Title page**Title:**

Tangled evolutionary processes with commonality and diversity in plastidial glycolipid synthesis in photosynthetic organisms

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Abstract

In photosynthetic organisms, the photosynthetic membrane constitutes a scaffold for light-harvesting complexes and photosynthetic reaction centers. Three kinds of glycolipids, namely monogalactosyldiacylglycerol, digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol, constitute approximately 80–90% of photosynthetic membrane lipids and are well conserved from tiny cyanobacteria to the leaves of huge trees. These glycolipids perform a wide variety of functions beyond biological membrane formation. In particular, the capability of adaptation to harsh environments through regulation of membrane glycolipid composition is essential for healthy growth and development of photosynthetic organisms. The genome analysis and functional genetics of the model seed plant *Arabidopsis thaliana* have yielded many new findings concerning the biosynthesis, regulation, and functions of glycolipids. Nevertheless, it remains to be clarified how the complex biosynthetic pathways and well-organized functions of glycolipids evolved in early and primitive photosynthetic organisms, such as cyanobacteria, to yield modern photosynthetic organisms like land plants. Recently, genome data for many photosynthetic organisms have been made available as the fruit of the rapid development of sequencing technology. We also have reported the draft genome sequence of the charophyte alga *Klebsormidium flaccidum*, which is an intermediate organism between green algae and land plants. Here, we performed a comprehensive phylogenetic analysis of glycolipid biosynthesis genes in oxygenic photosynthetic organisms including *K. flaccidum*. Based on the results together with membrane lipid analysis of this alga, we discuss the evolution of glycolipid synthesis in photosynthetic organisms.

Keywords

Glycolipid; Galactolipid; Sulfolipid; Photosynthetic organisms; Phosphate starvation; Plant evolution

1. Introduction

Lipid bilayers form the basis of biological membranes, which constitute one of the fundamental structural components of living cells. Many biological membranes of eubacteria and eukaryotes contain mainly phospholipids that carry fatty acids in ester linkage (reviewed in [1]). However, glycolipids are the major lipids of photosynthetic membranes—particularly the thylakoids of cyanobacteria and chloroplasts (reviewed in [2]). Expansion of the thylakoid membrane is important for ensuring a large surface area for photosynthesis, which provides energy and several organic compounds for

growth. The utilization of glycolipids for this purpose by photosynthetic organisms is considered reasonable because photosynthesis can supply plentiful carbohydrates and energy for the synthesis of glycolipids with conservation of phosphate, which is utilized in phospholipids but is in fact a limited resource yet absolutely necessary in living systems (reviewed in [3–6]).

Glycolipids can have various types of carbohydrate structures and thus are quite diverse, e.g., they include glycoglycerolipids, glycosphingolipids, lipopolysaccharides, and sterol glycolipids, among others. The most abundant glycolipids in plants are glycoglycerolipids, principally monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), which comprise approximately 80–90% of thylakoid membrane lipids in the oxygenic photosynthetic organisms—cyanobacteria, algae, and plants [6–10]. These glycolipids and metabolic pathways have multiple functions beyond their fundamental roles in membrane formation; these functions include photosynthesis [3–6,11–13], chloroplast development [14–17], defense against freezing stress [18,19], low-temperature adaptation [20], phosphate starvation response [11,21–31], and triacylglycerol biosynthesis [32,33]. Although glycolipid functions are widely divergent in photosynthetic eukaryotes, the importance of glycolipids is common in all photosynthetic organisms. However, glycolipid metabolic pathways and the lipid trafficking machinery have changed during the evolution of these organisms, particularly with the emergence of eukaryotic photosynthetic organisms, land plants, and seed plants [2,6]. In addition to the accumulated knowledge on lipid metabolism in land plants, substantial progress has been made in the last five years concerning the elucidation of glycolipid metabolism in cyanobacteria and algae. Moreover, our group recently reported the draft genome analysis of the charophyte algae *Klebsormidium flaccidum* [34], which consists of simple non-branching filamentous cells. This analysis revealed that *K. flaccidum* has acquired more than 1000 land plant-specific genes. Charophyte algae are therefore suitable model organisms for investigating the evolution of biological systems from green algae to land plants. Indeed, in many aspects, charophyte algae have characteristics intermediate between these two classes of organisms [35–38].

In this article, we present results from our phylogenetic analysis of glycolipid biosynthesis genes in photosynthetic organisms based on available genome sequences, and we provide an overview of the recent elucidation of glycolipid metabolism in these organisms. In addition, we present an analysis of the major membrane lipids and lipid synthesis genes of *K. flaccidum*.

2. Materials and Methods

2.1. Phylogenetic analysis

Protein and expressed sequence tag (EST) sequences (Supplementary Table 1) were collected from datasets by BLASTP and BLASTX [39] for subsequent phylogenetic analysis. After removing sequences that were inadequate for phylogenetic analysis (short sequence length, low quality, large deletion, etc.), sequences were aligned using MAFFT v7.220 [40]. trimAl v1.2 [41] was used to remove any poorly conserved regions, and the amino acid substitution model was calculated by Aminosan52 [42]. Phylogenetic analyses were performed using the maximum likelihood method and the neighbor-joining method in MEGA6 [43] and with Bayesian analysis, which was performed with MrBayes 3.2.3 [44] using the LG model + G (eight categories) for 1,000,000 generations. Every 500 generations were sampled, and the first 200 trees were discarded as burn-in.

2.2. Culture conditions of *K. flaccidum* for lipid analysis

The *K. flaccidum* strain NIES-2285 was cultured for 2 weeks (nearly stationary phase) in liquid C medium [45] under continuous light ($10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) with the cells circulated by bubbling air through the medium. Cells were harvested by vacuum filtration through filter paper (No. 3, 70 mm diameter, Advantec, Tokyo, Japan). The retained sample was frozen in liquid nitrogen and kept at -80°C .

