Relationship between acyl-lipid and sterol metabolisms in diatoms

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Abstract

Diatoms are a phylum of unicellular photosynthetic eukaryotes living in oceans and fresh waters, characterized by the complexity of their plastid, resulting from a secondary endosymbiosis event. In the model diatom *Phaeodactylum tricornutum*, fatty acids (FAs) are synthesized from acetyl-CoA in the stroma of the plastid, producing palmitic acid. FAs are elongated and desaturated to form very-long chain polyunsaturated fatty acids (VLC-PUFAs) in domains of the endomembrane system that need to be identified. Synthesis of VLC-PUFAs is coupled with their import to the core of the plastid *via* the so-called "omega" pathway. The biosynthesis of sterols in diatoms is likely to be localized in the endoplasmic reticulum as well as using precursors deriving from the mevalonate pathway, using acetyl-CoA as initial substrate. These metabolic modules can be characterized functionally by genetic analyzes or chemical treatments with appropriate inhibitors. Some 'metabolic modules' are characterized by a very low level of metabolic intermediates. Since some chemical treatments or genetic perturbation of lipid metabolism induce the accumulation of these intermediates, channeling processes are possibly involved, suggesting that protein-protein interactions might occur between enzymes within large size complexes or metabolons. At the junction of these modules, metabolic intermediates might therefore play dramatic roles in directing carbon fluxes from one direction to another. Here, acetyl-CoA seems determinant in the balance between TAGs and sterols. Future lines of research and potential utilization for biotechnological applications are discussed.

1. Introduction

Diatoms are major components of the microbial food web in oceans and fresh waters, playing an essential role in the carbon geochemical cycle, as primary producers [1, 2]. They contribute to at least 20% of global primary production (through the fixation of CO_2 by photosynthesis), representing about fourty-five gigatons of organic carbon per year [3, 4]. Their lifestyle exposes these organisms to permanent environmental variations and stresses, often marked by an imbalance in some elements, such as nitrogen and phosphorus, triggering an accumulation of carbon storing molecules [5-10]. Diatoms store carbon in two forms, triacylglycerols (TAGs) and to some extent chrysolaminarin, a soluble linear polymer of $\beta(1\rightarrow3)$ and $\beta(1\rightarrow6)$ linked glucose [11, 12]. TAGs are a class of glycerolipids composed of three fatty acids esterified to a glycerol backbone (for review, [8]). Because of their high hydrophobicity, TAGs generally accumulate inside lipid droplets [9]. TAGs extracted from microalgae, including diatoms, have attracted an increasing interest for multiple biotechnological applications. TAGs enriched in medium-chain fatty acids with little or no unsaturations (or double bonds) are considered a promising feedstock for biofuel production or green chemistry. In contrast, TAGs enriched in very long-chain polyunsaturated fatty acid (VLC-PUFAs such as eicosapentaenoic acid (EPA, 20:5 n-3) or docosahexaenoic acid (DHA, 22:6 n-3) are of high interest for nutrition and human health [8]. Microalgae are also interesting for the synthesis of pigments (carotenoids for food, cosmetic and pharmaceutical industries), sterols (for nutraceuticals), proteins and enzymes for the conversion of EPA into DHA (for review, [13]).

The establishment of models, such as the pennate diatom *Phaeodactylum tricornutum* or the centric diatom *Thalassiosira pseudonana*, through genome sequencing [14, 15], development of methods for transformation (biolistics, electroporation or conjugation) [16-19] and genetic engineering (TALEN and CRISPR/Cas9) [20-24], has allowed to address the carbon metabolic pathways in this important clade of microalgae. This mini-review focuses on pathways leading to the synthesis of TAGs and sterols, based on studies conducted mainly in *P. tricornutum*. Most recent studies have highlighted that these two metabolic pathways are in direct competition with respect to carbon, especially at their common point of entry, acetyl-CoA.

2. Overview of fatty acid and glycerolipid metabolism in diatoms

Building blocks for glycerolipids consist of glycerol-3-phosphate, providing the three-carbon backbone (termed *sn*-1, *sn*-2 and *sn*-3 carbons) found in all glycerolipids, fatty acids and different types of polar head precursors (for review, [25]). Positions *sn*-1 and *sn*-2 are esterified to fatty acids, whereas position *sn*-3 can be linked either to a polar head or to a third fatty acid. Knowledge on glycerolipid metabolism in diatoms, a secondary endosymbiont, is still incomplete, and mainly based on the translation of what has been shown in primary endosymbionts, such as plants and green algae. Indeed, an important feature of diatoms is the complexity of the plastid, resulting from a secondary endosymbiosis event, following the engulfment of a red alga within a secondary eukaryotic host [9, 26]). As illustrated in **Figure 1**, four membranes delineate their plastid ([26, 27]). In addition, the outermost membrane of the plastid (cERM in **Figure 1**) is in direct continuity with the outermost membrane of the nuclear envelope, meaning therefore a contact between the plastid and the endomembrane system [9, 28].

2.1. Synthesis of fatty acids in the stroma of secondary plastids

Fatty acids are carboxylic acids having an aliphatic chain of variable length (for review, [8]) In diatoms, the synthesis of fatty acids occurs in the stroma of the plastid, following the same processes as those deciphered in primary endosymbionts, *i.e.* by the action of a FAS II (Fatty Acid Synthase type II) system. It requires a pool of acetyl-CoA [29].

