Unraveling algal lipid metabolism: Recent advances in gene identification

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** A B S T R A C T **

Microalgae are now the focus of intensive research due to their potential as a renewable feedstock for biodiesel. This research requires a thorough understanding of the biochemistry and genetics of these organisms’ lipid-biosynthesis pathways. Genes encoding lipid-biosynthesis enzymes can now be identified in the genomes of various eukaryotic microalgae. However, an examination of the predicted proteins at the biochemical and molecular levels is mandatory to verify their function. The essential molecular and genetic tools are now available for a comprehensive characterization of genes coding for enzymes of the lipid-biosynthesis pathways in some algal species. This review mainly summarizes the novel information emerging from recently obtained algal gene identification.

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1. Introduction

This review covers the recent discoveries in the lipid metabolism of eukaryotic photosynthetic microalgae, an extremely diverse group of unicellular organisms dwelling in various environments. Our goal is to cover some aspects which have not been described in previous comprehensive reviews on algal lipid metabolism [1–3, and ref therein]; thus mainly novel information on recently identified genes will be summarized. Numerous microalgal species are used for the production of high-value lipid compounds, such as long-chain polyunsaturated fatty acids (LC-PUFA) and carotenoid pigments, mainly as a feed source in aquaculture and for nutraceutical applications. Oleaginous algae accumulate high quantities of neutral storage lipids, mainly triacylglycerols (TAG), in response to environmental stresses, such as nitrogen limitation, salinity or high temperature, and their TAG are composed largely of saturated and monounsaturated fatty acids [2,4 and ref. therein]. A rare exception is the freshwater chlorophyte *Parietochloris incisa* which accumulates an unprecedented proportion (60% of total fatty acids) of the ω6 LC-PUFA arachidonic acid (ARA) in its TAG [4,5]. We hypothesized that one of the roles of LC-PUFA-rich TAG in certain algal species, rather than serving only as an energy store, is to serve as a reservoir that can be used for the rapid construction of PUFA-rich chloroplastic membranes, particularly under low temperatures or during growth recovery after nitrogen starvation [4,6]. Indeed, the role of lipid bodies in eukaryotic cells is now thought to be much more complex than simply serving as a carbon store, because stores of fatty acid moieties are also required for membrane synthesis and remodeling [7,8]. Due to the high potential of microalgae as a renewable feedstock for biodiesel production [for further information refer to [9–12]], isolation and functional characterization of genes encoding enzymes that mediate the key steps in fatty acid and TAG biosynthesis, as well as in lipid catabolism, are of particular importance. The anticipated progress in elucidating these steps is supported by the wealth of genetic information on algae that has been revealed in the last decade.

Genome sequence information is currently available for seven eukaryotic algae, including the red microalga *Cymidiosphyzon merolae*, two marine diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum*, several green microalgae, including *Chlamydomonas reinhardtii*, and marine picoeukaryotes of the class Prasinophyceae: *Ostreococcus tauri*, *Ostreococcus lucimarins*, *Micromonas pusilla* and *Micromonas sp*. A few more projects on algal genomes are currently in progress (e.g. of the haptophyte *Emiliania huxleyi*) which, together with numerous expressed sequence tag (EST) studies of various algal species, are expected to provide substantial novel information [11,13–15]. *C. reinhardtii* is a model microalga for which microarray gene expression data are available and tools for the transformation of plastids and nuclear genomes, as well as for silencing by RNA interference (RNAi), have been developed [13,16]. New approaches for analyzing gene expression are expected to allow more efficient evaluation of the conditions that favor biosynthesis of energy-rich products, such as starch and TAG (http://www.jgi.doe.gov/sequencing/why/chlamy).
Moreover, genetic transformation tools have also been recently developed for diatom species [17,18], and the presence of functional silencing machinery and feasibility of targeted gene knockdown have been demonstrated in *P. tricornutum* by introducing constructs that express anti-sense or inverted-repeat-containing RNAs [19]. Diatom expression vectors for the validation of subcellular localization, immunodetection of proteins of interest and overexpression studies have also been developed [20]. Thus, the essential molecular and genetic tools are now available for a comprehensive characterization of genes coding for enzymes of the lipid-biosynthesis pathways.

2. Fatty acid biosynthesis and production of precursors in the plastid

Genome information has enabled in silico analysis of fatty acid and glycerolipid metabolism and a prediction of the genes encoding proteins involved in membrane and storage lipid biogenesis in some microalgae [21–24]. For example, the major pathways of fatty acid synthesis were reconstructed for *C. reinhardtii* [21–23]: based on their similarity to proteins with experimentally verified functions in higher plants, all components of the plastidial multisubunit acetyl coenzyme A (acetyl-CoA) carboxylase (ACCase), catalyzing the first committed step in fatty acid biosynthesis, carboxylation of acetyl-CoA to malonyl-CoA, and fatty acid synthase (FAS) complexes were identified (Fig. 1). Higher plant orthologs of key enzymes of the plastidial FAS complex are present in the genomic and EST data bases of other algae [24,25]. Examination of EST sequences of the thermo-acidophilic red microalga *Galdieria sulphuraria* [25] and of the genome of a primitive red microalga *C. merolae* [24] revealed that their fatty acid biosynthesis is likely similar to that of the green lineage, which includes green algae and higher plants. Two types of ACCase were identified in these algae: a prokaryotic-type multisubunit enzyme in the plastid and a multifunctional homomeric enzyme in the cytosol [24]. The plastid localization of the nuclear-encoded subunit of a multi-subunit ACCase was confirmed by green fluorescent protein (GFP)-fusion experiments. Moreover, genes encoding enzymes involved in acetyl-CoA generation in the plastid (Fig. 1), such as pyruvate kinase and pyruvate dehydrogenase complex (PDC), can also be readily identified, for example, in the *Chlamydomonas* genome, by sequence similarity to those of higher plants. PDC is a multienzyme complex that catalyzes the oxidative decarboxylation of pyruvate, leading to acetyl-CoA. The plastidial pyruvate kinase, which converts phosphoenolpyruvate into pyruvate, was shown to be important as a pyruvate supply for FAS via PDC. It should be noted that the cellular localization of the putative plastid proteins is generally predicted by web-based protein-targeting algorithms, such as TargetP (http://www.cbs.dtu.dk/services/TargetP), and sometimes these predictions produce uncertain probabilities of the protein being targeted either to mitochondria or chloroplasts. Moreover, it was suggested that activities of dual-targeted proteins in *Chlamydomonas* are required in both organelles [23]. Hence, functional validation and characterization of annotated genes in algal genomes requires further experimental evidence.

