revealed diminished proportions of eicosapentaenoic acid (from 41 to 30%) and of the eukaryotic molecular species (from 38 to 28% of monogalactosyldiacylglycerol (MGDG) and elevated proportion (10 vs. 2%) of triacylglycerols (TAG) in the mutant, as compared with the wild type. Pulse labeling of the wild type cells with radioactive fatty acid precursors indicated an initial incorporation of the fatty acids into phosphatidylcholine (PC) and TAG. Following the pulse, the label of PC and TAG decreased with time (from 25 to 5% of the total dpm in TAG) while that of chloroplastic polar lipids, mainly MGDG, continued to increase. In the mutant, however, the labeling of TAG after the pulse was higher (30% of the total dpm) than that of the wild type and decreased only slightly to 20%. This may indicate that in *P. cruentum*, TAG can contribute to the biosynthesis of eukaryotic species of MGDG.

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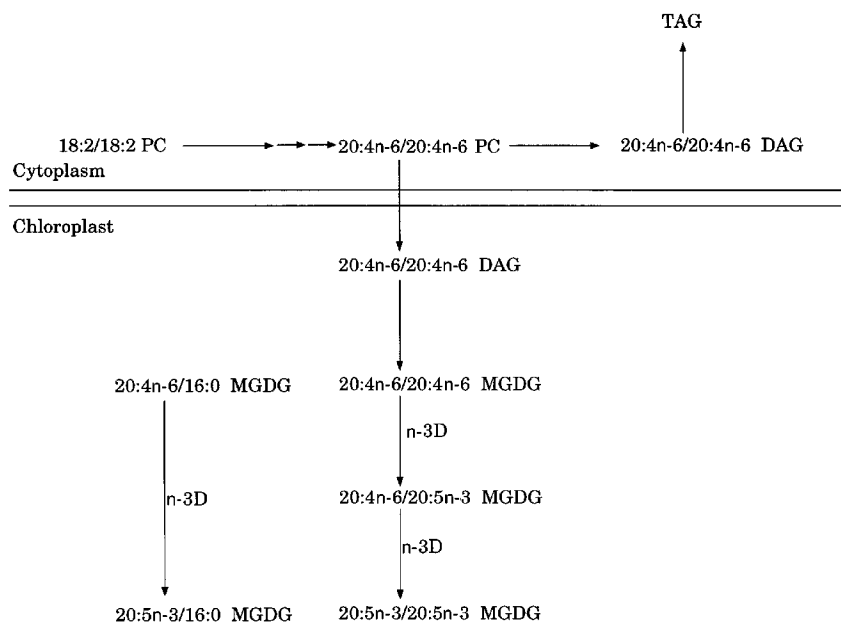
The complex biosynthetic pathways leading to the formation of the polyunsaturated fatty acid (PUFA) 18:3n-3 in higher plants' leaf lipids were reviewed by Browse and Somerville (1). The model consists of a prokaryotic pathway and a eukaryotic pathway. In the former, the fatty acids, which are synthesized *de novo* in plastids, are used as building blocks for the production of chloroplastic lipids. These lipids are characterized by the presence of a C₁₆ acyl group at the *sn*-2 position of the glycerol moiety of the lipid. In the eukaryotic pathway,

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Abbreviations: AA, arachidonic acid; C_n, fatty acid, fatty acid with *n* carbon atoms; DAG, diacylglycerol; DAGAT, diacylglycerol acyltransferase; DGDG, digalactosyldiacylglycerol; EPA, eicosapentaenoic acid (20:5n-3); MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; WT, wild type; X:Y, a fatty acyl group containing X carbon atoms and Y double bonds (*cis*). Pairs of numbers representing the fatty acids, when separated by a slash, designate the components in the *sn*-1 and *sn*-2 positions, respectively, of the molecular species.

acyl, groups, which are synthesized *de novo* in the chloroplast, are exported from the chloroplast to the cytoplasm and incorporated into phospholipids. After desaturation, most of the diacylglycerol (DAG) moieties of the phospholipids are transported back into the chloroplast to be galactosylated and further desaturated (1). These galactolipids typically contain a C₁₈ acyl group at their *sn*-2 position. DAG can also be acylated in the cytoplasm at their *sn*-3 position by a diacylglycerol acyltransferase (DAGAT) to produce (TAG). It is generally accepted that this reaction is not reversible and that TAG are end products that do not participate in any known pathway of fatty acid or lipid metabolism (1).

Although PUFA of leaf lipids and many algae contain 16 or 18 carbon atoms, some algae are unique in producing PUFA with longer carbon chains, such as 20:4n-6 [arachidonic acid (AA)], 20:5n-3 [eicosapentaenoic acid, (EPA)], and 22:6n-3 [docosahexaenoic acid, (DHA)]. The biosynthetic pathways leading to the production of 18:3n-3 in algae are believed to be similar to those suggested for higher plants (2). However, the biosynthesis of C₂₀ and C₂₂ PUFA from C₁₈ fatty acids in algae is still rather obscure (3,4). Following our study of EPA biosynthesis in *Porphyridium cruentum* (5), one of its promising sources (5,6), we recently proposed (7,8) several possible pathways leading to the biosynthesis of EPA in *P. cruentum*. In the major, n-6 pathway (Scheme 1), 18:2-bound phosphatidylcholine (PC) is converted to 20:4n-6-PC by a sequence of reactions that includes a Δ6 desaturation, an elongation step, and a Δ5 desaturation. In the minor n-3 pathway (not shown), first 18:2-PC is apparently desaturated to 18:3n-3-PC, which is further converted to 20:5n-3-PC, presumably by the same enzymes involved in the n-6 pathway. The products of both pathways are exported, as their DAG constituents, to the chloroplast to be galactosylated into the respective monogalactosyldiacylglycerol (MGDG) molecular species. Apparently, 20:4n-6 is also imported from an extra-chloroplastic lipid and inserted into the *sn*-1 position to form 20:4/16:0 MGDG, which is structurally analogous to prokaryotic species of higher plants' galactolipids. The source of 20:4 for prokaryotic lipids and its mode of transfer from the cyto-