Acetyl-CoA is a precursor for several metabolic pathways (lipids, proteins, carbohydrates). Acetyl-CoA can be synthesized first from free acetate as a substrate of the Krebs Cycle by an acetyl-CoA synthetase (ACS), thus generating a mitochondrial acetyl-CoA pool [30] (**Figure 2**). Acetyl-CoA can also be synthesized by the oxidative decarboxylation of pyruvate by the action of a plastidial pyruvate dehydrogenase complex (PDH), composed of three enzymes: pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2) and dihydrolipoyl dehydrogenase (E3) [31]. This route is preferentially used for the synthesis of the acetyl-CoA pool used for fatty acid synthesis in plants and algae [32]. Therefore, pyruvate is also an important substrate for fatty acid synthesis. Pyruvate is synthesized by many metabolic pathways such as glycolysis, the pentose phosphate pathway or by the conversion of malate by the malic enzyme (**Figure 2**, ME). Several studies have been conducted on ME (called respectively PtME and ME1) in *P. tricornutum* [33, 34]. The PDH activity is inhibited by a pyruvate dehydrogenase kinase (PDK). Antisense knockdown of PDK in *P. tricornutum* (PtPDK) was shown responsible for the accumulation of neutral lipids raising the importance of mitochondrial acetyl-CoA pool in diatoms [35]. Transcriptomic analyzes carried out in *P.*

tricornutum in the presence of 3 and 10 µM of nitric oxide [6] or 10 and 20 µM of ethynylestradiol [7] (**See 5**) show an increase in the expression of the E2 (Plastid-DHLATa: Phatr3_J23850 and Plastid-DHLATb: Phatr3_EG02309) and E3 (Plastid-LPD: Phatr3_J30113) subunits of the PDH.

Acetyl-CoA can also be produced by the degradation of fatty acids during β-oxidation and by disproportionation of pyruvate to acetyl-CoA and formate by a pyruvate formate lyase (PFL). A gene coding for this enzyme has been found in the centric diatom *T. pseudonana* (THAPSDRAFT_36689) but not in other Stramenopiles [36] (**Figure 2**).

In the stroma, acetyl-CoA is converted into malonyl-CoA by an acetyl-CoA carboxylase (ACCase) adding a carboxyl group (**Figure 2**). Metazoa present a unique ACCase homomeric form localized in the cytoplasm and the mitochondria whereas plants and algae, making up the Viridiplantae superclade, have a homomeric and heteromeric ACCase forms localized in the plastid (ACC1) and cytoplasm (ACC2) [13]. Several genetic studies have been conducted in diatoms to better characterize this enzyme. The overexpression of plastid ACCase in the diatoms *Cyclotela cryptica* and *Navicula sapuvila* leads to an increase in enzymatic activity but does not modify the amount of lipids [37, 38]. Opposite results were obtained in *P. tricornutum*. The plastid ACCase (PtACC-1) of *P tricornutum*, overexpressed in *Escherichia coli*, led to an increase in enzymatic activity of 1.72 fold, a 2-fold increase in the amount of neutral lipid determined by Nile red staining and an increase of 13% in monounsaturated fatty acids synthesis [39].

Then, malonyl-CoA is converted into malonyl-acyl carrier protein (malonyl-ACP) by a malonyl-CoA:acyl-carrier protein transacylase (MCAT). From malonyl-ACP, a series of four enzymatic reactions transfer two carbons per elongation cycle. The first reaction, catalyzed by a *β*-ketoacyl-ACP synthase III (KASIII), consists in the condensation of an acetyl-CoA with a malonyl-ACP, forming after decarboxylation of a molecule of 3-ketoacyl-ACP. Three genes are predicted to encode ketoacyl-ACP synthases in *P. tricornutum* (Plastid-KASa, Phatr3_J37367; Plastid-KASb, Phatr3_J52648 and Organelle-KAS, Phatr3_J18940). 3-ketoacyl-ACP is then reduced into 3-hydroxyacyl-ACP by a 3-ketoacyl-ACP-reductase, NADPH-dependent (KAR, Phatr3_J13073). The last two reactions consist in the synthesis of an acyl group, by the stepwise action of an hydroxyacyl-ACP dehydratase (HAD, Phatr3_J55157) forming 2,3-transenoyl-ACP, and of an enoyl-ACP reductase (ER, Phatr3_J10068). The final acyl group is thio-esterified to an acyl carrier protein by an ACP acyltransferase. It has been proposed that the FAS II system was the main machinery

synthesizing fatty acids in diatoms [40], resulting in the formation of 16:0-ACP after seven cycles [41, 42]. It remains unclear whether diatom FASII could form 18:0-ACP as in plants.

Subsequently, acyl-ACPs are likely to follow two directions, i) an incorporation inside the plastid *via* the so-called "prokaryotic" pathway (**see 2.2**) or ii) an export outside the plastid to the cytosol, where elongation/desaturation processes occur leading to the production of VLC-PUFAs [42-44] (see below). In the endoplasmic reticulum (ER), fatty acids can serve for the synthesis of glycerolipids *via* the so-called "eukaryotic" pathway [45, 46] (**see 2.3**). It is considered that the export of fatty acids outside the plastid requires the release of free fatty acids (FFA), by the action of an acyl-ACP thioesterase. Only one acyl-ACP thioesterase has been identified to date in *P. tricornutum* (PtTE, Phatr3_J33198) but it does not share sequence similarities with plant or bacterial thioesterases. Overexpression of PtTE in *P. tricornutum* does not alter the profile of total fatty acids but causes a 72% increase in total lipids [47]. Heterologous expression of a lauric acid biased thioesterase (C12-TE) from *Umbellularia California* and a myristic acid biased thioesterase (C14-TE) from *Cinnamomum camphora* in *P. tricornutum* led to an increase in short chain fatty acid synthesis in *P. tricornutum* [48]. The same kind of result has been obtained in several species of green microalgae [49-51].