Bioinformatics analyses have indicated that the key regulatory steps identified in eukaryotic microalgae for fatty acid biosynthesis (Fig. 1) and for the import of fatty acids to the endoplasmic reticulum (ER) for further glycerolipid biosynthesis, are largely similar to those in higher plant plastids [21–23]. Thus, several key strategic steps for the metabolic engineering of microalgae aimed at increasing TAG production, as required for sustainable biodiesel production, have been proposed [9–11]. Among these are overexpression of ACCase and FAS enzymes, increased availability of acetyl-CoA precursor for FAS by overexpression of the enzymes involved in its production or downregulation of phosphoenolpyruvate conversion to oxaloacetate; interference of competing pathways leading to TAG degradation, e.g. inhibition of β-oxidation and

![Fig. 1. A simplified scheme for the control of FAS formation in the plastid of green algae. ACCase, acetyl-CoA carboxylase; ACL, ATP-citrate lyase; FAS, fatty acid synthase; ME, malic enzyme; MCAT, malonyl-CoA:ACP transacylase; PDC, pyruvate dehydrogenase complex; PEP, phosphoenolpyruvate; PEPC, phosphoenolpyruvate carboxylase; PK, pyruvate kinase.](image-url)
lipoysis (Fig. 1). Modification of fatty acid chain length and saturation levels to produce monounsaturated and saturated TAG, the ideal form for biodiesel, can be further achieved via expression of thioesterases and silencing of desaturases. These strategies are based on the recently obtained thorough understanding of lipid metabolism in higher plants [26] and the success in manipulating lipid-biosynthesis pathways with molecular tools. Moreover, these strategies can be applied to algae which produce TAG under conditions of nutrient deficiency or environmental stress, and are amenable to genetic transformation [10]. Another feasible approach is the regulation of gene expression by transcription factors identified in algae to modulate lipid metabolism [27,28].

3. Neutral lipids

There is a significant body of research on the effects of environmental stresses on neutral lipid biosynthesis in algae [1,2]. Conversely, the enzymes and pathways that trigger and control the accumulation of storage TAG in microalgae have been little studied at the molecular level. The first genetic engineering effort to manipulate algal lipid production was made in the diatom Cyclotella cryptica by overexpressing the endogenous gene encoding ACCase [29]. It was previously shown that an increase in ACCase activity plays a significant role in the induction of TAG accumulation in nutrient-deprived C. cryptica [30]. However, as reported by Sheehan et al. [31], overexpression of the additional copies of ACCase did not have a significant effect on lipid synthesis in either C. cryptica or a different diatom strain, Navicula saprophila.

C. reinhardtii has been recently used in several studies relating to the isolation of genes and identification of proteins involved in neutral lipid biosynthesis. Nitrogen depletion induces significant ultrastructural changes in the cells of C. reinhardtii, such as reduction of stacked thylakoid membranes, accumulation of starch granules, and the appearance of lipid droplets (otherwise referred to as oil bodies) [32]. A lipid droplet-enriched fraction was purified from C. reinhardtii, and 16 proteins potentially involved in lipid biosynthesis were identified by mass spectrometry. These proteins included predicted acyl-CoA synthetases and acyltransferases, involved in the biosynthesis of TAG or the transfer of acyl groups between the membrane and neutral lipids. A major protein, designated major lipid droplet protein (MLDP), was isolated and its mRNA abundance appeared to correlate with the accumulation of TAG during the time course of nitrogen deprivation. The protein was found to be specific to the green algal lineage of photosynthetic organisms. MLDP was found to be a very hydrophobic protein, although it was not similar to olesin—the main structural hydrophobic protein of plant oil bodies. The functional importance of MLDP was further examined by RNAi silencing; for the first time this technique had been employed to characterize a lipid gene in algae. To this aim, an expression cassette containing the nitrate reductase (NIT1) promoter, inducible by nitrogen deprivation, was used to drive the expression of an MLDP genomic sense cDNA antisense RNAi hairpin. An increase in average lipid droplet size was observed in the MLDP-RNAi lines, but the levels of TAG or rate of TAG mobilization upon nitrogen replenishment did not change, indicating a structural role for MLDP in oil-body formation.

Stressful conditions, such as nutrient limitation or high light intensity, trigger both starch and TAG accumulation in some algal species. There is now substantial evidence that the shift of carbon fluxes from starch to TAG biosynthesis in those species represents an alternative and efficient approach to increasing oil production in microalgae [33,34]. This is supported by the fact that the block in starch biosynthesis leads to enhanced formation of lipid bodies and TAG [33,34]. Starch biosynthesis is impaired in the sta6 mutant of C. reinhardtii harboring a mutation that leads to inactivation of ADP-glucose pyrophosphorylase [35]. In the nitrogen-starved sta6 mutant, the cellular content of lipid bodies increased 30-fold [32], providing direct evidence that genetic interference of the starch biosynthesis pathway can enhance lipid accumulation. A further study by Li et al. [34] reported that the starchless mutant of C. reinhardtii enhances storage lipid accumulation under stressful conditions, resulting in a 10-fold increase in cellular TAG production (i.e., from 2 to 20.5% of DW).

