Opinion

Plastids with or without galactoglycerolipids

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In structural, functional, and evolutionary terms, galactoglycerolipids are signature lipids of chloroplasts. Their presence in nongreen plastids has been demonstrated in angiosperms and diatoms. Thus, galactoglycerolipids are considered as a landmark of green and nongreen plastids, deriving from either a primary or secondary endosymbiosis. The discovery of a plastid in *Plasmodium falciparum*, the causative agent of malaria, fueled the search for galactoglycerolipids as possible targets for treatments. However, recent data have provided evidence that the *Plasmodium* plastid does not contain any galactoglycerolipids. In this opinion article, we discuss questions raised by the loss of galactoglycerolipids during evolution: how have galactoglycerolipids been lost? How does the *Plasmodium* plastid maintain four membranes without these lipids? What are the main constituents instead of galactoglycerolipids?

What are galactoglycerolipids?

All photosynthetic membranes (thylakoids) analyzed to date from cyanobacteria to chloroplasts of land plants are characterized by a low proportion of phospholipids, mainly phosphatidylglycerol (PG), and high levels of three nonphosphated glycoglycerolipids [i.e., the negatively charged sulfoquinovosyldiacylglycerol (SQDG) and the neutral mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively)] [1,2] (Figure 1). These galactoglycerolipids constitute up to 80% of thylakoid lipids [3]. Therefore, based on the natural abundance of photosynthetic organisms, galactoglycerolipids constitute the most profuse lipid class on Earth [4].

Galactoglycerolipid function

Galactoglycerolipids are not only building blocks for membranes. Structural studies have shown specific interactions with core functional systems for growth and development, such as subunits of photosystems [5] and the chloroplast import machinery [6,7]. Based on crystallographic analyses and characterization of lipids from purified protein–lipid complexes [5,8], galactoglycerolipid interactions are more important at the level of components of photosystem II (PSII) [5]. Genetic studies in the cyanobacterial model, *Synechocystis*, and the angiosperm model, *Arabidopsis* (*Arabidopsis thaliana*), enabled the functional characterization of mutants deficient in MGDG or DGDG content. *Arabidopsis* containing less MGDG was obtained by knocking down or knocking out (KO) *MGD1*, one of the three genes encoding MGDG synthases [9–12]. Levels of MGDG could also be altered by chemical treatment with galvestine-1, a specific inhibitor of MGDG synthases [13,14]. The *mgd1-1* mutant exhibited a severe growth phenotype and a disrupted chloroplast biogenesis [9], whereas the *mgd1-2* KO was unable to grow unless supplemented with sucrose. *Arabidopsis* treated with galvestine-1 showed a similar phenotype [13,14]. Thus, MGDG content is directly linked to thylakoid development, as would be expected for such an abundant lipid. Functional analyses of PSII in the *mgd1-1* mutant suggested that the remaining MGDG (40% of wild type level) is sufficient to maintain the function of this complex in normal light exposure [15]. Nevertheless, the *mgd1-1* mutant suffered from increased PSII photoinhibition, an inefficient thermal dissipation of excess light energy during short-term high-light stress [16,17].

Concerning DGDG, *Synechocystis* mutants were obtained by knocking out the *dgdA* gene (the cyanobacterial DGDG synthase gene) [18–20]. Growth of *dgdA* mutants was not affected in low-light conditions, and neither was photosynthesis. Under high-light or high-temperature conditions, DGDG was required to shape the PSII structure through the binding of extrinsic proteins [18,19]. *Arabidopsis* mutants affected in their DGDG composition were obtained by disruption of *DGD1* and *DGD2* [21–23]. The *dgd1* mutant was pale green, contained structurally altered chloroplasts and exhibited a decreased PSII:PSI ratio [21]. Stoichiometry of pigments and pigment-binding apoproteins was also impaired and an increased number of peripheral light-harvesting complex II subunits, relative to the inner antenna and PSII core complexes, was detected [22]. The *dgd1* × *dgd2* mutant contained traces of DGDG, produced by an alternative pathway (a galactolipid:galactolipid galactosyltransferase producing DGDG with a different sugar anomery) and had a more severe phenotype [23]. Functional analyses of photosynthesis in *mgd1-1*, *dgd1*, and *dgd1* × *dgd2* mutants were consistent with

