STRESS-INDUCED NEUTRAL LIPID BIOSYNTHESIS IN MICROALGAE – MOLECULAR, CELLULAR AND PHYSIOLOGICAL INSIGHTS

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ABSTRACT

Photosynthetic microalgae have promise as biofuel feedstock. Under certain conditions, they produce substantial amounts of neutral lipids, mainly in the form of triacylglycerols (TAGs), which can be converted to fuels. Much of our current knowledge on the genetic and molecular basis of algal neutral lipid metabolism derives mainly from studies of plants, i.e. seed tissues, and to a lesser extent from direct studies of algal lipid metabolism. Thus, the knowledge of TAG synthesis and the cellular trafficking of TAG precursors in algal cells is to a large extent based on genome predictions, and most aspects of TAG metabolism have yet to be experimentally verified. The biofuel prospects of microalgae have raised the interest in mechanistic studies of algal TAG biosynthesis in recent years and resulted in an increasing number of publications on lipid metabolism in microalgae. In this review we summarize the current findings on genetic, molecular and physiological studies of TAG accumulation in microalgae. Special emphasis is on the functional analysis of key genes involved in TAG synthesis, molecular mechanisms of regulation of TAG biosynthesis, as well as on possible mechanisms of lipid droplet formation in microalgal cells.

Keywords: triacylglycerol (TAG), lipid, microalgae, diacylglycerol: acyltransferase (DGAT), phospholipid:diacylglycerol acyltransferase (PDAT), lipid droplet (LD).

1. Introduction

Photosynthetic algae, especially microalgae have been of considerable interest as a possible biofuel resource thanks to their ability to produce and store substantial amounts of energy-rich compounds, primarily triacylglycerol (TAG) and starch, which can be utilized for the production of several distinct biofuels such as biodiesel and ethanol [1]. A key feature of algae is that rapid formation of lipid droplets (LDs) occurs in response to different environmental stresses, while in plants TAG accumulation is generally under developmental control, although it can also be induced in response to freezing or aging in photosynthetic tissues [2, 3]. A commonly discussed and biotechnologically used cue to induce TAG biosynthesis is nitrogen (N) deficiency. Thus, identification of key genes governing TAG synthesis in algal cells has been extensively pursued by comparing global gene expression in N-replete and N-deprived cells of different microalgal species [2, 4]. Functional analysis of potential candidate genes predicted by these studies is currently one of the leading approaches in deciphering the mechanisms of algal lipid metabolism.

Reconstruction of TAG biosynthesis pathways and cellular trafficking of lipids in algal cells is mostly based on genome predictions and orthologous relationship to enzymes of known function in animals, yeast, and especially seed plants. However, closer scrutiny starting many years back with studies by Giroud and colleagues in *Chlamydomonas reinhardtii* [5], and more so during the last decade has provided evidence that fatty acid and TAG biosynthesis in algal cells has several features distinct from that of plants. As will be highlighted below, the bulk of TAG biosynthesis in microalgae occurs in photosynthetic cells, not specialized cells as predominantly in plants, complicating matters with regard to the regulation of carbon partitioning within a single cell. Accumulation of TAGs in response to stress conditions in unicellular microalgae encompasses gradual chloroplast degradation and re-organization of membrane structures and remodeling of the lipidome. With exceptions already mentioned above, TAGs are generally synthesized in specialized tissues of seed plants including developing seeds, fruits, or pollen grains and are stored in LDs, which are mobilized during active periods of metabolism (e.g. germination) [6, 7]. Many aspects of lipid metabolism seem to differ also between distinct species of oleaginous microalgae, reflecting the high evolutionary diversity

among algal genera [8]. In this review we present the most recent data on neutral lipid metabolism in model microalgal species and compare it to that of seed plants where appropriate.

2. TAG synthesis pathways in algae

By analogy to plant cells the synthesis of fatty acids in microalgae is generally thought to take place in the plastid (Figure 1). Following the export to the cytosol, fatty acids enter the TAG assembly pathway catalyzed by enzymes associated with the endoplasmic reticulum (ER) [8].

In addition, *de novo* TAG assembly in the chloroplast has been recently proposed to occur in *C. reinhardtii* following N deprivation of a starch-deficient mutant, raising the question of novel TAG assembly pathways in microalgae not observed in seed plants [9]. Until this hypothesis is further corroborated or refuted, one has to consider that parallel TAG assembly pathways in the plastid envelopes and the ER may exist (Figure 1). In fact, an increasing number of putative plastid-targeted acyltransferases in distinct microalgal genomes provides potentially supporting evidence for a chloroplast TAG assembly pathway [10, 11]. Unlike phospholipids found in biological membranes, TAGs do not perform a structural role in cells, but instead serve as a storage form of energy and carbon. After being synthesized, TAGs are deposited in discrete spherical structures, the LDs, located in the cytoplasm or chloroplast (Figure 1). Whether the latter are distinct from plastoglobuli, carotenoid-rich LDs present in plant chloroplasts remains to be seen.

Based on predicted orthology of genes identified in algal genomes, at least two major pathways of TAG synthesis have been proposed to function in microalgae: one is the Kennedy or glycerol phosphate pathway, the other is the monoacylglycerol pathway (Figure 2). In both pathways TAGs are formed by esterification between an acyl-CoA and a glycerol hydroxyl group. In the Kennedy pathway, the first acyl chain is esterified to glycerol-3-phosphate derived from the glycolytic intermediate dihydroxyacetone phosphate (DHAP). The enzyme is glycerol-3-phosphate-acyltransferase (GPAT) which forms lysophosphatidic acid, the substrate for the second acyltransferase, lysophosphatidic acid acyltransferase (LPAAT). This second reaction leads to the formation of phosphatidic acid, which is a central lipid metabolite giving rise to phospholipids and TAGs. Prior to conversion to TAGs, phosphatidic acid is dephosphorylated by a phosphatase to form diacylglycerol (DAG). The monoacylglycerol pathway starts with 2-

1 monoacylglycerol (*sn*2-MAG), which is converted to DAG by a monoacylglycerol
2 acyltransferase (MGAT). *De novo* synthesis of monoacylglycerol (MAG) in plant cells has been
3 previously described [12, 13] and was shown to be catalyzed by GPAT4 and GPAT6 enzymes,
4 which use $\alpha\omega$ -dicarboxylic and ω -hydroxy acyl-CoA as acyl-donors for production of *sn*2-MAG
5 [12, 14]. The MAGs have been considered initially as precursors for synthesis of waxy polymers;
6 however, their conversion to DAGs has been reported in *Nicotiana bethamina* leaves [14] and
7 recently in fruits of *Myrica pensylvanica* [15].

8 The DAG synthesized by either pathway is then converted to TAG. This enzymatic
9 reaction is catalyzed by an acyl-CoA:DAG acyltransferase (DGAT), which is considered to be
10 the committed step in the biosynthesis of TAGs [16].

11 The third pathway of TAG synthesis is catalyzed by phospholipid:diacylglycerol
12 acyltransferase (PDAT), in which a phospholipid [17] or, as recently shown, a galactolipid [18,
13 19] acts as an acyl donor in defiance of the original name of the enzyme. The PDAT-mediated
14 pathway of TAG formation has been shown in yeast and plants [17, 18]. PDAT activity in plants
15 was demonstrated in a few oil seed species, however its contribution to TAG biosynthesis varied
16 between them. PDAT-encoding genes have been identified in all microalgae studied (Figure 1),
17 suggesting that acyl-CoA-independent mechanisms of TAG synthesis exist also in microalgae.

