Genomic and Biochemical Analysis of Lipid Biosynthesis in the Unicellular Rhodophyte *Cyanidioschyzon merolae*: Lack of a Plastidic Desaturation Pathway Results in the Coupled Pathway of Galactolipid Synthesis†‡

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The acyl lipids making up the plastid membranes in plants and algae are highly enriched in polyunsaturated fatty acids and are synthesized by two distinct pathways, known as the prokaryotic and eukaryotic pathways, which are located within the plastids and the endoplasmic reticulum, respectively. Here we report the results of biochemical as well as genomic analyses of lipids and fatty acids in the unicellular rhodophyte *Cyanidioschyzon merolae*. All of the glycerolipids usually found in photosynthetic algae were found, such as mono- and digalactosyl diacylglycerol, sulfolipid, phosphatidylglycerol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. However, the fatty acid composition was extremely simple. Only palmitic, stearic, oleic, and linoleic acids were found as major acids. In addition, 3-trans-hexadecenoic acid was found as a very minor component in phosphatidylglycerol. Unlike the case for most other photosynthetic eukaryotes, polyenoic fatty acids having three or more double bonds were not detected. These results suggest that polyunsaturated fatty acids are not necessary for photosynthesis in eukaryotes. Genomic analysis suggested that *C. merolae* lacks acyl lipid desaturases of cyanobacterial origin as well as stearoyl acyl carrier protein desaturase, both of which are major desaturases in plants and green algae. The results of labeling experiments with radioactive acetate showed that the desaturation leading to linoleic acid synthesis occurs on phosphatidylcholine located outside the plastids. Monogalactosyl diacylglycerol is therefore synthesized by the coupled pathway, using plastid-derived palmitic acid and endoplasmic reticulum-derived linoleic acid. These results highlight essential differences in lipid biosynthetic pathways between the red algae and the green lineage, which includes plants and green algae.

The acyl lipids that make up the plastid membranes are highly enriched in polyunsaturated fatty acids in most plants and algae. In flowering plants, linoleic (18:2) and linolenic (18:3) acids are the most common unsaturated acids, whereas in many algae, highly unsaturated long-chain acids, such as arachidonic (20:4), eicosapentaenoic (20:5), and docosahexaenoic (22:6) acids, are found as major fatty acid components, depending on the species (4, 15, 17, 38, 50). (Fatty acids are expressed by a combination of the number of carbon atoms [X] and the number of double bonds [Y], such as X:Y. The positions of double bonds are specified in parentheses.) Unsaturated fatty acids, especially polyunsaturated ones, are important in maintaining membrane fluidity during the cold acclimation of photosynthesis (37). Linolenic acid was also found to be important in tolerance to high temperatures (32). The plastid lipids consist of two types of molecular species, namely, 1-C18:2-C16 species, or prokaryotic molecular species, and 1-C18:2-C18 species, or eukaryotic species. The relative abundance of these two types of molecular species varies with plants and algae. In flowering plants, the prokaryotic and eukaryotic types of lipid molecular species are synthesized by two distinct pathways, known as the prokaryotic and eukaryotic pathways, which are located within the plastids and the endoplasmic reticulum (ER), respectively (for a review, see reference 10). However, lipid biosynthesis in red algae, which constitute another major lineage of photosynthetic eukaryotes (11, 30, 42, 56), remains largely unknown.

*Cyanidioschyzon merolae*, a unicellular rhodophyte isolated from an Italian hot spring, has a very simple cell structure consisting of one mitochondrion, plastid, and microbody per cell (25). Its normal habitat is warm (up to 50°C) and acidic (pH 1.5 to 2.5) water containing sulfuric acid. The size of the nuclear genome is 16.5 Mbp (28). The cell proliferates by binary fission. These characteristics, as well as phylogenetic analyses (34), suggested that *C. merolae* is one of the most primitive red algae, probably diverged from near the root of the red lineage. The red lineage includes red algae, whereas the green lineage includes green algae and land plants (42). The single origin of plastids in the red and green lineages is believed to be highly probable (27, 42), and the single origin of plastid-harboring cells in these two lineages is gaining supporting evidence (30, 34). In addition, the chromists (brown algae, diatoms, cryptophytes, etc.) are believed to originate from secondary endosymbiosis by an ancestral red algal cell (11, 42, 56). *C. merolae* is therefore a good target of comparative biochem-

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istry to reveal similarities and differences in the red and green lineages.