3.1. Sterols

Ergosterol is the predominant sterol in the membranes of C. reinhardtii, which is rarely found in higher plants but is common in fungi. Bioinformatics analysis revealed, however, that C. reinhardtii likely synthesizes sterols via a pathway resembling the higher plant pathway that uses cycloartenol as a precursor, rather than lanosterol as in fungi [36]. This assumption was supported by the fact that two key enzymes in the cycloartenol pathway are found in the C. reinhardtii genome, namely cycloartenol cyclase and cyclopropyl isomerase. This pathway also involves the action of the C5 sterol desaturase (ERG3) responsible for introducing a double bond at C5 in the B-ring of episterol—an essential step in ergosterol biosynthesis. The ERG3 ortholog of C. reinhardtii was cloned and functional validation was performed by heterologous expression in the yeast Saccharomyces cerevisiae. To this aim, the ERG3 gene was knocked out by homologous recombination. Functional complementation in ERG3-knockout strains of S. cerevisiae was able to restore ergosterol biosynthesis and abolish the mutant’s characteristic sensitivity to cycloheximide.

3.2. Waxes

In higher plants, extracellular waxes play an important role as a barrier controlling the flux of water and environmental hazards. A unicellular phytoflagellate Euglena gracilis accumulates wax esters to up to 62% of its total lipid content under anaerobic growth conditions as an energy reserve. Wax esters produced by E. gracilis are 20 to 36 C atoms in length, comprised of 12- to 18C-chain saturated fatty acids and alcohols with myristyl myristate (14:0–14:0) as the major species [37]. Two genes involved in the biosynthesis of wax esters in E. gracilis were recently identified and characterized: a fatty acyl-CoA reductase (EgFAR) involved in the conversion of acyl-CoAs to fatty alcohols, and a wax synthase (EgWS) catalyzing the esterification of acyl-CoAs and fatty alcohols to yield wax esters. Functional expression in S. cerevisiae confirmed their activity and medium-chain substrate preference, and the wax biosynthesis pathway was reconstituted by coexpressing both genes.

3.3. Acyltransferases

TAG biosynthesis and assembly is a complex process involving diverse enzymatic activities. Acyltransferases of the Kennedy pathway [38] reside in the ER and catalyze a sequential transfer of acyl groups from the acyl-CoA pool to the different positions of the glycerol-3-phosphate backbone. These enzymes play a key role in determining the acyl composition of glycerolipids and the final content of TAG [39–41]. Candidate genes which putatively encode acyltransferases of the Kennedy pathway can be found in the genomic databases for the sequenced algal species. These include the acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT), the acyl-CoA:lysophosphatidic acyltransferase (LPAAT), and the acyl-CoA:diacylglycerol acyltransferase (DGAT). In addition, acyl-CoA-independent reactions can contribute significantly to the production of TAG in some plant species. The activities of
phospholipid:diacylglycerol acyltransferase (PDAT) [42] and DAG:DAG transacylase (DGTA) utilize diacylglycerol (DAG), a central lipid intermediate, for direct incorporation into the TAG molecule. The candidate genes for PDAT are identifiable in some algal genomes; as for DGTA, no gene candidates have been identified in either higher plants or algae. The TAG assembly enzymes play an important role in establishing the fatty acid patterns of the major polar membrane lipids and TAG, and therefore the genes that encode them are potential targets in the metabolic engineering of lipid-biosynthesis pathways in both higher plants and algae.

As far as algae are concerned, one GPAT and two DGATs were cloned and characterized based on the genome information and annotation of the diatom T. pseudonana and the chlorophyte O. tauri [43–45]. Detailed analysis of candidate genes of C. reinhardtii is currently underway [Q. Hu, personal communication]. Proteomic analysis of the lipid body-enriched fraction of C. reinhardtii as well as of the eyespot apparatus, which is composed of carotenoid-rich lipid globules inside the chloroplast, revealed the presence of several proteins with similarity to acyltransferases [32,46].

Microsomal GPAT catalyzes the initial acylation at the sn-1 position of glycerol-3-phosphate and produces lysophosphatic acid in the Kennedy pathway. In mammals, four homologous isoforms of GPAT, each the product of a separate gene, catalyze this reaction, two of them residing in the ER [39 and references therein]. All four isoforms are thought to be able to initiate glycerolipid biosynthesis and at least three of them are able to increase the incorporation of fatty acids into TAG. There are several indications of GPAT3’s high importance for TAG formation: GPAT3’s mRNA expression increases 60-fold in adipocytes after differentiation, indicating a key role in TAG formation [39,47]. In Arabidopsis thaliana, a family of eight related genes encodes GPAT proteins that are similar in size and deduced amino acid sequence [48], and that contain four conserved amino acid motifs characteristic of acyltransferases [49]. None of these isoforms were implicated in the biosynthesis of storage TAG. However, a new ER-localized GPAT isoform from A. thaliana was recently identified, and designated GPAT9 [48]; the deduced protein was shown to be most similar to the mammalian GPAT3 inferred to have role in TAG biosynthesis [47]. Both human GPAT3 and A. thaliana GPAT9 contain an additional fifth conserved motif. It is suggested that in plants GPAT may not exhibit a strong substrate preference and acyl-CoA substrate availability determines the acyl composition of sn-1 position of TAG [41].

Due to the limited information on algal acyltransferases, in this section we provide a detailed description of the pertinent research efforts (Table 1). A candidate GPAT of T. pseudonana was identified by searching the genome for DNA sequences with similarity to the yeast GPATs (Gat1 and Gat2) [43]. The amino acid sequence of GPAT of T. pseudonana showed 24% and 23% identity to yeast GPATs and little homology to bacterial, mammalian or Arabidopsis GPATs. Topology analysis predicted five transmembrane domains located in both the N and C termini of the deduced protein and separated by a stretch of more than 400 amino acids, including four catalytically important domains common in acyltransferases. To provide evidence for the predicted function, TpGPAT was expressed in a yeast GPAT-deficient mutant (gat1), and a GPAT assay using [14C] glycerol-3-phosphate as the acyl acceptor and palmitoyl-CoA as the acyl donor was performed. This resulted in restoration of GPAT function in the mutant, evidenced by a sevenfold increase in GPAT activity relative to the control transformed with empty vector. A similar approach was used to characterize an activity encoded by a putative GPAT of C. reinhardtii (CrGPAT): its overexpression in a gat1 mutant increased TAG and phosphoinositol content [Q. Hu, personal communication]. Moreover, the gene’s expression level was upregulated under stress conditions, high light or nitrogen starvation, inducing TAG production, indicating CrGPAT’s contribution to TAG biosynthesis in C. reinhardtii.