In the cytosol, FFAs need to be thioesterified to a coenzyme A by an acyl-CoA synthase (ACS) to be used as substrates for the eukaryotic pathway. This export outside the chloroplast is a major subject of study because it is almost unknown in diatoms. Eventually, VLC-PUFAs are imported back to the plastid to serve as precursors for glycerolipid syntheses. The VLC-PUFA import mechanism remains to be established (see below) and has been called the “omega” pathway [13, 26] (**see 2.3**).

2.2. Synthesis of glycolipids in the secondary plastids

From cyanobacteria to secondary endosymbionts, the glycerolipid composition in the chloroplast is remarkably conserved. It consists of a quartet of four lipid classes, *i.e.* monoglactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG) [52] (**Figure 1**). These four lipid classes are conserved in diatoms. Nevertheless, the precise composition of each of the four membranes is still unknown [5, 26].

The "prokaryotic" pathway classically begins with the esterification of fatty acid originating from an acyl-ACP at the *sn*-1 position of the glycerol-3-phosphate backbone by a glycerol-3-phosphate acyltransferase 1 (ATS1) forming lysophosphatidic acid (LPA). A second acyl

group is esterified, at the *sn*-2 position, by 1-acylglycerol-3-phosphate-acyltransferase (ATS2) forming phosphatidic acid (PA) [53-55]. Respectively, one and two homologs of ATS1 (Phatr3_J3262) and ATS2 (ATS2a, Phatr3_J11916 and ATS2b, Phatr3_J43099) were identified in *P. tricornutum* [5]. PA can be used for i) the synthesis of CDP-DAG by a plastid CDP-DAG synthase (CDP-DAGS, Phatr3_J7678 in *P. tricornutum*), which substitutes the polar head of PA by a cytidine diphosphate (CDP). CDP-DAG can then be converted into PG by a phosphatidylglycerol phosphate synthase (PGPS, Phatr3_J8663). Alternatively, PA can be utilized to synthesize diacylglycerol (DAG) by the action of a plastid phosphatidate phosphatase (PAP) (for review, [26, 52]).

DAG is then the precursor of the synthesis of SQDG and MGDG. The polar head of SQDG is classicaly formed from UDP-glucose by condensation with a molecule of sulfite (SO_3^{2-}) by a UDP-sulfoquinovose synthase (SQD1, Phatr3_J21201 in *P. tricornutum*) [56]. Transfer of sulfoquinovose to DAG is then achieved by a UDP-sulfoquinovose:DAG sulfoquinovosyltransferase (SQD2) forming SQDG [57]. Four genes code for putative UDP sulfoquinovose: DAG sulfoquinovosyltransferases in *P. tricornutum* (SQD2, Phatr3_J50356; SQD2-Like-A, Phatr3_EG02454; SQD2-Like-B, Phatr3_J54068 and SQD2-Like-C, Phatr3_J22554).

MGDG synthesis is catalyzed by MGDG synthases (MGDs). These enzymes transfer galactose from uridine diphosphogalactose (UDP-Gal) to DAG [58-60]. The formation of DGDG is catalyzed by DGDG synthases (DGDs), adding a second galactose from a UDP-Gal molecule. Homologues for all these enzymes can be detected in the genome of *P. tricornutum*, *T. pseudonana*, *Fistulifera*, and other diatoms [5] and a clear future challenge will be to establish their precise localization and roles in diatoms. Due to the predominance of photosynthetic organisms on Earth, MGDG and DGDG are the two most abundant lipids in the biosphere indispensable for the operation of the photosynthetic machinery (for review, [52]). MGDG and DGDG are probably main components of thylakoids in secondary plastids, possibly present in some of the plastid limiting membranes, but their precise localization remains to be demonstrated [26, 61].

2.3. Synthesis of phospholipids and betaine lipids in the endoplasmic reticulum

The ER is the second important site for glycerolipid metabolism. The ER is part of the endomembrane system (ER, Golgi, trans-Golgi network, nuclear envelope lysosomes, vacuoles, vesicular trafficking network, plasma membrane, etc.) and is connected therefore with the outermost membrane of the secondary plastid [9, 28] (**Figure 1**). The ER is the site

of elongation of fatty acids leading to chain lengths greater than 20 carbons [62]. The production of VLC-PUFAs such as eicosapentaenoic acid (EPA, 20:5) involves elongases (ELO) adding two carbons to the aliphatic chain, but also desaturases (FADs) adding double bonds [42-44]. Desaturation of fatty acids takes place both within chloroplast and in the ER. A number of desaturases have been identified in *P. tricornutum* by sequence homology with other organisms [42] and an update of the fatty acid desaturation pathways in diatoms has recently been performed [13]. Several desaturases (PtD5p, PtD6p, PtFAD2, PtFAD6) have been functionally characterized in *P. tricornutum* by heterologous expression in *Saccharomyces cerevisiae* and *Synechococcus* [63, 64]. Overexpression of a delta 5 desaturase (called PtD5b) in *P. tricornutum* led to an increase in PUFAs synthesis [65]. Similarly, the heterologous expression of delta 5 elongase (OtElo5) and delta 6 desaturase (OtD6) from the green picoalga *Ostreococcus tauri* in *P. tricornutum* led to an increase in DHA synthesis in TAGs [66].