SCHEME 1

plasm to the chloroplast still are not clear. The 20:4n-6 in both eukaryotic and prokaryotic molecular species of MGDG can be further desaturated to EPA by a chloroplastic n-3 desaturase (8).

Elucidation of the biosynthesis of PUFA in higher plants was made possible by the use of mutants of *Arabidopsis thaliana*, deficient in various steps of the biosynthesis (1). Similar mutants could be very valuable tools in the elucidation of biosynthetic pathways of long-chain PUFA in algae. Indeed, using a mutant deficient in EPA production, Schneider *et al.* (9) pointed to the existence of an extrachloroplastic $\Delta 17$ desaturase in *Nannochloropsis*. Wada *et al.* (10) showed that PUFA are necessary for growth and tolerance to photoinhibition in cyanobacteria at low temperatures. Based on the assumption that in *P. cruentum* EPA fulfills a role similar to that of 18:3n-3 in cyanobacteria and *Arabidopsis* (11), we employed the strategy utilized by Wada and Murata (12) to select for chill-sensitive mutants of *P. cruentum*. We anticipated that some mutants deficient in EPA biosynthesis could be found. Indeed, we describe here the successful selection of an EPA-deficient mutant of *P. cruentum* that is sensitive to low temperature and provides some biochemical characterization. Based on the data we have obtained, we suggest that TAG participate in the eukaryotic pathway of EPA biosynthesis in *P. cruentum*. Furthermore, HZ3 is the first described mutant of a higher or lower plant, that appears to be deficient in the ability to utilize TAG for lipid biosynthesis.

MATERIALS AND METHODS

Organism and Culture Conditions. *Porphyridium cruentum* strain 1380.1d was obtained from the Göttingen Algal Culture Collection (Göttingen, Germany) and was grown on

Jones' medium (13) as previously described (11) in Erlenmeyer flasks under an air/CO₂ (99:1) atmosphere at 25°C, unless otherwise stated. The flasks were placed in an incubator shaker and illuminated from above at a light intensity of 115 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Cultures were grown exponentially (with proper dilution) for at least 4 d prior to the onset of the experiment.

Selection of mutants. Cultures of *P. cruentum* in the logarithmic phase of growth ($1.4 \cdot 10^7$ cells mL⁻¹) were treated with 1-methyl-3-nitro-1-nitroso guanidine at a final concentration of 25 $\mu\text{g mL}^{-1}$ for 30 min. This concentration of the mutagen caused less than 5% survival. The surviving cells were plated on solid medium at a density that resulted in about 200 colonies per 90 × 15 mm plate. Then they were incubated at 25°C for 15 d. The surviving colonies were then plated onto two plates using toothpicks and incubated at 25 and 15°C, respectively. Colonies that failed to show appreciable growth at 15°C as compared to wild type (WT) cells were scored and inoculated into a small volume of liquid medium in test tubes and incubated at 25°C. When the cultures of the putative chill-sensitive lines reached comparable density, as judged by observation, the low-temperature screening was repeated by streaking equal volumes of the scored cultures on sectors of two plates side by side with aliquots of WT cultures treated similarly. One of the plates was incubated at 25°C and the other at 15°C. Cell lines that were inhibited at 15°C as compared to the WT were scored again and the screening was repeated once more.

Pulse label experiments. Ammonium salts of (1-¹⁴C)AA (5 μCi , specific activity 58 mCi/mmol) (Amersham, Little Chalfont, United Kingdom), (1-¹⁴C)linoleic acid (25 μCi , specific activity 53 mCi/mmol), and (1-¹⁴C) α -linolenic acid (25 μCi , specific activity 53 mCi/mmol) (NEN Research

Products, Mississauga, Ontario, Canada) were used in this work. Cultures were pulse-labeled for 30 min, centrifuged, washed twice with label-free medium, resuspended in one-half of the original volume, and cultivated as previously described (11). Aliquots were taken at various times after the end of the pulse. Experiments were repeated three times. The data in the figures depict the average of duplicate analyses in a representative experiment.

Nitrogen starvation. Cultures were resuspended in nitrate-free medium for 3 d. Ferric ammonium citrate was substituted with ferric citrate. After 2 d of nitrogen starvation, the cultures were labeled for 24 h with 5 μCi [$1\text{-}^{14}\text{C}$]AA and resuspended in label-free control medium. Labeling of lipids and their fatty acids was determined at various times.