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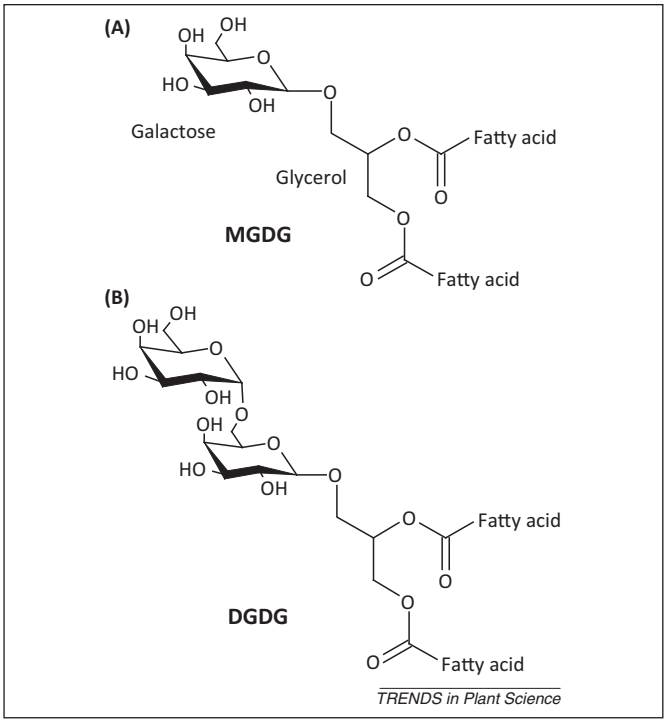

Figure 1. Plastid galactoglycerolipids. The structure of **(A)** monogalactosyldiacylglycerol (MGDG) and **(B)** digalactosyldiacylglycerol (DGDG) is based on a glycerol backbone, esterified by two fatty acids and harboring a polar head containing one or two galactosyl residues. Fatty acids differ in carbon chain length and desaturation levels depending on the species, the physiological and developmental statuses, and their position on the glycerol backbone. The first galactose residue is bound by a β-glycosidic bond to diacylglycerol, whereas the second is linked by a (1→6) α-glycosidic bond. MGDG is synthesized by the action of MGDG synthases (three enzymes in *Arabidopsis*, called MGD1, MGD2, and MGD3). DGDG is synthesized by the action of DGDG synthases (two enzymes in *Arabidopsis*, DGD1 and DGD2). These two lipids have been identified in all photosynthetic membranes analyzed to date.

the binding of the small fraction of DGDG molecules left in the thylakoid membranes to PSII [15]. Altogether, mutant analyses showed a critical role of DGDG in the structure and function of PSII from cyanobacteria to angiosperms. Therefore, in structural, functional, and evolutionary terms, galactoglycerolipids are considered signature lipids in all photosynthetic plastids [24].

Could galactoglycerolipids be absent in nongreen plastids?

This question has been addressed in plastids found in nonphotosynthetic organs and tissues of angiosperms or when plants are grown in the dark. Nongreen plastids include proplastids (in meristems) [25], etioplasts (in etiolated tissues) [26], amyloplasts (in storage tissues, filled with starch) [27], and more exotic forms, globally called leucoplasts, including elaioplasts (in some floral parts, with platoglobules containing sterols) [28] (Figure 2). The chromoplasts (filled with carotenoid-rich plastoglobules) [29] can contain thylakoids and are here considered to be 'green'. All nongreen plastids analyzed to date in vascular plants contained high levels of MGDG and DGDG [26]. The persistence of a galactoglycerolipid-rich envelope during the interconversion of plastids (Figure 2) is considered as one of the features that characterize the uniqueness of plastids in their diversity. Thus, they are considered landmarks of all types of plastid, green or nongreen.

The presence of galactoglycerolipids in nongreen plastids indicates a physiological role that is not directly linked to photosynthesis. On the one hand, when plants are subjected to phosphate (Pi) deprivation, galactoglycerolipids synthesis increases [30] and DGDG is exported to various extraplastidial membranes [31–33], where it can substitute phospholipids [1,31,34,35]. This phenomenon has been observed in roots (containing nongreen plastids) and green tissues (containing chloroplasts). Thus, in response to some environmental changes, galactoglycerolipids become critical for the biogenesis of nonplastidial membranes. On the other hand, analysis of pollen treated with galvestine-1 also highlighted a novel role of galactoglycerolipid for pollen tube elongation [36]. Based on genetic analyses, galactoglycerolipids also have a role in embryogenesis and development, although the underlying processes have not yet been fully elucidated [10,37].