The ER is also the site of synthesis of membrane glycerolipids, including phosphoglycerolipids or phospholipids (**Figure 1**, PL). Phosphatidic acid (PA) and DAG are at the origin of all PLs. PA is the precursor of phosphatidylcerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS) especially through a cytosolic CDP-DAG pathway, whereas DAG is a precursor of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) through the Kennedy pathway. PC is the most abundant phospholipid in diatoms, and possibly mainly present in the endoplasmic reticulum [5].

Betaine lipids (**Figure 1**, BL) are a class of glycerolipids occuring naturally in lower eukaryotic organisms such as algae, bryophytes, fungi and some primitive protozoa, and in some photosynthetic bacteria [67]. The three main BL detected in microalgae are 1,2-diacylglyceryl-3-O-2'-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-*β*-alanine (DGTA), 2-diacylglyceryl-3-O-4'-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS) and 1,2-diacylglyceryl-3-O-carboxy-(hydroxymethyl)-choline (DGCC). BL have two fatty acids esterified at the *sn*-1 and *sn*-2 positions, as well as a betaine polar group linked by an ether bond at the *sn*-3 position. Knowledge about BL in microalgae is still scarce. The synthetic route of DGTS was mainly studied in *Chlamydomonas reinhardtii*, a primary endosymbiont and in *Nannochloropsis oceanica*, a secondary endosymbiont [68]. Synthesis of DGTS is carried out by a betaine lipid synthase (BTA1) [69]. The synthesis of DGTA is still elusive, as multiple enzymatic activities might be involved. It is currently considered that synthesis of DGTA in *P. tricornutum* could be achieved by a betaine-lipid synthase-like (BTA-like – Phatr3_J42872) enzyme [6, 7, 68]. A phosphate deficiency is responsible for an accumulation of BL in compensation of the

degradation of PLs [5, 70]. Specific molecular species of DGTA have been detected at the surface of lipid droplets purified from *P. tricornutum* deficient in nitrogen [9].

Productions of VLC-PUFAs and of glycerolipids are tightly connected. In the pennate diatom *P. tricornutum*, both PC and DGTA are thus considered as platforms for the synthesis of EPA [5]. EPA is then directed to the plastid for the synthesis of MGDG, DGDG, SQDG and PG. Our knowledge on the machinery involved in this import, called the "omega" pathway [13, 26], is very poor and its dissection represents one of the most important challenges for the future.

2.4. Where is synthesized triacylglycerol?

TAGs are highly hydrophobic glycerolipids accumulating in the form of lipid droplets in the cytosol of *P. tricornutum* in case of nutritional starvation and abiotic stress [5-7, 9, 10, 71]. Two different pathways can carry out the synthesis of TAGs in the ER or ER-related compartment: i) an acyl-CoA dependent pathway commonly referred to as the Kennedy pathway (**Figure 2**) and ii) an acyl-CoA independent pathway involving phosphatidylcholine (PC) as an acyl donor.

The Kennedy pathway corresponds to a *de novo* synthesis of TAGs from fatty acids derived from the cytosolic pool of acyl-CoA. The route begins with an esterification of the glycerol-3-phosphate backbone with an acyl-CoA by a glycerol-*sn*-3-phosphate acyltransferase (GPAT) forming lyso-phosphatidic acid (LPA). Overexpression of GPAT in *P. tricornutum* (Phatr3_J54709) causes an increase in the amount of neutral lipids (determined by Nile Red staining) by a factor 2 as well as a higher proportion of unsaturated fatty acids [72]. LPA is esterified with a second acyl-CoA by a lysophosphatidate acyltransferase (LPAAT) forming phosphatidic acid (PA). One LPAAT called AGPAT1 (Phatr3_J45551), presenting four conserved acyltransferase motifs I-IV, has been identified in *P. tricornutum* [73]. Overexpression of AGPAT1 is responsible for an increase in TAGs and a decrease in the content of carbohydrates and soluble proteins. PA is then dephosphorylated by a phosphatidic acid phosphatase (PAP) producing diacylglycerol (DAG). Two putative PAP genes were identified in *P. tricornutum* [46]. An acyl-CoA can also serve as substrate for a diacylglycerol acyltransferase (DGAT) esterifying a third fatty acid to DAG, leading to the formation of TAG (**Figure 2**). Unlike higher plants where three types of DGAT were discovered (called DGAT1, DGAT2 and DGAT3) [74], only two types of DGAT were described in diatoms (DGAT1 and DGAT2) [46, 75-77]. The genome of *P. tricornutum* codes for 1 isoform of

DGAT1 (PtDGAT1, Phatr3_J9794) and 4 isoforms of DGAT2 (PtDGAT2A, Phatr3_J49462; PtDGAT2B, Phatr3_J49544; PtDGAT2C, Phatr3_J31662 and PtDGAT2D, Phatr3_J43469). PtDGAT1 contains at least 8 transmembrane domains [75] and isoforms of PtDGAT2 possess 1 to 4 transmembrane domains [46, 77]. Overexpression of PtDGAT2A in *P. tricornutum* causes a 34.8% increase in the amount of neutral lipids [76]. Likewise, a second study showed that the overexpression of PtDGAT2D led to a 1.4-fold and 2-fold increase in the amount of TAGs and total lipid content, respectively [78]. The authors also showed by dynamic incorporation of ^{13}C-labeled bicarbonate that the overexpressing lines exhibited an increase in carbon flux towards pyruvate and acetyl-CoA that may explain the increase in the amount of lipids observed [78].