Lipid extraction and analysis. Lipids were extracted using the procedure of Bligh and Dyer (14). Fatty acid methyl esters of total and individual lipids were obtained by transmethylolation with 2% sulfuric acid in methanol. Fatty acid methyl esters were separated by reversed-phase high-performance liquid chromatography (HPLC) on an RP-18, 5 μm (250 mm, Lichrospher 100; Merck, Darmstadt, Germany) column using a solvent system of methanol/acetonitrile/water, 76:12:12 (by vol), detected at 205 nm, and identified using authentic standards. Radioactivity of individual peaks was determined by a Flo-One/Beta series A-100 detector (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL). Distribution of radioactivity among individual lipids was assessed by thin-layer chromatography (TLC) on 10 \times 10 cm plates (Silica Gel 60, 0.25 mm thickness, Macherey-Nagel, Duren, Germany). Two-dimensional separations of polar lipids were carried out using a solvent system of chloroform/methanol/water, 65:25:4 (by vol) for the first direction and chloroform/methanol/1-ethylpropylamine/concentrated ammonia, 65:35:0.5:5 (by vol) for the second direction. To estimate distribution of label in neutral lipids, aliquots of total lipid extracts were separated by TLC using a solvent system of petroleum ether/diethyl ether/acetic acid, 80:20:1 (by vol). Lipids were visualized by brief exposure to I_2 vapors. Radioactivity was detected by autoradiography with x-ray films (X-OMAT AR; Kodak, Rochester, NY) exposed to the TLC plates for 17 h. Lipid spots were scraped directly into scintillation vials containing 1 mL of methanol, a scintillation cocktail was added, and radioactivity was measured in a liquid scintillation counter (Rackbeta LKB, model 1217; Wallac Oy, Turku, Finland). MGDG and digalactosyldiacylglycerol (DGDG) extracted from the silica gel plates were separated to the constituent molecular species by reverse-phase HPLC (column as mentioned above) with a solvent mixture of methanol/water, 95:5 (vol/vol) (15) on a Waters (Millipore, Milford, MA) chromatograph, equipped with ultraviolet and radioactivity detectors.

RESULTS

Selection of mutants and growth characteristics. By comparing the growth of putative mutants of *P. cruentum* on agar plates at 15 and 25°C to that of the WT, we were able to select

a series of mutants defective in growth at low temperatures. We chose the HZ3 mutant for further studies. The growth characteristics of the mutant and the WT at two temperatures are summarized in Figure 1. The growth of the mutant line at 15°C and at the optimal growth temperature of 25°C was severely inhibited. At 30°C however, inhibition of the mutant line could be observed only when cultures of low density biomass were compared, whereas at higher density, mutant cultures attained a final cell concentration similar to that of the WT cultures (data not shown).

Lipid and fatty acid composition. The proportion of TAG in the HZ3 mutant increased from 2 (in the WT) to 10% (of total fatty acids) with MGDG decreasing from 37 to 32% (Table 1). The proportion of EPA decreased from 41% (of total fatty acids) in the WT to 30% in the mutant, while the proportions of 16:0, 18:2, 18:3n-6 and 20:4n-6 increased (Table 1). The most affected lipids in the mutant were TAG, where the proportion of EPA decreased from 17 to 5%, and MGDG, with a decrease from 63 to 47%. In *P. cruentum*, the molecular species of DGDG and sulfoquinousyl diacylglycerol (SQDG) are almost entirely of prokaryotic structure, i.e., they contain C_{20} (or C_{18}) and C_{16} fatty acids in the *sn*-1 and *sn*-2 positions, respectively, while those of MGDG are partly eukaryotic and contain C_{20} fatty acids in both positions. Since MGDG was predominantly affected by the mutation, we suspected that only the synthesis of eukaryotic species was impaired. Therefore, separated MGDG and DGDG into their constituent molecular species. Indeed, the proportion of the major eukaryotic molecular species of MGDG, 20:5/20:5, decreased while that of the prokaryotic species 18:2/16:0, 20:4/16:0, and 20:5/16:0 increased (Table 2). The composition of DGDG, however, was not significantly changed. We have interpreted this as an indication that the mutation affected the eukaryotic pathway.

Incorporation of exogenously supplied radiolabeled fatty acids. Using exogenously supplied radiolabeled fatty acids, we

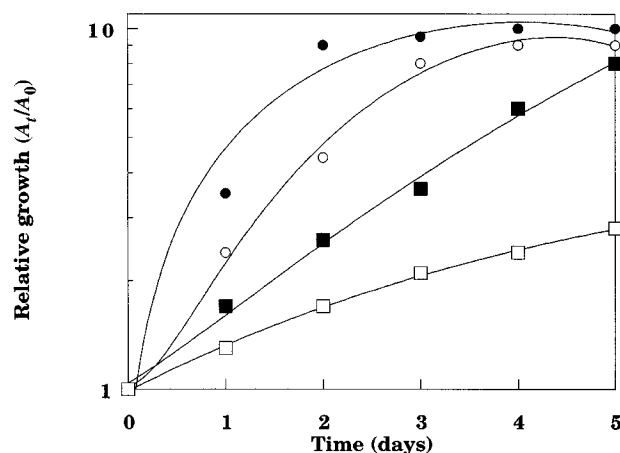


FIG. 1. Growth (expressed as chlorophyll concentration relative to day 0) of wild type (filled symbols) and HZ3 mutant (open symbols) of *Paraphrydium cruentum* cultivated at 25 (circles) and 15°C (squares) and at a light intensity of 100 $\mu\text{mol quanta m}^{-2} \text{sec}^{-1}$.