There is a short report on the total fatty acids of *C. merolae* (31). Among thermoacidophilic red algae, *Cyanidium caldarium* and *C. merolae* contained no detectable ω-linolenic acid (18:3), while this acid was abundant in *Pleurococcus sulfuratus* (currently called *Gallidiera sulfuraria*) at a low temperature. There was a report on α-18:3 in *Cyanidium caldarium* (23), but the current understanding is that several different algae were called *Cyanidium* in the past. No further analysis of the composition and biosynthesis of *C. merolae* has been attempted since the paper by Moretti and Nazzaro (31).

Apart from experimental analyses, one postgenomic study is to find all possible candidate enzymes of a metabolic pathway. For *Arabidopsis thaliana*, an attempt to make functional annotations for all proteins involved in lipid metabolism is in progress (7). For *Chlamydomonas reinhardtii*, draft sequence data were used to predict proteins that might be involved in lipid biosynthesis (36). The functional annotation of *C. merolae* has also been started (28). We have been trying to compare the genome contents of *A. thaliana* and *C. merolae* as well as cyanobacteria, and many proteins that are conserved in all of these photosynthetic organisms are being identified (45). Comparative genomics of these photosynthetic organisms are now conveniently analyzed through a web interface called the Gclust server (43; http://gclust.c.u-tokyo.ac.jp/). Based on such informatics, we are now able to predict probable proteins that are involved in acyl lipid metabolism in *C. merolae*.

In this report, we try to find distinct features of the red lineage with respect to lipid biosynthesis, using *C. merolae* as a model organism. We present results on the analysis of lipids and fatty acids in *C. merolae*. Next, lipid biosynthetic pathways of this alga are summarized based on the genomic data. The intracellular localization of some key enzymes was confirmed by green fluorescent protein (GFP) experiments. The origin of the minimal set of desaturases of this alga was established by comprehensive phylogenetic analysis. Finally, tracer experiments were conducted to validate the lipid biosynthetic pathway estimated by the genomic analysis. The coupled pathway for the synthesis of galactolipids is proposed, involving plastid-derived palmitic acid and extraplastidically synthesized linoleic acid. All of these data indicate distinct differences between the red and green lineages in lipid biosynthesis, in spite of their monophyletic origin.

**MATERIALS AND METHODS**

**Growth of organism.** Cells of *Cyanidioschyzon merolae* strain 10D (55) were grown under continuous white light (about 20 μE m⁻² s⁻¹) in the AA medium described by Allen (2), with aeration in 1% CO₂ at 35°C or 25°C.

**Analysis of lipids and fatty acids.** All analytical methods used were essentially the same as those described in previous publications (44, 48). Briefly, lipids were extracted from the cells by the method of Bligh and Dyer (8) and were separated by two-dimensional thin-layer chromatography (TLC). Lipids were quantified by measuring the amounts of fatty acids, determined as their methyl esters, by gas chromatography. A fused silica capillary column (0.25-mm internal diameter by 50 m) coated with SS-10 (equivalent to Silic 10C; Siwama Kako, Kyoto, Japan) was used. The following temperature program was used: 0.5 min at 180°C, a linear increase to 230°C at a rate of 3°C min⁻¹, and then 10 min at 230°C. Under these conditions, most commonly occurring isomers of fatty acid methyl esters were clearly separated. Fatty acid methyl esters from total lipids of *Ablantia capitata* (44) and those from monogalactosyl diacylglycerol (MGDG) of *Anabaena variabilis* (46) were used as references. The positional distribution of fatty acids within individual classes of lipids (including phospholipids) was analyzed by specific hydrolysis of the C-1 acyl ester linkage with the lipase from *Rhizopus delemar* (16) or the C-2 acyl ester linkage with phospholipase A₂.

**Radiolabeling of lipids.** *C. merolae* cells (25-ml culture) that had been grown at 38°C were incubated with [2,14C]acetate (2.0 MBq) at 38°C for 1 h in the light, with vigorous shaking, in a tightly closed 100-ml flask. Unlabeled acetate (3 mM) was then added, and the cells were harvested by centrifugation. They were washed once with fresh medium and then resuspended in fresh medium. The cells were allowed to grow under normal growth conditions for 20 h. Aliquots were withdrawn at 0, 2, 6, 10, and 20 h, and lipids were extracted. Lipids were separated by two-dimensional TLC, and then the lipid spots were detected with primuline under UV light. The analysis of radioactive lipids was performed essentially as described previously (39). Radioactivity was located by autoradiography. Radioactive lipid spots were scraped off, and the radioactivity was measured by liquid scintillation counting.

