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Review

Evolution of galactoglycerolipid biosynthetic pathways – From cyanobacteria to primary plastids and from primary to secondary plastids

Dimitris Petroutsos [a], Souad Amiar [b], Heni Abida [c], Lina-Juana Dolch [a], Olivier Bastien [a], Fabrice Rébeillé [a], Juliette Jouhet [a], Denis Falconet [a], Maryse A. Block [a], Geoffrey I. McFadden [d], Chris Bowler [c], Cyrille Botté [b], Eric Maréchal [a,*]

[a] Laboratoire de Physiologie Cellulaire et Végétale, CNRS, CEA, INRA, Univ. Grenoble Alpes, UMR 5168, Institut de Recherches en Sciences et Technologies pour le Vivant, CEA Grenoble, F-38054 Grenoble, France
[b] ApicoLipid Group, Laboratoire Adapation et Pathogenie des Microorganismes, CNRS, Univ. Grenoble Alpes, UMR 5163, Institut Jean Roget, F-38042 Grenoble, France
[c] Environmental and Evolutionary Genomics Section, Institut de Biologie de l'École Normale Supérieure, CNRS UMR 8197, INSERM U1024, 46 rue d'Ulm, 75005 Paris, France
[d] Plant Cell Biology Research Centre, School of Botany, University of Melbourne, Victoria 3010, Australia

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ABSTRACT

Photosynthetic membranes have a unique lipid composition that has been remarkably well conserved from cyanobacteria to chloroplasts. These membranes are characterized by a very high content in galactoglycerolipids, i.e., mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively). Galactoglycerolipids make up the bulk of the lipid matrix in which photosynthetic complexes are embedded. They are also known to fulfill specific functions, such as stabilizing photosystems, being a source of polyunsaturated fatty acids for various purposes and, in some eukaryotes, being exported to other subcellular compartments. The conservation of MGDG and DGDG suggests that selection pressures might have conserved the enzymes involved in their biosynthesis, but this does not appear to be the case. Important evolutionary transitions comprise primary endosymbiosis (from a symbiotic cyanobacterium to a primary chloroplast) and secondary endosymbiosis (from a symbiotic unicellular algal eukaryote to a secondary plastid). In this review, we compare biosynthetic pathways based on available molecular and biochemical data, highlighting enzymatic reactions that have been conserved and others that have diverged or been lost, as well as the emergence of parallel and alternative biosynthetic systems originating from other metabolic pathways. Questions for future research are highlighted.

Contents

* Corresponding author. Tel.: +33 438784985.
E-mail address: eric.marechal@cea.fr (E. Maréchal).

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1. Introduction

1.1. Galactoglycerolipids are a landmark of oxygen-evolving photosynthetic organisms

Photosynthetic eukaryotes (algae, plants and some protists) are characterized by the presence of a chlorophyll-containing organelle, the chloroplast, whose origin dates back to a primary endosymbiotic event, when an ancestral cyanobacterium was engulfed within or invaded a primary eukaryotic host (for review, [1–8]). The membrane architecture of cyanobacteria and primary chloroplasts are similar: both are delimited by a two-membrane envelope and contain flattened membrane sacs, or thylakoids, in which the photosynthetic complexes are embedded. These membranes have a lipid composition, which has been remarkably well conserved through evolution. In particular, they are characterized by a very high content in galactoglycerolipids, i.e., mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively). The anomery of the terminal galactosyl groups differs in these two lipids: in MGDG, the galactose is in β conformation, forming the 1,2-diacyl-3-O-(β-D-galactopyranosyl)-*sn*-glycerol structure, whereas in DGDG, the second galactose is in α conformation, forming 1,2-diacyl-3-O-(α-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl)-*sn*-glycerol [9,10] (Fig. 1). In this review, we shall refer to these structures as β−MGDG and αβ−DGDG. The transfer of galactose from one galactolipid to another, which occurs in Angiosperms during certain environmental stresses including exposure to ozone or cold, and leading to the production of ββ−DGDG, βββ−triGDG and ββββ−tetraGDG [11,12] shall not be discussed here.

MGDG and DGDG were first isolated from the benzene extract of wheat flour (*Triticum aestivum*) by Carter et al. in 1956 [9]. The systematic inventory of lipids in photosynthetic organisms was initiated a decade later, taking advantage of the thin-layer chromatography separation methods developed by Nichols in 1963 [13] and Allen et al. in 1966 [14]. The ubiquity of galactolipids in all photosynthetic organisms emerged as they were discovered successively in cyanobacteria, e.g., *Anacystis nidulans* and *Anabaena variabilis* [15], various green algae, firstly *Chlorella vulgaris* [16,17] and then *Chlamydomonas reinhardtii* [18,19], various embryophyta (plants), e.g., the moss *Hypnum cupressiforme* [20], the fern *Adiantum capillus-veneris* [21], the gymnosperm *Pinus sylvestris* [22] and the angiosperm *Spinacia oleracea* [23], and eventually to various photosynthetic protists deriving from green algae, such as *Euglena gracilis* [24] or deriving from red algae, such as the diatom *Phaeodactylum tricornutum* [25]. The presence of MGDG and DGDG was recognized as a hallmark of all oxygen-evolving photosynthetic organisms [10], and consequently as the most abundant lipid classes on Earth [26]. Analytical technologies (mass spectrometry, NMR) have increased in sensitivity and throughput the last 15 years. Lipidomic

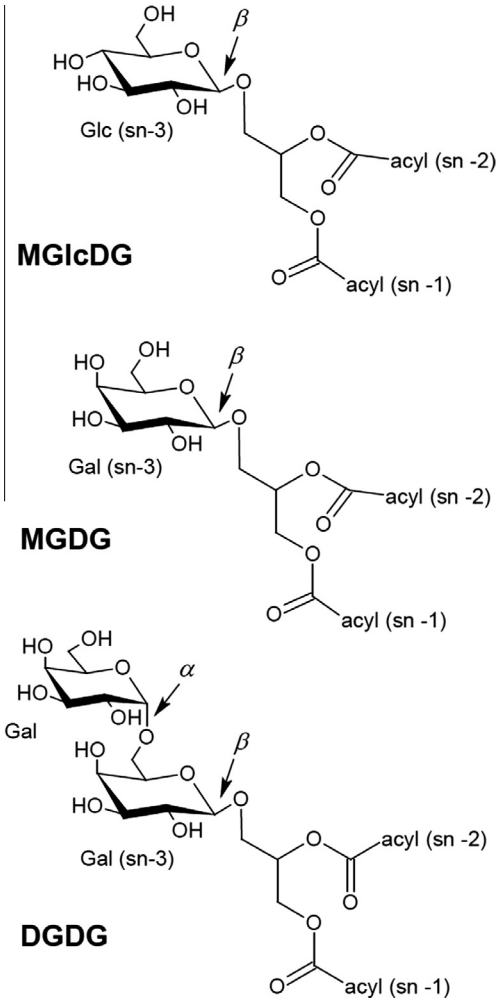


Fig. 1. Galactoglycerolipids conserved in photosynthetic membranes from cyanobacteria to primary chloroplasts of algae and plants. Positions *sn*-1 and *sn*-2 of the glycerol backbone are esterified to fatty acids and position *sn*-3 harbors the polar head. The α and β anomery are indicated. The precursor of galactolipids in cyanobacteria, MGlcDG, is also shown. MGlcDG, monoglucosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, diagalactosyldiacylglycerol.

characterization of numerous photosynthetic microorganisms in a large variety of genetic backgrounds, growth conditions and stresses have been made available [27] and provide precious information to start reconstructing the evolution of glycerolipid metabolism.

Together with phosphatidylglycerol (PG) and sulfoquinovosyldiacylglycerol (SQDG), MGDG and DGDG make up the lipid matrix hosting the photosystems [29]. Besides their role as a membrane component, galactoglycerolipids are also known to fulfill specific molecular functions. They stabilize photosystem subunits [29,30], bind to the plastid protein import machinery [31], are a source of polyunsaturated fatty acids for various purposes and, in some eukaryotes like plants, DGDG was furthermore shown to be exported to extra-plastidial membrane compartments [32–34], where it could substitute for phosphoglycerolipids [32,35–37]. These roles shall not be detailed here and the reader is invited to refer to some recent reviews [12,28,29].

Considering the ubiquity of MGDG and DGDG, one might expect that the enzymes involved in their biosynthesis have been conserved through evolution. Surprisingly, this is not the case: the biosynthetic machinery producing MGDG and DGDG in eukaryotes has strongly diverged from that found in extant cyanobacteria.

Here, we summarize the evolution of galactolipid metabolism beginning with the cyanobacteria, although this was not the first system to be elucidated. Indeed, genes coding for galactolipid synthesis enzymes were initially characterized in angiosperms by Shimojima et al. in 1997 [38] and Dörman et al. in 1999 [39]. Subsequently, other organisms were explored based on sequence similarity. Frustratingly, bioinformatic searches for galactolipid orthologs of plant genes in cyanobacteria provided no candidates, and their identification was eventually achieved by Awai et al. in 2006 [40] and 2007 [41] and by Sakurai et al. in 2007 [42] through more classical means. We shall therefore describe the evolution of these pathways, regardless of the timeline of scientific discovery.

1.2. How has galactoglycerolipid metabolism evolved in photosynthetic organisms, following primary and secondary endosymbioses?

The question of the evolution of galactoglycerolipid metabolism has to be formulated in the context of photosynthetic eukaryote evolution, which is characterized by dramatic transitions in subcellular architecture. Important evolutionary transitions comprise primary and secondary endosymbiotic events, from an ancestral cyanobacterium to a primary chloroplast (Fig. 2A), and then from a symbiotic unicellular alga to a secondary plastid (Fig. 2B), respectively (for reviews, [1–7]).

If we first consider the simplest situation observed in plants and algae (the Archaeplastida kingdom, Fig. 2A), cells contain "simple plastids" and molecular evidence supports the view that all these plastids trace back to a single event of endosymbiosis [1]. An envelope comprising two membranes (the inner envelope membrane, IEM, and the outer envelope membrane, OEM) delineates the chloroplasts and derives from the two limiting membranes of the Gram-negative cyanobacterial ancestor. Based on photosynthetic pigments, storage material and cell walls, three lineages of these primary plastid bearing eukaryotes have diverged: a blue lineage (Glaucophyta), a red lineage (Rhodophyta), and a green lineage (green algae and plants) (Fig. 2A). In the "blue lineage", in which chlorophyll *a* is associated to phycocyanin and allophycocyanin, there is a small group of unicellular organisms (Glaucophyta), including *Cyanophora paradoxa*, in which the chloroplast still contains a peptidoglycan cell wall between the inner and outer envelopes. The "red lineage", in which chlorophyll *a* is energetically coupled to phycobilin, includes the red algae or Rhodophyta, such as *Cyanidioschyzon merolae*. Lastly, the "green lineage", in which chlorophyll *a* is associated to chlorophyll *b*, contains green algae or Chlorophyta, such as *C. reinhardtii*, and plants, or Streptophyta, such as *Arabidopsis thaliana*.

Recently it has emerged that the primary endosymbiotic creation of plastids was not a unique event. Reduced endosymbiotic cyanobacteria within cells of the amoeba Paulinellia (in the Rhizaria taxon) are now recognized as a second, independent origin of plastids [43]. This organelle, also called the chromatophore, is therefore derived from a cyanobacterium, but is not ontogenetically related to chloroplasts found in all other species examined to date.