Based on the presence of galactoglycerolipids in all plastids studied so far, their occurrence could be hypothesized in the plastid discovered two decades ago in the cells of *Plasmodium falciparum*, the causative agent of malaria [38,39]. Have galactoglycerolipids been conserved or lost in this parasitic organism? If lost, how would the *Plasmodium* plastid maintain four membranes without this class of lipid? What would be the main constituents instead of galactoglycerolipids? What would a loss of this class of lipid in the Apicomplexa phylum inform on the role of galactoglycerolipids in eukaryotes?

Evolution of galactoglycerolipid biosynthesis

The biosynthesis of MGDG, DGDG, SQDG, and PG, before their incorporation into photosynthetic membranes, occurs in the membranes that delineate cyanobacteria or chloroplasts. A chloroplast bound by two membranes is the most basic structure for this category of organelle, which is well known from algae to plants (the Archaeplastida kingdom). Such plastids are called 'primary plastids' and the two limiting membranes are known as the 'plastid envelope' [2]. Molecular evidence reveals that primary plastids originated from a single event of endosymbiosis (Figure 3) [40]. The envelope derives from the two limiting membranes of the cyanobacterial ancestor [40].

Three lineages have evolved from this initial endosymbiosis. The green lineage, in which chlorophyll *a* and *b* are associated, includes green algae (Chlorophyta) and plants (Charophyta and Embryophyta); the red lineage, where chlorophyll *a* is coupled to phycobilin, comprises the red algae (Rhodophyta); and the blue lineage, where chlorophyll *a* is associated with phycocyanin and allophycocyanin, is a small group (Glaucocystophytes), in which chloroplasts still contain a peptidoglycan cell wall [40].

The localization of galactoglycerolipid synthetic enzymes in primary plastids has been best characterized in angiosperms, using appropriate models for membrane fractionation, that is, pea (*Pisum sativum*) [41], cucumber (*Cucumis sativus*) [42], and spinach (*Spinacia olearacea*) [43]. The localization in *Arabidopsis* has benefitted from the improvement of methods to purify chloroplast envelope membranes [44], advances in proteomic techniques [45],