In higher plants and yeast, alternative routes to the Kennedy pathway can involve PC as an acyl donor [79, 80]. For instance, a PC:acyltransferase (PDAT) can transfer a fatty acid from the *sn*-2 position of PC to the *sn*-3 position of DAG, thus forming a TAG molecule and Lyso-PC as a co-product [81]. There is no demonstration of the existence of this pathway in Heterokonta but a gene encoding a putative PDAT (Phatr3_J8860) has been detected in the genome of *P. tricornutum*, and has been proposed to also act in TAG biosynthesis [42, 82].
The recent purification of the lipid droplet from *P. tricornutum* cells starved in nitrogen has highlighted the presence of PC, SQDG and specific molecular species of DGTA in the lipid droplet glycerolipidome [9]. The authors propose that these three glycerolipids are components of the polar lipid monolayer of the lipid droplet. By comparison with other eukaryotes, PC and DGTA could be markers of the ER. However, the presence of SQDG, classically synthesized in plastid membranes, is puzzling and may suggest a connection between the lipid droplet and the plastid. Since the outermost membrane of the plastid is connected with the endomembrane system, one could speculate that it is a site of production of TAG, in the specific condition of nitrogen starvation. Other site(s) might be involved in other stress conditions.

3. Overview of the metabolism of steroids in diatoms

Steroids are essential building blocks for eukaryotic organisms especially for the curvature and the flexibility of the membranes [83]. Diatom sterol metabolism was initially reconstructed *in silico* using the DiatomCyc v2.0 model, based on genome annotations and validations by functional genomics [84, 85]. The synthesis of sterols could theoretically be fueled by two independent metabolic pathways, *i.e.* the mevalonate (MVA) pathway and the

2-C-methyl-$_D$-erythritol 4-phosphate/1-deoxy-$_D$-xylulose 5-phosphate (MEP/DOXP) pathway, also known as the non-mevalonate pathway (**Figure 1** and **Figure 2**). In diatoms the MVA pathway localized in the cytosol seems the major route for the synthesis of sterols, while the MEP pathway, located in the plastid, appears to preferentially feed the synthesis of carotenoids [85-88]. These two routes allow the synthesis of the precursors required for the synthesis of sterols, the five-carbon building blocks isopentenyl-pyrophosphate (IPP) and its isomer dimethylallyl-pyrophosphate (DMAPP) [85, 87, 88] (**Figure 1** and **Figure 2**).

The MVA pathway operates in six steps initiated by the condensation of two molecules of acetyl-CoA into acetoacetyl-CoA by acetyl-CoA C-acetyltransferase (ACAT) [85]. A third acetyl-CoA molecule is condensed to form acetoacetyl-CoA by the action of a hydroxymethylglutaryl-CoA synthase (HMGS) forming S-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Then, HMG-CoA is reduced by an HMG-CoA reductase (HMGR) into mevalonate. HMGR is localized in the endoplasmic reticulum in a large number of model organisms [89-91], however the precise localization of this enzyme is not known in diatoms. Finally, mevalonate is converted in three steps into IPP, *i.e.* two successive phosphorylation reactions catalyzed by a mevalonate kinase (MK) and a phosphomevalonate kinase (PMK), forming mevalonate-P and mevalonate-pyrophosphate, and an ATP elimination reaction catalyzed by a diphosphomevalonate decarboxylase (MVD) [87].

DMAPP and IPP are condensed, forming geranyl-pyrophosphate (GPP). The final addition of IPP to GPP generates farnesyl-pyrophosphate (FPP). FPP is converted by a squalene synthase (SQS) in presqualene-PP then into squalene [85]. In *P. tricornutum*, the sequences of SQS and Isopentenyl-diphosphate δ-isomerase (IDI) are fused [85, 88]. However, the *P. tricornutum* genome encodes an independent second IDI (Phatr3_J12533) [85]. Whatever the organism studied, the 30-carbon squalene is the precursor of all steroids. Squalene is oxidized by a squalene epoxydase (SQE) into 2,3-oxidosqualene. However, this enzyme was not detected in the genomes of the pennate diatom *Phaeodactylum tricornutum* or the centric diatom *Thalassiosira pseudonana* [85, 92]. It was suggested that an alternative SQE may exist in *P. tricornutum* based on pharmacological analyzes showing the no inhibitory effect of terbinafine, an SQE inhibitor [85]. The existence of this alternative *P. tricornutum* SQE (AltSQE – Phatr3_J45494) has recently been demonstrated [83]. Unlike SQE which is a flavoprotein, AltSQE is a fatty acid hydroxylase, localized in the ER using a fusion with a mVenus yellow fluorescent protein (YFP) protein, coinciding therefore with the location of SQE classically observed in eukaryotes [83, 93, 94]. Nevertheless, no difference could be

observed in sterol accumulation in *AltSQE* overexpressors or corresponding RNA interference (RNAi) lines in *P. tricornutum* [83]. Subsequently, 2,3-oxidosqualene is cyclized into cycloartenol by an oxidosqualene cyclase (OSC), which is a cycloartenol synthase (CAS) [85]. Our understanding of sterol metabolism in diatoms becomes fragmentary from this point. In *P. tricornutum*, twenty steps are necessary to reach the final product of sterol synthesis, brassicasterol [85]. Both end products, campesterol (24-methylcholest-5-en-3β-ol) and brassicasterol (24-methylcholest-5,22-dien-3β-ol) were detected by GC-MS [85, 95]. Conversion of campesterol into brassicasterol was confirmed by the use of imidazole, an inhibitor of cytochrome P450 proteins of the CYP70 subfamily [85]. Recently, the architecture (*i.e.* proteome, glycerolipidome, pigment and sterol composition) of the lipid droplet of *P. tricornutum* has been determined [9]. Only the presence of free brassicasterol (<5 mol% compared to sterol + glycerolipids) could be detected in the purified lipid droplets. Based on the polarity of brassicasterol and its proportion detected in the purified fraction of lipid droplets, a localization of brassicasterol at the level of the monolayer of polar lipids was proposed [9].