18 19 **3. Available genomes reveal the complexity of the TAG synthesis machinery in** 20 **microalgae**

21
22 To date, numerous microalgae have been screened for high lipid content and many oleaginous
23 species have been isolated and characterized under laboratory or outdoor culture conditions. At
24 time of writing, the genomes of 21 microalgae have been determined and partially annotated, and
25 undoubtedly more algal genome projects are currently in progress. The most promising
26 microalgae with deciphered genomes of great interest as models for research on lipid synthesis
27 and TAG accumulation include the green alga *C. reinhardtii* [20], several species of *Chlorella*
28 [21], *Ostroecoccus tauri* [22], several strains and species of the heterokont microalga
29 *Nannochloropsis* [10, 23-25], and the diatom *Phaeodactylum tricornutum* [26]. In spite of the
30 fact that it is generally not considered an oleaginous alga, *C. reinhardtii* is still the most
31 extensively studied microalga at the molecular and physiological level with regard to lipid

1 biosynthesis and TAG accumulation, mainly because of its simple life cycle, amenability to
2 genetic analyses due to the availability of sexual crossing, well developed transformation tools
3 for both nuclear and plastid genomes, as well as gene silencing by RNA interference [8, 27, 28].
4 Recently, considerable progress in the efficiency of genetic transformation has also been made
5 for other unicellular microalgae, including the diatom *P. tricornutum* [29, 30] and
6 *Nannochloropsis* [10, 31]. The availability of molecular and genetic tools will be essential for a
7 comprehensive characterization of key genes involved in lipid biosynthetic pathways in other
8 microalgae of interest.

9   In order to reconstruct lipid biosynthesis pathways, the genomes obtained for different
10 microalgae were primarily analyzed in comparison to genomes of plants for which biochemical
11 data are available. Indeed, most of the plant orthologues encoding key enzymes acting in fatty
12 acid biosynthesis in plastids were predicted in algal genomes. Those include genes for acetyl-
13 CoA carboxylase (ACCase), fatty acid synthase (FAS) subunits as well as pyruvate
14 dehydrogenase complex (PDC), which were present in *C. reinhardtii* [32-35], *Chlorella* [21],
15 *Nannochloropsis* [10] or *P. tricornutum* [26].

16   Both, plants and green algae seem to share all main acyl-CoA-dependent acyltransferases
17 that cooperate in TAG synthesis, however significant evolutionary divergence can be observed at
18 the level of their gene copies in non-green algae (Figure 2). Nine putative GPAT and LPAT
19 encoding candidates were identified in both *N. oceanica* [10] and *N. gaditana* [23] genomes.
20 Noteworthy, only one copy of a GPAT gene and one copy of an LPAT gene have been found in
21 the *C. reinhardtii* genome [25]. In turn, one gene encoding a putative GPAT and two encoding
22 putative LPATs have been identified in the genome of *O. tauri* [36]. In the Arabidopsis genome
23 nine extraplastidic GPATs and five LPAT genes have been found. In case of GPATs, eight of
24 them are unique to land plants, and are not present in animals, fungi or microalgae [37].
25 Surprisingly, only one gene encoding an ER-associated GPAT has been suggested as the best
26 candidate for the ER lipid assembly pathway in Arabidopsis [38] and no clear evidence for
27 LPAT activity in TAG synthesis has been obtained so far in Arabidopsis. Data on phosphatidic
28 acid phosphatase (PAP) homologues in algal genomes are to date still scarce. However, in the *C.*
29 *reinhardtii* genome three homologues of plant PAP-encoding genes were identified [39]. With
30 the exception of *C. reinhardtii*, the lack of known PAP homologues in oleaginous microalgae

1 results in poor functional characterization of these enzymes, which is an important gap in
2 reconstructing the TAG biosynthesis pathway.

3 Much more attention has been paid to identifying the algal homologues of key enzymes
4 directly responsible for TAG synthesis, the DGATs. In most of eukaryotic species DGATs have
5 been considered to be the primary enzyme for *de novo* TAG biosynthesis. At least one copy each
6 of two different types of DGATs have been identified, thus far, in most organisms and are
7 designated as DGAT1 and DGAT2 (or DGT1), respectively. They do not share high protein
8 sequence similarities, and they seem to play non-redundant roles in TAG synthesis and localize
9 to different domains of ER in plants [40]. Most probably DGAT1 and DGAT2 evolved
10 separately but they catalyze similar reactions as the result of convergent evolution [41].

11 In the genome of most model microalgae sequenced thus far, a single gene copy encoding
12 type 1 DGAT has been identified as found in plants and animals, with exception of *N. oceanica*
13 IMET1, where two isoforms of DGAT1 were found [11], and *O. tauri* where no DGAT1-
14 encoding homologue was identified [42]. Interestingly, a single DGAT1 predicted for the *P.*
15 *tricornutum* genome exhibits more similarity to plant DGAT1 than to other algal DGATs [43].
16 Moreover, *PtDGAT1* can give rise to two different transcripts, *PtDGAT1short* and
17 *PtDGAT1long*, differing by a 63 bp insertion, which causes the loss of DGAT1 activity in
18 *PtDGAT1long*. Therefore, only the shorter transcript encodes an active DGAT1 in *P.*
19 *tricornutum*. The alternative splicing of pre-mature *PtDGAT1long* mRNA is suggested to occur
20 in response to N deprivation, resulting in the formation of functional DGAT1 [43].

21 DGATs of type 2 (DGT2s) in microalgae are a remarkable exception among living
22 organisms, since most microalgae contain multiple copies of the respective genes, ranging from
23 2 in *O. tauri* [36] to up to 12 in *N. oceanica* CCMP1779 [10] (Table 1). However, it should be
24 noted that some of the predicted microalgal DGT2s could be in fact monoacylglycerol
25 transferases (MGATs), since it is impossible to distinguish them based on sequence information
26 alone. The latter could imply that a monoacylglycerol pathway of TAG synthesis exists in
27 unicellular algae. Moreover, DGT2s annotated thus far in algal genomes are very divergent from
28 each other, even among isoforms found in the same species. For example, of the three presumed
29 DGT2 paralogs identified in *Chlorella* sp. one is closer related to DGT2s from animal and fungi,
30 whereas two other show more similarity to their plant counterparts [41]. Divergent phylogenetic
31 relationships between DGT2s were also reported for *O. tauri* [42] and for *P. tricornutum*, where

four putative DGTTs encoding genes were found, but which lack extensive sequence similarity [44]. *N. oceanica* represents thus far an organism with the highest number of DGTT genes among known genomes [10, 25]. Originally, it was suggested that the amplification of DGTT genes found in the genomes of present-day microalgae might have arisen by repeated duplication events [41]. However, detailed phylogenetic analyses of algal DGATTs seem to speak against such a scenario and suggest their multiple origins [45]. Indeed, a recent study of evolutionary relationships among *Nannochloropsis* sp. DGATs [25] showed that separation of DGAT1s and DGTTs occurred probably very early during algae evolution and that the presence of exceptionally numerous copies of DGTTs in modern *Nannochloropsis* strains may be the result of repeated endosymbiosis events between a heterotrophic secondary host and green- or red algae.