For detailed analysis, MGDG, digalactosyl diacylglycerol (DDDG), and phosphatidylcholine (PC) were recovered from the TLC plate. Lipid molecular species were analyzed by argentation TLC (48), and radioactivity was detected by autoradiography. A precoated silica gel plate (Merck) was impregnated with AgNO₃, by immersing it in 5% AgNO₃ in acetonitrile for 30 min and then was dried at 60°C for 30 min. The developing solvents were aceton-benzoate-water (90:30:8, by volume) for MGDG and chloroform-methanol-water (60:30:5, by volume) for DDDG and PC. For fatty acid analysis of individual lipid classes, the isolated lipids were subjected to methanolysis. The resultant fatty acid methyl esters were analyzed by reversed-phase argentation TLC (26), a technique recently developed for the analysis of small amounts of radioactive fatty acids. A RP-18 HPTLC plate (Merck) was used. The developing solvent was 10% AgNO₃ in acetonitrile–1,4-dioxane-acetic acid (90:20:1, by volume). This TLC method clearly resolves 18:1 and 16:0, which comigrate in ordinary argentation TLC.

Incorporation of radioactive galactose was performed by incubating isolated plastids of *C. merolae*, which were prepared according to a published protocol (51), with modifications (T. Moriyama, K. Terasawa, M. Fujinawa, and N. Sato, unpublished data), with 37 kBq UDP-[14C]galactose (GE Healthcare/Amer sham) in 400 μl plastid isolation medium at 38°C for 1 h. Lipids were extracted and separated by two-dimensional TLC. MGDG and DDDG were recovered, and the molecular species were analyzed by argentation TLC.

**Genomic data and computational sequence analysis.** Genomic data for *C. merolae* were generated and annotated by the Cyanobichcyzon Genome Project (28), in which genes related to lipid metabolism were estimated by BLAST2 (3) searches, with known genes as queries. The sequences of the seed genes were retrieved from GenomeNet (ftp://ftp.genome.ad.jp/). The Gelust database, recently made publicly accessible in the Gelust server (43), was also used to find phylogenetically conserved proteins. Sequence manipulation was performed with the SIEVE package, version 1.30 (40). The sequence alignment of desaturases was prepared by the Clustal X program (12) after trimming of poorly conserved N and C termini. Phylogenetic analysis was done with MEGA2 software, version 2.1 (24), a DAP software, version 4.1 (26), ClustalX (5), and the MOLPHY package, version 2 beta 3 (1).

**Targeting of GFP fusion proteins.** DNA constructs consisting of the 35S promoter, a 5’ part of the putative Δ12 desaturase gene, and the NOS terminator were made by successive PCRs. The sGFP plasmid (13) was used as the template for the 5’ and the 3’ parts, while the PCR fragment corresponding to various parts of the *C. merolae* CMK291C gene was obtained by using the *C. merolae* genome as a template. The following three constructs were made: Met1-GFP, Met2-GFP, and Met3-GFP, containing residues 1 to 531, 202 to 531, and 364 to 531, respectively (numbers refer to the nucleotide count beginning from the most上游 ATG). The sGFP plasmid (13) was used as the template for the 5’ and the 3’ parts, while the PCR fragment corresponding to various parts of the *C. merolae* CMK291C gene was obtained by using the *C. merolae* genome as a template. The following three constructs were made: Met1-GFP, Met2-GFP, and Met3-GFP, containing residues 1 to 531, 202 to 531, and 364 to 531, respectively (numbers refer to the nucleotide count beginning from the most upstream ATG). The 5’ part includes the 35S promoter until the multiple cloning site, while the 3’ part includes the NOS terminator sequence beginning from the multiple cloning site in the sGFP plasmid. The constructs were introduced into a scaly leaf of an onion bulb by particle bombardment using a PDS-1000/He particle delivery system (Bio-Rad). Rupture disks for 1,100 lb/min and tungsten particles with a diameter of 1.1 μm were used. After 24 h of continued growth, the epidermis was peeled and examined under a fluorescence microscope (Olympus model BX-60) with an IB filter cube.