4. The impairment of sterol metabolism triggers an accumulation of TAGs

Understanding a metabolic pathway can be achieved through chemical engineering approaches, by the use of specific inhibitors of a particular pathway enzyme, in complement with genetic analyses of appropriate mutants. In the initial study of sterol synthesis [85], an intriguing question was the absence of a canonical SQE, raising the question on the way *P. tricornutum* used 2,3 epoxysqualene for sterol synthesis. To address this question, two chemical inhibitors were used, *i.e.* terbinafine, inhibiting SQEs, and Ro 48-8071, inhibiting OSCs. *P. tricornutum* cells did not appear to show any growth arrest after treatment with 40 µM terbinafine, whereas treatment with 30 µM Ro 48-8071 killed cells in 48 hours. The authors analyzed the sterol composition following the use of these inhibitors. A treatment with 10 µM of Ro 48-8071 was responsible for an accumulation of squalene and 2,3 epoxysqualene consistently with the cyclization of 2,3 epoxysqualene into squalene by an OSC in *P. tricornutum* (called PtOSC in this article). In addition, after Nile red staining, lipid droplets were observed in the cells treated with the OSC inhibitor [7, 85].

A second approach to dissect the steroid synthesis pathway in diatoms is the use of RNAi to silence the expression of target genes at the transcriptional level. RNAi corresponding to an oxidosqualene cyclase gene were generated**,** showing a decrease in cell growth coupled with a decrease in sterol synthesis and a high accumulation of lipid droplets [85]. Together, these

chemical and genetic analyses suggested that an alteration of the sterol synthesis pathway could lead to a relocation of carbon in *P. tricornutum* from sterols to TAGs.

Independently, a screening of small molecules was set up in the diatom *P. tricornutum* in order to discover biological processes that may be involved in the synthesis of TAG [7]. This chemical screening was performed using the Prestwick library of 1.280 compounds, for which biological targets had been previously identified in human or other organisms. This study was carried out with the underlying hypothesis of mimicry or structural relationship between obtained hit compounds and biomolecules involved in the *in vivo* signaling process, or metabolites. Among validated hit compounds, five molecules known to interact with the mevalonate pathway and sterol metabolism were identified: mevastatin and simvastatin, known to target HMGRs [96-98], ketoconazole targeting a sterol-14-α-demethylases [99], and eventually estrone and ethynylestradiol. The detection of a large number of inhibitors of the sterol pathway, together with whole genome expression analyses of treated cells, strongly suggested that there was a likely competition for carbon at the level of acetyl-CoA between sterol and TAG syntheses [7].

In parallel, it has also been shown that the inhibition of Target of Rapamycin (TOR) kinase by 2 µM of AZD-8055 in *P. tricornutum* affects the cell concentration and promotes the accumulation of TAG in the form of lipid droplets after different kinds of staining [100, 101]. The same type of response has been observed in the green algae *Chlamydomonas reinhardtii* [102]. A decrease in brassicasterol level in *P. tricornutum* following this treatment has also been observed, suggesting a convergence of TOR-mediated signaling and energy metabolism (lipid and sterol) in *P.tricornutum* [100].

5. The question of the effect of synthetic analogs of sterols on diatoms: case study of ethynylestradiol

Following the screening of small molecules in *P. tricornutum*, the effect of ethynylestradiol was further investigated [7]. This compound is a synthetic derivative of the natural hormones estrone and estradiol [103] used in birth control pills [104]. These molecules are well-known endocrine disruptors, with recorded utilization in aquaculture for the development of single-sex populations of fish [105]. The mode of action of ethynylestradiol in *P. tricornutum* is currently unknown but this compound may target cytochrome P450 (CYP) proteins [7, 106]. Following treatment an increase in Nile Red staining coupled with a decrease in *P. tricornutum* cell growth was observed (for concentrations >8 µM ethynylestradiol) [7]. A

small effect on the activity of photosystem II was observed (>30 µM ethynylestradiol). The glycerolipid profile of cells treated 48 hours in absence or presence of 30 µM ethynylestradiol highlighted a strong accumulation of TAG, confirming the Nile Red staining data, consistent with an accumulation of TAG following a modification of the sterol synthesis pathway. The same kind of response was observed with 15 µM simvastatin, an inhibitor of HMGR. For all glycerolipids, with the exception of SQDG, a decrease in the proportion of EPA was observed following treatment. In addition, PC and DGTA were enriched in C18 precursors of EPA (18:1 to 18:3). As the synthesis of EPA is a very fast mechanism, the presence of these intermediates suggests that treatment with ethynylestradiol could disrupt a metabolon involved in VLC-PUFAs production.