It is more difficult to understand how unicellular organisms may contain plastids limited by more than two membranes. Fig. 2B gives some examples of reasonable scenarios (adapted from [1]). Protists originating from a secondary endosymbiosis belong to at least three lineages: two independent green lineages (Chlorarachniophytes and Euglenids), and a red lineage (Chromalveolates). The Chlorarachniophytes, such as *Bigelowiella natans*, have a plastid surrounded by four membranes. They have retained a relic of the endosymbiont algal nucleus, called a nucleomorph, between the two innermost and the two outermost membranes of their plastid [44] (Fig. 2B). On the other hand, some Euglenozoa such as *E. gracilis* contain a plastid limited by three membranes, and they lack a nucleomorph. Parasites of the Trypanosomatidae phylum, such as *Trypanosoma bruceii*, belong to Euglenozoa, but have no plastid.

The "red lineage", in which a red alga is believed to have been engulfed by another eukaryote, is thought to account for all the other plastid-bearing protists. Significant biodiversity is represented in this lineage, including Cryptomonads, such as *Guillardia theta*, which have conserved a nucleomorph, Haptophytes, such as *Nannochloropsis gaditana*, Heterokonts, such as the diatoms *P. tricornutum* and *Thalassiosira pseudonana*, Chromerida, such as *Chromera velia*, and the closely related phylum of Apicomplexa, comprising human parasites such as *Toxoplasma gondii* and *Plasmodium falciparum*. Cavalier-Smith has proposed that most secondary endosymbionts of the red lineage can be grouped as Chromalveolata [45], a super-group which might not be monophyletic [46,47]. They were also shown to have been subjected to large transfers of genes from a green algal origin, proposed to be from a secondary endosymbiosis involving a green alga prior to the red algal endosymbiosis that is believed to be common to all Chromalveolates [48]. The nuclear genomes of Chromalveolata are therefore chimeric, a feature we shall consider further later on. Alveolata, including Apicomplexa and Chromerida, and Heterokontophyta, including Diatoms and Eustigmatophytes discussed here, seem to be monophyletic within the Chromalveolata.

With respect to lipid metabolism it is well known that cyanobacteria, primary and secondary plastids all contain a machinery to generate fatty acids in their stroma, the dissociated fatty acid synthase of type II (FASII) [49]. These fatty acids are used as building blocks for glycerolipids, including the galactoglycerolipids discussed here. Fatty acids are successively esterified to the *sn*-1 and *sn*-2 positions of glycerol-3-phopshate to generate phosphatidic acid (PA), which is then dephosphorylated to form diacylglycerol (DAG), the universal precursor for galactoglycerolipids (for review, [50,51]). In green algae and in plants, the assembly of galactolipids has progressively evolved from a utilization of PA/DAG precursors synthesized de novo within the plastid, like in cyanobacteria (the so called "prokaryotic" pathway), to the utilization of diacyl-moieties imported from the endoplasmic reticulum (the "eukaryotic" pathway) [52–54]. We shall therefore focus on the evolution of the synthesizing enzymes *per se*, which is of relevance for the first transition (cyanobacteria to primary chloroplasts), and also on the evolution of the upstream pathways, which generate the substrates for galactoglycerolipids.

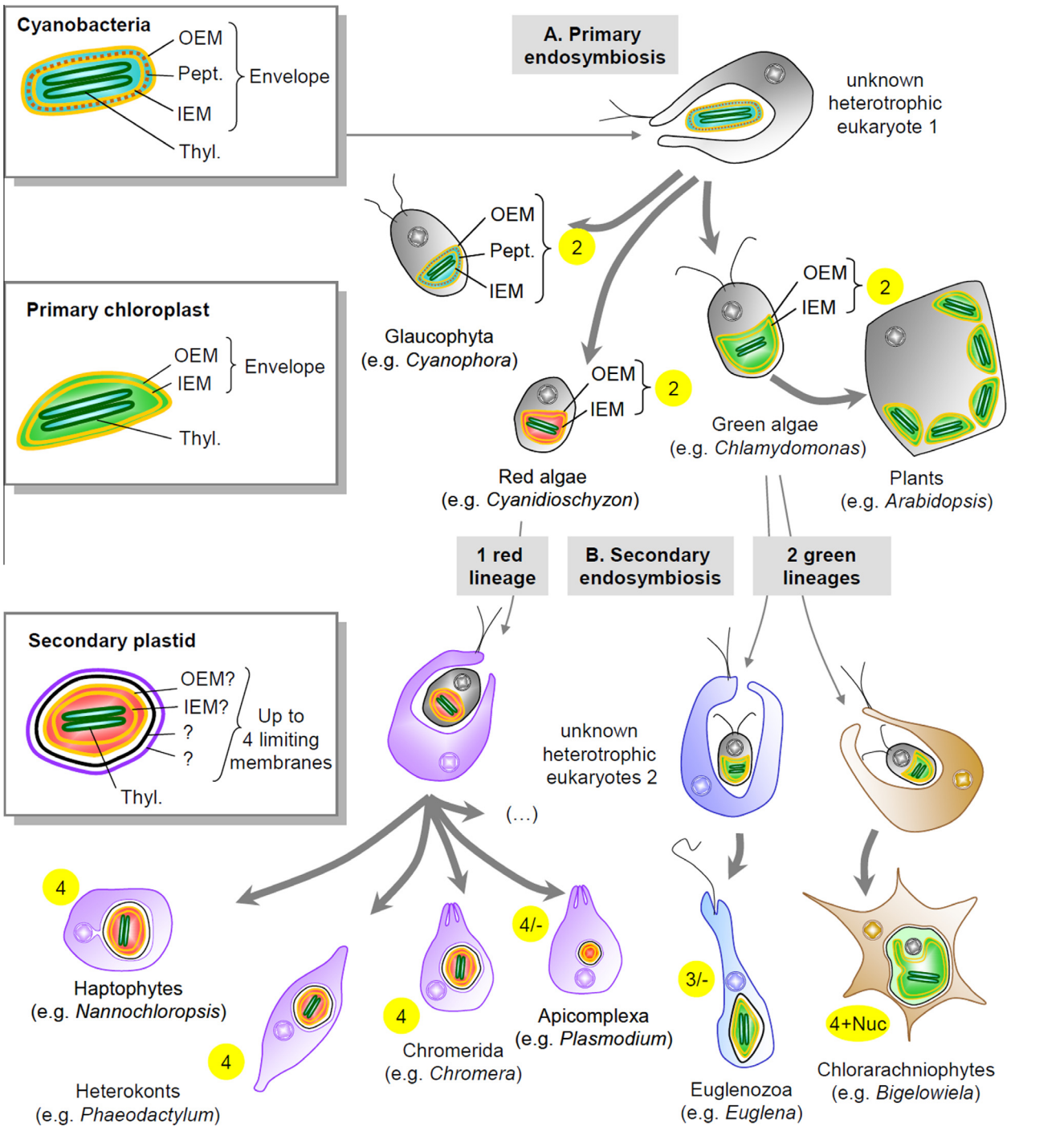


Fig. 2. Schematic representation of plastid evolution. (A) Primary endosymbiosis. In the upper panel, a single primary endosymbiosis between an unknown heterotrophic eukaryote and a Gram-negative cyanobacterium led to the three primary-plastid-bearing lineages, i.e., the "blue" lineage (glaucocystophytes), the "red" lineage (red algae) and the green lineage (green algae and plants, forming together the Viridiplantae clade). The primary plastid is always surrounded by an envelope containing two membranes, vertically inherited from the two membranes limiting the cyanobacterium (see schemes in figures on the left side). An independent endosymbiosis has led to the emergence of Paulinella, not shown in this figure. (B) Secondary endosymbiosis. Two types of secondary endosymbioses involving two different green algae and unrelated unknown heterotrophic eukaryotes led to Euglenozoa and Chlorarachniophytes. A single endosymbiosis between a red alga and a heterotrophic eukaryote probably led to all remaining plastid-bearing protists. Loss of photosynthesis is pervasive in several lineages. The number of membranes limiting primary and secondary plastids is highlighted in yellow: (2), (3) or (4). Phyla that include species that have lost their plastids are indicated: (3/−) and (4/−). Phyla in which the primary nucleus has been conserved as a nucleomorph are indicated: (4+Nuc). To maintain simplicity, the proposed origin of diatoms and other Chromalveolates from serial secondary endosymbioses involving both a green and a red alga [48] in (4) are not shown.

Concerning the second transition (primary to secondary plastids), information is scarce and the field of research is still open for a range of fundamental investigations. Little is known regarding the localization of galactoglycerolipids in the secondary plastid membranes, or about their biosynthetic machinery and their integration into the general scheme of cellular metabolism. We have thus compared pathways based on available molecular and biochemical data, highlighting enzymatic reactions that

appear to be conserved and some that may have diverged or been lost.

2. Biosynthesis of galactoglycerolipids in cyanobacteria

Cyanobacteria have been primarily classified as Gram-negative bacteria [55]. Their cell envelope is composed of an outer and a plasma membrane, separated by a peptidoglycan layer [56]. In addition to MGDG, DGDG, PG and SQDG, cyanobacteria were shown to also contain a very low proportion of a monoglucosyldiacylglycerol, or MGlcDG [55]. The glucosyl group is in β conformation, forming 1,2-diacyl-3-O-(β-D-glucopyranosyl)-*sn*-glycerol, or β−MGlcDG (see Fig. 1). This lipid has been detected in major phyla of cyanobacteria, including strains of the Pasteur Culture Collection of Cyanobacteria (PCC), a library initiated by Stanier in the late 1960s (see Supplementary Table 1 and Fig. 3). Although MGlcDG seems to be absent in some cyanobacteria (see Supplementary Table 1), suggesting that MGDG could be synthesized directly without this intermediate (see below), its occurrence, even at very low levels, has been assumed based on MGlcDG detection in other strains of the same taxonomic clusters. In *Gloeothece*, a group now called *Gloeobacter*, all tested strains seem to lack MGlcDG (see Supplementary Table 1 and Fig. 3). On average, cyanobacteria thus contain ~52% MGDG, ~15% DGDG, ~22% PG, ~9% SQDG and ~0–1% MGlcDG.

Because a unique primary endosymbiosis is at the source of all plastids (with the exception of the *Paulinella* example discussed above), the question of the uniqueness of a biosynthetic pathway to generate all galactoglycerolipids in cyanobacteria is critical. The biosynthetic scheme was first analyzed by biochemical approaches. Enzymatic activities responsible for the production of phosphatidic acid (PA), i.e., glycerol-3-phosphate acyltransferase and lyso-PA acyltransferase activities, were first characterized in *A. variabilis* [57–59]. These enzymes use fatty acids thio-esterified to the acyl carrier protein generated by the activity of FASII.