2.3. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of *K. flaccidum*

A frozen sample of *K. flaccidum* (100 mg) was ground to a fine powder in a mortar with liquid nitrogen and then dissolved in 600 μl of 100% methanol and homogenized twice using a TissueLyser (Qiagen, Valencia, CA, USA) set at 25 Hz for 2 min each time. The homogenate was centrifuged at $20,000 \times g$ for 10 min at 4°C . The organic phase was collected and filtered through a $0.2\text{-}\mu\text{m}$ polytetrafluoroethylene membrane. LC-MS was carried out using an LC-Orbitrap-MS (Ion trap Orbitrap XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) with electrospray ionization. The chromatographic column was a Halo C18, $3.0 \times 30 \text{ mm}$, $2.7 \mu\text{m}$ particle size (Advanced Materials Technology Inc., Wilmington, DE) maintained at 50°C and operated at 0.8 ml/min . The LC solvents were: solvent A (methanol, water, acetic acid, 25% aqueous ammonium solution, 500:500:1:1) and solvent B (isopropyl alcohol, acetic acid, 25% aqueous ammonium solution, 1000:1:1). Lidocaine, prochloraz, reserpine, bombesin and aureobasidin A were

incorporated into the LC solvent as internal standards. The LC gradient was 0–100% solvent B over 5 min. Mass spectra were acquired with an m/z range 100–2000 in electrospray ionization positive-ion mode. The electrospray voltage was set at 4 kV and the source temperature at 300°C. MS/MS spectra were collected at 30% collision energy.

2.4. Analysis of membrane lipid content and fatty acid composition of K. flaccidum

Total lipid was extracted from the frozen sample using the method of Bligh and Dyer [46]. Membrane lipid content was measured as described [47]. The lipids were separated by two-dimensional thin-layer chromatography (2DTLC) using the following solvent systems: first dimension, chloroform/methanol/7 M ammonia, 15:10:1 v/v; second dimension, chloroform/methanol/acetic acid/water, 170:20:17:3. Lipids were visualized with 0.01% w/v primuline in 80% v/v acetone under UV light, and the silica gel of selected spots was scraped for fatty acid extraction. Fatty acid methyl esters were prepared by incubating each lipid from the 2DTLC in 5% v/v HCl in methanol at 85°C for 1 h with 1 μ mol pentadecanoic acid as an internal standard; the esters were quantified by gas chromatography (GC) coupled with a flame ionization detector (GC-2014, Shimadzu, Kyoto, Japan). The GC column was ULBON HR-SS-10 (25 m, 0.25 mm; Shinwa Chemical Industries Ltd., Kyoto, Japan) held at 180°C. The carrier gas was helium at 0.53 ml/min.

3. Results and Discussion

3.1. MGDG biosynthesis

MGDG, in which one galactose residue is covalently linked to diacylglycerol (DAG), is the most abundant galactolipid in all photosynthetic organisms (Fig. 1). MGDG, with a small polar head, is a non-bilayer-forming lipid. MGDG, along with bilayer-forming lipids, carotenoids, and bilayer-spanning proteins, regulates the bilayer and non-bilayer lipid phases in thylakoid membranes and maintains the structural flexibility of these membranes [48,49,50,51]. X-ray crystallography studies have shown that MGDG is a component of both photosystem I (PSI) [52,53] and PSII complexes [54–57]. MGDG may be an important lipid for dimerization of the two PSII core complexes [58] and for energy transfer from the light-harvesting complex of PSII (LHCII) to PSII [59–61].

The plant-type MGDG synthase is highly conserved from algae to seed plants even through secondary symbiosis (Fig. 2). In cyanobacteria, however, MGDG is synthesized in a two-step process. First, monoglucosyl-DAG (GlcDG) is synthesized from DAG and UDP-glucose by GlcDG synthase A (MgdA) [62, 63]. Second, the glucolipid epimerase MgdE converts the glucosyl group of GlcDG to a galactosyl group [62,64] (Fig. 1). MgdA

is ubiquitous in cyanobacteria, whereas MgdE is absent in some cyanobacteria. Thus, it has been assumed that another epimerase exists in MgdE-lacking cyanobacteria [65]. Eukaryotic MGDG synthase produces MGDG directly from DAG and UDP-galactose [66] (Fig. 1), and homologs of this synthase are also found in some bacteria. Indeed, eukaryotic MGDG synthase might have been derived from ancestral Chloroflexi through horizontal gene transfer, as determined by phylogenetic analysis [67]. GlcDG exists widely in cyanobacteria [65]. It is unclear why cyanobacteria use an indirect pathway for MGDG biosynthesis, but one explanation was offered based on analysis of a GlcDG-deficient cyanobacterium whose MGDG synthesis is complemented by eukaryotic MGDG synthase [20]. The GlcDG-deficient mutant showed normal growth and photosynthesis under normal conditions, indicating that GlcDG is not required for normal growth. At low temperature, however, this mutant grew slowly, indicating that GlcDG may be necessary for appropriate response to environmental stress [20]. For eukaryotic cells, GlcDG-dependent environmental adaptation may have become unnecessary, perhaps owing to the evolutionary acquisition of other low-temperature adaptation mechanisms afforded by endosymbiosis. This hypothesis understandably does not explain the dominance of eukaryotic-type MGDG synthesis. Construction of transgenic plants with cyanobacteria-type MGDG synthesis will provide a means to validate this hypothesis.

Eukaryotic MGDG synthase has undergone functional divergence in land plants (Fig. 2, Fig. 3). Seed plants encode two types of eukaryotic MGDG synthase [30,68,69]. The type A MGDG synthase (MGD1 in *Arabidopsis thaliana*), which localizes to the chloroplast inner envelope, is the major MGDG synthase (Fig. 4) [68,70–72], and the *mgd1* mutation causes abnormal embryogenesis with critical underdevelopment of chloroplasts [14]. The type B MGDG synthase (MGD2 and MGD3 in *A. thaliana*) localizes to the chloroplast outer envelope and is induced upon phosphate starvation (Fig. 4) [68,70,71]. The galactolipid contents and fatty acid compositions of *mgd3* and *mgd2mgd3* double-mutant plants were not affected under normal growth conditions. However, mutations in *mgd3* and *mgd2mgd3* result in decreased DGDG content in concert with root and shoot growth inhibition upon phosphate starvation [28]. MGDG synthesized in the outer envelope by a type B MGD is converted to DGDG by the DGDG synthases DGD1 and DGD2 [24], which are described in section 3.2. This DGDG is transported to extraplastidic membranes and substitutes for phospholipids (mainly phosphatidylcholine, PC), and phosphate is supplied by degradation of phospholipids [6,24,25,73]. DGDG is a bilayer-forming lipid, as is PC, and is therefore able to functionally replace bulk PC. Recently, *A. thaliana* *MGD3* was also found to be

upregulated when carbon is in excess, i.e., to support enhanced growth [74]. It was also reported that expression of Type-B MGD genes are induced upon treatment of plants with hydrogen peroxide [75]. These results show that another signal(s) is present to induce outer-envelope MGDG synthesis except during phosphate deficiency.