Previous phylogenetic analyses of 117 DGATs from 70 organisms [46] comparing the amino acid sequences, showed that DGATs of type 1 and 2 cluster species-independently into two different clades. Furthermore, sequence motifs for either DGATs of type 1 or 2 were identified and proline and phenylalanine residues were found to be conserved among all DGATs [46]. The phylogenetic analysis of type 1 DGATs (Fig. 3a) comprised 19 DGATs from 14 different organisms, including animals, plants and algae (Supplemental Table 1). Most of the algal DGATs of type 1 are not closely related to their animal or plant counterparts (Fig. 3a) and form separate branches. Fig. 3b shows the evolutionary relationships between algal DGTTs and DGATs of type 2 from 24 different organisms (Supplemental Table 2). Type 2 DGATs from plants, animals and fungi cluster in distinct clades and none of the algal DGTTs groups directly with them (Fig. 3b). Similar observations have been previously reported for DGTTs of other microalgae [45]. Interestingly, multiple DGTTs from *N. oceanica* CCMP1779 are spread over the entire phylogenetic tree and do not form a distinct group. Moreover, they also do not directly cluster with their animal, plant or fungi counterparts, but rather with other algae and oomycete DGTTs and seem to be more closely related to DGTTs from other algal species than to each other. All these observations fit well with the heterogeneous origin of algal type 2 DGATs proposed previously in [45] and [25]. Consequently, a close phylogenetic relationship can be observed among DGTTs from organisms constituting the phylum Chromalveolates, including diatoms, oomycetes and Eustigmatophyceae, like *Nannochloropsis*, which is thought to have

1 evolved by secondary endosymbiosis of a secondary eukaryotic host, a cryptic prasinophyte-like
2 green algae and a red algae [47, 48].

3 One predicted PDAT encoding gene has been found in most of the available microalgal
4 genomes, with the exception of *Nannochloropsis* sp. where two putative PDAT-encoding genes
5 were identified. The PDAT of *C. reinhardtii* is similar in sequence to Arabidopsis PDAT1 and
6 yeast LRO1 [28] and was predicted to reside in chloroplasts [19]. Interestingly, the algal PDATs
7 are phylogenetically divergent from land plant PDATs and form a separate clade [49]. The
8 divergent gene structure of microalgal PDATs analyzed thus far suggests different mechanism of
9 TAG synthesis in the PDAT-mediated pathway in microalgae when compared to plants.

11 **4. Functional characterization confirms a diverse nature of microalgal TAG synthesis** 12 **enzymes**

14 Recent development of molecular tools has opened a new chapter in microalgae engineering and
15 has enabled state-of-the art algal cell biology. Considerable progress in the functional analysis of
16 genes involved in TAG synthesis has been made during the recent decade leading to novel
17 insights into the cellular and molecular mechanisms of this process in microalgae.

18 The functional characterization of the acyltransferases of the TAG assembly pathway,
19 GPAT, LPAT, and PAP is still limited to single studies in microalgae. In *C. reinhardtii* none of
20 the GPATs and LPATs has been characterized so far, however indirect evidence for the
21 importance of GPAT in TAG biosynthesis has been recently reported [50] as overexpression of
22 GPAT from the fresh-water algae *Lobosphaera incisa* in *C. reinhardtii* resulted in up to 50%
23 increase of TAG content. Transcriptomic studies of *C. reinhardtii* following N deprivation also
24 showed that an LPAT-encoding gene is strongly up-regulated. Its repression resulted in a 20%
25 decrease of neutral lipid content, suggesting its role in TAG biosynthesis [51].

26 Noteworthy, among three *C. reinhardtii* PAP - genes two, *CrPAP1* and *CrPAP2*, are up-
27 regulated following N deprivation, suggesting their potential role in TAG accumulation [2]. The
28 evidence for direct regulation of TAG synthesis by ER-localized PAP in *C. reinhardtii* has been
29 recently reported [39]. Silencing of the *CrPAP2* gene using an RNAi approach resulted in
30 reduced TAG content, whereas its over-expression was accompanied by an increase TAG
31 content following N deprivation. This suggests that the *CrPAP2* gene contributes to cellular

1 TAG synthesis in microalgae, probably acting as one of the ER-localized enzymes of the TAG
2 assembly pathway. On the other hand, studies based on the molecular species composition of
3 TAGs [9] seemed to suggest that chloroplast-derived DAGs may be the substrate for nutrient
4 stress-induced TAG formation in plastids of *C. reinhardtii*, giving rise to the hypothesis of a
5 plastid assembly pathway for TAG synthesis. In this context, it seems possible that the plastid
6 isoform of PAP can also be a key contributor during the switch from polar lipid to TAG
7 synthesis at chloroplast envelope membranes in response to nutrient deprivation.

8 DGATs and PDATs are considered the two major enzymes involved in the final steps of
9 TAGs synthesis in microalgae. Hence, recent studies focused on addressing their function in
10 heterologous eukaryotic systems, mostly plants and yeast. Identifying the best candidates from
11 multiple and often divergent DGAT-encoding genes in most microalgae for the engineering of
12 TAG content is essential for biotechnological purposes.

13 A set of new functional data became recently available for DGATs from several
14 microalgae, especially *C. reinhardtii* and *P. tricornutum*. The initial criterion for selecting the
15 potential DGATs of interest in microalgae is its expression profile after inducing TAG synthesis,
16 usually by N depletion of the culture medium. Interestingly, not all DGAT-encoding genes in
17 algae are induced following N deprivation, which is clearly visible in strains encoding multiple
18 DGAT isoforms, like *Nannochloropsis* or *C. reinhardtii* (Table 1). Thus, not all predicted algal
19 DGATs seem to be equally involved in TAG synthesis following nutrient stress. This is
20 consistent with different predicted cellular locations of DGATs (especially of DGTTs) and
21 suggests that some of them could act in other, as of yet unknown, cellular pathways of lipid
22 synthesis. Among 6 DGATs encoded by the *C. reinhardtii* genome, one was predicted to be
23 located in the chloroplast, three associated with the ER, one in mitochondria [34, 52]. Similarly,
24 of 13 putative DGATs found in *N. oceanica* IMET1, only four DGTTs were predicted to be ER
25 associated, and only one in the chloroplast (together with one of the two DGAT1 copies). For the
26 remainder of the DGTTs as well as for one DGAT1, other cellular locations were predicted [25].

27 Table 1 presents a summary of literature data accessible so far on the number of up-
28 regulated DGAT-encoding genes in several microalgae and their functional characteristics. Clear
29 transcriptional response following N deprivation has been shown for DGAT1 in *C. reinhardtii*
30 and in *P. tricornutum* as well as for one of two DGAT1-encoding genes in *N. oceanica* IMET1
31 [4, 11, 43]. A rapid up-regulation of DGAT1 and DGTT1-encoding genes has been also

confirmed by two independent studies in *C. reinhardtii* under different nutrient stress conditions, including N deprivation [2, 4], suggesting the prominent role for these respective enzymes in TAG synthesis.