The production of MGlcDG synthesis was then measured in *A. variabilis* [60–62]. Labeling experiments using [^{14}C]-bicarbonate showed that the synthesis of MGlcDG preceded that of MGDG suggesting that MGlcDG served as a precursor for the synthesis of galactolipids. Conversion of MGlcDG into MGDG by an epimerase was indeed characterized in both *A. variabilis* [60–62] and *A. nidulans* [63], i.e., by stereochemical isomerization of the carbon-4 of the glucosyl group, and not by a replacement of glucose by galactose. DGDG synthesis was then shown to occur by transfer of a galactosyl group (and not a repetition of a glucosyl transfer followed by an isomerization) to MGDG [60–62]. The complete biosynthetic pathway can therefore be summarized as in Fig. 4, at least as initially characterized biochemically in *Anabaena*, *Anacystis* and eventually in the best studied model *Synechocystis*.

Interestingly, one of the most ancient cyanobacterial lineages also contains enzymes that were shown to be related to eukaryotic MGDG synthases (called "MGDs"), capable of synthesizing MGDG in one step, from DAG and UDP-Gal, just like in eukaryotic algae and plants. For example, in *Gloeobacter violaceus* Yuzawa et al. reported the presence of a sequence forming a clade with different MGD homologues from green non-sulfur bacteria, *Chloroflexus aurantiacus* and *Chloroflexus aggregans* [64]. Supposing that chloroplasts derive from an ancient cyanobacterium, it could be hypothesized that a cyanobacterial MGD may be at the origin of algal and plant MGDs; this cyanobacterial MGD might be lost in modern cyanobacteria besides a few species such as *Gloeobacter*. But in contradiction with this hypothesis, rigorous phylogenetic analyses [64] rather support that the *Gloeobacter* MGD may find its origin in a more recent *Chloroflexi* → *Gloeobacter* horizontal gene transfer [64]. Nevertheless, one cannot exclude that such horizontal transfer might have occurred with the primal cyanobacteria, the mother of all plastids.

2.1. Enzymes synthesizing MGlcDG

Amongst prokaryotes, MGlcDG, with β-anomeric configuration of the glucose, is not unique to cyanobacteria. In particular, it has been found in *Bacillus subtilis*, where it accounts for 10% of the lipids [65]. Furthermore, in the cell-wall less bacterium *Acholeplasma laidlawii*, a MGlcDG in an α-anomeric configuration of glucose represents the most abundant lipid, accounting for about half of the lipids in these membranes [66]. Genes coding for the corresponding β-MGlcDG and α-MGlcDG synthases in these bacterial models have been cloned and characterized [67,68] but no ortholog has been identified in available complete cyanobacteria genomes. Awai et al. in 2006 [40] resolved the question with an elegant strategy. They first considered that the gene for MGlcDG synthase should be conserved between two sequenced cyanobacteria, the unicellular *Synechocystis* sp. PCC 6803 strain and the filamentous *Anabaena* sp. PCC 7120. They then assumed two characteristics of the enzyme, namely that its sequence should harbor a glycosyltransferase motif, and that its annotation should fall into the category of

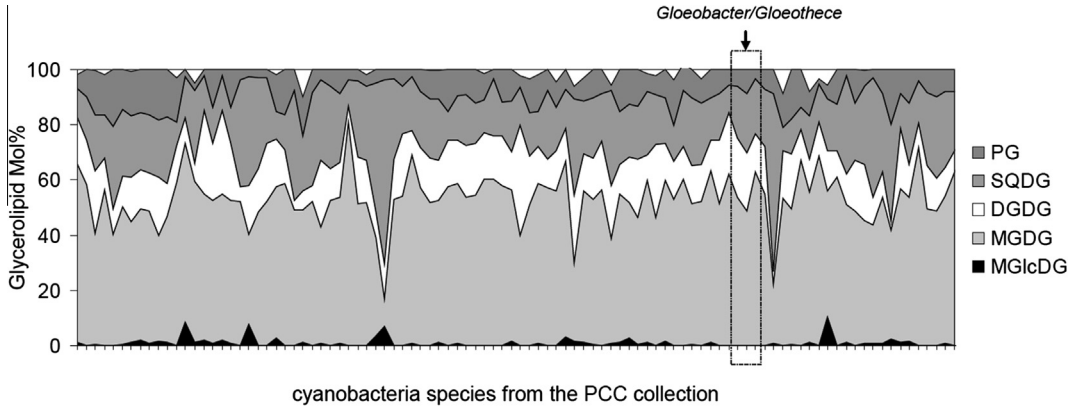


Fig. 3. Systematic analysis of the glycerolipid profiles of cyanobacteria from the PCC collection. MGDG, DGDG, SQDG, PG and MGlcDG have been analyzed in 98 strains of the Pasteur Culture Collection of Cyanobacteria, covering at least 2 representative groups of the following spiecies: *Anabaena*, *Arthrospira*, *Calothrix*, *Chamaesiphon*, *Chroococcus*, *Cyanothece*, *Cylindrospermum*, *Geitlerinema*, *Gloeocapsa*, *Gloeothece/Gloeobacter*, *Leptolyngbya*, *Microcystis*, *Nodularia*, *Nostoc*, *Oscillatoria*, *Pseudanabaena*, *Scytonema*, *Synechococcus* and *Synechocystis*. Doted frame highlight *Gloeothece/Gloeobacter* strains apparently missing MGlcDG. Graph was built with values reported in Supplementary Table 1, based on analyses kindly provided by A.J. Dorne.

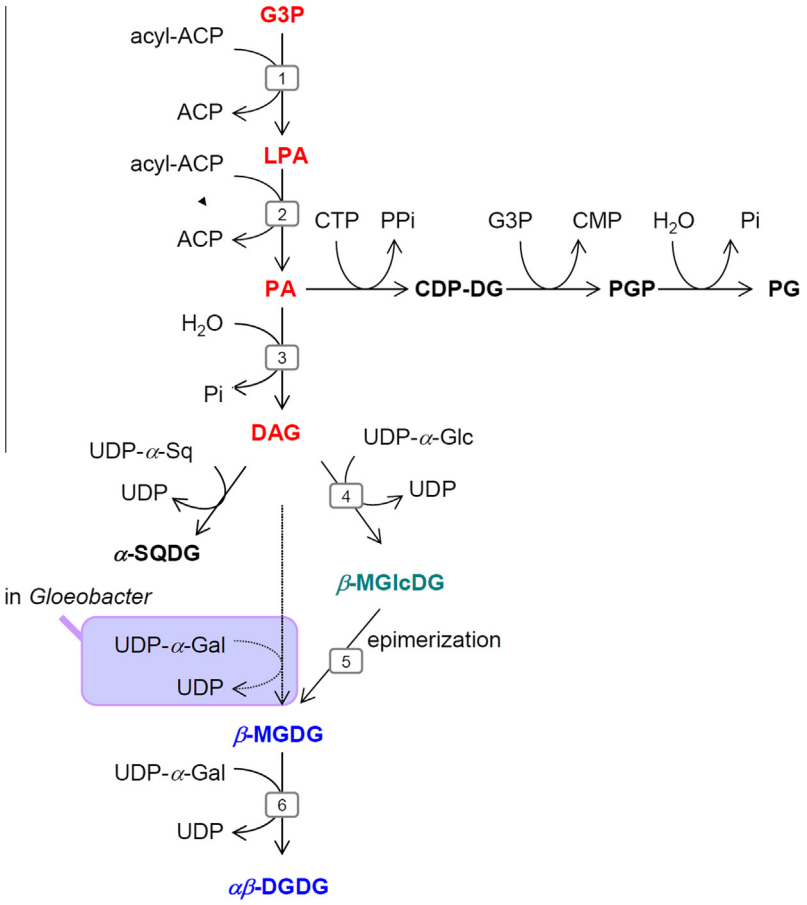


Fig. 4. Biosynthesis of galactoglycerolipids in cyanobacteria. (1) *sn*-glycerol-3-phosphate acyltransferase or ATS1, (2) 1-acylglycerol-phosphate acyltransferase or ATS2, (3) phosphatidate phosphatase, (4) MGlcDG synthase; (5) epimerase, (6) dgdA-type DGDG synthase. The SQDG synthase, CTP: phosphatidate cytosine transferase, glycerol-3-phosphate:CDP-diacylglycerol phosphatidyltransferase, phosphatidylglycerol synthase are also shown. In cyanobacteria, a major difference occurs in the synthesis of galactolipids, by a two-step process involving a synthesis of MGlcDG followed by an epimerization of the glucose polar head into galactose, thus forming MGDG (see text). In *Gloeobacter*, a MGD-like enzyme, catalyzing the direct synthesis of MGDG using UDP-Gal, has been acquired by a recent horizontal transfer.

genes with unknown function. Out of the four gene candidates identified based on these criteria in *Synechocystis*, only one, sll1377, led to the synthesis of β-MGlcDG when expressed in *Escherichia coli*. One ortholog was found in *Anabaena*, all3944. MGlcDG synthase was shown to use UDP-Glc and DAG as substrates [40]. A systematic survey of available cyanobacteria genomes subsequently showed the presence of an ortholog in all species, with >40% identity in amino acids.

MGlcDG synthase sequences show putative membrane spanning domains in the N- and C-terminal regions of the protein. MGlcDG synthases also contain two motifs (D...DXD and QXXRW) found in the GT2 family of glycosyltransferases classified in the CAZy database (carbohydrate-active enzymes database; http://www.cazy.org/) [69]. The GT2 family contains inverting glycosyltransferases such as cellulose synthases or chitin synthases. The activity of the MGlcDG synthase linking glucose in a β configuration is thus consistent with this classification. Glycosyltransferases containing D...DXD and QXXRW motifs also occur in the GT21 family. Glycosyltransferases containing such a sequence motif in the CAZy database include processive enzymes. In addition, like other GT2 proteins, MGlcDG synthase was shown to require divalent cations, in particular magnesium [40]. Interestingly, plant and algal MGDG synthases, which are also inverting enzymes, do not belong to either the GT2 or GT21 family (see below).

We found a putative ortholog of the MGlcDG synthase in *Paulinella chromatophora*, (RefSeq YP_002049084) with 43% identity and a Blast E-value of 5×10^{-97}, supporting the hypothesis that in the chromatophore of Rhizaria, MGlcDG is synthesized like in cyanobacteria. The *P. chromatophora* sequence is presently annotated as a glycosyltransferase of the GT2 family. Functional analysis is now required to confirm this activity in chromatophore organelles.

Concerning chloroplasts, no ortholog of MGlcDG synthases has been found in any algal or plant genome [40]. Based on comparative genomic surveys, MGlcDG synthases appear therefore restricted to cyanobacteria and possibly to the chromatophore of Rhizaria.

The localization of the cyanobacterial MGlcDG synthases has not been unambiguously assessed yet, although the presence of membrane spanning domains indicates a tight association with membranes. Based on membrane fractionation and enzymatic measurements, MGlcDG synthase activity was detected in both thylakoid and plasma membranes of *A. nidulans* [63].

2.2. Epimerases converting MGlcDG into MGDG

The search for the epimerase acting on the conversion of β-MGlcDG into β-MGDG is still open for investigation and is one of the last challenges to fully understand galactoglycerolipid synthesis in cyanobacteria. No epimerase candidate genes have been found associated with any MGlcDG synthase operon [40], and of the nine epimerases currently listed in the annotation of *Synechocystis* PCC6803 (according to the reference database Cyanobase, http://genome.microbedb.jp/cyanobase/Synechocystis), none appear to act on a glucolipid substrate.