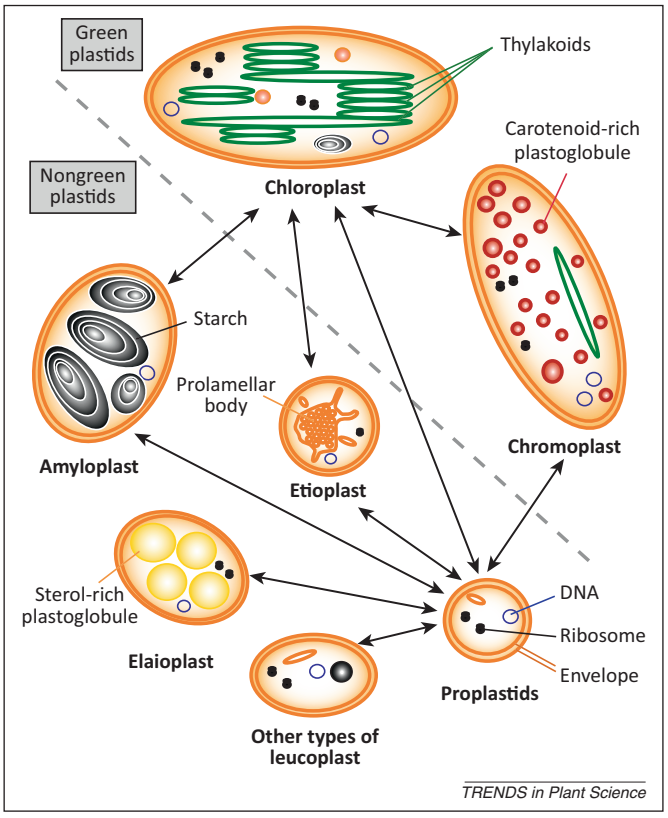

Figure 2. Plastid interconversion in angiosperms. In the zygote and meristematic cells, only embryonic plastids or proplastids are present. Proplastids can also occur in dedifferentiated cells and contain a circular DNA that is transmitted to all differentiated plastids, depending on the cell fate in the developmental program (such as the differentiation of chloroplasts in green tissues, chromoplasts in some organs such as tomato fruits, amyloplasts in some storage organs such as potato tubers, or elaioplasts in some floral parts, etc.) or on changes occurring in response to environmental changes (such as etioplasts in dark conditions). All green and nongreen plastids contain the relevant DNA and gene expression machinery (including ribosomes, tRNA, transcription system, etc.) and, thus, are considered to be semiautonomous organelles, with the ability of interconversion (reversible arrows). The differentiated function of the plastids can be dependent on the presence of plastoglobules with specific compositions. The surrounding envelope is the unique membrane structure conserved in all plastids.

and in-depth functional characterizations of mutants (reviewed in [46]).

In *Arabidopsis*, three MGDG synthase isoforms (EC 2.4.1.46; i.e., MGD1, MGD2, and MGD3 [47]), catalyze the transfer of a galactose from a UDP-galactose donor, to a diacylglycerol acceptor. Based on sequence similarities, MGDG synthases belong to the GT28 family of glycosyltransferases, following the carbohydrate-active enzymes (CAZy database) classification [48]. MGD1 is localized in the inner envelope membrane (IEM), is abundant in green tissues and is critical for the synthesis of lipids required for thylakoid expansion [24,47]. MGD2 and MGD3 are located in the outer envelope membrane (OEM) and are more abundant in nongreen tissues, such as roots [24,47]. MGD2 and MGD3 have partly overlapping roles, in particular for the increased production of MGDG upon phosphate starvation [14,24,46,49]. Using the sequence of plant MGDG synthases as a probe, homologs have been detected in all major taxa of Archaeplastidia [47,50]. Surprisingly, the origin of eukaryotic *MGD* genes cannot be easily traced in cyanobacteria, which use a distinct set of enzymes, first synthesizing monoglucosyldiacylglycerol (with a polar head containing glucose instead of galactose) and then converting it into MGDG via the action of an epimerase [51,52]. Based on phylogenetic studies, eukaryotic MGD is likely to be related to bacterial enzymes, acquired following a horizontal transfer, which has not yet been fully elucidated [50,53].

A multigenic family of two DGDG synthases (EC 2.4.1.241; i.e., DGD1 and DGD2) catalyzes the production of DGDG in *Arabidopsis* by transfer of a second galactose from UDP-galactose [54,55]. The major enzyme is DGD1, localized in the OEM [54,55]. DGD2 is also localized in the OEM and is involved in the synthesis of DGDG upon Pi starvation [23], in coordination with MGD2/MGD3 [14,24,46]. DGDG synthases belong to the GT4 family of glycosyltransferases, following the CAZy classification, and have been detected in most major Archaeplastidia taxa analyzed to date [48]. As for genes encoding MGDG synthetic enzymes, the eukaryotic *DGD* genes are distinct from their cyanobacterial counterparts [19] and, therefore, their origin is also puzzling. Thus, MGDG and DGDG appear to be so critical for photosynthesis that their synthesis has been conserved from prokaryotes to eukaryotes, although using distinct synthetic machineries.

Are galactoglycerolipids present in nongreen plastids of Apicomplexa parasites?

In 1996, the surprising discovery of a nongreen plastid, the apicoplast, in human pathogens of the Apicomplexa phylum [38,39,56], fueled the search for conserved features that could serve as targets for novel therapeutic treatments. This plastid was found to be essential for the parasites [57,58] and, therefore, the presence of galactoglycerolipids in this phylum became an important question regarding their evolution as well as a potential target for biomedical purposes. All Apicomplexa, with the noticeable exception of *Cryptosporidium* spp., were shown to contain this unique nonphotosynthetic plastid (Figure 4), referred to as the apicoplast (apicomplexan plastid) (reviewed in [59]).