A transcriptomic analysis of *P. tricornutum* treated with 0, 10 and 20 µM of ethynylestradiol was performed [7]. Expression of genes involved in the MEP/DOXP pathway was not impaired by treatment with ethynylestradiol. On the other hand, important alterations in the mevalonate pathway were noted, with increased expression of genes coding for a HMGS, a geranyl-diphosphate/farnesyl-diphosphate synthase, and a sharp decreased expression of genes coding for a MK and a putative geranyl-diphosphate synthase. Finally, an increase in the expression of genes putatively coding for enzymes of the sterol synthesis pathway, in particular cytochrome P450 (CYP) enzymes was detected following treatment with ethynylestradiol. At the glycerolipid metabolism level, some genes coding for TAG catabolism were downregulated whereas genes coding for the biosynthesis of acetyl-CoA were upregulated. The lipidome and transcriptome analyses performed in parallel were therefore consistent with a relocation of carbon from sterols to TAGs. Together these results suggested that ethynylestradiol might target specific CYP, which role needs to be determined in the future. An increase in gene expression of the VLC-PUFAs pathway was induced by ethynylestradiol, with the noticeable exception of a gene encoding Δ6-ELO-B1, coinciding with the interruption of the VLC-PUFAs pathway observed at the level of PC and DGTA. A more in-depth investigation of this Δ6-ELO-B1 may allow in the future to understanding the effects of ethynylestradiol in the VLC-PUFAs pathway.

6. Conclusion and future lines of research

Although knowledge of metabolic pathways deciphered in yeast, *Arabidopsis* or *Chlamydomonas* is extremely useful to mine the genome of diatoms and propose metabolic schemes, this mini-review highlight gaps that need to be filled, which cannot benefit from comparative analyses with previous models. Most importantly, the understanding of

metabolic processes occurring in the secondary plastid (**Figure 1**) cannot be addressed simply based on knowledge from plant and algae (algae = green algae, red algae and Glaucophytes) as the secondary plastid differs strongly in its structure and function with the primary chloroplast [26, 107, 108]. This question is critical when addressing the fatty acid and sterol metabolic networks. The proteomic and lipidomic compositions of the four limiting membranes of diatom's plastid are unknown; the connection between the two outermost membranes and the nuclear envelope is a real puzzle. The lipid droplet from *P. tricornutum* cells, starved in nitrogen, is the only purified subcellular compartment analyzed to date, highlighting a possible connection with the outermost membrane of the plastid. Future challenges include therefore the characterization of the proteomes of compartments that are specific to diatoms. In some instances, like the synthesis of VLC-PUFAs and its allocation to specific glycerolipid classes, the absence of metabolic intermediates between the initial substrate and the end products suggests a possible channeling *via* large-size complexes or structures, called "metabolons" [109, 110]. Metabolic modules can be pointed in a diatom cell: (1) fatty acids are synthesized in the stroma of the plastid; (2) fatty acid elongation and desaturation occur mainly on PC and DGTA, acting therefore like "lipid platforms" for VLC-PUFAs production (in a domain of the endomembrane system that needs to be identified); (3) the synthesis of VLC-PUFAs is tightly coupled with their import to the core of the plastid *via* the so-called "omega" pathway; (4) sterols are mainly synthesized using precursors deriving from the mevalonate pathway. These metabolic modules can be characterized functionally by genetic analyzes or chemical treatments with appropriate inhibitors. At the junction of these modules, metabolic intermediates can therefore play dramatic roles in directing carbon fluxes from one direction to another. Here, acetyl-CoA seems determinant in the balance between TAGs and sterols, although the mass ratio between these two carbon sinks are different. The impairment of sterol metabolism is not only compensated by an increase in TAG, as illustrated by the disruption of the VLC-PUFAs pathway and because a structural and functional impact on membranes is likely to occur following a sterol decrease. Acetyl-CoA might therefore not act simply as a limiting factor. Future studies will also need to assess the dynamics of the relationship between acyl-lipid and sterol metabolism. It will be important to determine if any physiological regulation of acetyl-CoA homeostasis is involved in the control of the acyl-lipid / sterol balance. Thus, the metabolic connections summarized in this review are valuable to propose strategies to control the level in TAG in microalgae for multiple biotechnological applications, but they also highlight important challenges to advance our understanding of lipid metabolism in secondary endosymbionts.

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Figure Legends

Figure 1: Hypothetical scenario of synthesis pathways of sterols and glycerolipids in the pennate diatom *Phaeodactylum tricornutum*. Cell compartments: cERM, chloroplast endoplasmic reticulum membrane (continuous with the outer nuclear envelope membrane); pPM, periplastidial membrane; oEM and iEM, outer and inner envelope membrane, respectively; ppc, periplastidial compartment. Glycerolipid metabolism: FAS, fatty acid synthase; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PL, phospholipid; BL, betaine lipid; TAG, triacylglycerol. Sterol metabolism: AltSQE,alternative squalene epoxydase; MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate; MVA, mevalonate; IPP, isopentenyl-pyrophosphate; DMAPP, dimethylallyl-pyrophosphate.

Figure 2: Competing sinks for carbon in diatoms. The red dashed arrows indicate several steps in the sterol synthesis pathway. Abbreviations in alphabetical order – Acetyl-CoA and Pyruvate precursors: ACL, acetyl-CoA lyase; ACS, acetyl-CoA synthetase; ME, Malic enzyme; PDH, pyruvate deshydrogenase; PEP, phosphoenolpyruvate; PFL, pyruvate formate lyase; PK, Pyruvate kinase. Glycerolipid metabolism: ACCase, acetyl-CoA carboxylase; DGAT, diacylglycerol O-acyltransferase; ER, enoyl-ACP reductase; GPAT, glycerol-*sn*-3-phosphate acyltransferase; HAD, hydroxyacyl-ACP-reductase; KAR, 3-ketoacyl-ACP-reductase, NADPH-dependent; KASI, ketoacyl-ACP synthase I; KASII, ketoacyl-ACP synthase II; KASIII, *β*-ketoacyl-ACP synthase III; LPAAT, 1-acylglycerol-3-phosphate O-acyltransferase; MCAT, malonyl-CoA:acyl-carrier protein transacylase; PAP, phosphatidate phosphatase. Sterol metabolism: ACAT, acetyl-CoA C-acetyltransferase; AltSQE, alternative squalene epoxydase; CDP-ME, 4-diphosphocytidyl-2-C-methylerythritol; CDP-MEP, 4-

diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate; CMK: 4-diphosphocytidyl-2-C-methyl-D-erythritol; CMS: 2-C-methyl-D-erythritol 4-phosphate; DMAPP, dimethylallyl-pyrophosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; FPP, farnesyl-pyrophosphate; G3P, glyceraldehyde-3-phosphate; GPP, geranyl-pyrophosphate; HDR, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate reductase; HDS, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate synthase; HMB-PP, (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate; HMG-CoA, S-3-hydroxy-3-methylglutaryl-CoA ; HMGR, HMG-CoA reductase; HMGS, hydroxymethylglutaryl-CoA synthase; IPP, isopentenyl-pyrophosphate; MCS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MEcPP,2-C-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-C-methylerythritol 4-phosphate; MEP/DOXP, 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate; MK, mevalonate kinase; MVA, mevalonate; MVD, diphosphomevalonate decarboxylase; OSC, oxidosqualene cyclase; PMK, phosphomevalonate kinase; SQS, squalene synthase.

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Figure 1 - Maréchal & Lupette

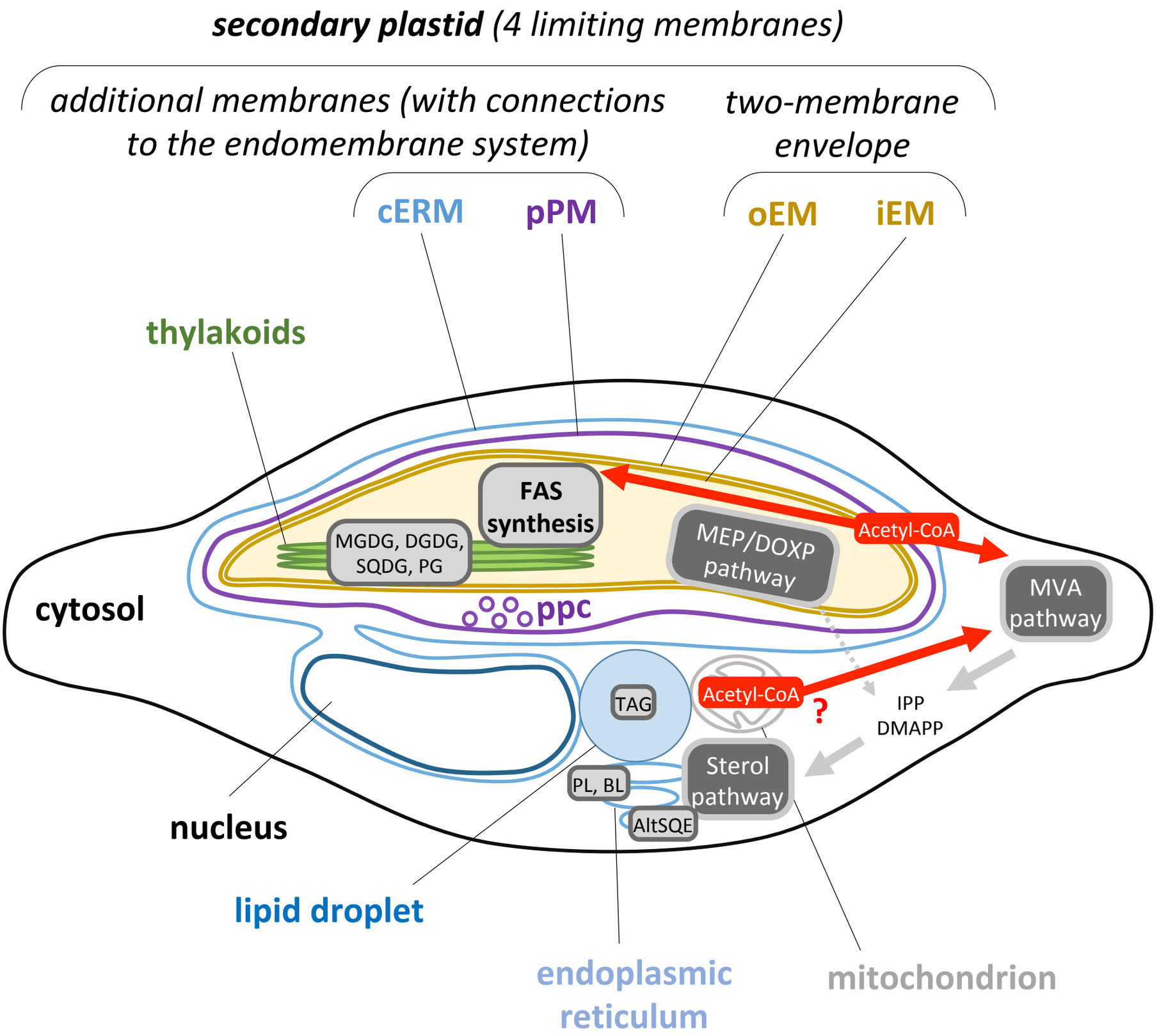

Figure 2 - Maréchal & Lupette