2.3. Enzymes synthesizing DGDG

As for MGlcDG synthase, the identification of the cyanobacterial gene coding for a DGDG synthase, called dgdA, was determined based on comparative genomic studies. In 2007, Awai et al. [41] and Sakurai et al. [42] simultaneously reported the discovery of the same *dgdA* gene in both *Synechocystis*, slr1508, and in *Anabaena*, alr4178. The first group had previously reported the identification of MGlcDG synthase amongst 4 genes of *Synechocystis* containing glycosyltransferase motifs and being functionally uncharacterized. They added a third criterion, specifically, the presence of a similar sequence in the genomes of Cyanidophytina, a red alga living in very acidic environments that lack the eukaryotic DGD gene, e.g., *C. merolae*, *Cyanidium caldarium* and *Galdieria sulphuraria*. They thus identified a unique candidate gene in *Synechocystis* and *Anabaena* and assessed function based on the lack of DGDG in knockout lines of cyanobacteria, and on synthesis of DGDG after heterologous expression in *E. coli* [41]. The second group had a similar approach, using a supervised phylogenetic profiling to identify putative glycosyltransferase genes shared by cyanobacteria and *Cyanidioschyzon*, and not present in green plants. They based the functional characterization on single gene knockout analyses [42]. The dgdA protein belongs to the GT4 family of the CAZy classification [69], just like the unrelated DGD proteins catalyzing the synthesis of DGDG in all algae and plants, with the notable exception of some red algae.

When *E. coli* expresses the *Synechocystis dgdA* gene, the synthesis of αβ-DGDG can only be detected if a plant β-MGDG synthase is coexpressed and UDP-Gal provided [41]. By contrast, when dgdA is coexpressed with a β-MGlcDG synthase, no diglycosyl-lipid is synthesized, showing that MGlcDG is not a substrate for dgdA. This specificity is, on the one hand, consistent with previous analyses showing that α-MGlcDG and α-MGDG produced in *Synechococcus* following genetic engineering are not used for the synthesis of α-Gal-α-GlcDG or αα-DGDG [70]. On the other hand, this result confirms the biosynthetic scheme shown in Fig. 4. In the absence of a reliable assay to measure the activity of dgdA, the subcellular localization has not been documented by enzymatic detection in subcellular fractions and should therefore be obtained by proteomic analyses of membrane fractions.

As for the conservation of a cyanobacterial gene synthesizing DGDG in eukaryotes, we could only identify a sequence in the genome of *P. chromatophora* (RefSeq YP_002049341) coding for a *dgdA* ortholog. This again supports the inheritance of biosynthetic pathways from modern cyanobacteria in the chromatophore of Rhizaria, with an evolutionary story that is distinct from that of chloroplast-containing eukaryotes.

Interestingly, whereas MGlcDG synthase, and possibly the associated epimerase, have been totally lost in chloroplasts, being replaced by a simple MGDG synthase, dgdA has been retained in some red algae even though it has been lost in the vast majority of algae and all plants. Thus, the loss of the enzymes of the cyanobacterial pathway has not occurred in a single step during the early evolution of primary plastids.

3. From cyanobacteria to primary plastids: emergence of a new galactolipid synthetic pathway

The galactolipid biosynthetic pathway in primary chloroplasts has been mainly characterized enzymatically in angiosperms and in a few green algal models. Once MGDG synthase (MGD) and DGDG synthase (DGD) genes had been identified in angiosperms [38,39], it was rapidly evident that this pathway did not derive from the ubiquitous cyanobacterial system, and that this innovation was shared by all eukaryotic plastids, from Archaeplastida (Glaucophyta, Red algae, Green algae, plants) to secondary endosymbionts. In all cases, the biosynthesis of MGDG and DGDG was reported to occur in the outer envelope of chloroplasts. We start the description of the evolution of this pathway with the source of the diacyl-precursors that are utilized because cooperation between the plastid and other compartments of the cell, most notably the endoplasmic reticulum, is an important innovation that has occurred here.

3.1. Diacyl-precursors for MGDG and DGDG synthesis

3.1.1. Neosynthesis of C18/C16 phosphatidic acid and diacylglycerol in the stroma of chloroplasts (prokaryotic pathway)

Some of the lipid biosynthetic machineries have been remarkably well conserved in the cyanobacteria → primary chloroplast transition, e.g. the biosynthesis of fatty acids, thio-esterified to the acyl carrier protein (in angiosperms: 16:0-ACP and C18:0-ACP), is carried out by the same enzymatic system found in prokaryotes, i.e., an acetyl-CoA carboxylase complex and a fatty acid synthase of type II (FASII) (see above and [49]). Furthermore, some fatty acids generated inside the chloroplast harbor features that make them useful as *signatures* allowing their detection when esterified to glycerolipids, such as the length (in general C16 or C18), the number of double bonds (like the desaturation of C16:0 to C16:3 or C16:4 in numerous algae and plants), as well as specific transfers to positions *sn*-1 and *sn*-2 of glycerol-3-phosphate (G3P). Desaturation of chloroplast fatty acids can be initiated by a stromal delta-9 acyl/stearoyl-ACP desaturase (FAB2) that catalyzes the synthesis of 18:1-ACP from 18:0-ACP [71]. In some tissues or in certain organisms the desaturation of 16:0-ACP into 16:1-ACP can also be catalyzed [72]. De novo fatty acids can then be used either in chloroplasts or exported to the cytosol where they feed the cytosolic pool of acyl-CoAs [35,49,73–75].

The synthesis of G3P, the other important building block of glycerolipids, can occur in the stroma [75]. Using acyl-ACP and G3P, the envelope is known to be the site of biosynthesis of PA and DAG, by the stepwise action of *sn*-glycerol-3-phosphate acyltransferase (ATS1) in the stroma, a 1-acylglycerol-phosphate acyltransferase (ATS2) in the IEM, and a phosphatidic acid phosphatase (PAP) in the IEM [76] (Fig. 5). In the case of angiosperms, the specificity of ATS1 and ATS2 leads to the production of PA and DAG with 18:1 and 16:0 at positions *sn*-1 and *sn*-2, respectively. The glycerolipids assembled in the plastid, with 16-carbon fatty acids at the *sn*-2 position, harbor a diacyl-structure similar to that observed in cyanobacterial glycerolipids, named therefore a 'prokaryotic' structure [35,37,75,77].

3.1.2. Import of extraplastidial C18/C18 and C16/C18 diacyl-precursors (eukaryotic pathway)

In the endoplasmic reticulum of angiosperm cells, acyl-CoAs and G3P are used for the stepwise synthesis of PA and DAG within the ER, with 16:0, 18:0 or 18:1 at *sn*-1 and 18:0 or 18:1 at *sn*-2 positions. Membrane glycerolipids assembled in the ER, with 18-carbon acyls at *sn*-2 position, have a diacyl structure defined as 'eukaryotic' [35,37,75,77]. Import of precursors from the ER to the chloroplast is reflected by the high proportion of this eukaryotic signature in galactoglycerolipids. This import system was first described by Ohlrogge and Browse in 1995 [78]: the plastid pathway is known as the prokaryotic pathway, whereas the endoplasmic route is known as the eukaryotic pathway.

An important issue concerns the *dual origin of PA/DAG substrates* in all primary plastids, and the presence of a *eukaryotic pathway* in all these organisms. In green algae, this has been particularly well studied in *Chlorella kessleri*, thanks to a careful analysis of fatty acids at the *sn*-1 and *sn*-2 positions of glycerolipids [79]. In this study, galactolipids contained almost exclusively C18 acyls at the

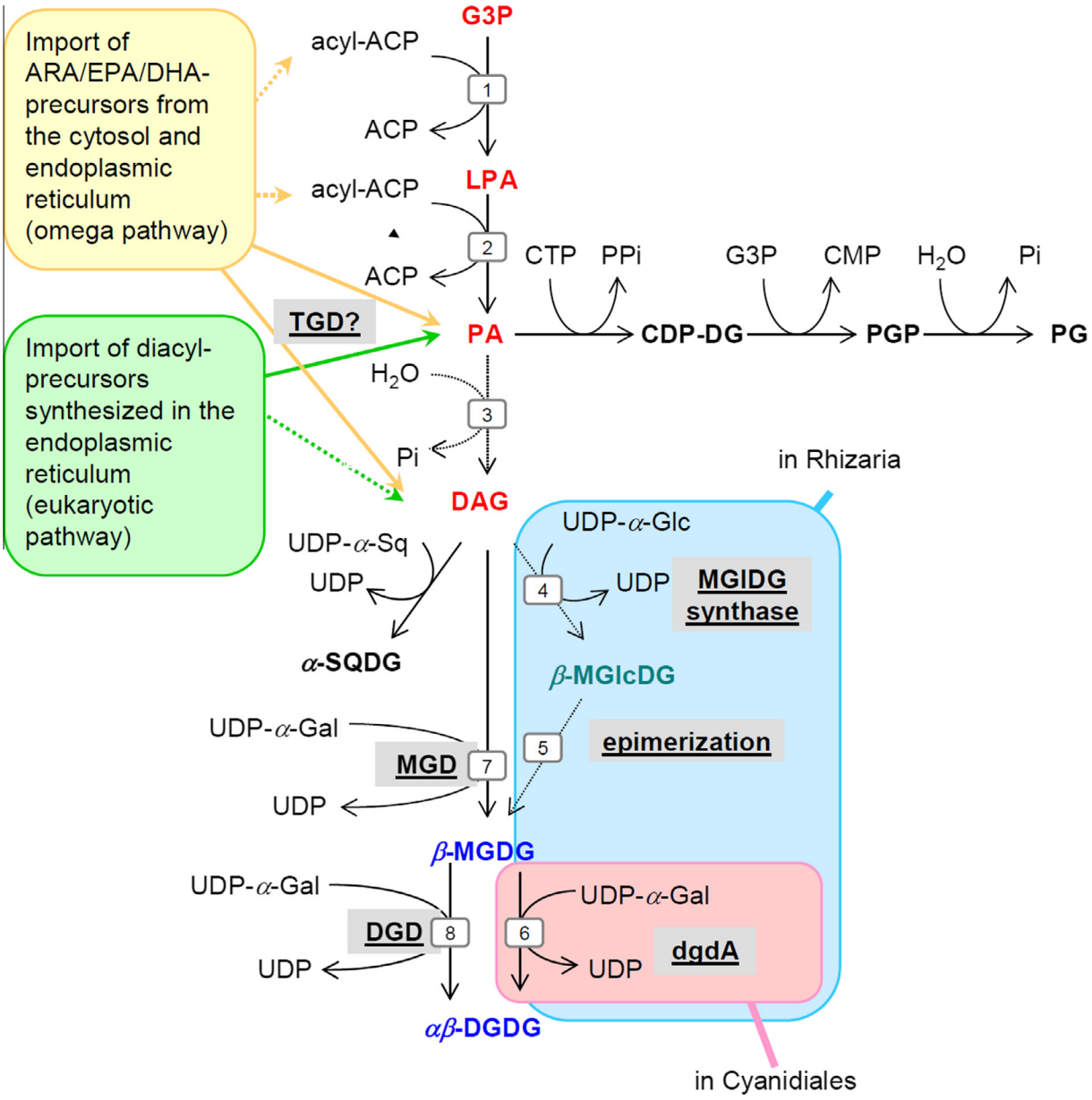


Fig. 5. Biosynthesis of thylakoid lipids in the chloroplast envelope of angiosperms. (1) *sn*-glycerol-3-phosphate acyltransferase or ATS1, (2) 1-acylglycerol-phosphate acyltransferase or ATS2, (3) phosphatidate phosphatase, (4) MGlcDG synthase; (5) epimerase, (6) dgdA-type DGDG synthase, (7) MGDG synthase or MGD, (8) DGDG synthase or DGD. The synthesis of PG and SQDG are also shown. The catalysis of DGDG via a cyanobacterial dgdA-type enzyme is unique to Cyanidiales, a sub-group of red algae. The genome of the chromatophore of Paulinella encodes the cyanobacterial enzymes. Dotted lines indicate hypothetical precursor transfers that still need to be demonstrated.