The most ancient type B MGD was found in the EST sequence of *Picea glauca*, a gymnosperm, although a type A MGD was not found in this species [67]. Type A/B divergence time is estimated as ~323 million years ago, based on a phylogenetic analysis [67]. The data for the *P. glauca* genome analysis were recently improved [76], and we found a type A MGD candidate in the EST data. Phylogenetic analysis supports the type A/B divergence of *Amborella*, which is the earliest-diverging angiosperm known so far [77]. Further, the type A/B divergence in gymnosperms is supported by three different methods even with weak support values (Fig. 3). In addition, it has been suggested that, compared with type B and certain type A MGDs, the type A MGD candidate of *P. glauca* has a longer variable domain, which probably corresponds to a subcellular localization signal (Supplementary Fig. 1). Type A/B divergence of MGD might have contributed to the relative abundance of seed plants in low-phosphorus environments [78].

Fig. 1 Chemical structures and biosynthetic pathway of the major glycolipids in photosynthetic organisms.

(a) The synthase for each glycolipid reaction is indicated as bold and underlined. Blue indicates a cyanobacteria-type synthase, and red indicates a eukaryotic type synthase. MGD, MgdA, SQD2, SqdX, and DGD are UDP-glycosyltransferases, which use UDP-hexose as a substrate (UDP-Gal and UDP-Glc denote UDP-galactose and UDP-glucose, respectively). It has been assumed that DgdA also uses UDP-Gal as a substrate [62]. SQD2 also synthesizes glucuronosyldiacylglycerol (GlcADG) under phosphate starvation. Galactolipid:galactolipid galactosyltransferase (GGGT) is a glycosyl hydrolase that transfers a galactose residue from MGDG to other galactolipids. (b) DGDG and SQDG can substitute for the phospholipids phosphatidylcholine (PC) and phosphatidylglycerol (PG), respectively. R1 and R2 represent alkyl groups at the *sn*-1 and *sn*-2 positions, respectively.

	Prokaryote	Eukaryote								
	Cyanobacteria	Glaucophytes	Red algae		Stramenopiles	Green algae	Chlorarachniophytes	Charophyte algae	Land plants	
		(<i>C. paradoxa</i>)	Cyanidiophytina	Rhodophytina			(<i>B. natans</i>)	(<i>K. flaccidum</i>)	Moss, Fern	Seed plants
MGDG synthase	MgdA (MgdE)	MGD	MGD	MGD	MGD	MGD	MGD	MGD	MGD	MGD TypeA TypeB
DGDG synthase	DgdA	DGD (LS) DgdA(nuclear)	DgdA (plastid)	DGD	DGD	DGD	DGD	DGD	DGD	DGD1 DGD2
SQDG synthase	SqdB (PCC 6803 type) (PCC 7942 type)	SQD1 (PCC 6803 type)	SQD1 (PCC 7942 type)	SQD1 (PCC 7942 type)	SQD1 (PCC 7942 type)	SQD1 (PCC 6803 type)	SQD1 (PCC 7942 type)	SQD1 (PCC 6803 type)	SQD1 (PCC 6803 type)	SQD1 (PCC 6803 type)
	SqdX	SQD2	SQD2	SQD2	SQD2	SQD2	SQD2	SQD2	SQD2	SQD2
GGGT	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	GGGT	GGGT	GGGT/SFR2
TGD complex	SltA	TGD1	TGD1 (plastid)	TGD1 (plastid)	(NF)	TGD1	(NF)	TGD1	TGD1	TGD1
	SltB	TGD2	TGD2 (LS) (plastid)	TGD2 (LS) (plastid)	(NF)	TGD2	(NF)	TGD2	TGD2	TGD2
	SltC	TGD3	TGD3	TGD3	(NF)	TGD3	(NF)	TGD3	TGD3	TGD3
	(NF)	(NF)	(NF)	(NF)	(NF)	TGD4 (LS)	(NF)	TGD4	TGD4	TGD4
	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	TGD5
PAH	(NF)	PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH	PAH
NPC	(NF)	(NF)	(NF)	(NF)	(NF)	(NF)	NPC	NPC	NPC	NPC

Fig. 2 Distribution of glycolipid synthases, transport proteins, and enzymes involved in glycolipid synthesis.

The proteins of glaucophytes, chlorarachniophytes, and charophytes were each searched against protein sequences from a single organism, namely *C. paradoxa*, *B. natans*, or *K. flaccidum*, respectively. Databases for organisms are indicated in Supplementary Table 1. Boxes of the same color represent an identical gene origin. Each protein ID is indicated in Supplementary Table 2. Each gray box indicates that the protein sequence was “not found” (NF) in each organism or group. The terms “nuclear” and “chloroplast” indicate that the genome encoding the gene differs from that of other organisms. The term “LS”, for “low similarity”, indicates that protein E-values (as determined with BLASTP) are markedly low compared with proteins of other organisms.

Fig. 3 Phylogenetic tree analysis of MGDs.

The analysis utilized the maximum likelihood, neighbor-joining, and Bayesian inference algorithms. The topologies and branch lengths were calculated using the maximum likelihood method based on the LG model +G (8 categories). Bootstrap values (maximum likelihood and neighbor-joining) and Bayesian posterior probabilities (Bayesian inference) are indicated under each branch (maximum likelihood/neighbor-joining/Bayesian inference). Support values of type A/B MGD divergence are indicated in red type. The symbol (–) indicates distinct topological arrangements. The scale bar represents 0.2 amino acid substitutions per site. BLASTP top hits for proteins in the *A. thaliana* database were all MGDs. Abbreviations for species are as follows. Primary algae are as follows. Glaucophyta: Cpa (*Cyanophora paradoxa*); red algae: Cyanidiophytina, Cme (*Cyanidioschyzon merolae*), Rhodophytina, Ccr (*Chondrus crispus*), Ppu (*Porphyra purpurea*); green algae: Micr (*Micromonas* strain RCC299), Ota (*Ostreococcus tauri*), Chl (*Chlorella variabilis* NC64A), Vca (*Volvox carteri f. nagariensis*), Cre (*Chlamydomonas reinhardtii*); and charophyte algae: Kf

(*Klebsormidium flaccidum*). Ferns and moss are as follows. Pp (*Physcomitrella patens* subsp. *patens*), and Sm (*Selaginella moellendorffii*). Seed plants are as follows. Os (*Oryza sativa* subsp. *japonica*), Pop (*Populus trichocarpa*), and Ath (*Arabidopsis thaliana*). Secondary endosymbionts are as follows. Stramenopiles, Esi (*Ectocarpus siliculosus*), Ptr (*Phaeodactylum tricornutum*), Tps (*Thalassiosira pseudonana* CCMP 1335); chlorarachniophytes, Bna (*Bigelowiella natans* CCMP2755). Other organisms are indicated by their scientific names.