TABLE 1
Lipid and Fatty Acid Composition of Wild Type and HZ3 Mutant of *P. cruentum* at 25°C^a

Strain	Lipid	% of total lipids	Fatty acid composition (% of fatty acids)									
			16:0	16:1 ^b	18:0	18:1	18:2	18:3	20:2	20:3	20:4	20:5
						n-9	n-6	n-6	n-6	n-6	n-6	n-3
WT	Biomass	100	25.9	7.6	0.3	0.3	4.9	0.8	0.3	0.5	17.9	41.3
HZ3		100	30.4	7.7	0.4	0.4	7.5	1.2	0.3	0.7	22.3	30.3
WT	MGDG	37	26.2	0.8	0.3	0.3	4.1	0.1	—	0.1	5.5	62.6
HZ3		32	32.0	2.2	0.5	1.0	9.3	0.2	—	0.3	7.2	47.0
WT	DGDG	22	45.9	—	0.5	0.5	5.2	0.1	—	0.1	2.1	45.3
HZ3		22	50.1	0.3	0.5	0.5	4.1	0.1	—	0.1	2.9	41.2
WT	SQDG	13	49.8	0.7	1.3	0.9	1.5	—	2.0	0.2	4.4	37.1
HZ3		14	53.5	0.2	1.3	0.9	2.0	—	2.2	0.3	3.7	32.5
WT	PC	9	26.9	0.6	0.8	1.0	3.6	3.1	0.1	1.7	56.6	4.6
HZ3		7	18.5	0.9	0.9	0.8	2.0	3.0	0.1	1.9	67.7	3.7
WT	PE	2	35.0	2.3	4.4	2.8	3.4	3.4	—	0.2	33.7	13.4
HZ3		4	45.6	0.4	1.5	0.8	1.2	5.6	—	1.2	33.5	8.9
WT	PG	9	23.5	40.3	0.6	0.5	0.7	—	—	—	2.5	35.0
HZ3		6	25.4	49.9	0.9	0.3	1.0	0.1	0	0	6.2	30.0
WT	PI	2	54.0	1.7	1.3	1.5	29.6	1.2	0	0.5	5.8	3.2
HZ3		2	53.4	0.9	2.8	1.6	24.6	1.8	0	0.8	12.5	1.1
WT	PA	5	15.5	1.5	3.0	1.7	2.9	2.2	0	1.4	63.4	7.4
HZ3		3	16.6	0.8	1.5	1.0	2.1	2.8	—	1.5	68.2	3.9
WT	TAG	2	21.2	1.6	2.0	1.1	20.9	1.7	—	0.7	32.7	17.4
HZ3		10	24.6	1.7	1.2	1.4	24.6	2.2	—	1.4	37.9	4.8

^aThe data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 15% for minor peaks, of three independent samples, each analyzed in duplicate.

^bSum of two isomers. In PG 16:1Δ3t constituted 36.5 and 35.5% of fatty acids in WT and HZ3, respectively. Abbreviations: WT, wild type; MGDG, monogalactosyldiacylglycerol; DGDG, dialactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PA, phosphatidic acid; TAG, triacylglycerol.

recently showed (8) that 18:2 is converted to EPA through a major n-6 and a minor n-3 (not shown) pathway in *P. cruentum* (Scheme 1). Thus, to obtain more insight into the biosynthesis of EPA in the mutant, WT and mutant cells were pulse-labeled with [¹⁴C]18:2n-6 or [¹⁴C]20:4n-6 and the redistribution of label in the lipids and fatty acids was followed.

Incorporation of [¹⁴C]linoleic acid. Immediately after the pulse of [¹⁴C]18:2, most of the label in both the WT and the HZ3 mutant was in PC and TAG (Fig. 2A,B). Total counts did not change significantly in both cases. In the WT, the label in the lipids decreased with time in favor of chloroplastic lipids (Fig. 2C,D). While the label of PC in the mutant decreased in time in a similar pattern to that of the WT, the extent of label in TAG was relatively stable and decreased much more slowly over a period of 22 h (Fig. 2). In TAG of the WT, most of the radioactivity coming from 18:2 disappeared after 22 h, whereas

in the mutant, the decrease was much milder (Fig. 3). Radiolabeled 20:4n-6, which appeared after 4 h, accumulated faster in the mutant than in the WT. In MGDG of the mutant, 18:2 was more highly labeled, whereas 20:4 and 20:5 were less than in the WT (Fig. 4).

In comparison to the WT the molecular species analysis of MGDG of the mutant revealed a delay in the accumulation of radioactivity and eventually a decrease in all eukaryotic species, (Fig. 5). The proportion of the labeled prokaryotic species 20:4n-6/16:0 and 20:5n-3/16:0 was similar to that of the WT while that of 18:2/16:0 increased.

We also labeled the WT and the mutant with [¹⁴C]18:3n-3. The label pattern was rather similar to that obtained following the incorporation of radioactive 18:2 (data not shown).