**RESULTS**

**Composition of lipids and fatty acids.** We started by analyzing the compositions of lipids and fatty acids in *C. merolae* cells (Table 1). Major lipids included common chloroplast lipids, such as MGDG, DDDG, and sulfoquinovosyl diacylglycero-
TABLE 1. Fatty acid composition of individual lipid classes at two different growth temperatures

| Fatty acid          | MGDG | DGDG | SQDG | PG | PE | PC | PI |
|---------------------|------|------|------|----|----|----|----|
| 14:0                | 0.2  | 0.0  | 0.0  | 0.0| 0.0| 0.0| 0.0|
| 16:0                | 3.5  | 1.6  | 2.44| 0.0| 0.0| 0.0| 0.0|
| 16:1(9)             | 0.9  | 0.0  | 1.55| 1.5| 0.0| 0.0| 0.0|
| 18:0                | 1.5  | 2.44| 0.0  | 0.0| 0.0| 0.0| 0.0|
| 18:1(9)             | 1.4  | 0.0  | 0.0  | 0.0| 0.0| 0.0| 0.0|
| 18:2(9,12)          | 1.5  | 0.0  | 0.0  | 0.0| 0.0| 0.0| 0.0|

Each value is an average percentage for three different determinations. Standard deviations are shown for nonzero values. For lipid composition, each value represent an average percentage for two (25°C) or three (38°C) different determinations.

18:2 was attached to the C-1 position. This is the prokaryotic id, 16:0 was primarily bound to the C-2 position, whereas MGDG, DGDG, SQDG, and PG (chloroplast or plastid lipids) positional distribution of fatty acids in individual classes of lipids. Cells grown at 38°C and 25°C were analyzed. All classes of lipids contained 16:0 and 18:2 as the major acids, although 16:0 accumulated to about 70% in SQDG. 18:0 and 18:1 were also abundant in PE, PC, and PI. 16:1(9), which is commonly found in lipids of cyanobacteria (Fig. 1D) (22, 46) and algae (17), was detected only in PC, at a very low level. The level of 3-trans-16:1 in PG (Fig. 1C) was significantly lower in C. merolae (ca. 5%) than in land plants and algae analyzed to date (15 to 40%). Effects of growth temperature were noted for the fatty acid compositions of MGDG, PE, PC, and PI. In MGDG, the level of 18:2 was higher, while that of 16:0 was lower, at 25°C than at 38°C. For the three classes of phospholipids analyzed, the level of 18:2 was higher, while that of 18:1 was lower, at 25°C. The level of 18:0 was also lower in PE and PC. However, the growth temperature did not change the qualitative compositions of fatty acids.

Positional distribution of fatty acids. An analysis of the positional distribution of fatty acids in individual classes of lipids revealed marked differences between the lipids known as chloroplast lipids and other phospholipids (Table 2). In MGDG, DGDG, SQDG, and PG (chloroplast or plastid lipids), 16:0 was primarily bound to the C-2 position, whereas 18:2 was attached to the C-1 position. This is the prokaryotic type of distribution (1-C18-2-C18) found in the chloroplast lipids of plants and algae. In addition, a significant level of 18:2 was also found at the C-2 position in MGDG and DGDG. This points to the presence of the eukaryotic type of molecular species (1-C18-2-C18). SQDG contained 1-C16-2-C18 molecular species as well. The 3-trans-16:1 species was exclusively bound to the C-2 position of PG, as in the case of plants and algae. A totally different distribution was found in PE and PC. 16:0 and 18:0 were bound to the C-1 position, whereas the C-2 position was occupied mainly by 18:1 and 18:2. A low level of 18:2 also bound to the C-1 position. Therefore, PE and PC consisted mainly of the 1-saturated-2-unsaturated type of molecular species, as in many eukaryotes. These results suggest that the
plastid lipids of *C. merolae* consist of prokaryotic and eukaryotic molecular species, as occurs in plants.