sn-1 position whereas the *sn*-2 position contained both C16 and C18 acyls. Based on the structural definition given above, MGDG was found to contain 65% of prokaryotic molecular species and 35% of eukaryotic ones. By contrast, and with a similar trend to that observed in higher plants, DGDG contained 68% of eukaryotic structures. Pulse-chase experiments using [^{14}C] acetate showed that MGDG and DGDG were primarily labeled as C18/C16 (prokaryotic) molecular species, and subsequently as C18/C18 (eukaryotic) molecular species, with a concomitant decrease of C18/C18 labeling in extraplastidial phosphatidylcholine [79]. This study supports the existence of a eukaryotic pathway in green algae functionally related to that described in Angiosperms, with a route of lipid intermediates coming from PC and feeding the chloroplast with precursors for the synthesis of galactolipids. Today, besides the TGD machinery that is conserved in green algae, components of the machinery that specifically divert PC to feed galactolipid synthesis are unknown and it is therefore difficult to speculate on the evolution of this system.

3.1.3. Import of extraplastidial ω3/ω6 very-long chain polyunsaturated acyl precursors (omega pathway)

In addition to the ER → chloroplast route that has been studied in great detail in Chlorophyta and Embryophyta, the elaboration of fatty acids with more than 20 carbons, containing multiple double bounds and forming the so called very long chain poly-unsaturated fatty acids (VL-PUFAs), requires elongase and desaturase activities that are associated with the cytosol and the endoplasmic reticulum [27]. The presence of such fatty acids like all-*cis*-$\Delta^{5,8,11,14}$ 20:4 (C20:4 ω-3, arachidonic acid, or ARA), all-*cis*-$\Delta^{5,8,11,14,17}$ 20:5 (C20:5 ω-3, eicosapentaenoic acid, or EPA) and all-*cis*-$\Delta^{4,7,10,13,16,19}$ 22:6 (C22:6 ω-3, docosahexaenoic acid, or DHA) in galactolipids is a clear evidence of an ER/cytosol → chloroplast

import (Fig. 5). These fatty acids have been identified in primary plastids in the green lineage (e.g., *Chlorella minutissima* [80]) as well as the red lineage (e.g., *Porphyridium purpureum*, previously known as *P. cruentum* [81]). The biosynthesis of ω3/ω6-VL-PUFAs in the cytosol of *P. purpureum* has been exquisitely dissected using externally supplied fatty acids and precursors [82,83], and the identified activities serve as a reference to better understand the conservation of this pathway in the cytosol of other organisms. Interestingly, based on a specific inhibitor response, it was shown that part of the synthesis of ω3/ω6-VL-PUFAs requires FAs to be linked to PC, for the C18:1 → C18:2ω6 desaturation catalyzed by a Δ12-desaturase. Following desaturation of C20, FAs would then occur on various phospholipid classes (for review, [27]). ARA/EPA-rich-PC could then be a source of ARA/EPA-rich DAG, used as precursor for other lipid classes including MGDG. In the chloroplast, a Δ17-(ω3) desaturase was proposed to convert ARA-MGDG into EPA-MGDG [82]. Regardless of this activity that allows ARA → EPA conversion in chloroplasts, all of the ω3/ω6-VL-PUFAs found in galactolipids are likely to originate from the cytosol.

In the case of the green alga *C. minutissima*, diacylglyceryl-N,N,N-trimethylhomoserine (DGTS) is composed of up to 44% of total lipids, which is accompanied by PC as the major phospholipid. In *C. minutissima*, both positions of DGTS are acylated with EPA (>90% of total). The DGTS level shows a rhythmic fluctuation with time which is inversely correlated with the level of MGDG and it has thus been proposed that EPA in galactolipids might originate from EPA in extraplastidial DGTS [80]. In the case of the red alga *P. purpureum*, pulse-chase labeling with radioactive fatty acid precursors showed incorporation into EPA-rich PC and TAG and, during the chase, a decrease of the labeling of these lipids and an increase of that of MGDG. It was thus concluded that TAG could supply EPA precursors for the biosynthesis of MGDG [81].

Could ω3/ω6-VL-PUFAs found in galactolipids be imported via a similar mechanism as the C18/C18 eukaryotic system? Is there a specific import of free fatty acids for ARA, EPA of DHA? Alternatively, is there a transfer of a diacyl-moiety from extra-plastidial phospholipids to feed MGDG synthesis? This overall import system is called here the omega pathway. Is the TGD machinery involved in this route? To our knowledge, these questions are still unresolved.

3.1.4. Evolution of plastidial and extra-plastidial pathways

The evolution of the integrated prokaryotic/eukaryotic systems has not been investigated extensively in green and red algae. In particular, understanding of the evolution of the ω3/ω6-VL-PUFA import system requires more data. It will be extremely important to address this question in the future since the omega pathway for the synthesis of MGDG and DGDG has been conserved in secondary endosymbionts (see later).

In angiosperms, Mongrand et al. (1998) have provided the most comprehensive analysis, by searching for correlations between the existence of the prokaryotic and eukaryotic pathways and the evolutionary position of different organisms (Fig. 6) [54,84]. By using all-*cis*-$\Delta^{7,10,13}$ C16:3 as a marker for the existence of the plastidial pathway, they studied the overall fatty acid composition of 468 plant species distributed among 141 botanical families. The synthesis of galactoglycerolipids using prokaryotic precursors was found to have been lost during evolution and, in the case of dicots, this loss occurred independently in numerous groups and at different rates [54]. A trend can therefore be noted in the upstream pathway feeding galactolipids, characterized by an increasing dependence on imported precursors from the ER. The importance of this phenomenon has been established in angiosperms, but it remains to be investigated in algae, in particular concerning the analogous import of eukaryotic diacyl-substrates and the specific case of VL-PUFA precursor import.

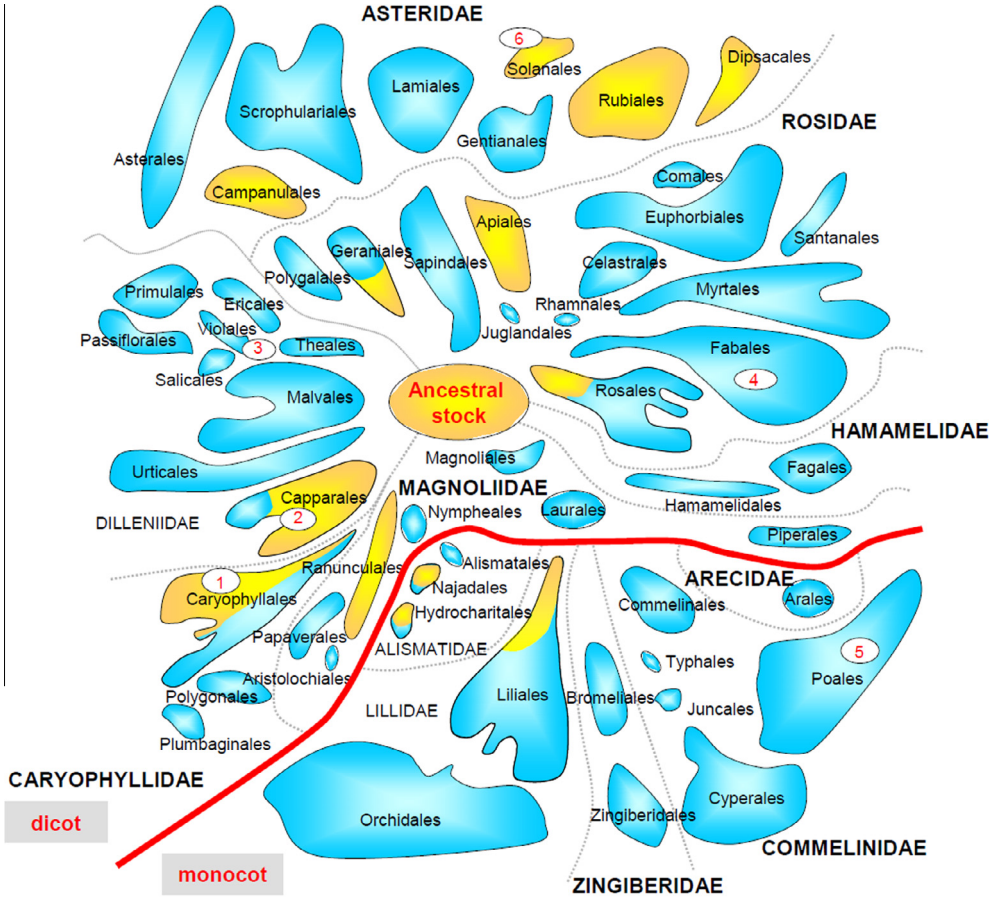
In conclusion, there has been an intense adaptation of the pathways feeding galactolipids with PA and DAG precursors, with a clear trend towards an import from the cytosol. Indeed, besides plastidial PG, whose synthesis seems to be highly dependent on the conservation of a prokaryotic production of PA [76], it seems that during the course of evolution MGDG and DGDG synthesis relies more and more on extraplastidial substrates. The "long" path for FAs, first synthesized in the stroma, then reaching the endoplasmic reticulum where they are incorporated into phosphoglycerolipids, and then imported back into the chloroplast, appears to be a costly system compared with the local production and utilization of FAs for chloroplast lipids *via* the prokaryotic route. The emergence of a specific import system might therefore provide benefits that could ensure a better fitness. One such benefit could be an improved integration of biosynthetic pathways in the ER and the chloroplast at the whole cell level [51,85]. This has been proposed in the context of the activation of MGDG synthases from angiosperms by extraplastidial PA [51,85]. This feature appears therefore to be important in order to understand the evolution of galactolipid synthesizing enzymes.

3.2. Evolution of galactoglycerolipid synthesis in primary endosymbionts

3.2.1. MGDs

As detailed above, in cyanobacteria MGDG is not synthesized by a homolog of MGDG synthase, but by a two-step process. This raises several questions, such as (i) the importance of conserving MGDG in photosynthetic membranes, (ii) the reasons for the loss of the MGlcDG synthase/epimerase system, and (iii) the origin of MGDG synthases in eukaryotes following the primary endosymbiosis. MGD enzymatic activities have been analyzed in great detail in Angiosperm models. MGD proteins belong to the GT28 family of glycosyltransferases classified in the CAZy database [69]. The presence of MGD orthologs in all primary endosymbionts analyzed to date, except Rhizaria, has been reported in numerous comparative phylogenies. In green and red algae, only one *MGD* gene is usually detected [64,86]. In the green alga *C. reinhardtii*, it has thus been annotated as *MGD1*, although no other paralog has yet been detected [87].