The apicoplast was acquired by the secondary endosymbiosis of a red alga that had been engulfed within a second eukaryote (Figure 3) [40,59–61]. As a consequence, the apicoplast is delimited by four membranes of three different origins: (i) the two innermost membranes are likely to correspond to the initial plastid envelope, inherited from the cyanobacterial ancestor; (ii) an intermediate periplastid membrane, which might have derived from the plasma membrane of the engulfed red alga, although the mechanism for the conservation of this membrane remains unresolved; and (iii) the outermost membrane, whose origin is thought to be the phagotrophic membrane that engulfed the alga during the secondary endosymbiosis event. Given its origin from the endomembrane system, this membrane is in direct interaction with the parasite endoplasmic reticulum [60,62].

The apicoplast has completely lost photosynthetic capacities. Interestingly, a protist isolated in association with a coral, *Chromera velia*, was shown to be closely related to the Apicomplexa and to be photosynthetic [61,63]. Thus, the comparison of Chromerida and Apicomplexa is considered a key to this puzzling evolutionary history of plastid-containing eukaryotes.

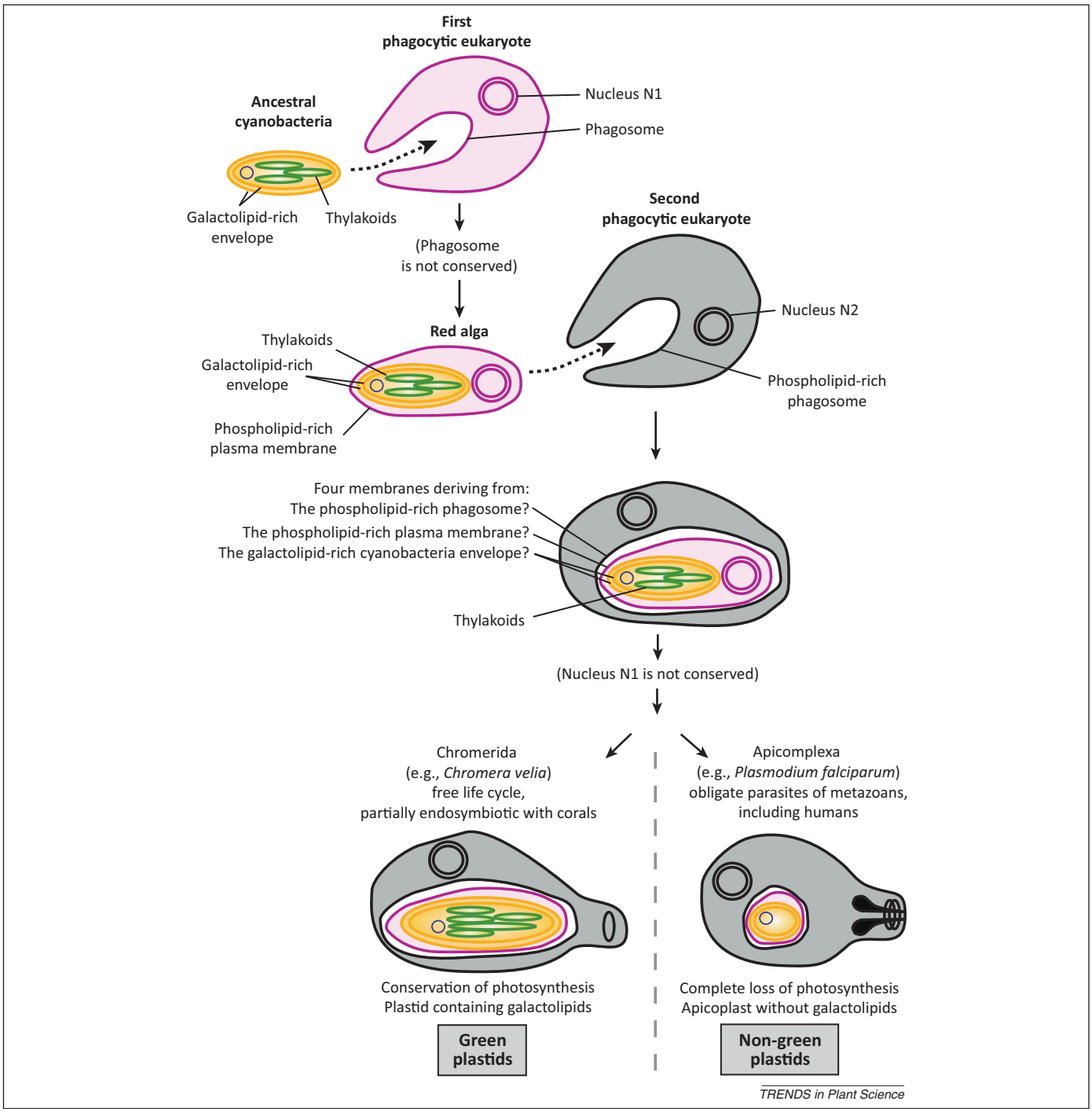

Figure 3. Origin of the secondary plastid in the photosynthetic Chromerida and nonphotosynthetic Apicomplexa phyla. In the upper part of the scheme, the primary endosymbiotic event at the origin of all plastids is shown. An ancestral cyanobacterium is engulfed and 'enslaved' within a first eukaryote (nucleus N1). After loss of the phagosome, the chloroplast is bound by a two-membrane galactolipid-rich envelope deriving from the membranes delineating the cyanobacteria. A secondary endosymbiosis occurred after a red alga was engulfed and 'enslaved' within a second eukaryote (nucleus N2). After loss of cellular structures of the alga, including the nucleus N1, the secondary plastid is bound by four membranes. In Apicomplexa, the plastid is nongreen and is called the 'apicoplast'. Chromerida are the closest relatives of Apicomplexa and contain four membrane-bound chloroplasts.

In search of lost lipids and synthetic enzymes in a nonphotosynthetic human parasite

Ever since the apicoplast was shown to be related to algal chloroplasts, multiple attempts have been made to search for galactoglycerolipids in *P. falciparum* and another common Apicomplexa, *Toxoplasma gondii* [64–67]. However, the amount of parasites for biochemical investigation is limited compared with the kilograms of fresh material that plant scientists have at their disposal. Therefore, the biochemical characterization of whole cells or isolated organelles was challenging. In a first approach, the incorporation of radiolabelled galactose into a lipid co-migrating with MGDG and DGDG in thin layer chromatography was reported in *Plasmodium* and *Toxoplasma* [64]. However, such labelled lipids were not detected in similar analyses performed on *Plasmodium* membranes released after