Functional assignment of type 1 DGATs in microalgae is still relatively poor when compared to type 2 DGATs (DGTTs). The most complete set of data on the functional nature of algae DGTTs comes so far from studies in *C. reinhardtii*. Among five DGTT-encoding genes found in this microalgae, three of them - *CrDGTT1*, *CrDGTT2* and *CrDGTT3* exhibit higher expression in response to N deprivation [2, 4]. However, overexpression of DGTT-encoding genes in *C. reinhardtii*, did not result in a strong increase in TAG content, neither under standard growth condition nor following N-deprivation [53]. Heterologous expression of *CrDGTT1*-*CrDGTT3* in a yeast *dgal1 lro1* TAG-deficient mutant restored the TAG phenotype, however the highest efficiency in TAG production was observed for *CrDGTT2* (Table 1) [54]. The overexpression of this DGTT-encoding gene in Arabidopsis resulted in more than 20-fold increase in TAG content in the leaves [55]. This strongly suggests that *CrDGTT2* is one of the most promising DGTT-encoding candidates for further experimental strategies to maximize oil production not only in algae, but also in other eukaryotic systems. Interestingly, silencing of *CrDGTT1* and *CrDGTT5* resulted in the decrease of lipid content by 16%–24% or 28%–37%, respectively, confirming their critical role in TAG synthesis. The fact that no detectable change in TAG content was observed after down-regulation of *CrDGTT2* and *CrDGTT3* is consistent with the assumption that not all DGTTs are equally involved in TAG synthesis in microalgae and it likely attests of their redundancy.

Relatively little information on the functional nature of DGTTs can be found for other microalgae. Early studies on the functional characterization of three putative DGTTs identified in the *O. tauri* genome showed that only *OtDGAT2B* is able to restore the TAG phenotype in the TAG-deficient yeast mutant, exhibiting low specificity towards the acyl substrates [42]. In *P. tricornutum* only one of the DGTT-encoding genes, *PtDGAT2B*, has been effective in restoring the TAG phenotype in the TAG-deficient yeast mutant (Table 1) [44]. However, considerable differences were observed in expression levels between *PtDGAT1* and *PtDGAT2B*. A clear response following N deprivation was observed for *PtDGAT1* expression, but not for *PtDGAT2B*, whereas under N-replete conditions *PtDGAT2B* was strongly expressed. One must assume that *PtDGAT1* is directly involved in TAG synthesis in response to N deprivation

1 whereas *PtDGAT2B* could play a role in TAG accumulation under nutrient-replete growth
2 conditions [44]. Depletion of N has also been shown to be a potent inducer of TAG synthesis and
3 DGAT gene expression in *Chlorella pyrenoidosa* [56]. Strong positive correlation between
4 expression profiles and TAG content has been shown for three putative DGATs encoded in the
5 genome of this algae, however no data on their functional characterization has been reported so
6 far. The dramatic increase of DGAT enzyme abundance in response to N limitation was reported
7 also at the proteome level of *C. vulgaris* [57]. A functional analysis of the abundant DGAT genes
8 in *N. oceanica* CCNP1779 is currently under way in our laboratory.

9   Functional analysis of TAG synthesis by PDAT in unicellular microalgae is lagging
10 behind. So far, only in *C. reinhardtii* a direct role of PDAT in TAG formation has been reported.
11 Expression of *CrPDAT* is induced by starvation deprivation and its silencing results in reduced
12 TAG content under both, N-replete and N-depleted conditions [4, 19]. Interestingly, null mutants
13 of *CrPDAT* are still able to synthesize TAG, however at lower levels, suggesting a functional
14 cooperation with DGAT enzymes. The apparent cooperation of PDAT and DGAT enzymes in
15 TAG biosynthesis was previously described for Arabidopsis and yeast [18, 58]. *CrPDAT*
16 possesses transacylase and/or lipase activities towards a wide range of substrates, including
17 TAG, phospholipids and galactolipids [19]. Analysis of *CrPDAT* mutants strongly suggests that
18 PDAT is essential for membrane lipid turnover and TAG synthesis in *C. reinhardtii*. Indeed, the
19 recycling of acyl chains from membrane lipids as substrates for TAG synthesis fits well with the
20 observation that the content of major membrane lipids decreases concurrently with TAG
21 accumulation [59]. The role of polar membrane lipids, especially MGDG, in TAG synthesis has
22 been recently shown for the *pgd1* (PLASTID GALACTOLIPID DEGRADATION 1) mutant of
23 *C. reinhardtii*, which has lost a galactoglycerolipid lipase encoding gene [60]. The *pgd1* loss-of-
24 function mutant showed reduced rates of TAG accumulation in response to N deprivation [60].
25 Increase of PDAT gene expression in response to N deprivation has recently been shown for *P.*
26 *tricornutum* and was accompanied by relatively weak increase in DGAT gene expression [61].
27 Thus, a prominent role of PDAT in TAG accumulation in N-deprived *P. tricornutum* cells has
28 been proposed.

5. Induction of TAG synthesis in microalgae following stress

Oleaginous microalgae produce small amounts of neutral lipids, mainly TAG, under favorable growth conditions, and they start to accumulate large amounts of TAG in LDs upon stresses such as nutrient limitation, elevated temperatures, and high light intensities [6, 62, 63]. N deprivation is the best investigated inducer of TAG synthesis to date, which leads to the cessation of cell division (cellular quiescence) [8, 28, 64] and causes oleaginous algae to produce substantial amounts of TAG, up to ~20-50% of dry weight [6]. Depletion of other nutrients also stimulates oil accumulation, such as silicon deprivation in case of the diatom *Cyclotella cryptica* [65] [66], sulfur deprivation in the green algae *Chlorella ellipsoidea* [67] and *C. reinhardtii* [68], phosphorus deprivation in the marine diatom *P. tricornutum*, the marine haptophyte *Isochrysis galbana* [69, 70], and the fresh water alga *Monodus subterraneus* [71], and zinc and iron deprivation in *C. reinhardtii* [72, 73]. Besides nutrient limitations, chemical stimuli such as treatment with Brefeldin A, an ER-stress inducer, led to TAG accumulation in *C. reinhardtii* and *Chlorella vulgaris* [74]. Many microalgae accumulate TAG in LDs in response to physical environmental stresses including unfavorable light intensity, temperature, high salinity, alkaline pH, and dehydration. For example, high light conditions stimulate TAG formation in the green alga *Cladophora* sp. [75], the diatom *Thalassiosira pseudonana* [76] and the red alga *Tichocarpus crinitus* [77], whereas darkness with hypoxia or 24 h extended darkness alone increases TAG in *C. reinhardtii* [78]. In *C. reinhardtii*, heat stresses rapidly induces TAG formation [79] [80], while raising the temperature up to 30°C causes the elevation of total lipids in the freshwater phytoflagellate *Ochromonas danica* [81] and the marine alga *Nannochloropsis salina* [82]. Synthesis of TAG is also induced by other environmental stresses such as high salinity as seen in the green algae *Dunaliella salina* [83] and *C. reinhardtii* [59], alkaline pH in *P. tricornutum* [84], and dehydration in the green alga *Chlorella kessleri* [85]. Recent studies on *P. tricornutum* revealed a strong and very complex impact of N deprivation, alkaline pH stress, bicarbonate supplementation, and organic acid supplementation on lipid metabolism. However, for all conditions tested, N deprivation was a prerequisite for accumulation of TAGs and the other factors only enhanced lipid synthesis [84]. Noteworthy, one of the observed responses to all stress conditions explored in this study was increased expression of genes encoding a putative PDAT or putative phospholipases accompanied by remodeling of major polar lipids