Molecular phylogenetic analysis of MGD sequences has been recently published by two teams [64,86] drawing similar conclusions on the origin of contemporary plant MGDs. Both were based on amino acid sequence alignments, maximum likelihood and Bayesian inference. The more thorough analysis [64] included four million iterations, rather than the classical 10–20 thousands to determine phylogenetic trees. These studies support the lateral transfer of a MGDG synthase ancestral gene from a chlorobacterium ancestor of the Chloroflexi type. Based on these phylogenetic reconstructions, the analysis of MGDs from Rhodophyta, Chlorophyta and Embryophyta show a monophyly and a single ancestor. The modern group of Chloroflexi comprises anoxygenic phototrophic bacteria, having diverged before cyanobacteria. The *in vitro* analysis of the substrate specificity and anomeric configuration of the sugar head group for the three prokaryotic genes closely related to eukaryotic *MGDs* in *Roseiflexus castenholzii* showed one member that encoded a MGDG synthase [64]. Other studies have explored the possibility that a Chlorobacculum type gene might have been transferred [88]. When and how this horizontal transfer of a *MGD* gene from a Chloroflexi ancestor to the nuclear genome of an early chloroplast-bearing eukaryote has occurred is unknown. A large-scale phylogenetic analysis has revealed that a considerable amount of non-cyanobacterial genomic material has been acquired prior to red and green lineage divergence [89]. The analysis of the genome of *C. paradoxa* has also highlighted important gene transfers from a *Chlamydia*-like ancestor, also supporting



1, *Spinacia*; 2, *Arabidopsis*; 3, *Cucumis*; 4, *Pisum*, *Medicago*, *Lotus*, *Glycine*; 5 *Zea*, *Hordeum*; 6, *Lycopersicon*.

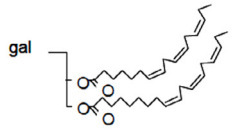
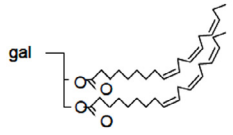
sn-1/*sn*-2	C16:3 plants	C18:3 plants
	C18:3/C16:3	-
	C18:3/C18:3	C18:3/C18:3

Fig. 6. Evolution of the plastidial (prokaryotic) and extra-plastidial (eukaryotic) pathways, generating substrates for galactoglycerolipids in Angiosperms. Two kinds of angiosperms have been distinguished based on their ω3-trienoic fatty acid composition in photosynthetic tissues. The *cis*-9,12,15-octadecatrienoic acid/*cis*-7,10,13-hexadecatrienoic acid (or C18:3/C16:3) balance reflects the pathways that produce diacyl-precursors for galactoglycerolipids: a plastidial or prokaryotic one and an extra-plastidial or eukaryotic one. Mongrand et al. [54,84] have analyzed the correlation between the existence of these pathways and the evolutionary classification of Angiosperms. By using *cis*-7,10,13-hexadecatrienoic acid as a marker for the existence of the plastidial pathway, they studied the fatty acid composition of 468 plant species distributed among 141 botanical families. The lower part illustrates the distribution of major MGDG molecular species in C16:3 and C18:3 plants. The upper part of the figure shows the extrapolated distribution of so called C16:3 plants (in yellow) and C18:3 plants (in blue) in the classification of angiosperms. The blue color thus reflects the loss of the prokaryotic pathway in galactoglycerolipids. Number allows localizing major plants analyzed in the literature, 1, spinach; 2, *Arabidopsis*; 3, cucumber; 4, pea, alfalfa, soybean; 5, corn, wheat; 6, tomato.

that the primary endosymbiosis with a cyanobacteria had been accompanied by some associations with other prokaryotes who provided substantial portions of genetic material [90]. In the absence of any trace of the cyanobacterial system in eukaryotes, we thus propose that acquisition of Chloroflexi-type *MGD* likely occurred very early during the primary endosymbiotic process.

In angiosperms, *MGD* genes have evolved into two main types, denoted A and B, that were first characterized in spinach [91] and *Arabidopsis* [92]. Type A is characterized by a cleavable N-terminal region (about 100 amino acids) allowing targeting to chloroplasts via the translocon protein import system. By contrast, Type B is characterized by a very short non-cleavable N-terminus. In the

case of *Arabidopsis*, type A has only one member, MGD1, whereas type B has two members, MGD2 and MGD3 [92]. Based on *in vitro* enzymatic studies, analyses of subcellular fractions of chloroplast membranes, and GFP-fusion localization by epifluorescence microscopy [91–96], MGD1 was shown to be the most abundant and most active enzyme, localized in the inner envelope membrane, and to be essential for the expansion of thylakoids. This subcellular localization is consistent with the presence of an N-terminal transit peptide. A knockout of *MGD1* [97] is lethal in the absence of an external source of carbon substrate [98]. MGD2 and MGD3 are in the outer envelope membrane and their expression is triggered in some stress conditions, such as a shortage of phosphate [92]. Simple and combined genetic disruptions of *MGD2* and *MGD3* genes have no striking phenotype in normal growth conditions [99], so these enzymes seem likely to act mostly in specific contexts like in response to phosphate starvation or in specific cell types such as elongating pollen tubes [100,101]. Type B seems therefore to be an invention of Angiosperms.

Based on the gene structures in *Arabidopsis*, *MGD2* lacks two introns (Fig. 7), suggesting that the ancestral sequences for *MGD1* and *MGD3* originated from an early divergence marked by a deep modification of the N-terminal region and that *MGD2* originated likely from the duplication of a *MGD3* ancestral sequence [92].

A comprehensive phylogenetic analysis has shown that Bryophytes had only one type of *MGD* and that the divergence between Type A and B preceded that of Gymnosperms and Angiosperms [64]. Based on phylogenetic tree calibrations, the A/B divergence was dated to around 320 million years ago, during the Carboniferous period, and so after the emergence of Spermatophyta. By comparing functional and phylogenetic studies, this recent evolution of *MGD* genes in Spermatophyta suggests an increased specialization of a novel type (Type B), at the periphery of chloroplasts, to respond to environmental changes (e.g., nutrient shortages) in the context of an intense membrane lipid remodeling within plant cells.

3.2.2. Cyanobacterial-type dgdA in Cyanidiales, a subdivision of Rhodophyta

By contrast with the synthesis of MGDG, two systems exist in eukaryotes to synthesize DGDG. A small number of sequenced red algae contain, in their plastid genome, orthologs of the cyanobacterial *dgdA* gene. The presence of *dgdA* is thus clear in the

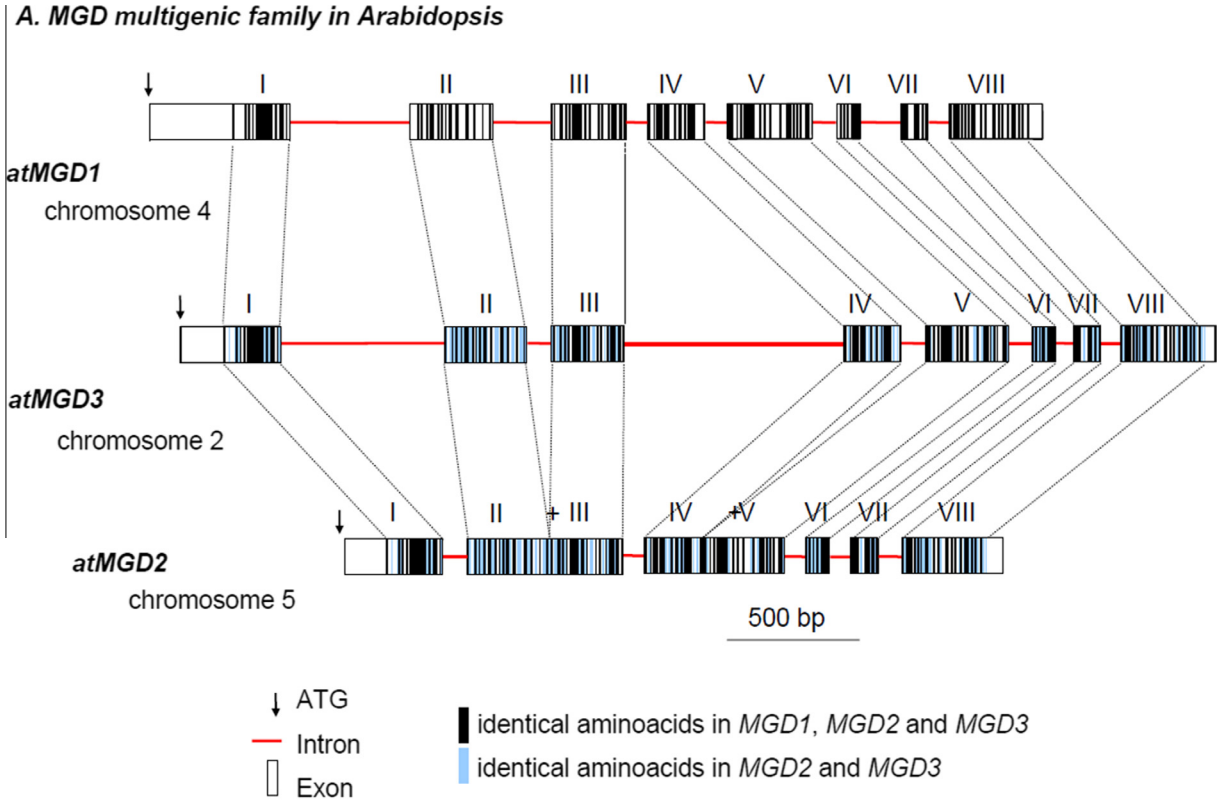


Fig. 7. Type A and Type B *MGD* genes. (A) Genomic organization of *MGD1*, *MGD2* and *MGD3* in *Arabidopsis*. Introns are presented as lines and exons as rectangles. Exons were compared for identical translated amino acids. Black rectangles, identical amino acids among the 3 proteins; blue rectangles, identical amino acids between MGD2 and MGD3 (not observed in MGD1). MGD1 has a longer N-terminal domain corresponding to a chloroplast transit peptide. (B) Model of evolutionary divergence leading to the two types of MGD proteins.

genomes of *C. merolae*, *C. caldarium* and *G. sulphuraria* [41,42]. Interestingly, *C. merolae* contains a typically eukaryotic MGD enzyme that was confirmed to have a MGDG synthase activity by expression in *E. coli* [41]. Cyanidiales contain species living in very acidic environments, which might have been isolated early after the primary endosymbiosis. The conservation of the *dgdA* gene in Cyanidiales supports an early divergence of this group amongst red algae and provides a clue about the loss of cyanobacterial galactolipid biosynthesis genes.

3.2.3. DGDs in all other primary endosymbionts (Rhodophyta, Glaucophyta, Chlorophyta and Plants)

Based on previous reports and on systematic surveys of genomic databases, no *dgdA* ortholog can be detected in the plastid genomes of multicellular red algae, including in *Gracilaria* or *Porphyra*. No *dgdA* gene could be found in the glaucophyta *Cyanophora*, the green alga *Chlamydomonas*, the moss *Physcomitrella*, nor in higher plants [41,87]. By contrast, orthologs of the *DGD* genes identified in Angiosperms [39] have been unambiguously identified in Glaucophyta, Chlorophyta and most Rhodophyta (except Cyanidiales, discussed above) [41,87]. DGD genes have also been found in the genome of the Bryophyte *Physcomitrella patens* [102].