Fig. 4 Upregulation of glycolipid synthases and membrane remodeling during phosphate starvation in *A. thaliana*.

Ellipses represent major proteins involved in glycolipid biosynthesis. Red type indicates enzymes whose expression is upregulated and lipids whose levels are increased, and blue type indicates lipids whose levels are decreased during phosphate starvation. Increased DGDG and SQDG are substituted for PC and PG, respectively, levels of which are decreased during phosphate starvation. oE and iE denote the outer and inner envelope of the chloroplast, respectively. Transport of PA by the TGD complex is controversial. Although DAG, which is produced from PA by PAH1/2 and from PC by NPC5, is transported to the plastid envelope membranes, its transport mechanisms remain to be clarified. Each transport pathway is indicated by a dashed line, although the mechanism remains unclear in each case.

3.2. DGDG biosynthesis

DGDG is a bilayer-forming lipid and the second-most abundant galactolipid in photosynthetic organisms, after MGDG. Along with MGDG, DGDG regulates the bilayer and non-bilayer lipid phases of thylakoid membranes. [50,51]. Further, DGDG is involved in the stacking of thylakoid membranes [50,51]. DGDG is found in the core of the LHCII [79] and PSII complexes [54–57] and is involved in the formation of LHCII trimers [79].

Two kinds of DGDG synthase, namely DgdA [26,80] and DGD [81], which emerged independently during evolution, have been reported in photosynthetic organisms. These synthases produce DGDG by linking a second galactose moiety to MGDG via a α -glycosidic bond (Fig. 1). The distribution of these two DGDG synthases in organisms differs from that of the two MGDG synthases (Fig. 2). First, DgdA is found in both cyanobacteria and Cyanidiophytina. The *DgdA* genes of *Cyanidioschyzon merolae* and *Cyanidium caldarium* (Cyanidiophytina) are found in the plastid genome (Fig. 2). By contrast, we identified a nuclear *DgdA* gene in the glaucophyte *Cyanophora paradoxa* [82] (Fig. 5). Maida and Awai also identified the same gene in *C. paradoxa* (*CpDgdA*) (also see Maida and Awai in this issue [83]). They reported that *CpDgdA* has DGDG synthase activity. These results suggest that *CpDgdA* was transferred to the host-cell nucleus via endosymbiotic gene transfer. The amino acid sequence of DgdA is quite similar to that of sqdX/SQD2 (SQDG synthase). Based on a phylogenetic analysis of DgdA and SqdX/SQD2, it has been proposed that DgdA was derived from an sqdX-like gene before the emergence of cyanobacteria [84]. Moreover, the *DGD* genes are distributed in *C. paradoxa*, Rhodophytina, Stramenopiles, green algae, *Bigeloviella natans*, *K. flaccidum*, and land plants. *C. paradoxa* likely has at least one candidate DGDG synthase, although this candidate shares low amino acid sequence similarity with other DGDs (Fig. 6). Thus, two types of DGDG synthases probably coexisted in early photosynthetic organisms, and one type probably was independently selected early during the evolution of these organisms and then DGD was subsequently utilized in the green-algae lineage.

DGD genes underwent further functional divergence in land plants, as occurred with eukaryotic MGDG synthases. *A. thaliana* has two DGD genes (*DGD1* and *DGD2*) that localize to the chloroplast outer envelope [24,85], and the two encoded enzymes share a common C-terminal glycosyl transferase domain. The N-terminal portion of DGD1 is lacking in DGD2 [23] (Supplementary Fig. 2). DGD1 is the predominant DGD synthase under normal conditions [81,84], and the DGDG level in a *dgd1* mutant was found to be >90% lower compared with wild type [86]. *DGD2* is induced upon phosphate starvation

[23], but under normal conditions the growth, photosynthesis, and lipid composition of a *dgd2* mutant were found to be equivalent to those of wild type; however, phosphate starvation-induced DGDG accumulation was decreased in this mutant [24]. Furthermore, analysis of the fatty acid composition of DGDG in a *mgd2/mgd3* double mutant indicated that MGD2/MGD3 and DGD2 are functionally linked during phosphate starvation (MGD2/MGD3-DGD2 pathway, type B pathway) i.e., in a manner distinct from the synthesis of bulk DGDG (MGD1-DGD1 pathway, type A pathway) [28,70] (Fig. 4). Perhaps gene duplication and recombination of domains generated a new, functionally divergent pathway [34,87,88].

We found that DGD1 and DGD2 diverged before than emergence of *Amborella* (Fig. 6). Moreover, there are two DGD genes in the EST data of *P. glauca*. Our three different phylogenetic analyses support the divergence of DGD1/2 in gymnosperms even though support values were weak (Fig. 6). However, the N-terminal coding region of one *P. glauca* EST sequence was found to be slightly longer than that of DGD2 of other plants, and the remaining N-terminal sequence remains unclear because only a partial gene sequence was available (Supplementary Fig. 2). Biochemical and molecular biological analyses of MGDs and DGDs in gymnosperms and ferns will reveal the origin of adaptation to phosphate starvation by the type B pathway. One hypothesis posits that MGDs and DGDs are co-evolutionarily linked [2]. If this hypothesis is correct, type B MGD and DGD2 should be found in the same lineage.

Fig. 5 Phylogenetic tree analysis of DgdA.

The analysis utilized the maximum likelihood method based on the LG model +G (8 categories) with 500 bootstrap replicates. The scale bar represents 0.2 amino acid substitutions per site. DgdA (slr1508) was the top BLAST hit for each of the glaucophyte and Rhodophytina DgdA candidates based on the *Synechocystis* sp. PCC 6803 database. Refer to Figure 3 for species abbreviations.

Fig. 6 Phylogenetic tree analysis of DGDs.