(phosphatidylcholine, phosphatidylethanolamine or galactoglycerolipids), which probably serve as donors of acyl groups for TAG formation. Similar induction of expression was observed for the *PtDGAT* encoding gene, with exception of organic acid addition [84]. In general, carbon source availability seems to play a crucial role after induction of TAG accumulation in microalgae. Besides the higher biomass growth rates, an increase in TAG accumulation in response to an external organic carbon source such as acetate was shown for *C. reinhardtii* [86] as well as for *P. tricornutum* [87]. In the latter case, reallocation of membrane lipids in response to N deprivation was accompanied also by massive redirection of general cellular carbon flux into lipid biosynthesis [88]. The crucial role of carbon flux in TAG accumulation was also shown for *P. tricornutum* lines with a silenced methylcrotonyl-CoA Carboxylase encoding gene (*mcc2*). These lines exhibited disturbed carbon metabolism but were able to synthesize and accumulate TAGs even after N resupplementation, indicating that the presence of N, in this case in the form of nitrate, in the medium is not a crucial factor for TAG accumulation *per se* once the cells are programmed for TAG production [89]. All these findings imply important functions of TAG metabolism in stress tolerance of microalgae. Furthermore, since the various stresses will induce TAG while concomitantly cause the inhibition of growth, reducing biomass and overall oil yield, an insight into the detailed mechanisms of TAG biosynthesis and its induction in response to various stresses would provide strategies for the engineering of oil production in microalgae under favorable conditions for biomass yield.

Induction of TAG synthesis in microalgae in response to N deprivation is accompanied not only by formation of TAG-enriched LDs but also by a gradual degradation of thylakoid membranes [4, 19, 90]. The tight link between chloroplast degradation and TAG formation in microalgal cells has been previously observed and an increasing number of recent reports are trying to make biological sense of this inverse relationship. The *pgd1* knockout-mutant of *C. reinhardtii* showed a significant reduction in TAG accumulation following N deprivation, accompanied by a chlorosis phenotype that can be reversed by addition of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a specific inhibitor of linear electron transport at photosystem II [60]. This finding appears to support the hypothesis that *de novo* TAG synthesis sequesters excess electrons from photosynthesis as an electron sink and protects thylakoid membrane against overreduction and release of toxic reactive oxygen species, especially under conditions when the carbon supply exceeds the capacity of starch biosynthesis in *C. reinhardtii*

[6, 91]. Indeed, it has been reported that a *C. reinhardtii* starchless mutants accumulates more TAG [92-94], suggesting an essential role of TAG synthesis in energy storage and survival during stress. Intriguingly, TAG synthesis in some species of microalgae is coordinated with carotenoid synthesis such as β -carotene and lutein in cytosolic carotenoid-rich LDs, which could serve as sunlight reflectors to prevent excess photons from striking photosynthetic membranes [95, 96].

As photosynthetic organisms, microalgae rely on solar energy and under natural conditions their metabolism oscillates with light/dark cycles. At this time, the cellular response to diel dark/light cycles was analyzed at the transcriptome level in *O. tauri* [97], *C. reinhardtii* [98] and recently in *N. oceanica* [99]. The latter report discusses complex physiological and transcriptomic changes relevant to lipid metabolism under dark/light conditions. In *N. oceanica* TAG accumulation occurs only during the light phase, reaching the highest levels at the end of the day with TAG being one of the most abundant lipid classes and the only one showing oscillations under light/dark cycles, suggesting its role as a transient carbon storage form [99-101]. Expression patterns of *N. oceanica* DGAT- and PDAT-encoding genes also reflected the diel TAG accumulation, but peaked at different times during the light phase [99].

6. Regulation of TAG synthesis and LD formation – how do microalgae measure up to plants?

6.1 Different regulation, distinct pathways but a common final outcome

Most of our current knowledge on TAG synthesis and LD formation in plants comes from studies on seed tissues and has been summarized in detail in numerous of reports. Here we provide only a brief outline of TAG accumulation pathways in developing seeds as a point of reference to possible mechanisms in microalgal cells and photosynthetic plant tissues (Fig. 4). Among known transcription factors affecting fatty acid biosynthesis and TAG accumulation in seeds (Fig. 4A), WRINKLE1 (WRI1) has been well characterized and seems to be most directly involved. An Arabidopsis loss-of-function mutant, *wri1-1*, shows an 80% reduction in TAG content in seeds [102]. WRI1 is an APETALA2 (AP2) type transcription factor [103, 104]. Microarray analysis indicated that many of the genes with reduced expression in the *wri1-1*

1 mutant encode fatty acid biosynthesis and glycolytic enzymes [105]. Some of these genes have
2 been characterized as direct *AtWRI1* targets and *AtWRI1* binding *cis*-elements are present in the
3 promoter regions of the *AtWRI1* target genes [106-108].

4 Ectopic expression of *AtWRI1* has been shown to increase TAG content in Arabidopsis
5 and tobacco leaves [109, 110] but the molecular basis of *WRI1* regulation, e.g. control of *WRI1*
6 expression and *WRI1* posttranslational modifications need to be determined, and the dissection
7 of *WRI1* functional domains (or motifs) still needs to be completed. Three intrinsically
8 disordered regions (IDRs) have been recently identified in *AtWRI1* [111]. C-terminal IDR
9 (IDR3) of *AtWRI1* has been shown to play an important role in mediating *WRI1* stability. A
10 PEST motif is present in IDR3 of *AtWRI1* and its removal from the IDR3 of *AtWRI1* resulted in
11 increased protein stability and TAG production [111]. Intriguingly, mutagenesis of putative
12 phosphorylation sites in IDR3-PEST of *AtWRI1* led to increased TAG production in transient
13 expression assays in tobacco leaves and in stable transgenic Arabidopsis plants, which suggests
14 that phosphorylation of IDR3-PEST in *AtWRI1* might be a potential regulatory mechanism of
15 *AtWRI1* stability [111]. Moreover, studies described in [112] showed that *AtWRI1* is a target of
16 *LEAFY COTYLEDON2*, which positively affects the expression *AtWRI1* during seed
17 development.

18 Fatty acids for TAG synthesis are produced *de novo* in the plastids and then exported to
19 the cytosol in the form of acyl-CoAs. Synthesis of TAGs using those precursors takes place in
20 the endoplasmic reticulum by coordinated action of DGAT enzymes (Kennedy pathway).
21 Additionally, the action of PDAT enzymes also leads to TAG formation. In this reaction
22 phospholipid serves as acyl donor and DAG as acceptor. Moreover, a subpool of phospholipids
23 might be derived from plastid membrane lipids, which are gradually degraded by yet unidentified
24 lipases during seed development. These TAGs are then packed into LDs, formed by the ER and
25 equipped with several abundant LD-associated proteins – oleosin, caleosin and steroleosin -,
26 which stabilize LDs and mediate their mobilization during seed germination [113]. The
27 expression of oleosin genes is controlled by transcriptional factors *ABSCISIC ACID*
28 *INSENSITIVE3* (*ABI3*) and *ABSCISIC ACID INSENSITIVE5* (*ABI5*) and thus depends on
29 abscisic acid levels during specific stages of seed development [114].