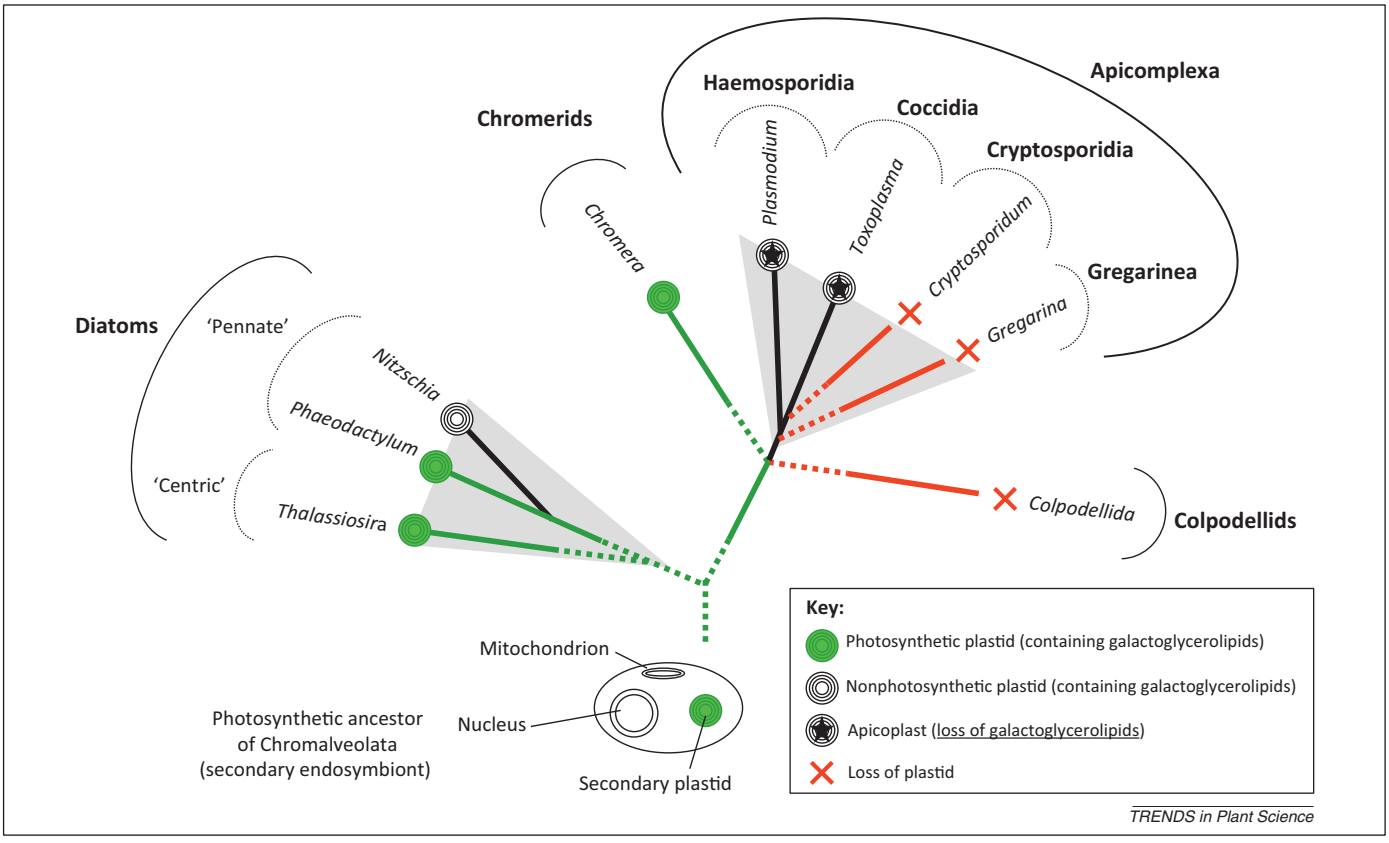

Figure 4. Tree of Chromerida and Apicomplexa within the Chromalveolata supergroup. In this simplified view, orders of the Apicomplexa phylum are shown, with some representative genera (e.g., the Haemosporidia order containing *Plasmodium* species). Apicomplexa are closely related to chromerids, which are free-living photosynthetic unicellular protists, and to colpodellids, which are free-living single cell predators. Chromalveolata represents one of the largest fractions of protists diversity. Diatoms are also shown, with genera containing photosynthetic and nonphotosynthetic species. The conservation of photosynthetic plastids is shown by green circles and lines, whereas the presence of nonphotosynthetic plastids is shown by black circles and lines. The presence of an apicoplast without galactoglycerolipids is indicated by a star, whereas the loss of the plastid is shown by red lines and crosses.

disruption of the cells [67]. In a second series of experiments, an epitope reacting with an anti-DGDG polyclonal antibody was detected in a membrane structure at the periphery of the parasites [65], but showed no labeling at the level of the apicoplast. Lipid analyses of the purified peripheral membrane structure, as well as on whole parasites, could also not confirm the presence of chloroplast-like galactoglycerolipids [65,66]. These results suggested that, if present, galactoglycerolipids were a minor lipid class in the parasitic cell. Overall, these analyses were not fully conclusive and, given the conservation of galactoglycerolipids in all plastids analyzed to date, a low-level presence in one of the apicoplast membranes, below detection thresholds, could not be excluded at that time.