Incorporation of [¹⁴C]arachidonic acid (AA). At the end of the pulse, PC of the WT was the most highly labeled lipid,

TABLE 2
Molecular Species Composition of Galactolipids of Wild Type and HZ3 Mutant of *P. cruentum*^a

Culture	Lipid	Molecular species composition (% of total)					
		20:5/20:5	20:4/20:5	20:4/20:4	20:5/16:0	20:4/16:0	18:2/16:0
WT	MGDG	37.7	2.5	tr	55.5	0.8	3.5
HZ3	MGDG	22.6	2.2	tr	55.9	2.8	16.5
WT	DGDG	tr	—	—	94.2	0.7	5.1
HZ3	DGDG	tr	—	—	94.6	1.6	3.8

^aGalactolipids were isolated by 2D thin-layer chromatography. Molecular species of galactolipids were separated by reversed phase high-performance liquid chromatography as detailed in the Materials and Methods section and are arranged in the order of their elution. The data shown represent mean values with a range of less than 10% for major peaks of three independent samples. See Table 1 for abbreviations.

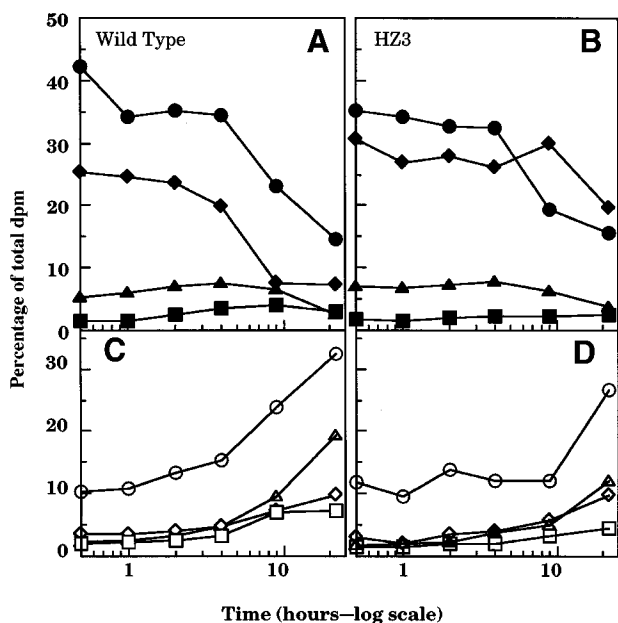


FIG. 2. Redistribution of radioactivity in lipids of wild type and HZ3 mutant of *P. cruentum* after labeling with 25 μ Ci of [$1-^{14}$ C]linoleic acid. Lipids were separated by two-dimensional thin-layer chromatography, ●, phosphatidylcholine (PC); ◆, triacylglycerol (TAG); ■, phosphatidylethanolamine (PE); ▲, phosphatidylinositol (PI); ○, monogalactosyldiacylglycerol (MGDG); △, digalactosyldiacylglycerol (DGDG); ◇, sulfoquinovosyldiacylglycerol (SQDG); □, phosphatidylglycerol (PG).

accounting for 76% of total radioactivity, while TAG constituted only 9% (Fig. 7). Gradually, the label in these lipids decreased to 15 and 1%, respectively. In the mutant, the label of PC was slightly less at the end of the pulse, but much higher after 22 h (22 vs. 15%). The label of mutant TAG was higher than that of the WT throughout the time course and showed very little decrease. Chloroplastic lipids of the mutant were more highly labeled in the first 2 h, in comparison to the WT, but less labeled after 22 h. In the mutant, the fatty acid com-

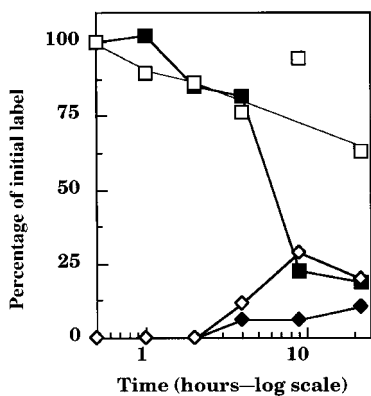


FIG. 3. Redistribution of radioactivity in fatty acids of TAG of wild type (filled symbols) and HZ3 mutant (open symbols) after labeling with [$1-^{14}$ C]linoleic acid. Data presented as percentage of initial label. Fatty acids were determined by radio-high-performance liquid chromatography, ■, 18:2; ◆, 20:4n-6. For abbreviation see Figure 2.

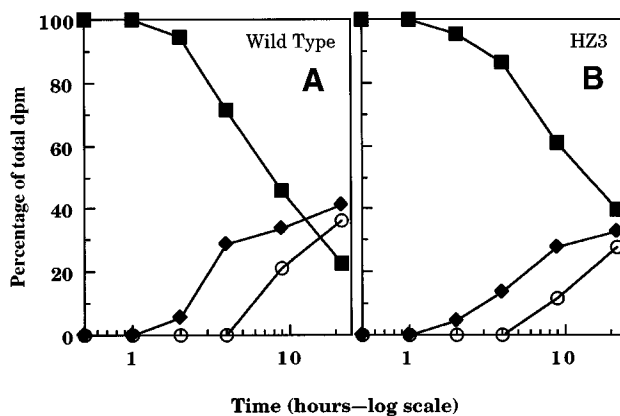


FIG. 4. Redistribution of radioactivity in fatty acids of MGDG of wild type and mutant after labeling with [$1-^{14}$ C]linoleic acid. ■, 18:2; ◆, 20:4n-6, ○, 20:5n-3. For abbreviation, see Figure 2.

position of total lipids and MGDG showed a decrease in the conversion of 20:4n-6 to 20:5 (data not shown).