30 Non-seed tissues are also known to accumulate TAGs (Fig. 4B), mainly in response to
31 stress factors, such as freezing and desiccation, ozone fumigation or reduced nitrogen levels

[115-119]. TAG accumulation under such conditions is thought to be the result of conversion of polar lipids, mainly MGDGs, into DAGs and their further acylation into TAGs. This conversion has been observed for freeze-treated *Arabidopsis* leaves [2, 120, 121] or ozone-fumigated spinach leaves [117, 122]. An increase in TAG content concomitant with a decrease in MGDG was also observed in response to water deficiency in desiccation-tolerant plants such as *Craterostigma plantagineum* (blue carpet), *Lindernia brevidens* and *Ramonda serbica* (Serbian-phoenix flower), as well as in desiccation-sensitive plants such as *Arabidopsis* and several crops, e.g. *Gossypium hirsutum* (cotton), *Triticum aestivum* (wheat) and *Zea mays* (maize) [115, 116, 123]. However, knowledge of the transcriptional regulation and possible pathways involved in this phenomenon is still scarce. In vegetative tissues of *Arabidopsis* seedlings exposed to low nitrogen levels the ABSCISIC ACID INSENSITIVE4 (ABI4) transcription factor has been shown to participate in the abscisic acid-mediated increase in TAG accumulation by direct regulation of DGAT1 expression [119]. Intriguingly, although WRI1 activates TAG accumulation only in seed tissues, three other proteins from the WRI family (WRI2, WRI3 and WRI4) were recently identified and found to be expressed in non-seed tissues of *Arabidopsis* [124]. Their functional characterization showed that all three WRIs activate genes to provide acyl chains for cutin biosynthesis in floral tissues and prevent adherence of the flower organs, which could lead to semi-sterility [124]. The fact that WRI2-4 have been shown to be directly involved in lipid synthesis in floral tissues does not exclude their similar role in fatty acid biosynthesis and TAG accumulation in other non-seed tissues. Under freezing conditions the cells of *Arabidopsis* photosynthetic tissues undergo a complex process of membrane lipid remodeling, which is a first line of defense against freeze damage caused by cellular dehydration. The critical enzyme involved in this process is the SENSITIVE TO FREEZING 2 PROTEIN (SFR2), which removes MGDGs from the chloroplast outer envelope and transforms them into oligogalactolipids and DAGs [118]. The latter ones are used then for synthesis of TAGs, finally deposited in LDs formed under freezing conditions.

Since no oleosins were found in non-seed tissues, an oleosin-independent model of LD formation has been proposed [113], where mature LDs are formed as the consequence of fusion between small LDs, but the mechanisms of this process and its control remain elusive. It cannot be excluded that other, yet to be identified types of LD-associated proteins (LDAPs) may function in LD formation in non-seed tissues. Recently, a novel class of perilipins associated

1 with LDs and probably involved in their biogenesis or mobilization has been identified in the
2 mesocarp of avocado (*Persea americana*) [125]. Thus, the presence of other LD-associated
3 proteins distinct from oleosin cannot be currently excluded for non-seed plant tissues.

4 Unlike plants, microalgae are unicellular organisms and the complete process of lipid
5 synthesis, processing and accumulation in response to stress takes place within a single cell.
6 Given that most of the current models of TAG metabolism are based on findings with non-
7 photosynthetic plant tissues (*i.e.* seed), one needs to ask the question to which extent these
8 findings can be applied to microalgal cells. As mentioned above, TAG accumulation following
9 plant exposure to various environmental stresses shows similarities to TAG accumulation
10 following stress exposure in microalgae, including the degradation of chloroplast polar lipids, an
11 increase in DAG content, and its conversion to TAGs (Fig. 4C). N deprivation is widely used to
12 induce TAG accumulation in microalgae in laboratories and microalgal cultivation facilities.
13 TAG synthesis utilizing precursors derived from membrane lipid degradation during
14 environmental stresses in microalgae is similar to the mechanisms of TAG accumulation
15 observed for plant photosynthetic tissues in response to stress. Thus, findings on algal TAG
16 metabolism and its engineering may be relevant for the engineering of TAG metabolism in
17 vegetative photosynthetic tissues of plants and *vice versa*.

18 Despite recent progress in our understanding of lipid metabolism in microalgae as
19 outlined above, few mechanistic insights into the transcriptional regulation of TAG metabolism
20 related genes (Fig. 4A) is currently available and this area provides opportunities for further
21 studies. One of the first and, thus far, the only functionally characterized transcription factor
22 candidate is NRR1 (Nitrogen Responsive Regulator 1) identified in *C. reinhardtii* [4, 126]. The
23 NRR1 protein belongs to the *SQUAMOSA* promoter binding protein (SPB) family and possesses
24 a DNA-binding domain. Its gene is highly expressed following N deprivation. Importantly, the
25 *nrr1* loss-of-function mutant showed over 50% reduction in TAG levels in response to N
26 deprivation [4]. Further efforts to identify putative transcriptional factors required for lipid
27 accumulation by transcriptomic analysis showed that genes for one of the orphan family of
28 transcription factors as well as for one transcription factor belonging to the SPB family are
29 highly up-regulated during TAG accumulation and might be part of the regulatory mechanism in
30 *C. reinhardtii* [2, 51]. Recent genome-wide prediction of encoded transcription factors in six
31 *Nannochloropsis* strains [127] led to the identification of several putative transcription factors

1 potentially involved in TAG biosynthesis. Three possible transcription factors involved,
2 including one putative Arabidopsis WRINKLED1 (WRI1) orthologue were identified in *N.*
3 *oceanica* IMET1, *N. oceanica* CCMPP1779 and *N. gaditana* CCMP526 transcriptomes. Whether
4 the recently predicted algal WRI1-encoding gene is a true orthologue of plant WRI1 remains to
5 be seen.

6 Interestingly, among other 11 transcription factors potentially involved in TAG accumulation
7 in *N. oceanica* IMET1, five belong to the bZIP family. In the currently hypothesized
8 transcriptional network governing TAG accumulation in *N. oceanica* IMET1 it was proposed
9 that some of the genes might be up- or down regulated due to the activity of multiple
10 transcription factors. LPAT gene up-regulation was predicted to involve two transcription factors
11 of the bZIP family and one MYB transcription factor, whereas DGAT-2A gene expression was
12 suggested to involve a single transcription factor of the MYB family [127]. Nevertheless, all
13 predicted interactions of the putative *Nannochloropsis* transcription factors need to be further
14 experimentally verified.