The analysis utilized the maximum likelihood, neighbor-joining, and Bayesian inference algorithms. (a) The topologies and branch lengths were calculated using the maximum likelihood method based on the LG model +G (8 categories). Bootstrap values (maximum likelihood and neighbor-joining) and Bayesian posterior probabilities (Bayesian inference) are indicated under each branch (maximum likelihood/neighbor-joining/Bayesian inference). The symbol (–) indicates distinct topological arrangements. (b) A part of the tree calculated using the neighbor-joining method is represented. Refer to Figure 3 for species abbreviations. Support values for DGD1 and DGD2 divergence are indicated in red type. The scale bar represents 0.1 amino acid substitutions per site. DGD1 or DGD2 were the top BLASTP hits for all proteins to the *A. thaliana* database.

3.3. Oligogalactolipid biosynthesis

Trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG) are oligogalactolipids involved in freezing resistance in *A. thaliana* [18]. Under normal conditions, TGDG and TeGDG levels are very low. These oligogalactolipids are not synthesized during cold acclimation but rather just after the onset of freezing stress by the action of galactolipid:galactolipid galactosyltransferase (GGGT) [18], which is identical to the gene *SENSITIVE TO FREEZING 2 (SFR2)* [89,90]. It has been proposed that TGDG and TeGDG accumulate in the outer envelope to protect chloroplasts from membrane fusion caused by freezing-induced dehydration [19]. Whereas MGDs and DGDs transfer a galactose moiety from UDP-galactose to DAG or MGDG via their glycosyltransferase activities, GGGT transfers a galactose from MGDG to other galactolipids via its glycosyl hydrolase activity (Fig. 1) [18]. The two galactose moieties are linked via a β -glycoside bond, which differs from the $\alpha\beta$ -linkage in DGDG by DgdA and DGDs [5]. Although it has been reported that *Oscillatoria*, *Tolypothrix* (cyanobacteria) [91], and *Chlorella* (Chlorophyta) [92,93] also accumulate TGDG, unequivocal SFR2 homologs have been found only in land plants and charophyte algae (Fig. 2, Supplementary Fig. 3). Indeed, we detected TGDG from *K. flaccidum*, a terrestrial charophyte alga, by LC-MS/MS (Supplementary Fig. 4). The membrane lipids of *K. flaccidum* under normal conditions were determined with 2DTLC-GC (Fig. 7), which revealed that TGDG comprises 3.12 ± 0.39 mol% of all membrane lipids. However, the anomeric configuration of *K. flaccidum* TGDG was not defined. It has been reported that *A. thaliana* DGD2 synthesizes α,α,β -TGDG *in vitro* [23], and α,α,β -TGDG has also been found in rice bran [94]. Thus, the possibility cannot be excluded that *K. flaccidum* TGDG is synthesized via a GGGT-independent pathway. Assignment of the anomeric configuration will probably help elucidate the TGDG synthesis pathway of *K. flaccidum*.

In higher plants such as *A. thaliana*, TGDG is hardly produced under normal conditions. It is thus unclear why TGDG is constitutively present at such a high level in *K. flaccidum*. It has been reported that some Klebsormidium species are highly tolerant to both drought and freezing [95–98]. Indeed, in addition to freezing, dehydration is induced by several abiotic stresses such as salinity and drought. Terrestrial algae and land plants are frequently exposed to these harsh stresses. Thus, constitutive TGDG in *K. flaccidum* probably facilitates the rapid response to environmental changes. Analysis of the oligogalactolipid synthesis pathway of *K. flaccidum* might shed light on one of factors that enabled ancient water-borne plants to adapt to terrestrial environments.

Fig. 7 *K. flaccidum* NIES-2285 membrane lipid composition and fatty acid composition of MGDG.

K. flaccidum NIES-2285 was cultured for 2 weeks, and then lipids were extracted and separated using 2DTLC. (a) *K. flaccidum* membrane lipid composition. Abbreviations: diacylglyceryltrimethylhomoserine (DGTs), phosphatidylethanolamine (PE), phosphatidylinositol (PI). (b) Fatty acid composition of MGDG.

3.4. SQDG and glucuronosyldiacylglycerol (GlcADG) biosynthesis

The sulfur-containing glycolipid SQDG also is distributed in many photosynthetic organisms. SQDG is found in the PSII [54–57] and cytochrome *b₆f* complexes [99]. SQDG is an anionic lipid, as is phosphatidylglycerol (PG). A SQDG-deficient mutant of *A. thaliana* grew normally under normal conditions [100], but a SQDG/PG double-deficient mutant exhibited severely limited growth even compared with the parental line *pdp1-1* mutant that produced 30% less PG [101]. Thus, SQDG can partly compensate for PG deficiency. In several organisms, SQDG production increases in response to a PG decrease during phosphate starvation [21,22]. This membrane remodeling is also important for phosphate sparing as well as substitution of PC with DGDG in extraplastidial membranes. The phenotypes of SQDG-deficient mutants differ among organisms. SQDG-deficient mutants of *Synechococcus elongatus* PCC 7942 [102,103] and *Rhodobacter sphaeroides* [104] showed no detectable phenotype compared with wild type under normal conditions but exhibited reduced growth under phosphate-limiting conditions. Nevertheless, in *Synechocystis* sp. PCC 6803, impairment of SQDG synthesis affected not only PSII activity [105] but also DNA synthesis, and growth of an SQDG-defective mutant was arrested under both photoautotrophic conditions and light-activated heterotrophic growth conditions [105]. In addition, the SQDG-defective mutant *hf-2* of *Chlamydomonas reinhardtii* had 42% reduced PSII activity compared with the parent [106].

The SQDG biosynthesis pathway is common among photosynthetic organisms [107]. The enzyme SQD1 (SqdB in prokaryotes) synthesizes UDP-sulfoquinovose from UDP-glucose and a sulfur donor [108]. SQD2 (SqdX in prokaryotes) transfers sulfoquinovose to DAG in the chloroplast inner envelope of plants [109] (Fig. 1). SQD2 also synthesizes glucuronosyldiacylglycerol (GlcADG) in *A. thaliana*. GlcADG is found in bacteria, fungi, algae, and plants [31,110–114]. During phosphate starvation, *A. thaliana* SQD2 transfers glucuronic acid (GlcA) to DAG from UDP-GlcA [31] (Fig. 1). GlcADG is essential for tolerance to phosphate deprivation. The levels of GlcADG and phosphatidylinositol/phosphatidylethanolamine are reciprocally maintained [31].