The search for galactoglycerolipid synthesis genes in Apicomplexa was also inconclusive, although both *P. falciparum* and *T. gondii* genomes have been sequenced [68,69]. Despite strong homologies between Apicomplexa, plants, and algae, the genomes of these parasites have not yet been completely annotated. The genome of *P. falciparum* is particularly difficult to mine because of a strong nucleotidic compositional bias [68]. More than 80% of the genome comprises adenosine and thymidine, leading to protein sequences with uncommon amino acid compositions [70,71]. Approximately half of the genes have been functionally annotated, because of improved bioinformatic mining tools [72]. Potential homologous genes encoding enzymes acting upstream of MGDG synthases have been identified, but neither MGD nor DGD putative homologs have yet been found. No glycosyltransferases of the GT28 family and only one of the GT4 family, unrelated to DGD, could be identified in the *Plasmodium* genome following the CAZy classification [48]. As stated above, it is also known that Archaeplastida MGDG synthases are not homologous to the enzymes that catalyze the synthesis of MGDG in cyanobacteria [50,52]. Taken together, it could not be excluded that synthetic enzymes might be encoded by a gene in the unannotated half of the genome or that a synthesis of galactoglycerolipid might occur via the action of a nonhomologous set of glycosyltransferases in Apicomplexa.