**Genes involved in the synthesis of lipids and fatty acids.** We then searched the genomic data of *C. merolae* to infer genes involved in the synthesis of fatty acids and lipids (Table 3). All common genes for the synthesis of long-chain saturated fatty acids were detected. Two types of acetyl-coenzyme A (acyl-CoA) carboxylases, namely, a prokaryotic multisubunit enzyme of the plastid and a multifunctional cytoplasmic enzyme, were found. The plastid localization of the nuclear *accC* gene (*CMS299C*) product was confirmed by GFP experiments (Table 3; see Fig. S12 in the supplemental material). Two copies of the condensing enzymes, CMM286C and CML329C, were detected, but the former was confirmed as the plastid enzyme (see Fig. S12 in the supplemental material). Only three genes for putative desaturases were found. The gene for the stearoyl acyl carrier protein (ACP) desaturase, which is essential in producing oleate in flowering plants, was not detected. According to the GcLust database (data set CZ35, cluster 4024), putative genes for this enzyme were detected in the green alga *Chlamydomonas reinhardtii* (36) and the diatom *Thalassiosira pseudonana* (5).

Genes encoding the enzymes involved in the synthesis of complex lipids were also identified by homology searches (Table 3). The eukaryotic MGDG synthase (encoded by CMI271C) (9) was found, and its intracellular localization was confirmed with a GFP fusion protein (Table 3; see Fig. S12 in the supplemental material). Curiously, a homolog of the Synecocystis enzyme Sll1377, which was recently found to be the enzyme involved in the synthesis of monoglucosyl diacylglycerol (GlcDG) (6), was also detected (CMT267C) (GcLust data set CZ20x0, cluster 2792). However, we could not detect a spot of putative GlcDG migrating slightly faster than MGDG in TLC after a short (10 min) pulse-labeling period (data not shown), which normally can detect a significant amount of GlcDG (equivalent to the amount of MGDG) in *Anabaena* (47). The function of CMT267C therefore still has to be determined.

Another point is the lack of a plant-type DGDG synthesis enzyme (DGD1/DGD2). No homolog of DGD1/2 (GcLust data set CZ23, cluster 5665) has been detected in cyanobacteria. Until now, a cyanobacterial DGDG synthesis enzyme has not
been reported. This raises the possibility that *C. merolae* has a cyanobacterial DGDG synthesis enzyme (see Discussion).

Cardiolipin was not detected (see above), but genomic data suggested the presence of an enzyme for cardiolipin biosynthesis (CMN196C) (Gclust data set CZ20x0, cluster 4414). The family of cluster 4414 was originally annotated as a phosphatidylglycerophosphate synthase, but Katayama et al. (21) showed that the *Arabidopsis* homolog is involved in the synthesis of cardiolipin. To increase the sensitivity of detection of phospholipids, *C. merolae* cells were incubated with $[\ulsecond P]$ phosphate, and the lipids were analyzed by two-dimensional TLC (results not shown). However, the putative spot of cardiolipin was still obscure, and we cannot definitively confirm the presence of this lipid at a very low level.

The genomic analysis suggested that *C. merolae* has the capability of synthesizing some sterols (Table 3), although this is not the main topic of the present study. In Table 3, lanosterol synthase is listed because of its homology, but the exact specificity of the enzyme must be determined experimentally.

Sterol methyltransferases with unknown specificity are also predicted (not listed in Table 3).

**Phylogenetic analysis of desaturases.** To obtain further information on the biosynthesis of fatty acids, a phylogenetic analysis of desaturases was performed (Fig. 2A). An uncompressed version of the identical tree is available upon request. The $\Delta9$ desaturases are divergent from the $\Delta12$ and $\omega3$ desaturases, and all of these groups of desaturases, though still significantly homologous, diverged from each other before the separation of prokaryotes and eukaryotes (Fig. 2A). Other types of desaturases, such as $\Delta6$ desaturases, were also included in the published large phylogenetic tree of desaturases (54), but they are too divergent to allow construction of a reliable tree. Among the acyl-CoA $\Delta9$ desaturases, one group of enzymes have an extra cytochrome $b$ domain (29, 33). One of the *C. merolae* enzymes (CMN045C) belongs to this type (20). Acyl lipid $\Delta9$ desaturases are typically found in cyanobacteria (DesC), and homologs are also found in plants, which are known to function as $\Delta7$ desaturases acting on MGDG (19).
Cyanobacterial DesC and plant DesC-like proteins are sister groups (Fig. 2A). This suggests that the DesC-like enzymes in plants originated from the cyanobacterial endosymbiont. However, the second Δ9 desaturase (CMJ201C) in C. merolae is outside the DesC group and close to bacterial enzymes.