Recovery from nitrogen starvation. Under nitrogen starvation, cells of *P. cruentum* accumulate TAG (16). Thus, to evaluate the extent of the involvement of TAG in the biosynthesis of eukaryotic MGDG, we studied growth resumption following recovery from nitrogen starvation. When nitrogen is replenished, growth is resumed and chloroplastic lipids, especially eukaryotic MGDG, are actively produced (16,17). AA was used for labeling since it is one of the major fatty acids of TAG, especially under nitrogen starvation. Furthermore, the acyl moieties exported from the cytoplasm are predominantly C_{20} fatty acids, AA and EPA. We resuspended cultures of WT and HZ3 *P. cruentum* in nitrogen-free medium and kept them in this medium for 3 d. Two d after the medium change, the cultures were labeled with [$1-^{14}$ C]AA for 24 h, as

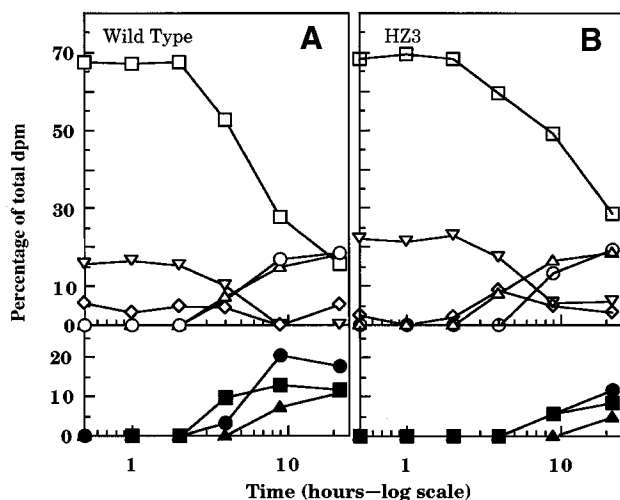


FIG. 5. Redistribution of radioactivity in molecular species of MGDG of wild type and HZ3 mutant after labeling with [$1-^{14}$ C]linoleic acid. □, 18:2/16:0; ○, 20:4/16:0; ▽, 20:4/18:2; △, 20:5/16:0; ◇, 20:5/18:2; ■, 20:4/20:4; ●, 20:4/20:5; ▲, 20:5/20:5. See Figure 2 for abbreviation.

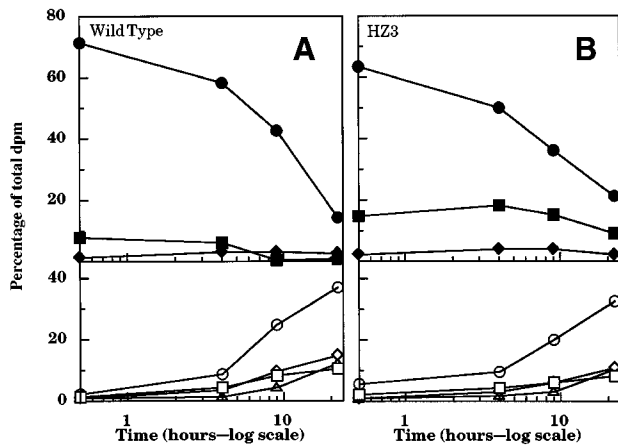


FIG. 6. Redistribution of radioactivity in lipids of wild type and HZ3 mutant *P. cruentum* after labeling with 10 μCi [$1\text{-}^{14}\text{C}$]20:4n-6. ●, PC; ◆, TAG; ■, PE; ○, MGDG; △, DGDG; ◇, SQDG; □, PG. See Figure 2 for abbreviations.

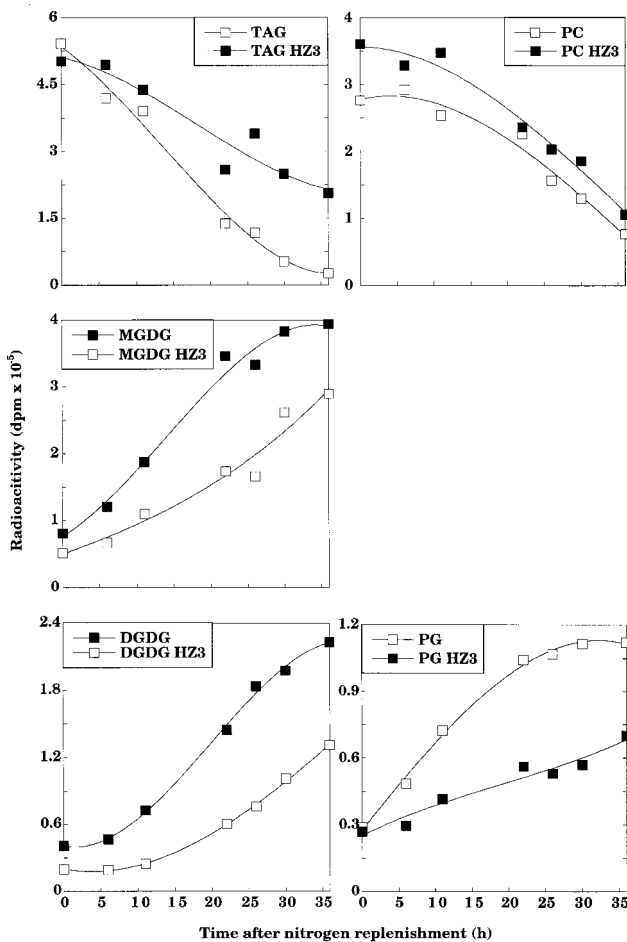


FIG. 7. Transfer of radioactivity from cytoplasmic (TAG and PC) to chloroplastic (MGDG, DGDG, and PG) lipids in wild type and HZ3 mutant *P. cruentum* following recovery from nitrogen starvation and labeling with 5 μCi [$1\text{-}^{14}\text{C}$]20:4n-6. See Figure 2 for abbreviations.