It is difficult to understand how SQD1 evolved because there are two types of SQD1 in photosynthetic organisms, and a similar scenario exists for algal DGDG synthases [4,115,116]. SqdB in cyanobacteria is separated into the PCC 7942 type and PCC 6803 type [4] (Fig. 2, Fig. 8). It is unclear whether these SqdBs diverged in cyanobacteria or were independently acquired. SQD1 of red algae and its secondary symbionts was inherited from the PCC 7942 type. Another SQD of the PCC 6803 type accounts for the origin of SQD1 of glaucophytes, green algae, and land plants. Two possibilities exist

that could explain the presence of these two different types of SQD1 in red and green algae. First, SQD1 of the ancestor of red algae could have been replaced with a PCC 7942-type SQD1 through horizontal gene transfer (Supplementary Fig. 5a). However, SQD1 of the chlorarachniophyte alga *B. natans*, for which plastids were derived from a secondary endosymbiosis of Chlorophyta, is a PCC 7942 type. This hypothesis requires the occurrence of at least two independent horizontal gene transfers. Second, two types of SQD1 may have been acquired during primary endosymbiosis, after which one SQD1 was independently selected. Subsequently, the ancestor of *B. natans* might have acquired a PCC 7942-type SQD1 (Supplementary Fig. 5b). On the other hand, SQD2 was derived from a single origin through endosymbiotic gene transfer, and therefore it has been highly conserved in photosynthetic eukaryotes [117] (Fig. 2). Some non-photosynthetic bacteria (such as *Rubrobacter xylanophilus*) acquired the cyanobacterial *sqdX*-like glycosyl transferase after divergence of *sqdX* and *SQD2* [116,117], and these genes may have been acquired from cyanobacteria via horizontal gene transfer.

Fig. 8 Phylogenetic tree analysis of SqdB and SQD1.

The analysis utilized the maximum likelihood method based on the LG model +G (8 categories) with 500 bootstrap replicates. The scale bar represents 0.2 amino acid substitutions per site. Refer to Figure 3 for species abbreviations.

3.5. Membrane lipid trafficking

3.5.1. Prokaryotic pathway

In the plastid membrane of photosynthetic organisms, glycolipids are synthesized from DAG as the first substrate (Fig. 1). In plants, two pathways that supply DAG have been proposed—the prokaryotic and eukaryotic pathways [70,118–120] (Fig. 9). It is assumed that the prokaryotic pathway in plastids probably originated from an endosymbiotic event that resulted in the acquisition of plastids. In *A. thaliana*, plastidial glycerol-3-phosphate acyltransferase (plastidial GPAT, ATS1) first converts glycerol-3-phosphate to lysophosphatidic acid via acylation of the *sn*-1 position with an 18-carbon fatty acid covalently linked to acyl carrier protein (C18-ACP) [121,122]. The plastidial lysophosphatidic acid acyltransferase (plastidial LPAAT, ATS2) converts lysophosphatidic acid to phosphatidic acid (PA) via acylation of the *sn*-2 position with C16-ACP [123,124]. Subsequently, lipid phosphate phosphohydrolase dephosphorylates PA to produce DAG [125]. However, the pathway that supplies DAG from PA is absent in many plants, especially among angiosperms, which are called 18:3 plants [126,127]; these plants synthesize glycolipids from DAG exclusively supplied from the endoplasmic reticulum (ER) via the eukaryotic pathway and contain large amount of C18:3 and no C16:3 in glycolipids. On the other hand, plants having both the prokaryotic and eukaryotic pathways contain both C16:3 and C18:3 and are called 16:3 plants [120,126,127].

Fig. 9 Scheme for prokaryotic and eukaryotic pathways in *A. thaliana* (16:3 plants). Fatty acid synthase (FAS) synthesizes acyl carrier protein in plastids. The prokaryotic pathway produces PA from two acyl carrier proteins and glycerol-3-phosphate (G3P) by ATS1 and ATS2. PA in the plastid inner envelope (iE) is converted to DAG by the plastidial lipid phosphate phosphohydrolase (LPP). The plastidial LPP activity is absent in 18:3 plants. The eukaryotic pathway involves the release of fatty acids (FA) by FATA and FATB with their subsequent export from plastids to the ER. GPAT, LPAAT, and LPP synthesize DAG in the ER. Transport of PA from ER to iE by the TGD complex is controversial and is indicated with a dashed line. oE, outer envelope of the plastid.

3.5.2. DAG biosynthesis in the ER via the eukaryotic pathway

The eukaryotic pathway of galactolipid biosynthesis produces PA at the cytoplasmic face of the ER and provides DAG to not only the ER but also plastids for galactolipid synthesis [70,118–120] (Fig. 9). Acyl-ACP thioesterase is crucial for the export of C16 and C18 fatty acids from plastids to the ER [128]. Acyl-ACP thioesterase genes in land plants are highly diverged compared with those in algae (Supplementary Fig. 6). Acyl-ACP thioesterase variants, namely FATA and FATB, have been reported in *A. thaliana* [129,130]. FATB is involved in the supply of C16:0 fatty acids and seed development [131,132] as well as in wax biosynthesis [132,133].

The synthesis of PA in the ER proceeds via acylation of glycerol-3-phosphate with ER-localized GPAT and LPAAT, similar to the prokaryotic pathway. In the eukaryotic pathway, C16 or C18 fatty acids are covalently linked at the *sn*-1 position, whereas C18 fatty acids are attached to the *sn*-2 position according to the substrate specificity of ER-localized LPAAT (Fig. 9). The major GPAT of the ER for membrane lipid biosynthesis has not been identified in plants until recently. However, it has been reported that *A. thaliana* GPAT9 in the ER, which is similar to mammalian GPAT, is essential for the storage and membrane lipid synthesis [134,135]. The mammalian or yeast GPAT-like protein is widely distributed in photosynthetic eukaryotes [136] (Supplementary table 1). On the other hand, plant-specific GPATs (GPAT1–8) are involved in suberin and cutin biosynthesis [137,138]. Plant-specific GPAT homologs have not been found in *K. flaccidum* or other algae.