The phylogeny of the Δ12 (and ω3) desaturases is complicated (Fig. 2A). The cluster that diverges from the root includes bacterial and some marine cyanobacterial enzymes of unknown specificity (cluster I). Cluster II includes cyanobacterial DesA and plant FAD6 localized in the chloroplast. Another large cluster includes plant FAD2, which is localized in the ER (cluster IV). The Δ12 enzymes in marine cyanobacteria, C. merolae, and Phaeodactylum tricornutum (diatom) are related to the FAD2 group. The desaturases of nematodes (cluster III) diverge from both cluster II and cluster IV. Interestingly, ω3 desaturases (except the nematode enzyme) diverge from cluster IV of Δ12 desaturases. This is supported by a high bootstrap confidence level (89%). The cyanobacterial DesB protein was identified as the origin of the plant ω3 desaturases, including both chloroplast and ER isozymes.

These results show close relationships of cyanobacterial DesA and DesB with FAD6 and FAD7 in chloroplasts of plants and green algae (green lineage), respectively. Among cyanobacteria, marine species, such as Prochlorococcus marinus, have a cluster IV enzyme but no DesA or DesB. These enzymes were probably acquired by horizontal gene transfer, and this result should not be considered evidence that the C. merolae Δ12 desaturase originated from marine cyanobacteria. This is in clear contrast with Δ9 desaturases, for which both marine and freshwater species of cyanobacteria have orthologous DesC proteins.

These results suggest that the desaturases of C. merolae are unrelated to the enzymes of the cyanobacteria and the green lineage. Assuming the monophyletic origin of the red and...
green lineages (27, 28, 42), this indicates that the red alga does not keep cyanobacterial desaturases and retains the desaturases that existed before the cyanobacterial endosymbiosis. In addition, the Δ12 desaturases of the diatom *P. tricornutum*, PtFAD2 and PtFAD6, also clustered with the Δ12 desaturases of *C. merolae* and marine cyanobacteria (Fig. 2B and C). Neighbor joining (Fig. 2B) and maximum likelihood (Fig. 2C) as well as maximum parsimony (Fig. 2B) did not give a consistent relationship of these desaturases within this subcluster. PtFAD2 and PtFAD6 are known to be targeted to the ER and plastids, respectively (14).

**Localization of Δ12 desaturase.** The N terminus of PtFAD2 is similar to the N terminus of other ER-localized FAD2 proteins of plants and fungi (Fig. 2D), suggesting the presence of similar signal sequences. However, the Δ12 desaturase of *C. merolae* has an N-terminal extension, which is partially similar to the N-terminal extension of PtFAD6. Here we show the entire N-terminal sequence of the putative Δ12 desaturase (CMK291C). However, in the current version of annotation given in the *Cyanidioschyzon* website (http://merolae.biol.s.u-tokyo.ac.jp/), the CMK291C sequence begins from the second methionine. There is an in-frame upstream methionine codon, which could act as the initiation codon, and we used the entire sequence for the analysis in the present study. Intracellular localization of the putative Δ12 desaturase of *C. merolae* (CMK291C) was examined using GFP fusion constructs (Fig. 3). The polypeptide starting from Met1 was targeted to mitochondria, the polypeptide starting from Met2 was targeted to the ER, and the polypeptide beginning from Met3 showed no clear localization. It is interesting that the fluorescence of the Met2 construct is localized to the ER as well as to the membranes surrounding the nucleus (ER and nuclear envelope). It is therefore reasonable that the desaturase is translated from the second methionine, as described in the current database, and targeted to the ER.

**Labeling of lipids.** The computational analysis suggested that there is no desaturase in the plastid, but our lipid analysis showed that typical plastid lipids containing 18:2, such as MGDG, exist in this red alga. To solve this problem, we analyzed the flow of lipid synthesis within the cell by pulse labeling with radioactive acetate, followed by a chase period (Fig. 4). About 40% of added radiocarbon was incorporated into the total lipid fraction. The results of two-dimensional TLC indicated that PC and MGDG were the major labeled lipids. Interestingly, DGDG was not labeled efficiently after the 1-h labeling period, but it was densely labeled after the 20-h chase period. The radioactivity in PC decreased steadily during the chase period, while the radioactivity in MGDG increased. These results are consistent with the flow of carbon from PC to MGDG and then to DGDG, as documented for higher plants (10).