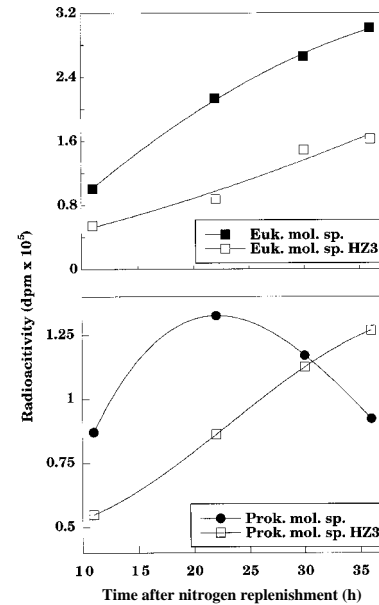


FIG. 8. Increase in label of prokaryotic (prok.) and eukaryotic (euk.) molecular species (mol. sp.) of MGDG in wild type and HZ3 mutant *P. cruentum* following recovery from nitrogen starvation and labeling with 5 μCi [$1\text{-}^{14}\text{C}$]20:4n-6. See Figure 2 for abbreviation.

preliminary studies have shown that after that period, TAG were already maximally labeled. Following the pulse, there were no significant differences between the level of radioactivity incorporated into the WT and the mutant, or in the distribution of the label between cytoplasmic and chloroplastic lipids (Figs. 7,8). However, with time, the label in the cytoplasmic lipids of the WT declined faster than that of the mutant, while that of the chloroplastic lipids increased to a greater extent in the WT compared to the mutant. After 36 h, WT cytoplasmic lipids retained only 15% of total counts in comparison to 40% in the mutant (data not shown). While PC of both cultures lost about 70% of their label, WT TAG lost 95% of its original label in comparison to only 59% in the mutant (Fig. 7). Molecular species analysis of MGDG showed that, whereas the prokaryotic species (20:4n-6/16:0 and 20:5n-3/16:0) were similarly labeled throughout the time course, there were large differences in the labeling of the eukaryotic species. Labeling of all the eukaryotic species (20:4n-6/20:4n-6, 20:4n-6/20:5n-3, 20:5n-3/18:2, and mostly 20:5n-3/20:5n-3) of the mutant was about half of the WT (Fig. 8).

DISCUSSION

C_{18} PUFA contribute to chill tolerance in higher plants (18) and cyanobacteria (19). In *P. cruentum*, the proportion of the main PUFA, EPA, increases in MGDG, its major depot, from 10% at 30°C to 51% at 20°C (17). This is reflected in an enhancement in the proportion of the ultimate eukaryotic molecular species, 20:5/20:5, from 6 to 40%. Furthermore, we have shown that the proportion of EPA at each temperature is

correlated with growth rate (11). We have thus hypothesized that the comparison of the growth rates at optimal and low temperatures would be a tool for the isolation of mutants of *P. cruentum* deficient in EPA content. Indeed, we were able to select such mutants. One of these mutants, HZ3, displayed the most severe growth impairment at reduced temperatures (Fig. 1) and was selected for further studies. The lipid and fatty acid analysis of the HZ3 mutant indicated a reduced level of eukaryotic molecular species of MGDG, which could be the consequence of a deficiency in the eukaryotic pathway. The reduced growth rate at 15°C (Fig. 1) of the HZ3 mutant and the low level of EPA, suggest that this fatty acid may have a role in the growth of microalgae at lower temperatures. However, further studies are required in order to exclude the possibility that the inhibition of growth and the impairment of lipid metabolism are not the result of a pleiotropic effect of a single mutation or the result of two or more independent mutations.

The labeling experiments with each of the different radioactive fatty acids revealed that mutant TAG accumulated a higher percentage of the initial label and were severely limited in turning over the label in comparison to the WT. One may argue that the higher TAG content of the HZ3 mutant represents a larger pool size, which is responsible for the delay in the turnover of the label. However, some decrease would have been expected in the first h after the pulse, but there was no decrease whatsoever in the label of mutant TAG for the first 10 h (Figs. 2,6). Under nitrogen starvation, the TAG content was similar in the WT and the mutant, and yet the same differences were observed (Fig. 7). That the disappearance of label from PC in the mutant was similar to that of the WT indicates that any contribution of DAG moieties of PC to the eukaryotic pathway is not impaired. We interpret our *in vivo* radiolabeling studies as showing that, in addition to PC, there is a notable contribution of TAG to the synthesis of chloroplastic lipids of *P. cruentum*. Possibly, the mutant is deficient in its ability to mobilize DAG (or acyl) moieties from TAG for the production of eukaryotic molecular species of MGDG. In oilseeds, which accumulate PUFA, TAG share a common DAG pool with phospholipids, primarily with PC. The conversion of DAG to TAG in oil-accumulating tissues is generally considered to be unidirectional (1) and TAGs are regarded metabolically as end-products that are used only as an energy store. However, Garcés *et al.* (20) showed that when the growth temperature of developing sunflower seeds is reduced, the oleate acyl groups of TAG are superseded by linoleates. Recently, Stobart *et al.* (21) obtained evidence that supports a transacylation mechanism that can account for the TAG turnover in microsomal membranes of developing safflower seeds.