16 **6.2 Algal LDs differ from those of plants**

18 Similar to plants, algae store neutral lipids in discrete spherical LDs, which structurally,
19 consist of a hydrophobic core, composed mainly of TAGs, surrounded by a single layer of polar
20 lipids, mostly glycerolipids, with a few embedded unique proteins [93] (Figure 1). In plants, LDs
21 serve not only as energy supply for active periods of metabolism (e.g. seed or pollen grain
22 germination), but were shown to participate in many cellular events, including signal
23 transduction, cytoskeletal rearrangements and membrane trafficking [128, 129]. LDs from some
24 microalgae have been purified and characterized at both, lipidome and proteome level. Those
25 include LDs from *C. reinhardtii* and *N. oceanica*, however LDs from *C. reinhardtii* are the best
26 characterized among all microalgae models, thus far [93, 130-132]. As mentioned above, LDs
27 are proposed to form in both, the chloroplast and cytosol in the *C. reinhardtii* starch-less mutant
28 [9]. Proteomic analysis of cytosolic LDs from *C. reinhardtii* showed the presence of more than
29 30 LD-associated proteins, including lipid synthesis enzymes (GPAT, LPAT and PDAT) as well
30 as proteins involved in lipid signaling and trafficking [130, 132]. Of the most abundant LD-
31 associated proteins in non-algal cells, perilipin-like proteins and oleosins, none were found in

LDs isolated from algae, suggesting evolutionary divergent LD-associated factors mediate LD dynamics in algal cells. Indeed, the identification of a 28 kDa major lipid droplet protein (MLDP) seems to support such a scenario in photosynthetic microalgae [132]. Orthologues of MLDP have been found in other green algal lineages, such as *Chlorella vulgaris*, *Volvox carteri*, *Dunaliella salina*, *Haematococcus pluvialis* and in *Dunaliella bardawil* but not in red algae, diatoms or plants [132-134]. Detailed analysis of MLDP showed its essential role in LD dynamics and function. MLDP associates with both, ER membranes and mature LDs, stabilizes them and acts as a hub for recruitment of other proteins, in particular tubulin [135]. Repression of MLDP gene expression results in enlarged LDs, suggesting also that it acts as a factor preventing LDs from fusing and, thus, provides a larger surface-to-volume ratio for accessibility of lipases directly involved in LD degradation [132].

Proteomic studies of the LDs isolated from *N. oceanica* revealed another abundant protein associated with LDs, Lipid Droplet Surface Protein (LDSP) [131]. LDSP function still needs to be characterized as it is present in all *Nannochloropsis* species. No homologs of LDSP can be found in other heterokont algae or in the green or animal lineage [131]. LDSP is a hydrophobic protein with a unique primary sequence and its levels are highly correlated with the TAG amount in *Nannochloropsis* during both, TAG accumulation and degradation. Functional characterization of LDSP in an *Arabidopsis* OLEOSIN1-deficient mutant *oleo1* showed that it affects LD size but does not restore the mutant phenotype related to TAG hydrolysis during seed germination [131].

Particularly interesting data regarding the diverse cellular origin of LDs came from recent studies in the halotolerant microalgae *Dunaliella bardawil* [136, 137]. Following N-deprivation, two populations of LDs are produced in *D. bardawil* cells – cytosolic (CLDs) and chloroplastic, designated as β -carotene rich plastoglobuli (β C-plastoglobuli). Proteomics showed that both types of LDs differ in protein composition. In CLD, similarly to *C. reinhardtii*, a rich pool of MLDP protein as well as enzymes involved in lipid and sterol metabolism were found [137]. In turn, the β C-plastoglobuli proteome showed similarity to that of the *C. reinhardtii* eyespot as well as to that of plastoglobuli from *Arabidopsis* and included several plastid-specific proteins, like carotene-globule-associated protein, VESICLE-INDUCING PLASTID PROTEIN1 or the EYE3 and SOUL3 proteins, involved in eyespot assembly. The latter strongly suggest that β C-plastoglobuli are related to eyespot lipid droplets [137]. Both types of LDs seem to share most of

1 the lipid components, including TAG species and total fatty acids. However, labeling studies
2 suggest that precursors of TAGs deposited in CLDs are formed at the ER from *de novo*
3 synthesized fatty acids, whereas TAGs present in β C-plastoglobuli most probably are derived
4 from products of chloroplast membrane degradation [136].

5 Thus far, currently available data strongly suggest evolutionary differences between
6 structure, metabolism, and function of plant and algae LDs. The most striking difference between
7 algal and plant LDs (especially those in non-photosynthetic tissues) is that the latter ones serve
8 mainly as a stable long-term storage of carbon and energy supply and are irreversibly mobilized
9 during active periods of metabolism (e.g. seed or pollen germination). In turn, algal LDs are
10 more transient reservoirs of cellular lipids converted into TAGs and deposited in LDs in response
11 to environmental stress. Restoring favorable environmental conditions results in rapid
12 degradation of LDs and renewal of plastid and ER membranes. The dynamic nature of algal LDs
13 is supported also by the presence of enzymes involved in neutral and polar lipid synthesis on
14 their surface together with lipid degradation enzymes as well as by abundant LD-specific
15 proteins [130, 132]. The broad molecular equipment of LDs likely reflects an efficient repertoire
16 of mechanisms enabling a rapid metabolic response of the algal cell to changing environmental
17 conditions.

18 19 **7. Subcellular localization of the TAG synthesis machinery in microalgae is still** 20 **ambiguous**

21
22 By analogy to plant cells, it was originally proposed that TAG assembly occurs mainly at the ER.
23 However, increasing evidence suggest that at least in some microalgae a plastid pathway of *de*
24 *novo* TAG biosynthesis takes place (Figure 1). This conclusion is supported by predictions of
25 target sequences in DGATs and PDATs identified so far in algal genomes as well as microscopic
26 studies on microalgal cells. Evidence for a chloroplast pathway of TAG synthesis is derived from
27 studies on the *C. reinhardtii* starch-less mutant, BAFJ5, in which chloroplast-derived DAGs are
28 converted to TAGs, which are stored in LDs. These LDs are present in both, the chloroplast and
29 the cytosol, where they are primarily found in close association with the chloroplast envelope
30 membrane [9, 138]. Moreover, the proteome of LDs from *C. reinhardtii* contains selected
31 chloroplast envelope membrane proteins suggesting their origin, at least in part, from chloroplast

1 envelope membranes. LD surface lipids are rich in *de novo* synthesized fatty acids, and are
2 mainly composed of galactolipids typical for chloroplast membranes [135]. These findings
3 together with further predictions of chloroplast location of major enzymes of TAG biosynthesis –
4 *CrPDAT1*, *CrDGAT1* and *CrDGTT4* support the presence of a plastid pathway for TAG
5 accumulation in *C. reinhardtii* [4, 19, 86]. It seems likely that an ER pathway operates in parallel
6 in *C. reinhardtii*, since for *CrDGTT1* and *CrDGTT2* an ER location has been predicted [34] and
7 it currently cannot be ruled out that the LDs associated with chloroplast envelope membranes in
8 the starch-less mutant BAFJ5 originate in the ER. It is plausible that both pathways
9 concomitantly exist in microalgal cells. However, their specific contribution to TAG synthesis
10 may vary depending on the actual growth conditions.

11 As it was mentioned above, among 12 DGATs encoded by the *Nannochloropsis* IMET1
12 genome, three DGTTs are designated as ER-associated whereas for DGAT1-1B and DGAT2-2C
13 chloroplast localization was predicted [11]. This suggests multiple pathways of TAG synthesis in
14 IMET 1, which may act in parallel in response to N deprivation leading to a rapid increase in
15 TAG content. It should be noted that in *Nannochloropsis* *sp.* like in other heterokonts the plastid
16 is surrounded by four envelope membranes raising additional questions regarding TAG enzyme
17 location and lipid trafficking in these organisms.