Figure 5 shows the results of fatty acid analysis using a recently developed reversed-phase argentation TLC method. In the total lipid fraction, desaturation of fatty acids was clearly detected. In MGDG, however, only 16:0 and 18:2 were detected, with a decrease in 16:0 and an increase in 18:2. No possible intermediate, such as 18:0, 18:2, or 16:1, was detected as a labeled acid. For DGDG, labeling in the fatty acids was noted after the 6-h chase period. 16:0 was labeled first, and after the 20-h chase period, 18:2 was also labeled. In PC, various fatty acids were labeled, and the changes were explained by desaturation, except for the initial high level of labeling of 18:2, which might suggest rapid turnover of a small pool of PC-bound 18:2.

Lipid molecular species were also analyzed (Fig. 6). In MGDG, 18:2/16:0 was the major labeled molecular species.
from the beginning. At later times, 18:2/18:2 was also labeled. The rapid labeling of the 18:2/16:0 species of MGDG was not found in similar labeling experiments with plants (18), algae (17, 38, 50), or cyanobacteria (47) and seemed quite strange. This molecular species was recovered from the plate, and the fatty acids were analyzed (Fig. 5E). It was clear that the initially labeled 18:2/16:0-MGDG was labeled only in its 16:0 part, not in its 18:2 part. After the chase, both fatty acids were labeled. The delay in 18:2 labeling can be explained by the large size of the pool that provides 18:2. This suggests that MGDG is synthesized from 16:0 and 18:2 but that the two acids are supplied from different compartments, namely, the rapidly labeled 16:0 is supplied within the plastids, whereas the slowly labeled 18:2 is supplied from outside the plastids, possibly from the ER. Labeling of DGDG molecular species was similar to that for MGDG. In PC, various molecular species were labeled, and the radioactivity shifted from less unsaturated to more unsaturated species. This suggests that the desaturation of fatty acids occurs mainly on PC, as in other eukaryotes (10, 17). The initial molecular species of galactolipid synthesis was
also confirmed by incorporation of UDP-[U-14C]galactose, using isolated plastids (Fig. 7). 18:2/16:0 and 18:2/18:2 were the major labeled molecular species in both classes of lipids, although their exact proportions were not similar.

DISCUSSION

Lack of trienoic acids. The results presented above indicate that *C. merolae* does not contain polyunsaturated acids with more than three double bonds. This is unusual for a photosynthetic eukaryote. Among the eukaryotes, *Saccharomyces cerevisiae* and some species of yeasts are the only organisms that possess only saturated and monounsaturated acids (15). Photosynthetic organisms in general have polyunsaturated acids such as 18:3, 20:4, or 22:6. The only known photosynthetic organisms that lack polyunsaturated acids are *Synechococcus* sp. strain PCC 6301 (formerly called *Anacystis nidulans* [49]) and its close relative PCC 7942 as well as some unicellular species of cyanobacteria (22). Polyunsaturated acids such as 18:3 are known to be important for two major reasons. First, 18:3 and 20:4 are precursors to physiologically active signaling compounds, such as jasmonate and prostaglandins in plants and animals, respectively. The lack of 18:3 implies that jasmonate might not act as a signal in this microalga. Second, 18:3 is required for maintaining photosynthetic activities at low and high temperatures. At low temperatures, this acid keeps the fluidity of membrane lipids to increase the tolerance to chilling and freezing (37). In addition, 18:3 is known to increase tolerance to high temperatures in plants (32). This does not mean that *C. merolae* does not have a tolerance to both cold and high temperatures. In fact, this alga can grow in a wide range of temperatures, from 25 to 50°C.

Pathway of lipid and fatty acid biosynthesis in *C. merolae*. *C. merolae* contains common glycerolipids that are usually found in plants and algae. Genomic analysis supports the hypothesis that this alga possesses a standard pathway of biosynthesis of these glycerolipids, except for DGDG (see Fig. S9 in the supplemental material). The biosynthesis of DGDG in plants is known to be catalyzed by the products of the *DGD1/DGD2* genes. However, *C. merolae* does not have a homolog of these plant-type galactosyltransferases. A survey of the Gclust database (data set CZ20x0, cluster 2825) identified a putative glycosyltransferase (Ycf82) shared by cyanobacteria and the *C. merolae* plastid genome. The disruption of the *Synechocystis* homolog (sr1508) resulted in a lack of DGDG (I. Sakurai, N. Mizusawa, H. Wada, and N. Sato, unpublished data). This gene is also being analyzed in different laboratories, and we hope that this is the structural gene for the cyanobacterial and red algal DGDG synthesis enzyme. We also note that further studies are needed to obtain a conclusion about the presence of cardiolipin in *C. merolae*.