Nevertheless, we cannot exclude the possibility that the genetic lesion occurred in a different site that is responsible for the production of a component requiring high levels of eukaryotic MGDG. In the mutant, lower levels of this component would require lower levels of eukaryotic MGDG, resulting in a down regulation of the contribution of TAG to the production of these molecular species.

Under nitrogen starvation, much of the acyl flux of *P. cru-*

entum is diverted from the production of chloroplastic lipids, predominantly eukaryotic MGDG, to the accumulation of TAG (16). Replenishing the nitrogen to the algal cells results in a quick return to exponential growth. This, in turn, requires the synthesis of new chloroplastic membranes. The massive transfer of label from TAG to eukaryotic MGDG supports our hypothesis that C₂₀ PUFA deposited in TAG of *P. cruentum* can be utilized as a reservoir for the swift production of eukaryotic MGDG when necessary.

Algal TAG are generally characterized by saturated and monounsaturated fatty acids, thought to serve as storage material (22). These characteristics seem to be common to most algal species studied for their potential to produce C₂₀ PUFA. The TAG of the eustigmatophyte *Nannochloropsis*, an EPA producer, contains mainly 14:0, 16:0, and 16:1 (23). Similar findings were reported for other EPA-producing algae such as *Monodus subterraneus* (24) and *Phaeodactylum tricorutum* (25). Likewise, the TAGs of the DHA-rich cryptomonad *Chroomonas salina* (26) are almost entirely made of C₁₈ fatty acids. However, certain algae are able to produce TAG rich in EPA and AA, e.g., *Ectocarpus fasciculatus* (27), *Pavlova lutheri* (28), *Nitzschia frigida*, and *Melosira antarctica* (29). A high content of AA in TAG appears to be a feature of many, if not all, rhodophytes. Thus, the proportion of AA in TAG was reported to be 36% in *Chondrus crispus*, 49% in *Polysiphonia lanosa* (30), 40 to 64% in various *Gracilaria* sp. (31), and 33% in *P. cruentum* (as well as 17% EPA) (11). The ability of *P. cruentum* to utilize its TAG may explain the unique fatty acid composition of these lipids. However, it remains to be seen whether other microalgae having PUFA-rich TAG are able to utilize their TAG for similar purposes.

At present, we have no data to support any hypothesis concerning the mechanism by which the reutilization of TAG may take place. Nonetheless, several possibilities can be considered: i) a transacylation of monoacylglycerol by TAG to produce two molecules of DAG (21); ii) a lipase activity that hydrolyzes TAG to DAG; and (iii) a DAGAT activity that is also capable of operating in the reverse direction. However, in the case of the latter, one would have expected a lower than normal level of TAG since this enzyme is likely to affect the incorporation of label into TAG, not just its turnover.

In higher plants, especially in *Arabidopsis*, mutants deficient in the production of either the prokaryotic or the eukaryotic molecular species of chloroplastic lipids demonstrated normal behavior at normal or low temperatures. Furthermore, it was shown that these mutants were able to ameliorate the damage to their membranes by adjusting the fatty acid flux leading to the prokaryotic and the eukaryotic pathways (32). In higher plants, the ultimate prokaryotic molecular species of MGDG, 18:3/16:3, differs from the eukaryotic species, 18:3/18:3, by only two carbon atoms. We speculate that for this reason, prokaryotic and eukaryotic molecular species of MGDG of higher plants are, to a certain extent, interchangeable. In *P. cruentum*, however, the eukaryotic molecular species, 20:5/20:5, contain four more carbon atoms and five more double bonds than the prokaryotic species, 20:5/16:0.

P. cruentum is found mainly in shallow marshes where temperature fluctuations are rapid and more pronounced than in deeper water bodies. The increase in the proportion of EPA in MGDG at low temperatures, and especially in that of the eukaryotic component of MGDG, 20:5/20:5, could possibly be attributed to the organism's attempt to cope with the stress inflicted by sudden drops in temperature. However, the *de novo* synthesis of EPA is apparently not fast enough to accommodate the increased demand for EPA. When *P. cruentum* was labeled with radioactive acetate, labeled EPA appeared only after 10 h (data not shown, see also Fig. 4). We hypothesize that TAG can be utilized as a buffering system for 20:4- and 20:5-containing DAG, which can be mobilized relatively rapidly for the production of eukaryotic molecular species of the major chloroplastic lipid MGDG. The role of these PUFA is not just maintenance of membrane fluidity. In various algae, enhanced n-3 desaturation was shown to be correlated with the activity of photosystem I (33). This hypothesis is supported by the work of Wanner and Kost (34) who found that rapid formation of cellular membranes from lipid bodies of different intracellular localization (cytoplasmic oil bodies and chloroplastic plastoglobules) has been observed during regeneration of starved cells of *P. cruentum*.

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