In vitro, TpGPAT could accept a range of tested acyl-CoA substrates, with the preference for 16:0-CoA. TpGPAT did not utilize 14:0-CoA or 16:1-CoA; however, it should be noted that substantial activity was also measured for 20:4-CoA, and lower levels determined for 18:0-, 18:1- and 22:6-CoAs. Fatty acid composition of the transformed yeast also indicated TpGPAT’s preference for 16:0: a significant increase in the share of 16:0 was determined in phospholipids and TAG with a concomitant decrease in the proportions of monounsaturated 16:1 and 18:1. The fatty acid composition of T. pseudonana is characterized by a predominance of 16:0, 16:1 and the ω3 LC-PUFA EPA (20:5ω3); C18 fatty acids are present at low levels. Almost 60% of the molecular species of TAG in the starved T. pseudonana contain 16:0 in combination with 16:1 and 14:0 [50]. This alga also contains some DHA and is capable of partitioning ω3 LC-PUFA (EPA and DHA) into TAG in the stationary growth phase [51]. Indeed, these LC-PUFA were shown to be major components of the higher molecular weight molecular species of TAG detected in T. pseudonana cells that had been nitrogen- or

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<th>Table 1</th>
<th>Updated available information on algal acyltransferases involved in phospholipid and TAG biosynthesis.</th>
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<tr>
<td>Gene</td>
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<td>GPAT</td>
<td>T. pseudonana</td>
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<td>C. reinhardtii</td>
<td>S. cerevisiae (gat 1 mutant)</td>
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<tr>
<td>P. incisa</td>
<td>S. cerevisiae (gat 1 mutant)</td>
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<tr>
<td>DGAT2</td>
<td>O. tauri</td>
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<tr>
<td>T. pseudonana</td>
<td>S. cerevisiae H1236</td>
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<tr>
<td>C. reinhardtii</td>
<td>S. cerevisiae H1236</td>
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<tr>
<td>PDAT</td>
<td>C. reinhardtii</td>
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<tr>
<td>LPCAT</td>
<td>T. pseudonana</td>
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* Personal communication.
silica-starved [50]. The ability of TpGPAT to incorporate LC-PUFA into phospholipids and TAG was tested in yeast-feeding experiments. The expression of TpGPAT did not affect the incorporation of supplied EPA and DHA into either lipid group in comparison to the empty-vector control. It was thus proposed that TpGPAT catalyzes the incorporation of 16:0 at the sn-1 position of glycerol-3-phosphate and may be of value for the production of saturated TAG in genetically modified algae that have inherently higher PUFA content than needed for biodiesel production.

Extraplastidic LPAAT catalyzes sn-2 acylation of LPA and the formation of phosphatidic acid, a key intermediate in the biosynthesis of both membrane phospholipids and storage TAG. The key role of the seed-specific LPAAT in channeling unusual fatty acids to the sn-2 position of TAG in certain species of oilseed plants is well established [41 and references therein]. Moreover, increases in seed oil content have been reported following expression of foreign microsomal LPAATs; in A. thaliana, for example, expression of two microsomal LPAAT isoforms of Brassica napus resulted in enhanced oil content in the seeds [52]. Overexpression of yeast LPAAT (SLC1-1) in A. thaliana and B. napus was shown to increase seed oil content as well as alter the fatty acid composition, with higher accumulation of very long-chain fatty acids [53]. It should be noted that bioinformatics approaches have revealed the presence of candidate LPAATs in several algal genomes; however, their functions and acyl specificities have yet to be elucidated.

DGAT catalyzes the final, committed step in TAG assembly—the final acylation of diacylglycerol. DGAT is regarded as a bottleneck in TAG biosynthesis since it has the lowest activity among the Kennedy pathway enzymes [41]. Two major isoforms, DGAT1 and DGAT2, mediate this step in various organisms, including mammals, higher plants and fungi. The genes encoding the putative DGAT are present in the Chlamydomonas genome, but their functional assignment needs further characterization. Three putative DGAT candidates were identified in the genome of O. tauri, all of which appeared to belong to the DGAT2 family [44]. O. tauri is able to synthesize LC-PUFA by sequentially alternating desaturation and elongation steps which have been partially characterized by molecular tools [54,55]. The major ω3 LC-PUFA DHA (22:6) accounts for more than 25 mol% of the TAG in this microalgla and is significantly less abundant within phospholipids and glycolipids (less than 5 mol%) [44], indicating its specific channeling to TAG. Among three putative DGAT2-like gene sequences, OtDGAT2A and OtDGAT2B were most similar to those DGAT2 of higher plants that engage in the channeling of unusual fatty acids from phospholipids to TAG and thus prevent their accumulation in membrane lipids [56]. OtDGAT2C showed high homology to mammalian monoacylglycerol acyltransferases. All three candidate genes were expressed in a yeast mutant lacking DGAT1 and PDAT. Importantly, significant codon optimization was necessary to obtain a high level of algal protein expression in the heterologous host; nevertheless, two of the candidate genes, OtDGAT2A and OtDGAT2C, were virtually not active in the yeast system, even after complete codon optimization. Only expression of OtDGAT2B with a codon-optimized N terminus resulted in successful complementation of the TAG-deficient mutant phenotype, as determined by the appearance of a TAG spot on TLC plates. Moreover, the expression of OtDGAT2B in the quadruple S. cerevisiae mutant H1246, which is unable to produce neutral lipids, TAG or sterol esters [57], restored oil-body formation. TAG production was also observed in enzymatic DGAT assays in homogenates of transformed yeast cells; this activity did not discriminate between dioleoyl- and linoleoyl-CoA. By feeding the recombinant yeast with various non-radioactive fatty acids, it was also shown that OtDGAT2B accepts a wide array of acyl substrates, ranging from saturated fatty acids to PUFA and LC-PUFA, indiscriminately incorporating both ω3 and ω6 substrates into TAG. Thus, at this point, it is not likely that OtDGAT2B is responsible for the targeting of DHA to TAG. However, the results of this work suggest the emerging role of an acyl-CoA-independent pathway in this process. Research in our laboratory has shown that both acyl-CoA-dependent and independent pathways are potentially involved in TAG formation in P. tricornutum [58]. Microsomes and oil bodies of P. tricornutum were able to synthesize TAG when 1,2-[14C]oleoyl-DAG or 1-stearoyl-2-[14C]arachidonoyl-DAG were supplied as the sole acyl donors, indicating transacylase activity. DGAT with lower specific activity was also observed in both cellular fractions.