The PA synthesized via the eukaryotic pathway is converted to several membrane lipids and triacylglycerol. In *A. thaliana*, the transport of lipids from the ER to plastids for glycolipid synthesis is thought to be accomplished through PA provided by the PC pool [27]. Although PC is not detected in *C. reinhardtii* and some green algae, diacylglyceryltrimethylhomoserine (DGTS) is thought to replace PC; indeed, DGTS is a zwitterionic lipid like PC. The coexistence of DGTS and PC has been reported in many green algae, moss, and ferns [139–141]. It is thought that DGTS substitutes for PC and limits phosphate incorporation into phospholipids in membranes [142,143]. A recent screen of 13 *Chlamydomonas* strains revealed the presence of PC in four strains regardless of phylogenetic relationship [144]. The levels of PC and DGTS vary greatly among photosynthetic organisms. We also found that the *K. flaccidum* genome has both PC and DGTS biosynthesis genes (Supplementary table 1). We identified both lipids by 2DTLC-GC and LC-MS/MS in *K. flaccidum* (Fig. 7a, Supplementary Fig. 7), suggesting that the coexistence of PC and DGTS is rather common in photosynthetic eukaryotes except for seed plants. The substitution for phospholipids by DGDG as result of type A/B

MGD divergence may have led to loss of DGTS in seed plants.

3.5.3. Lipid transport from the ER to plastids via the eukaryotic pathway

In *A. thaliana*, lipids synthesized via the eukaryotic pathway are transported to the inner envelope membrane of plastids. The TGD complex, which comprises five TGD proteins (TGD1–5), is involved in lipid transport from the ER to plastids [72,145–150] (Fig. 2, Fig. 9, Supplementary Fig. 8–10). Mutations of these TGD genes suppress the decrease of DGDG observed in *dgd1* mutants via activation of oligogalactolipid synthesis by GGGT [145]. These five proteins are involved in lipid trafficking from the ER to plastids. The TGDs 1–3 form a bacteria-type ABC transporter in the chloroplast inner envelope [151–153]. The photosynthetic eukaryotes, except for secondary symbionts, commonly have *TGD1–3*-like genes. In red algae, TGD1 and TGD2 are encoded by plastid genes, whereas TGD3 is encoded by a nuclear gene [154], suggesting their bacterial origin. Indeed, TGD1–3 homologs in *Synechocystis* sp. PCC 6803, namely SltA, SltB, and SltC, are involved in environmental stress tolerance and intracellular lipid transport [155,156]. However, we identified nuclear-encoded TGD1–3 in *C. paradoxa* (Fig. 2). In red algae, fatty acid synthesis and lipid trafficking have been proposed to be co-regulated because genes encoding TGD2 and the α -subunit of the acetyl-CoA carboxylase in plastids are located in the same operon [157]; the close correlation between this carboxylase and TGD2 in red algae may have hindered the transfer of *TGD1* and *TGD2* genes to the nucleus during evolution. These TGD1–3 candidates of photosynthetic eukaryotes likely had a single origin from a cyanobacterial endosymbiosis event, and they are highly similar. However, taking into account the functional diversity of the ABC transporter, experimental evidence may be needed to confirm that these TGD candidates are indeed functional homologs. Meanwhile, we found no TGD candidate in chlorarachniophytes, *B. natans*, or stramenopiles [157]. These secondary plastids lack ER-plastid membrane contacts because of the presence of periplastid membranes. Thus, TGDs in secondary endosymbionts may have been lost during evolution. TGD4 is a beta-barrel protein in the chloroplast outer envelope [153]. TGD4-like proteins are found in some green algae and land plants [148], and we also found them in *K. flaccidum*. TGD4-like proteins in *C. reinhardtii* were also identified via BLASTP using the *K. flaccidum* TGD4 sequence, although the sequence similarity to land-plant TGD4 is very low (Fig. 10). TGD5 is a small glycine-rich protein found only in seed plants [149]. The kind of lipids transported by the TGD complex is controversial. Although the enzyme that converts PA to DAG in plastids of 18:3 plants is unclear, binding of TGD2 and TGD4 to PA in vitro supports the transport of PA synthesized in

ER [146,150]. On the other hand, a massive influx of DAG to the plastid inner envelope has been rationalized by mathematical modeling [158].

It is clear that these TGDs are involved in transport of glycolipid precursors via the eukaryotic pathway in *A. thaliana*, yet the origin of the eukaryotic pathway is uncertain. The eukaryotic pathway has been reported in some other algae, namely *Parachlorella kessleri* (Trebouxiophyceae) [159], *Porphyridium cruentum* (Rhodophyta) [160], and *Cyanidioschyzon merolae* [161]. In many algae, however, lipids derived from the eukaryotic pathway have not been identified based on the fatty acid composition of their MGDGs [159]. We analyzed lipid content to determine whether *K. flaccidum* acquired the ability to mediate lipid transport from the ER to plastids. As measured by 2DTLC-GC (Fig. 7b), the C18 fatty acid content of MGDG was determined to be 1.79 times that of C16 fatty acids. In addition, C18/C18 fatty acid species at the *sn1/sn2* positions of MGDG were identified by LC-MS/MS (Supplementary Fig. 7). If the ATS2 in *K. flaccidum* introduces a C16 fatty acid into the *sn-2* position as in *A. thaliana* [123,124], the MGDG that has a C18 fatty acid at the *sn-2* position is certainly synthesized via the eukaryotic pathway. However, this estimation is based on the substrate specificities of acyltransferases in *A. thaliana*. The substrate specificities of acyltransferases in algae might differ from those of *A. thaliana*. Thus, the demonstration of the eukaryotic pathway in these algae may need further validation.

Although the green algae *C. reinhardtii* clearly has *TGD1-3*, there are only traces of C18 fatty acids at the *sn-2* position in MGDG [162], and therefore it seems clear that *C. reinhardtii* has no apparent eukaryotic MGDG. However, a recent analysis of a *Chlamydomonas tgd2* mutant suggests the existence of TGD2-dependent lipid transport from the ER to plastids [163]. In this regard, the eukaryotic pathway in algae remains to be clarified. Accordingly, future studies of TGDs in several algae will be necessary to clarify the origin and evolution of the lipid transport system from the ER to plastids in land plants and to possibly identify other unknown components of the transport machinery.