Biochemical analysis clearly indicated that *C. merolae* can synthesize saturated fatty acids and mono- and diunsaturated fatty acids. The gene involved in the synthesis of Δ3-trans-16:1, which is a typical fatty acid present at the C-2 position of PG
in photosynthetic eukaryotes, is still unknown and is not a subject of the current discussion. The genomic analysis indicated that the pathway of fatty acid desaturation in this alga must be very different from that in flowering plants. Although fatty acid biosynthesis certainly occurs only in the plastids in *C. merolae*, as evidenced by the localization experiments using GFP (Table 3; see Fig. S12 in the supplemental material), the synthesis of oleate occurs in the ER and in the form of both acyl-CoA and acyl lipid (Table 3). This is fundamentally different from the Δ9 desaturation in flowering plants, in which oleate is produced only by the stearoyl ACP desaturase in the chloroplast, an enzyme that catalyzes desaturation by a different mechanism from that of acyl lipid desaturases (53). The genes encoding Δ7 acyl lipid desaturases have also been reported for plants, with some of their products acting as MGDG desaturases (19).

The second desaturation, namely, Δ12 desaturation, is catalyzed by the only Δ12 acyl lipid desaturase, which is likely to be localized in the ER (Fig. 3). However, MGDG, DGDG, and PG consist mainly of 1-(18:2)-2-(16:0) species, with small amounts of 1-(18:2)-2-(18:2) molecular species (Table 2). This indicates that the molecular species of these plastid lipids are synthesized by the coupled supply of 16:0 within the plastid and 18:2 from the outside. The role of the ER in acyl group desaturation was suggested by the results of tracer experiments with the cryptophyte *Cryptomonas* sp. (39), a descendant of secondary red algal endosymbiosis. In *Cryptomonas*, the radiolabel was initially incorporated into PC and then moved to...
MDGD. This and other results indicate that PC is the major substrate of desaturation in Cryptomonas. An essentially similar flow of carbon from PC to MGDG was found in C. merolae (Fig. 4).

**Origins of desaturases.** The desaturases in plants and algae have two different origins, namely, either the eukaryotic host or the cyanobacterial endosymbiont. The diversification of ∆9 and ∆12 desaturases had already occurred before the creation of eukaryotes, but some cyanobacterial enzymes were also transferred to plants and algae during endosymbiosis. It is clear of eukaryotes, but some cyanobacterial enzymes were also identified. Fig. 2A clearly shows that the cyanobacterial origins is illustrated in Fig. 8. Biosynthesis of MGDG there- is an interesting characteristic of C. merolae. The coupled pathway of MGDG synthesis. All of the results of tracer experiments clearly indicate that MGDG is synthesized in the ER (Fig. 7). 16:0 can be supplied within the plastids, whereas 18:2 cannot be supplied within the plastids because of the lack of desaturases within the plastids. It is most likely that PC in the ER is the site of desaturation, because all intermediate fatty acids were detected in the PC. A summary of galactolipid biosynthesis in C. merolae is illustrated in Fig. 8. Biosynthesis of MGDG therefore requires a supply of extraplastidic 18:2. This is the most interesting characteristic of C. merolae. We call this the coupled pathway, as opposed to the prokaryotic and eukaryotic pathways that were explained in the introduction. The coupled pathway of MGDG synthesis is a result of the total lack of desaturases of cyanobacterial origin and of stearoyl ACP desaturase.

In red algae other than those in the Cyanidiales (17, 35), C₁₈ unsaturated fatty acids are not abundant. In Porphyra yezoensis, for example, C₁₈ polyunsaturated acids amount to <1% of the galactolipids (4). This can be explained if, as in C. merolae, the synthesis of unsaturated fatty acids takes place mostly in the ER rather than in the plastids. The elongation of unsaturated fatty acids is also active in the ER, which results in vast production of C₂₀ or C₂₂ unsaturated fatty acids that characterize non-Cyanidiales red algal galactolipids. Although we still do not know if red algae in general lack stearoyl ACP desaturase, this enzyme could be an interesting switch that changes the metabolic flow of fatty acids in red algae.

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