Another example of the DGAT cloned from algae is described in a patent application by Zou et al. [45]. The gene coding for T. pseudonana DGAT2 was isolated and used to transform the quadruple S. cerevisiae mutant H1246; the same strain, transformed with DGAT1 of A. thaliana, served as a positive control. It was reported that the recombinant TpDGAT2 could efficiently incorporate DHA into TAG. There is also evidence of two ways of transacylating DHA to TAG in the hyper-accumulation of TAG in a starchless mutant of this alga that was demonstrated by the increased transcription of the gene in the mutant, relative to the wild type, under conditions that induce TAG biosynthesis [Q. Hu, personal communication]. Another recent report underscored the intensive search for algal genes mediating TAG biosynthesis relates to a putative PDAT from C. reinhardtii (CpPDAT). The annotated gene, with homology to PDAT, phospholipid:sterol acyltransferase and lecithin:cholesterol acyltransferase was cloned, and its truncated sequence was expressed in the methylotrophic yeast Pichia pastoris [Q. Hu, personal communication]. Multiple catalytic functions are suggested for the recombinant CpPDAT, including transacylation of phospholipids and DAG, or of two molecules of DAG, and even lipase activity. Further information on such multifunctional enzymes is definitely of interest to the scientific community.

The identification of acyltransferases with a very strong substrate preference for EPA or DHA is important in the genetic engineering of higher plants and algae to produce desired LC-PUFA [59]. Lysophosphatidylcholine acyltransferase (LPCAT) plays a critical role in remodeling fatty acid and phosphatidylcholine (PC) pools and is important for the effective channeling of LC-PUFA to TAG. This enzyme mediates bidirectional exchange between the PC and acyl-CoA pools, and the remodeled fatty acyl chains in the form of acyl-CoA or esterified at the sn-2 position of PC can be used for TAG biosynthesis [for further information refer to 59]. LPCAT was identified in the alga T. pseudonana by a homology search of the genome with LPCAT from S. cerevisiae [60], and the deduced protein of T. pseudonana showed 27% identity to the yeast LPCAT. To confirm its function, a LPCAT-deficient yeast strain was transformed with the coding region of TpLPCAT. As described in the patent application, LPCAT function was restored in the mutant, and its sensitivity to 1-O-alkyl-sn-glycero-3-phosphocholine, which is characteristic of this mutant, was reduced. The LPCAT activity assay with yeast microsomal fractions using [14C]lysoph-PC and a range of unlabeled acyl-CoAs revealed that TpLPCAT is capable of incorporating a wide range of saturated, monounsaturated acyl-CoA, as well as 22:6 (DHA)-CoA; it showed highest preference, however, for 18:1-CoA.

3.4. Acyl-CoA synthase

Long-chain acyl synthase (LACS) activate free fatty acids to produce acyl-CoAs and play an important role in regulating the size, composition and availability of the cytoplasmic acyl-CoA pool for various enzymatic activities, including TAG biosynthesis pathway. Eight putative LACS genes were identified by analysis of the T. pseudonana genome [61]. One of these putative genes (TpiLacA) was characterized in detail and appeared to encode LC-PUFA-specific acyl-CoA synthase [61]. The ORF was heterologously
expressed in the yeast deletion strain (faa4D) featuring low background levels of acyl-CoA activation and, particularly, for the LC-PUFA, 20:5ω3 and 22:6ω3. It was shown that the recombinant protein was efficient in the activation of both ω6 and ω3 PUFA: 18:3ω6, 20:4ω6, as well as 18:4ω3 and 20:5ω3. It was thus suggested that the algal LC-PUFA synthase activity might be of use to improve the flux of acyl intermediates through the LC-PUFA biosynthetic pathway to ensure the accumulation of DHA in the genetically modified plant storage lipids.

4. Membranal glycerolipids

The biosynthesis of membrane lipids in eukaryotic microalgae often involves cooperation between the plastid and the extraplastidial compartment, with the participation of enzymes of the ER and chloroplast envelope. The current knowledge on membrane lipid biosynthesis in a model alga C. reinhardtii is summarized and described in detail elsewhere [21–23]. Extraplasmidial membranes of C. reinhardtii do not contain PC, but they do contain the non-phosphorous betaine lipid diacylglycerol-3-phosphate (DGTS), which has similar physico-chemical properties, as a major membrane component [1,21–23]. A bioinformatics approach identified a putative protein in the genome of C. reinhardtii, BTA1Cr, which is sufficient for the biosynthesis of DGTS. It was predicted by its sequence similarity to two proteins, BtaArq and BtaBms, of the bacterium Rhodobacter sphaeroides, required for the biosynthesis of DGTS [21]. BTA1Cr appeared to encode a bifunctional protein that demonstrates functional homology to the bacterial orthologs. Heterologous expression of BTA1Cr alone led to DGTS accumulation in Escherichia coli. Interestingly, BTA1Cr was present as the second major protein in the purified lipid droplet fraction of C. reinhardtii [32]. It was therefore suggested that DGTS may have role in lipid-droplet formation similar to that of PC for the oil bodies of other organisms. This assumption seems very likely since DGTS, which possesses properties analogous to PC, replaces the latter in the extraplasmidial membranes of C. reinhardtii. However, contamination in the oil-body preparations of the microalgae can not be excluded.