In Angiosperms, two types of *DGD* genes could be identified based on primary sequences. One type, known as *DGD1* in *Arabidopsis*, is a two-domain protein with an N-terminal segment of unknown function, and a C-terminal galactosyltransferase domain [103,104]. The second type, known as DGD2 in *Arabidopsis*, corresponds to a shorter amino acid sequence, consisting only of a galactosyltransferase domain. The DGD1 and DGD2 galactosyltransferase domains belong to the GT4 family defined in the CAZy classification [69].

The expression patterns of *MGD1*, *MGD2*, *MGD3*, *DGD1* and *DGD2* in different tissues of *Arabidopsis* or in response to some stresses, such as phosphate shortage, indicate that MGDs and DGDs act as specific pairs [34,92,100,104–107]. On the one hand, *DGD1* and *MGD1* are expressed in green tissues and the corresponding enzymes act together in the biogenesis of thylakoid lipids. On the other hand, *DGD2* is expressed together with *MGD2* and/or *MGD3* and the corresponding enzymes act together to produce galactolipids in non-green tissues (including in roots and flower organs) and in response to environmental stress. Because MGD1 and DGD1 are localized in different membranes, the inner and outer envelope membranes, respectively, a specific transfer of MGDG from one membrane to the other is necessary. Furthermore, because MGD2/MGD3 and DGD2 are found in the outer envelope membrane, a metabolic channeling is supposedly made easier thanks to the co-localization of enzymes. Since Type B MGDs are unique to Spermatophyta, the emergence of this system might be optimal to respond to signaling processes occurring in the cytosol and at the periphery of the chloroplast.

The DAG → MGDG → DGDG chanelling via the MGD1/DGD1 or the MGD2;MGD3/DGD2 system does not mean that all MGDG is used as substrates for DGDG. Only a fraction of MGDG, that with a eukaryotic structure, is converted into DGDG by the action of DGDG synthase [37]. In angiosperms, the fatty acid compositions of MGDG and DGDG are therefore different, with MGDG being 16:3-rich and DGDG being 16:3-poor. This observation suggests that (i) a desaturase (FAD5) catalyzes the very rapid desaturation of 16:0 into 16:1 at the *sn*-2 position of MGDG, and that (ii) 16:1, 16:2 and 16:3-MGDG species are not utilized by DGD1 and DGD2. This being said, one would expect that a correlation of the evolution of DGDs might have occurred, linking the evolution of MGD1 with DGD1, MGD2/MGD3 with DGD2 and possibly MGD1/DGD1 with FAD5. These hypothetical co-evolutionary processes have not been explored yet.

In the genome of the Bryophyte *P. patens*, the four orthologous sequences (Phypadraft_162919, Phytadraft_137342, Phytadraft_216055 and Phytadraft _218058) have longer N-terminal domains, like the angiosperm DGD1. Similarly, sequences in Chlorophyta, such as the unique DGD protein in *C. reinhardtii* (XP_001693597; Cre13.g583600), harbor longer N-terminal extensions like DGD1. By contrast, some sequences in Rhodophyta, like the unique DGD protein in *Chondrus crispus*, consist only of the GT4 domain, like the angiosperm DGD2. It seems therefore that the evolution of DGD genes is not simple. The conservation level of the GT4 domain seems furthermore to be higher than that of the N-terminal domain. The emergence of DGD2 in Spermatophyta might therefore correlate with the emergence of MGDs of Type B, but this hypothesis still needs confirmation.

4. From primary plastids to secondary plastids

4.1. The puzzling question of the lipidome of secondary plastids

Although the presence of MGDG and DGDG was confirmed decades ago in a range of important phyla including secondary endosymbionts deriving from green algae, e.g., *E. gracilis* [24], or Chromalveolata deriving from red algae, e.g., the non-photosynthetic diatom *Nitzschia alba* [108], the photosynthetic diatom *P. tricornutum* [25] and the eustigmatophyte *Nannochloropsis* [109,110], we still lack clear information about (i) the precise localization of galactoglycerolipids in the three to four membranes that delineate the secondary chloroplasts and (ii) the subcellular mapping of the biosynthetic enzymes. While it is common for secondary endosymbionts to have an intricate association of their chloroplasts and endoplasmic reticulum (often referred to as Chloroplast-ER) [111,112] various representatives of Chromalveolata such as *Ochromonas danica* and *P. tricornutum* also display continuity between the Chloroplast-ER and the nuclear membrane [111]. Other notable examples include Apicomplexans, which are characterized by the conservation of a reduced non-photosynthetic relict plastid, known as the apicoplast [113,114]. Apicomplexa is a phylum of unicellular eukaryotes that mainly comprises obligates intracellular parasites, responsible of major human diseases such as malaria and toxoplasmosis, respectively cause by *Plasmodium* spp. and *T. gondii*. To the exception of *Cryptosporidium* spp. and possibly free living gregarins, all Apicomplexa possess an apicoplast. Very recent analyses have provided evidence that neither MGDG nor DGDG were present in the apicoplast [115], indicating a rare but puzzling loss of the galactoglycerolipid biosynthetic pathway. Following secondary endosymbiosis, many genes were transferred from the endosymbiont to the host cell nucleus, which means that protein targeting mechanisms must have adapted accordingly to allow transport across the newly acquired membrane structure [116,117]. Here, we discuss how the metabolism of galactoglycerolipids has evolved following each of the secondary endosymbiotic events illustrated in Fig. 2.

Based on the variety of evolutionary scenarios, one possibility is that each lineage has had an independent evolution. The metabolic pathways in secondary endosymbionts combine metabolic routes inherited from the ancient cyanobacteria at the origin of chloroplasts, the primary endosymbiont genome, the secondary endosymbiont genome and multiple other organisms via horizontal transfers. Thus, in this part of the review, we do not try to define a unique scheme but rather address questions that in our opinion should be prioritized in future research.

4.2. Multiple possible systems to synthesize fatty acids de novo in the stroma and the cytosol

In most secondary endosymbionts studied so far, the neosynthesis of fatty acids occurs mainly via a FASII system likely to have

been inherited from the cyanobacterial progenitor of the plastid. In some species, e.g., *Nannochloropsis* and *Toxoplasma*, a cytosolic FASI also exists. In the case of a co-occurrence of both FASII and FASI, the most extensive biochemical studies have been performed in *T. gondii*, with apparently contradicting results [118,119] and in a galactolipid-free context. Current consensus is that the apicoplast pathway essential in Apicomplexa parasites as based on molecular disruption of the pathway. However, this essentiality differs depending on the parasite: whilst it is always essential for *T. gondii* [119], it appears to be only essential during *Plasmodium* asymptomatic liver stages or mosquito sporozoite division stages [120–122]. Initial biochemical analyses have suggested that the *T. gondii* FASII was not essential for bulk lipid synthesis but rather for the apicoplast biogenesis and maintenance [118,119,123], possibly due to a chloroplast-analogous necessity for the synthesis of these lipids to be utilized during the growth and division of this organelle. However, more refined metabolic labeling experiments showed that the *T. gondii* FASII was responsible for the majority of the long chain saturated fatty acids [123]. Lipid analyses of the apicoplast of *P. falciparum* confirmed that this organelle was highly enriched in saturated fatty acids (~90% of all fatty acids), mainly in C18:0 [115]. The C18:0-ACP desaturation activity might have been also lost in this organelle. Furthermore, the apicoplast seem to be able to utilize FA to generate PA by stepwise acylations of a G3-P backbone via a glycerol 3-phosphate acyltransferase (ATS1) and a lyso-phosphatidic acid (LPA) acyltransferase (ATS2/LPAAT), both predicted to be present in the apicoplast [51,121,124]. Recently, the disruption of the rodent malaria *P. yoelii* ATS1 gene led to the impairement of the biogenesis of the parasite intracellular compartments, including the apicoplast, during liver stages [125]. Together, these results suggest that the apicoplast might be involved in bulk lipid neosynthesis in Apicomplexa via the generation of FA, LPA and/or PA. The role of this prokaryotic pathway in the absence of galactoglycerolipids remains to be fully understood. The respective roles of FASII in the apicoplast and of FASI and fatty acid elongase in the cytosol of *T. gondii* has been recently analyzed in the context of a parasitic life style of this Apicomplexa [123,126].

The combination of functionally active plastid FASII and cytosolic FASI has been known in *E. gracilis* since the early 1970s [127], indicating that this dual FASII/FASI system exists in photosynthetic secondary endosymbionts of the green lineage. In the red lineage, recent efforts to sequence the genomes of different *Nannochloropsis* species (e.g., *N. oceanica* [128]) have highlighted that, in addition to a plastid FASII, a cytosolic FASI-like protein also exists (e.g., CCMP1779 in *N. oceanica*). FASI enzymes are similar to animal FAS, but also close to polyketide synthases and it is not always straightforward to infer their function from primary structure [129]. To our knowledge, the activity harbored by the *Nannochloropsis* FASI-like enzyme has not yet been fully characterized. It has been speculated that FASI in *Nannochloropsis* catalyzes the production of shorter fatty acids (C14:0) as proposed in *Schizochytrium* [130], a heterotrophic species belonging to the Labyrinthulomycetes, phylogenetically related to the Eustigmatophytes within the Heterokonts.

The origin of the fatty acids used as precursors for glycerolipids can therefore be far more complicated in secondary endosymbionts compared to primary ones. The fate of fatty acids, from their neosynthesis to their incorporation into galactoglycerolipids, is therefore obscure in most of the species that have so far been analyzed. It is difficult to address the question of the import of eukaryotic diacyl-precursors, synthesized outside secondary plastids, for the following reasons: (i) the simple C18/C18 or C18/C16 definition initially stated might not hold true with the set of acyl transferases acting in a given species, and (ii) the presence of 4 membranes around secondary chloroplasts makes the presence of an import system that would be homologous to that in the envelope of primary chloroplasts unlikely. It is therefore very difficult, based solely on the diacyl signature in glycerolipids, to infer the presence or absence of such a pathway. Metabolic labeling should thus be performed in each species of interest to assess the precise origin of precursors for plastid galactoglycerolipids. Nevertheless, regardless of this C16 and C18-based definition of eukaryotic molecular species, the large number of species accumulating ω3/ω6-VL-PUFAs in galactoglycerolipids, with C20 to C22 major molecular species, is striking [27,131,132], indicating that at least the omega pathway has been conserved.