In addition to TGDs, other factors involved in lipid transfer from the ER to plastids have been reported in *A. thaliana*. Phosphatidate phosphohydrolase (PAH1 and PAH2) and non-specific phospholipase C5 (NPC5) are thought to supply plastids with DAG derived from the ER when phosphate is limiting [27,164,165]. PAH1 and PAH2 are homologs of the mammalian lipins and yeast Pah1p, which are widely distributed among eukaryotes (Fig. 2, Supplementary table 1). PAH1 and PAH2 produce DAG from PA via the activity of phosphohydrolase. In *A. thaliana*, PAH1 and PAH2 are thought to be involved in DAG supply for galactolipid synthesis via the eukaryotic pathway under

phosphate starvation [27]; these enzymes also regulate PA turnover in phospholipid synthesis [166] (Fig. 4). The participation of PAH in the eukaryotic pathway has not been reported in other species. On the other hand, NPC5 directly produces DAG—possibly in the plastid outer envelope—from PC provided from extraplastidic membranes [165] (Fig. 4). This DAG is converted to DGDG via the type B MGD pathway and is increased during phosphate starvation. NPC-like genes are found in *K. flaccidum* and land plants, which also include organisms without type B MGD localized in the chloroplast outer envelope (Fig. 2, Supplementary table 1). The divergence of MGD has been proposed to have occurred during land plant evolution, probably in the period during which seed plants appeared [67]. However, MGD synthase activity in the plastid outer envelope has been reported in *C. reinhardtii*, which has only one MGD gene [163]. Thus, even in organisms without a type B MGD, an NPC-like protein likely facilitates the transfer of lipids from the ER to plastids.

According to the evidence noted above, it is expected that *K. flaccidum* has a lipid remodeling system that is genetically and functionally much closer to land plants than other algae without a type B MGD, although information on galactolipid synthesis via the eukaryotic pathway is currently insufficient—particularly for algae. More biochemical analyses of the lipid pathway in each species will be needed to elucidate the evolution of galactolipid synthesis via the eukaryotic pathway.

Fig. 10 Phylogenetic tree analysis of TGD4.

The analysis utilized the maximum likelihood method based on the LG model +G (8 categories) + F with 500 bootstrap replicates. The scale bar represents 0.5 amino acid substitutions per site. The right three bar graphs indicate bit scores of BLASTP using *A. thaliana* TGD4 AT3G06960 (left), *K. flaccidum* TGD4 candidate kf100100_0060 (middle), and *Synechocystis* sp. PCC 6803 sll0350 (right) as the query. Subject proteins (y axis) correspond to the phylogenetic tree, and each query is indicated in red. The x axis represents the bit score. ND: not detected by BLASTP search (cutoff E-value = $1e^{-3}$). Refer to Figure 3 for species abbreviations.

3.6. Common regulation of lipid remodeling during phosphate starvation in photosynthetic organisms

As described above, during phosphate starvation, the glycolipids DGDG, SQDG and GlcADG can partly substitute for the decrease of phospholipids to ensure proper membrane function. These membrane lipid remodeling events bring about adaptation to low phosphate in addition to other phosphate starvation responses, namely the enhancement of phosphate uptake, induction of phosphate transport, secretion of organic acids and phosphatases, root growth, and root hair formation [167–169].

Indeed, these membrane remodeling-deficient mutants are sensitive to phosphate starvation [27,28,31]. The above-mentioned *MGD2*, *MGD3*, *DGD1*, *DGD2*, *PAH1* and *NPC5* are upregulated during phosphate starvation to ensure adequate DGDG biosynthesis [170]. Similarly, *SQD1* and *SQD2* are upregulated to ensure SQDG biosynthesis [22,100]. These pieces of evidence suggest that the membrane remodeling mediated by these genes is controlled by a certain transcription factor(s). In *A. thaliana*, *phr1-1*, isolated as a mutant defective for broad responses to phosphate starvation [171], also suppresses substitution of phospholipids by glycolipids during phosphate starvation [165,170]. PHR1 is a myb transcription factor that upregulates all of these genes [170]. The *C. reinhardtii* PSR1 is also a myb transcription factor belonging to the subgroup that includes PHR1 [169]. Mutation of PSR1 inhibits the increase in inorganic phosphate uptake during phosphate starvation [172,173]. It remains unknown whether PSR1 regulates membrane remodeling of *C. reinhardtii* under phosphate starvation, but *SQD1* and *SQD2* are indeed upregulated under these conditions [174]. Furthermore, surprisingly, the *C. reinhardtii*-originated *SQD2* promoter is activated specifically when inorganic phosphate is limiting in the secondary symbiont, *Nannochloropsis* strain NIES-2145 [117]. These results raise the possibility that regulation of lipid remodeling genes by these transcription factors is a common attribute of eukaryotic organisms. Further work of the transcription factors will be helpful for understanding the evolution of glycolipid regulation.

4. Perspective

The defining characteristics of glycolipid evolution are the complex processes that exhibit commonality (characteristic composition of the major glycolipids, conserved response to phosphate starvation) and diversity (biosynthetic pathways, lipid transport). This commonality presumably arose via close functional association between photosynthesis (mediated by photosynthetic membrane proteins) and the membrane glycolipids produced by photosynthesis. In addition, it is also important that the two

plastidial glycolipids DGDG and SQDG can partly substitute functionally for the phospholipids PC and PG, supplying the inorganic phosphate that is an essential nutrient and tends to be insufficient in the environment. In this way, there is close relationship between glycolipids, photosynthesis, and response to phosphate starvation. Some organisms like Apicomplexa, which are protozoan parasites, have a degenerated non-photosynthetic plastid, termed the apicoplast, that was acquired via the secondary endosymbiosis of red algae. They rely on their hosts for nutrients and a stable environment. Nevertheless, Apicomplexa maintain four membranes in their apicoplast, and they lack galactoglycerolipids (reviewed in [2,175]). On the other hand, there are several variations of glycolipid biosynthetic pathways and transport mechanisms. It seems that the essential point is not how glycolipids are synthesized but rather where they localize, how their synthesis is regulated, and how they function.

Further genome sequencing of key organisms that reside at the basal points of evolution of photosynthetic organisms will link gene information and accumulating biochemical knowledge. Sequencing of the *A. thaliana* genome has resulted in remarkable advances in elucidating the roles and biosynthesis of glycolipids. These integrated data have made it possible to estimate glycolipid evolution based on comparative genome analysis. Genomic information for algae species is insufficient, however. *K. flaccidum* is an intermediate organism between green algae and land plants, and it is the only charophyte alga whose genome sequence has been reported. We expect that studies of *K. flaccidum* glycolipids will cast new light on glycolipid evolution from algae to land plants. Genomic information for other algae species will increase rapidly owing to improvements in high-throughput sequencing technology. Contrary to what would be expected based on their simple morphology, algae have greater gene diversity than land plants. The analysis of glycolipids and gene function by orthodox lipid analysis and mutant analysis with various algal species will thus become increasingly important for the elucidation of glycolipid evolution.

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