Biochimica et Biophysica Acta xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbalip

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

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ARTICLE INFO

Article history:
Received 17 December 2015
Received in revised form 9 April 2016
Accepted 15 April 2016
Available online xxxx

Keywords:
Glycolipid
Galactolipid
Sulfolipid
Photosynthetic organisms
Phosphate starvation
Plant evolution

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 phylogenic 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. This article is part of a Special Issue entitled: Plant Lipid Biology edited by Kent D. Chapman and Ivo Feussner.

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 Ref. [1]). However, glycolipids are the major lipids of photosynthetic membranes—particularly the thylakoids of cyanobacteria and chloroplasts (reviewed in Ref. [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 Refs. [3–6]).

Glycolipids can have various types of carbohydrate structures and thus are quite diverse, e.g., they include glycoglycerolipids, glycosphingolipids, lipopolysaccharides, and steryl 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

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http://dx.doi.org/10.1016/j.bbalip.2016.04.015
1388-1981/

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 phylogenic 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 μmol photons m^{-2} 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 × *g* for 10 min at 4 °C. The organic phase was collected and filtered through a 0.2-μm 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 × 30 mm, 2.7 μm 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 μmol 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 [48–51]. 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

Please cite this article as: K. Hori, et al., Tangled evolutionary processes with commonality and diversity in plastidial glycolipid synthesis in photosynthetic organisms, Biochim. Biophys. Acta (2016), http://dx.doi.org/10.1016/j.bbalip.2016.04.015