Previously, two other enzymes important for phosphoethanolamine biosynthesis were cloned and characterized from C. reinhardtii: diacylglyceryl-CDP-ethanolaminephosphotransferase (EPT) and CTP:phosphoethanolamine cytidylyltransferase (ECT) [62,63]. A CDNA encoding EPT was cloned from C. reinhardtii and its function was demonstrated by functional complementation in a yeast mutant deficient in both cholinephosphotransferase and ethanolaminephosphotransferase. Importantly, the EPT of C. reinhardtii was capable of synthesizing both PC and phosphoethanolamine; yet PC is not present in C. reinhardtii cells. The deduced protein of ECT of C. reinhardtii is longer than the same protein in yeast, rat or humans due to 75 amino acids forming a hydrophobic N-terminal extension. Analysis of ECT activity distribution in cellular fractions indicated mitochondrial localization of the protein in Chlamydomonas cells in agreement with the presence of a predicted mitochondrial-targeting sequence. Thus, the subcellular localization of ECT enzyme appeared to be different between mammals and photosynthetic C. reinhardtii. The function of ECT was confirmed by heterologous expression in E. coli which demonstrated the production of CDP-ethanolamine from phosphoethanolamine and CTP.

5. Desaturases and elongases

Due to the interest in metabolic engineering of oilseed plants for the production of nutritionally valuable LC-PUFA [59], a number of PUFA desaturases and elongases with various substrate and regio-selectivities have been cloned from LC-PUFA-producing algal species in the last decade [54,55,64–74]. Microalgae are primary producers of LC-PUFA, and many algal species therefore possess the machinery for sequentially alternating desaturation/elongation steps. The numerous efforts made to clone and characterize desaturases and elongases involved in LC-PUFA biosynthesis are described elsewhere, as well as their use in genetic modification of oilseed plants to reconstitute LC-PUFA biosynthesis [59 and references therein]. The main practical focus of these investigations is to provide a sustainable feedstock for human consumption as an alternative to fish oils, whose supply is decreasing and quality deteriorating. At the same time, such research has enabled an elucidation of the constraints and bottlenecks in LC-PUFA production in heterologous organisms [for further information refer to 75]. Microalgal PUFA elongases are structurally similar to the ELO family of enzymes that catalyze the condensation step of fatty acid elongation in animals and fungi [67]. These elongases are different from the higher plant condensing enzymes that participate in microsomal elongation of saturated and monounsaturated fatty acids. Three major types of PUFA elongases, regarding substrate specificity, were characterized from LC-PUFA-producing microalgae: Δ6 C18-PUFA-specific elongases were shown to be involved in the elongation of C18 PUFA (18:3ω6 and 18:4ω3) and the production of C20 LC-PUFA, ARA and EPA, while Δ5 C20-PUFA-specific elongases are engaged in the elongation of 20:5ω3 (EPA) in the pathway of DHA biosynthesis, in some marine species. In an alternative route, C18–Δ9–specific elongation of 18:2ω6 and 18:3ω3 to the respective C20 intermediates precedes sequential Δ8 and Δ5 desaturations to form ARA and EPA, respectively. For functional characterization, these enzymes are expressed in yeast where the recombinant proteins are suggested to accomplish a condensing step in conjunction with host activities of keto-reduction, dehydration and enoyl reduction. For example, an elongase gene from the marine haptophytes of the Pavlova family, being expressed in yeast, catalyzed the conversion of EPA into DPA (22:5ω3) [66,70]. This enzyme appeared to be specific towards both ω6 and ω3 C20-PUFA substrates and did not show activity towards C18- or C22-PUFA substrates. The consecutive activity of Δ4 desaturase acting on DPA is necessary to accomplish DHA biosynthesis in microalgae, differently from the Δ4 desaturase-independent Sprecher pathway [76] operating in mammals and fish. In the latter pathway, DPA is further elongated to 24:5ω3, followed by Δ6 desaturation to 24:6ω3 and chain shortening via β-oxidation to DHA in peroxisomes [67]. Several genes encoding for Δ4 desaturases were cloned from DHA-producing microalgal species [65,66,69]. The PsaD4Des gene of P. salina was able to desaturate both 22:4ω7,10,13,16 and 22:5ω7,10,13,16,19 at the Δ4 position when expressed in yeast [66]. The Δ4 desaturase of Euglena gracilis showed strict Δ4-regioselectivity and required the presence of a Δ7-double bond in the substrate; the enzyme featured a broad substrate specificity and activity was not limited to C22 PUFA and also efficiently desaturated C16 PUFA such as 16:3 ω3,ω7,ω10,13 [65]. Some those candidate genes were used as a terminal activity to reconstitute DHA biosynthesis in higher plants.

In some primitive marine eukaryotes of the Thraustochytridae family, the alternative polyketide synthase pathway (PKS) catalyses the production of LC-PUFA [77]. The PKS pathway does not require aerobic desaturation while the double bonds are introduced during the process of fatty acid synthesis. The PKS pathway is predominant in Schizochytrium, whereas a desaturation/elongation pathway acts in Thraustochytrium of the same family [78,79]. The homology between the prokaryotic Sphingobium and eukaryotic Schizochytrium PKS genes suggested that the PUFA PKS pathway has undergone lateral gene transfer [77]. Further, we provide a few examples of unusual algal desaturases that are not involved in LC-PUFA biosynthesis and an update of the
latest developments in the cloning of acyl-CoA-dependent desaturases from microalgae. An unusual front-end desaturase (containing a cytochrome b5-like domain at its N terminus) was cloned from the marine diatom *T. pseudonana* based on the set of genomic DNA sequences putatively encoding front-end desaturases in this alga [80]. The deduced amino acid sequence of TpDES displayed typical features of microsomal desaturases involved in the biosynthesis of LC-PUFA, i.e. a fused N-terminal cytochrome b5 domain and three conserved histidine-rich motifs. However, this desaturase was specifically active on saturated 16:0, desaturating it to 16:1<sup>15</sup>ω when expressed in yeast, but was not active on supplemented PUFA. Interestingly, this Δ11-desaturase activity had been previously detected only in insect cells. The identification of such a novel enzyme in photosynthetic organisms expands the functional repertoire of the membrane-bound algal desaturases.