18 19 **8. TAG accumulation vs. lipolysis in microalgae** 20

21 At the cellular level, stress conditions like N deprivation induce a quiescence phase in
22 algal cells, which is a reversible state of the cell cycle during which cell divisions are temporarily
23 suspended and cell metabolism is reprogrammed in order to survive the unfavorable condition
24 [139]. The latter is accompanied by disassembly of the photosynthetic membrane as well as a
25 massive TAG accumulation in the form of LDs, which can become the most prominent
26 structures in the cell under these conditions. Restoring of favorable conditions by N resupply
27 induces the exit from quiescence accompanied by restoring of cell divisions as well as
28 progressive lipid mobilization from LDs and restoration of the photosynthetic apparatus. Up to
29 date, the only identified protein directly connected to the regulation of TAG turnover is CHT7
30 from *Chlamydomonas* [64]. Ablation of this gene delayed TAG degradation after the N resupply
31 to *cht7* cells accompanied by a strong delay in cell regrowth. CHT7 is located in the nucleus and,

1 similarly to plant and mammalian DNA-binding proteins, likely interacts with DNA via two
2 cysteine-rich motifs of CXC domain. CHT7 has been proposed to be a repressor of N
3 deprivation-induced quiescence in *C. reinhardtii* [64].

4 The process of rapid TAG degradation requires undoubtedly a coordinated and efficient
5 action of TAG lipases, however none of them has yet been characterized in microalgae, with
6 exception of a TAG lipase in *P. tricornutum* [140]. The identified protein is composed of 813
7 amino acids and contains motifs distinct from characterized TAG-lipases of *Arabidopsis* and *S.*
8 *cerevisiae*. These divergent structural features might suggest that similar to microalgal DGATs',
9 an independent evolution of TAG lipases occurred leading to distinct mechanisms of TAG
10 degradation in algal cells. Putative TAG lipases, some of them with a partial similarity to yeast
11 (triglyceride lipase - TGL3-5) and *Arabidopsis* (sugar dependent 1- SDP1) TAG lipases, have
12 been found in the genomes of *C. reinhardtii* [2], *N. oceanica* CMP1779 [10], *O. tauri* [141] and
13 *Chlorella* sp. [21]. Interestingly, the expression of an acylglycerol lipase CrLIP1 has been shown
14 to be up-regulated in response to N resupply in *C. reinhardtii* cultures and was inversely
15 correlated with the level of TAG [142]. CrLIP1 possesses a lipase domain with high similarity to
16 two human DAG lipases - DAGL α and DAGL β and catalyzes the hydrolysis of DAG and polar
17 lipids. It has been suggested that CrLIP1 is involved in TAG turnover in *C. reinhardtii* probably
18 by degrading the DAG produced during TAG degradation [142].

19 20 21 **10. Conclusion and perspectives**

22 Recent advances in the molecular biology of microalgae have undoubtedly fostered the
23 development of microalgae as potential biofuel feedstocks and excellent models for studies of
24 diverse aspects of lipid metabolism. An increasing number of reports reveal substantial
25 differences in lipid metabolic pathways not only between microalgae and plants but also between
26 different algal species, indicating that our knowledge about how lipid biosynthesis and
27 metabolism are regulated in this diverse group of organisms is still scarce. The evolutionary and
28 molecularly distinct mechanisms of TAG synthesis, reported thus far, should motivate further
29 mechanistic studies on lipid metabolism in microalgae. The diversity of lipid metabolism in
30 microalgae provides rich opportunities not only for functional analysis of essential cell biological
31 process in microalgae, but also for novel strategies towards biofuel feed stock development. The
32

current advances in research on lipid biosynthesis and metabolism in oleaginous microalgae are only the beginning. Consequently, the verification of key hypotheses concerning lipid accumulation and trafficking in microalgal cells still remains limited. Among numerous questions awaiting an answer, the most fundamental concern the origin of TAGs, mechanisms of LD formation in both, cytosol and chloroplast, as well as identification of the TAG assembly machineries specific for the two compartments.

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Figure Legends

Figure 1. TAG synthesis pathways proposed for microalgae cells. Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DAG, diacylglycerol; DGAT, acylCoA:diacylglycerol acyltransferase, FA, fatty acid; FAS, fatty acid synthase, G-3-P, glycerol-3-phosphate; LD, lipid droplet; MLDP/LDSP – MAJOR LIPID DROPLET PROTEIN/LIPID DROPLET SURFACE PROTEIN, PDAT, phospholipid:diacylglycerol acyltransferase, PGD1, PLASTID GALACTOLIPID DEGRADATION 1 protein, PtdOH, phosphatidic acid; PL, phospholipids, TAG, triacylglycerol.

Figure 2. Enrichment of genes involved in key steps of TAG biosynthesis pathways predicted for model unicellular microalgae. Figure is based on references given in square brackets.

Fig 3. Phylogenetic trees of DGATs of type 1 (A) and DGATs of type 2 (B). Cladograms were generated using the MEGA 6 alignment program using default settings. A list of 19 DGATs of type 1 (A) and 78 DGATs of type 2 (B). Abbreviations and references are listed in Supplemental Table 1 and 2, respectively. Pink, green, brown and violet boxes indicate animal, plant, fungal and oomycete DGATs, respectively.

Fig. 4. Comparison of possible mechanisms in the regulation of TAG synthesis in microalgae, plant seeds and plant non-seed tissues. ABI3/5, Absciscic Acid-Insensitive 3/5, ABI4, Absciscic Acid-Insensitive 4, CTH7, Compromised Hydrolysis of Triacylglycerols 7, LDSP, Lipid Droplet Surface Protein, LEC2, Leafy Cotyledon 2, LCS2, long-chain acyl-CoA synthetase, MLDP, Major Lipid Droplet Protein, NRR1, Nitrogen Responsive Regulator 1, SFR2, sensitive to freezing 2, TFs, Transcription Factors, WRI1,3,4, Wrinkled 1,3,4.

Table

Table 1. Number, expression profile and functional characteristics of type 1 DGATs and type 2 DGATs (DGTTs) in model unicellular microalgae under N deprivation conditions. (-lack of known homolog/ortholog, n/a – not analyzed). Table is based on references given in square brackets.

	<i>DGATI</i>			<i>DGTT</i>			
	total	upregulated	Restoring TAG phenotype	total	up-regulated	restoring TAG phenotype	Highest efficiency
<i>Chlorella sp.</i> ^[56]	-	-	-	6	3	n/a	n/a
<i>Nannochloropsis</i> IMET1 ^[11]	2	1	n/a	11	6	n/a	n/a
<i>Phaeodactylum tricornutum</i> ^[43, 44]	1	1	1	4	1	1	<i>PtDGAT2B</i>
<i>Chlamydomonas reinhardtii</i> ^[28, 54]	1	1	n/a	5	3	3	<i>CrDGTT2</i>
<i>Ostreococcus tauri</i> ^[42]	-	-	1	3	n/a	1	<i>OtDGAT2B</i>

1
2

1
2

A

B

1