4.3. The conservation of the omega pathway

In numerous secondary endosymbionts, the presence of ω3/ω6-VL-PUFAs (ARA, EPA, DHA) has been observed in galactoglycerolipids in both the green and the red lineages [27]. The highest level of EPA in MGDG and DGDG has been recently reported in the Chromerid *C. velia*, a close photosynthetic relative of Apicomplexa, with 70–80% EPA in MGDG and DGDG [86]. Based on genomic surveys, the synthesis of ω3/ω6-VL-PUFAs seems always to be catalyzed by cytosolic enzymes. The corresponding enzymes have been predicted in the sequenced genomes from the diatoms *T. pseudonana* [133] and *P. tricornutum* [134], and from the eustigmatophyte *Nannochloropsis* sp., e.g., *N. oceanica* [128]. Some of these enzymes have been functionally characterized, in particular in *P. tricornutum* and *T. pseudonana* [135–138]. In the Eustigmatophyte *Monodus subterraneus*, metabolic labeling experiments were performed using radiolabelled acetate or linoleic (C18:2) acid [139]. PC was mostly involved in the desaturation of C18 acyls, whereas PE and DGTS were substrates for further desaturations of C20 acyls, resulting in the accumulation of C20:5 (EPA) in these protists [139]. Metabolic labeling results were consistent with PE and DGTS providing EPA, either as free C20:5 or as C20:5/C20:5-DAG for the synthesis of MGDG in the chloroplast [139]. The route from the cytosolic production of ω3/ω6-VL-PUFAs to their plastidial incorportation in galactoglycerolipids has been further assessed experimentally in *Phaeodactylum* [137] and *Nannochloropsis* [128]. The presence of an omega pathway, supplying the plastid with ω3/ω6-VL-PUFAs for galactoglycerolipids therefore seems to be one of the main features that has been conserved in secondary endosymbionts. As for the eukaryotic pathway in Angiosperms, it appears to have become the major provider of fatty acids for MGDG and DGDG in several species, whereas the prokaryotic route has assumed a minor role. This trend might be related to a better integration of plastid lipid synthases with extra-plastidial biosynthetic pathways. It might also highlight the importance of ω3/ω6-VL-PUFA specific functions.

4.4. Mapping galactolipid-synthesizing enzymes in the multiple membranes that delimit secondary plastids

Available genomic data indicate that secondary endosymbionts contain genes encoding eukaryotic MGDs and DGDs. In our surveys, we did not detect any orthologs of genes encoding cyanobacterial MGlcDG synthase or *dgdA*.

The question of the localization of MGD and DGD within the three to four membranes limiting secondary plastids has not been addressed experimentally, due to the lack of an accurate fractionation method for these organelles. The only secondary plastid that has been isolated to date is the apicoplast of *Plasmodium* [115], which does not contain any galactoglycerolipids (see below). The only secondary plastid in which galactoglycerolipids have been localized using anti-DGDG antibodies is from *Chromera* [86].

If we look more closely at the *P. tricornutum* example, we can predict three *MGD* genes. One of these (Phatr_14125) encodes a

protein harboring an unambiguous bipartite targeting sequence (signal peptide + chloroplast transit peptide) [140] consistent with its likely localization in the innermost membrane of the plastid. A second gene (Phatr_54168) encodes a protein with an N-terminal segment that shares some features of a bipartite targeting sequence. The third gene (Phatr_9619) encodes a protein whose subcellular localization cannot be predicted using available bioinformatic tools. These MGDs might be localized in different plastid membranes or possibly outside. The *P. tricornutum* genome is also predicted to contain three *DGD* genes, all having a longer N-terminal sequence than the angiosperm *DGD2*. One gene (Phatr_12884) encodes a protein with a bipartite targeting sequence, supporting a localization in the innermost membrane of the chloroplast. The two other genes (Phatr_11390 and Phatr_43116) have no known targeting sequences. They might be located in more peripheral membranes or even outside the plastid. Like in angiosperms, a Phatr_14125 (MGD1)/Phatr_12884 (DGD1) system might operate in the innermost membrane for the synthesis of thylakoids, whereas other combinations of enzymes might act in other locations for the expansion of specific membranes or to respond to some physiological or environmental contexts. The precise localization of these enzymes is therefore an important objective for future efforts, as are attempts to discover novel types of diatom targeting sequences [141].

4.5. Origin of MGDs and DGDs in Chromalveolates

The nuclear genomes of Chomalveolata contain genes deriving from the different actors of the secondary endosymbiosis, including the primary eukaryotic red alga and the secondary eukaryotic host. As mentioned above, a phylogenomic analysis of the diatom proteome using complete genome data from *Thalassiosira* and *Phaeodactylum* has allowed the identification of about 2500 genes likely derived from eukaryotic algae [48], with more than 70% of these genes being of green rather than red lineage provenance. These green genes seem to derive from an ancestor organism closely related to present-day Prasinophyta, e.g., *Micromonas* and *Ostreococus*. The green gene contribution constitutes ~16% of the diatom proteome and is also found in various Chromalveolata, including Apicomplexa and Haptophyta [48]. The occurrence of green genes together with genes inherited from the red algal endosymbiont seems to predate the split of Cryptophyta and Haptophyta from other Chromalveolata [48]. These conclusions have been recently reevaluated and the detection of green genes might have been overestimated due to a taxonomic sampling bias [142]. The detection of horizontal transfers of genes deriving from other actors than those strictly involved in the secondary endosymbiosis is reminiscent to the vast proportion of bacterial genes also found in primary endosymbiosis. In primary endosymbionts, *MGD* genes were thus shown to derive from Chloroflexi bacteria and the source of *DGD* genes is unknown.

A similar question can be raised about the red or green origin of *MGD* and *DGD* genes in Chromalveolata. In published phylogenetic analyses, MGD sequences from *Phaeodactylum*, *Thalassiosira* and *Aureococcus* are more closely related to the sequence from the Rhodophyta *Cyanidioschyzon*, than from *Ostreococcus* and *Micromonas*, supporting a red algal origin. Nevertheless, since we could not reproduce the bootstrap values of this study by reiterating the phylogenetic reconstruction, this origin should be re-evaluated. A refined analysis of the DGD sequences should also be undergone to answer this question.

4.6. Loss of galactolipids in the secondary plastid of Apicomplexa

The apicoplast in Apicomplexa is a very specific case amongst secondary plastids, as it contains the smallest known plastid circular DNA, with the most reduced genome [7,113,143–145]. This organelle has lost its photosynthetic capacity and no photosynthetic genes have been detected. Significant efforts have focused on the search for galactoglycerolipids by metabolic labeling with radioactive precursors [146,147], immunostaining strategies [148] or whole parasite lipidomic analyses [148,149].

The search for *MGD* or *DGD* orthologs in Apicomplexa has also been inconclusive, although both *P. falciparum* and *T. gondii* genomes are now available [150,151]. The genome of *P. falciparum* is particularly difficult to mine, due to a strong nucleotidic compositional bias [150,152,153]. Approximately half of the genes have been functionally annotated, due to improved bioinformatics tools [154]. Using the CAZy classification [69], no glycosyltransferase of the GT28 family and only one of the GT4 family, unrelated to DGD, could be identified in *Plasmodium*. In spite of the lack of these enzymes, it could not be excluded that galactoglycerolipids might be synthesized via the action of a non-homologous set of glycosyltransferases in Apicomplexa.

Other groups of secondary endosymbionts also contain non-photosynthetic species. For instance, the non-photosynthetic diatom *N. alba* was shown to contain galactoglycerolipids [108]. Additional evidence for loss of galactoglycerolipids in the apicoplast was therefore required. Very recently, purification of the apicoplast from *P. falciparum* has been achieved [115]. This is actually the first report of the isolation of a secondary plastid. The method, based on immunopurification with magnetic beads, yielded sufficient amounts of purified organelle to perform an in-depth lipidomic analysis. No galactoglycerolipid could be detected, providing the first evidence for the absence of this landmark lipid of green and non-green plastids. Other lipids, such as sphingomyelin, ceramides and cholesterol, were detected and, along with most apicoplast structural lipids, were proposed to be generated and imported from the endomembranes, at least during this life stage [115].As mentioned above, *C. velia*, a close relative of Apicomplexa, has large amounts of MGDG and DGDG [86] and its genome contains orthologs of *MGD* and *DGD* genes [86]. Bioinformatic predictions were confirmed by biochemical measurements and metabolic labeling [86]. Chromerida like *C. velia* and *Vitrella brassicaformis* [155] share a common photosynthetic ancestor with Apicomplexa. Comparative studies might therefore help us understand the evolutionary history of the galactoglycerolipid pathway in Apicomplexa in the context of the disappearance of photosynthesis combined with the dramatic simplification of the parasite's lipid metabolism.

5. Conclusion and perspectives

In conclusion, the evolution of galactoglycerolipid metabolism is marked by important transitions. In cyanobacteria, all species rely on a stepwise synthesis via a glucosyl intermediate, catalyzed by a MGlcDG synthase, incorporating glucose from UDP-Glc, an unknown epimerase converting glucose into galactose, and a dgdA-type DGDG synthase. As an exception to this rule, some cyanobacteria like *Gloeobacter* sp. have acquired a bacterial MGD enzyme by a recent horizontal transfer, enabling them to synthesize MGDG by direct incorporation of galactose.

In the conversion of a cyanobacterial endosymbiont into the primary plastid, the MGlcDG synthase/epimerase system was rapidly lost, and it can no longer be found in any present-day primary endosymbiont. On the other hand, a *MGD* gene from a Chloroflexi-like ancestor has been acquired by horizontal transfer. In some red algae living in acidic environments, the Cyanidiales, a DgdA-type DGDG synthase of cyanobacterial origin has been conserved, whereas in all other eukaryotes the synthesis of this lipid relies on *DGD* genes of unknown origin. The emergence of eukaryotic MGD and DGD enzymes is therefore not synchronous in all

phylogenetic groups. MGD and DGD are thus likely to have evolved by duplication and intense reorganization of N-terminal domains. Type A and B MGDG synthases have emerged in Spermatophyta, with Type A being dedicated to the production of lipids for thylakoids, and Type B operating mostly in non-green tissues and in response to some environmental stresses such as phosphate limitation. Pairs of MGD and DGD isoforms seem to act together, like MGD1/DGD1 and (MGD2;MGD3)/DGD2 in *Arabidopsis*. The evolution of primary endosymbionts seems to reflect an increasing level of integration with the lipid metabolism of the whole cell. In particular precursors for the synthesis of galactoglycerolipids seem to be more and more dependent on import from the cytosol, via the so-called eukaryotic pathway and/or the omega pathway.

With respect to the conversion from primary endosymbionts to secondary plastids, many important questions remain to be elucidated, in particular regarding the subcellular topology of MGD and DGD enzymes in the three to four membranes that bound secondary chloroplasts. Because secondary endosymbionts are not monophyletic, multiple scenarios can be envisaged. MGD and DGD genes seem to be conserved in all cases except Apicomplexa. Based on published phylogenetic studies, Chromalveolata *MGD* genes seem to derive from the red algal endosymbiont rather than from a cryptic Prasinophyta algal source. The origin of *DGD* genes in Chromalveolata remains to be established. The omega pathway feeding galactoglycerolipids with extraplastidial precursors has been conserved in numerous species. The machinery importing ω3/ω6-VL-PUFAs in the secondary chloroplast and the benefits for their high proportion of galactoglycerolipids are still unknown, and MGDG and DGDG have been lost in the apicoplast. How a plastid can be maintained without galactoglycerolipids is a major question for future research, and will require both the evolution of the upstream plastidial biosynthesis of PA and the import of all other membrane components to be addressed. There is no doubt that the analysis of galactoglycerolipid pathways in complex plastids will be a fascinating subject of research in the next decade.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plipres.2014.02.001.

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