An important element to address the question of the conservation or loss of lipids and MGD and DGD enzymes came with the discovery of *Chromera velia*, a 'green Apicomplexa-like organism'. Indeed, analysis of *C. velia* whole-lipid extract, using highly sensitive mass spectrometry, confirmed the full structural characterization and presence of typical MGDG and DGDG [36]. Assessing their localisation within the protist using anti-DGDG labeling and confocal imaging confirmed the expected plastid localization. Nevertheless, it was impossible to assess which of the four membranes delineating the chloroplast contained DGDG. Eventually, by mining available genomic sequences [73], homologs of green *MGD* and *DGD* genes

were identified [36]. *In silico* predictions were confirmed by biochemical measures and metabolic labeling [36]. Therefore, the Chromerida group, including *C. velia* and *Vitrella brassicaformis* [74], provides a reference to analyze the evolutionary story of the galactoglycerolipid pathway in Apicomplexa. In the case of Apicomplexa, therefore, the loss of galactoglycerolipids would be associated with the disappearance of photosynthesis in combination with simplification of the lipid metabolism of the parasite.

The loss of galactoglycerolipids from the apicoplast

In other groups of protists deriving from a secondary endosymbiosis, nonphotosynthetic organisms have been previously analyzed. For example, the nonphotosynthetic diatom *Nitzschia alba* was shown to contain high levels of MGDG and DGDG (Figure 4) [75]. Evidence for the loss of galactoglycerolipids in the nonphotosynthetic plastid of Apicomplexa had to be indubitable. Thus, a *P. falciparum* transgenic line expressing the only known metabolic transporter and gate keeper of the apicoplast, the outermost membrane triose phosphate transporter (TPT) tagged with hemagglutinin (HA), was used to immunopurify the apicoplast on magnetic beads coupled to anti-HA antibodies [76]. This technique yielded enough highly purified and intact apicoplasts to perform an in-depth lipidomic analysis using the latest mass spectrometry approaches. No galactoglycerolipid could be detected, providing the first evidence of the absence of this marker of green and nongreen plastids in this organelle. Other lipids atypical for primary plastids, such as sphingomyelin, ceramides, and cholesterol, were detected and their presence may be because of the endomembranous origin of the outermost apicoplast membrane [76]. So far, it is not known whether these lipids are also components of all secondary photosynthetic plastids found in other protists. The study also showed that the apicoplast is able to perform lipid neosynthesis to compensate for fatty acid-limiting conditions [76], a parasitic innovation that might have evolved from the chloroplast capacity to fuel lipid synthesis in response to environmental variations.

Concluding remarks

The loss of galactoglycerolipids in the apicoplast during the evolution of secondary plastids is surprising and possibly due to the parasitic lifestyle and ancient loss of photosynthetic capacities. *MGD* and *DGD* genes could not be detected, and it is likely that they might have been lost or diverged to fill other functions. Several questions remain: because galactoglycerolipids are major building blocks for chloroplasts, the machinery for synthesizing lipids that substitute for MGD and DGD for the apicoplast membrane biogenesis should be characterized, considering the importance of this organelle. Given the connection of the outermost membrane of the apicoplast to the endomembrane system, the endoplasmic reticulum might contribute substantially to the production of lipids required for the biogenesis of this organelle. It is more puzzling how the two innermost membranes of the apicoplast (originating from the ancestral algal plastid) can be generated in the absence of typical plant galactoglycerolipids. The occurrence of sphingomyelin, ceramides, and cholesterol, which had never been found in any plastid analyzed previously, raises the question of their role in this organelle. The presence of these components in secondary plastids of other taxa should also be investigated.

The role of apicoplasts is currently being reassessed and evidence for the synthesis of major lipid precursors, including fatty acids, phosphatidic acid, and diacylglycerol, which contribute to the biosynthesis of galactoglycerolipids in other plastids, has now been gathered [77]. The production of these precursors in the apicoplast might supply bulk lipid syntheses in the parasites and might be an accurate target for future therapeutic treatments to fight apicomplexan diseases, including malaria.

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