Another interesting case is a FAD6 from the diatom *P. tricornutum* [81]. This alga is widely used in aquaculture due to its content of ω3 eicosapentaenoic acid (EPA; 20:5<sup>ω3,6,9,12,15</sup>). The pathways of EPA biosynthesis have been previously described and genes involved in EPA biosynthesis have been cloned and characterized [64]. The chloroplastic lipids of this alga also contain an unusual isomer of ω6 hexadecatrienoic acid (16:3<sup>ω6,9,12</sup>), rather than the ω3 hexadecatrienoic acid (16:3<sup>ω3,6,9</sup>), and ω3-linolenic acid (18:3<sup>ω3,6,9</sup>), which are common in chloroplast membrane lipids of higher plants. The gene encoding PtFAD6 was cloned and expressed in a strain of the cyanobacterium *Synechococcus*, which is characterized by a simple fatty acid composition and the absence of di- and trienoic fatty acids. The heterologous expression of PtFAD6 in the photosynthetic organism resulted in the production of 16:2<sup>Δ6,Δ9</sup> in support of the suggestion that it encodes a plastidial enzyme which requires ferredoxin/ferredoxin oxidoreductase as electron donors. In contrast, the microsomal desaturase PtFAD2 was shown to be active when expressed in yeast and specific for oleic 18:1<sup>ω9</sup> acid, indicating that PtFAD2 requires cytochrome b5/cytochrome b5 oxidoreductase and is likely involved in the biosynthesis of EPA in the ER. PtFAD6 appeared to have a longer N-terminal extension than the protein encoding a plastidial FAD6 desaturase from *Brassica napus*. The N terminus of PtFAD6 contains two domains; one is basic with an Arg in the third position and contains a hydrophobic portion similar to a classical ER-targeting signal. This domain is followed by a domain rich in hydroxylated amino acids (Ser and Thr), which is typical for chloroplast transit peptides involved in transport into the complex plastids of diatoms, surrounded by four membranes. The plastid origin of PtFAD6 was confirmed when the N-terminal 113 amino acids were fused with EGFP (enhanced green fluorescent protein) and expressed in *P. tricornutum* cells.

Elucidation of algal genome sequences has accelerated the identification of PUFA desaturases and their functional characterization in yeast and higher plants. Recently, it was shown that some algal front-end Δ6 and Δ5 desaturases act on PUFA esterified to CoA, similar to mammalian front-end desaturases. Currently, the examples include Δ6 desaturases from the microalgae *O. tauri* [54], *O. lucimarinus* [71] and *M. pusilla* [74], and Δ6 and Δ5 desaturases from *Mantoniella squamata* [55]. The acyl-CoA-dependent enzymes insert a double bond into CoA-activated fatty acid from the extraplastidal acyl-CoA pool; this is distinctly different from phospholipid-linked desaturases, which require a fatty acid to be attached to PC or phosphoethanolamine. This feature is of great importance considering current intensive use of acyl-CoA-dependent desaturases in research on genetic modification of oilseed plants. The use of the acyl-CoA-dependent enzymes with the correct substrate specificities may eliminate the requirement for the rate-limiting acyl-exchange with PC and thus avoid “substrate dichotomy” for lipid-linked desaturases and acyl-CoA-dependent elongases [for further information refer to 59,75].

Moreover, most of the recently cloned algal acyl-CoA-dependent desaturases are highly specific for their ω3 substrates when expressed in yeast [55,74], apart from the Δ6 desaturase of the alga *O. tauri* which desaturated ω6 and ω3 substrates with similar efficiency [54]. The strong and beneficial preference for the ω3 substrate was utilized in research aiming to the metabolic engineering of ω3 LC-PUFA (EPA and DHA) biosynthesis in plants [55,74]. The Δ6 desaturase from the marine microalga *M. pusilla* was shown to be very efficient in heterologous systems: this enzyme displayed 3.5-fold higher conversion efficiency for the ω3 substrate ω3-linolenic acid (18:3<sup>ω3,6,9</sup>) over the ω6 substrate linolenic acid (18:2<sup>ω6,9</sup>) [74]. The preference for ω3 substrate was also confirmed in a competition experiment employing both substrates. This enzyme was used for metabolic engineering of an EPA-biosynthesis pathway in *plants*: it was coexpressed with two more algal enzymes, the Δ6 elongase of the microalga *Pyramimonas cordata* and the Δ5 desaturase of the haptophyte *Pavlova salina* (Fig. 2). This was performed by simultaneous expression of single genes in Cauliflower mosaic virus (35S CMV)-driven constructs in tobacco (*Nicotiana benthamiana*) leaf tissue in the presence of the P19 viral suppressor and DGA1 of *A. thaliana* to direct produced fatty acids into TAG. The novel leaf-based assay [82] was developed for the rapid<sup>3</sup>/<sup>4</sup> desaturase expression of multistep pathways to identify effective gene combinations for the production of desired VLC-PUFA and indeed, it resulted in 26% EPA in the leaf TAG [74]. In addition, preference for ω3 was also observed in stable Arabidopsis transformations with an ω3/ω6 ratio as high as 4.5 in T2 seeds. This pathway was further extended to the production of EPA by the addition of the Δ5 20:4 EPA elongase and the Δ4 desaturase [83] (Fig. 2). Earlier study by the same group demonstrated the production of ω6 and ω3 LC-PUFA, ARA, EPA and DHA from endogenous tobacco linoleic and α linolenic acid by five transgene-encoded reactions [82]. The recombinant ω6 and ω3 pathways were initiated with the action of *Isochrysis galbana* Δ9 elongase on

![Fig. 2. Transient reconstitution of the EPA- and DHA-biosynthesis pathway, consisting of algal enzymes, in tobacco leaf](74) [74]. The pathway initiates with the action of *M. pusilla* Δ6 desaturase with a ω3 preference on the endogenous ALA of tobacco leaves [82,83]; ALA, ω3-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid.
the endogenous fatty acids of tobacco leaves. The algal enzymes used in that study were the l. galbana Δ9 elongase, which elongates 18:2Δ9,12 to 20:2Δ11,14 and 18:3Δ9,12,15 to 20:3Δ11,14,17 in the so-called alternative pathway, and four sequential enzymes engaged in the sequential processing of 20:2Δ11,14 and 20:3Δ11,14,17, Δ8 desaturase, Δ5 desaturase, Δ5 elongase and Δ4 desaturase of P. salina. Analysis of leaf TAG demonstrated that it contained 37% newly synthesized LC-PUFAs, including 7% ARA, 6% EPA and 3% DHA.

The focus of current research into modifying the fatty acid composition of seed oils consists of the search for novel highly efficient enzymes from low eukaryotes, including algae, the search for efficient means to avoid bottlenecks in LC-PUFA production and for channeling them towards TAG assembly [59]. The similar approaches are applicable to the genetic modification of microalgae for a desired LC-PUFA composition.

6. Microsomal elongation of saturated and monounsaturated fatty acids

Elongation of saturated and monounsaturated fatty acids (mainly 16:0 and 18:1) to very-long-chain C20 and C22 fatty acids (VLC-FA) in higher plants occurs in the ER and is mediated by the fatty acid elongase complex. Fatty acid elongation involves the activities of four consecutive enzymatic reactions (condensation, reduction, dehydration, and a second reduction) and results in a two-carbon extension per cycle. In higher plants, microsomal elongation activity is important for the production of long-chain saturated and monounsaturated fatty acids (LC-FA), which are common in the cuticle, surface waxes and TAG of some oilseed plants. The first enzyme of the fatty acid elongase complex, β-ketoacyl-coenzyme synthase (KCS), catalyzes the rate-limiting condensation reaction of the acyl primer with malonyl-CoA [84]. The other three components of the elongase complex (the 3-ketoacyl-CoA reductase, the 3-hydroxyacyl-CoA dehydratase, the enoyl-CoA reductase) were identified and characterized in yeast and A. thaliana [26 and reference therein]. The activities of Arabidopsis genes were confirmed by means of a reverse-genetics approach utilizing yeast deletion mutants deficient in each of the activities.

In A. thaliana the KCS gene family, consisting of 21 members, was discovered by genome annotation [85]. Several of these enzymes were shown to be functional, as they were expressed in yeast [86] and revealed distinct patterns of substrate selectivity and product chain length, as well as differential sensitivity to oxysterol and chloroformanilide herbicides [87]. To date, relatively little is known about the genes and enzymes mediating microsomal elongation of saturated and monounsaturated fatty acids in microalgae. Some species of green algae demonstrate very high sensitivity to herbicides targeting KCS. Severe inhibition of radio-labeled 18:1 incorporation into the non-soluble “non-lipid” fraction occurred in some green algal species as a result of administration of chloroformamid [84]. This fraction appeared to contain sporopollenin, an acid-resistant complex material of the cell wall in green algae. It was further proposed that oleic acid serves as a precursor of sporopollenin and that KCS activity is important for its formation [88,89]. In addition, effects of chloroformamides on fatty acid composition of membrane lipids were also demonstrated.

Furthermore, it was suggested that C18 fatty acids are produced by KCS-mediated elongation of 16:0-CoA in the ER of the marine haptophyte Pavlova lutheri. The elongation of 16:0-CoA was implicated as a key intermediate step in the biosynthesis of the LC-PUFA EPA and DHA [90]. Immunodetection analysis with antibody raised against a fusion protein containing the central portion of the KCS of Brassica napus showed that a KCS-like enzyme is present in the microsomes of the alga. The activity of the condensing enzyme assayed in the microsomal fraction of the alga preferred 16:0-CoA over shorter, longer or monounsaturated acyl substrates. The gene Pleio 1 (Pavlova lutheri elongase 1) was cloned and its deduced protein showed strong similarities to higher plant KCS. It was further suggested that the highly active elongation reaction provides additional substrate (18:0-CoA) for further desaturation and consequently, for VLC-PUFA biosynthesis. Several lines of evidence supported this assumption: the abundance of 16:0-CoA in the acyl-CoA pool, and the increased transcript abundance of Pleio 1 in the stationary phase which was associated with a high level of microsomal elongase activity. However, no data on heterologous expression have yet been provided.

The salt-inducible KCS was cloned from the halotolerant green microalga Dunaliella salina exposed to 3.5 M NaCl [91]. Similar to the KCS activity of P. lutheri, that of D. salina favored saturated medium-chain acyl-CoA, with a preference for 16:0-CoA over the monounsaturated 16:1- and C18:1-CoAs. The function of the D. salina KCS gene product was demonstrated by the presence of condensing activity in the recombinant E. coli. It was suggested that the C16 elongase activity in conjunction with further desaturation steps is important for the adaptive membrane changes required for proper membrane function at high internal glycerol concentrations [91]. The putative KCS that was presumably involved in microsomal elongation of medium-chain fatty acids was also isolated from the green microalga P. incisa (Khozin-Goldberg, unpublished). Homologs of higher plant KCS can also be found in the genome of C. reinhardtii and of several diatoms. It should be noted that KCS genes of green algae code for deduced proteins with an unusual N-extension upstream of the two transmembrane regions, typical of higher plants’ microsomal KCS.

7. Conclusions

In conclusion, our knowledge on lipid metabolism at the molecular level in eukaryotic microalgae is largely incomplete. Genes encoding lipid-biosynthesis enzymes are present in various algal genomes. However, their biochemical analysis is essential to verify their function. We believe that in the near future, research into lipid metabolism of microalgae will advance rapidly, thanks to the development of indispensable molecular tools for algal gene characterization.

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