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Time-dependent transcriptome profile of genes involved in triacylglycerol (TAG) and polyunsaturated fatty acid synthesis in *Nannochloropsis gaditana* during nitrogen starvation

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Abstract

In this research, the gene expression of genes involved in lipid metabolism of the eustigmatophyte alga *Nannochloropsis gaditana* was measured by transcriptomic data. This microalga can be used as a source of triacylglycerol (TAG) and the omega-3 fatty acid eicosapentaenoic acid (EPA). Insight in TAG and EPA production and regulation are needed to improve their productivity. Nitrogen starvation induces TAG accumulation in *N. gaditana*. Previous research showed that during nitrogen starvation, EPA was translocated from the polar lipids to TAG and de novo synthesized in *N. gaditana*. Therefore, the expression levels of genes involved in fatty acid translocation and de novo TAG synthesis were measured. Furthermore, the genes involved in de novo EPA synthesis such as elongases and desaturases were studied. The expression levels were measured during the first hours of nitrogen starvation and the subsequent period of 14 days. One phospholipid:diacylglycerol acyltransferase (PDAT) gene involved in translocation of fatty acids from membrane lipids to TAG was upregulated. In addition, several lipases were upregulated, suggesting that these enzymes might be responsible for the translocation of EPA to TAG. Most desaturases and elongases involved in de novo EPA synthesis were downregulated during nitrogen starvation, except for Δ9 desaturase which was upregulated. This upregulation correlates with the increase in oleic acid. Due to the presence of many hypothetical genes, improvement in annotation is needed to increase our understanding of these pathways and their regulation.

Keywords Microalgae · *Nannochloropsis gaditana* · Triacylglycerol · Nitrogen starvation · Transcriptome · Polyunsaturated fatty acids

Introduction

Microalgae are a sustainable source of lipids which can be used for food, feed and fuel. Fatty acids can accumulate in triacylglycerol (TAG) lipid droplets and in membrane lipids. *Nannochloropsis gaditana* is a microalga known for its large TAG accumulation during nitrogen starvation and the production of omega-3 fatty acid eicosapentaenoic acid (EPA) during growth. The genome of *N. gaditana* has been sequenced (Radakovits et al. 2012; Corteggiani Carpinelli et al. 2014; Schwartz et al. 2018), which enables transcriptomic analysis by measuring the expression levels using RNA sequencing (RNA-seq). The expression levels give information about the transcriptional activity of the genes in the studied pathways.

In previous research, EPA present in TAG was proven to be translocated from the polar lipids and de novo synthesized during nitrogen starvation (Janssen et al. 2019), but the exact metabolic pathways are unknown. Therefore, we focussed on the genes involved in TAG and EPA synthesis during nitrogen starvation in this research.

In microalgae TAG can be produced via two pathways, the acyl-CoA dependent Kennedy pathway and the acyl-CoA independent pathway. These pathways start with the fatty acids synthesis by the fatty acid synthase (FAS) complex. After synthesis of C16:0, C18:0 and C18:1 by the FAS complex,

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they are converted to acyl-CoA. These can be used in the acyl-CoA dependent Kennedy pathway to produce TAG but also be used for the synthesis of polyunsaturated fatty acid (PUFA). An overview of the most important steps in the TAG synthesis pathways is given in Fig. 1.

TAG synthesis via acyl-CoA dependent pathway

The acyl-CoA synthesized by FAS can be transferred to glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to yield lysophosphatidic acid (Lyso-PA). The addition of another acyl chain to Lyso-PA by lysophosphatidic acid acyltransferase (LPAAT) produces phosphatidic acid (PA). The dephosphorylation of PA by phosphatidic acid phosphatase (PAP) yields diacylglycerol (DAG). The last step in the TAG synthesis is the acylation of DAG to triacylglycerol (TAG) by diacylglycerol acyltransferase (DGAT). *Nannochloropsis gaditana* has one gene copy of the DGAT-1 family and 11 gene copies of DGAT-2 gene family (Wang et al. 2014). This is the highest gene dose of DGAT-2 among sequenced algal genomes (Wang et al. 2014; Alboresi et al. 2016).

TAG synthesis by the acyl-CoA independent pathway

Next to TAG synthesis via the acyl-CoA dependent pathway, TAG can also be synthesized by an acyl-CoA independent pathway. In this pathway, membrane lipids can be translocated to TAG by phospholipid:diacylglycerol acyltransferase (PDAT) or lipases (Banerjee et al. 2017). A PDAT enzyme isolated from the microalga *Chlamydomonas reinhardtii* showed phospholipid and galactolipid diacylglycerol transferase activity, DAG: DAG transacylase activity (producing TAG and monoacylglycerol (MAG)) and lipase activity with broad substrate specificity (Yoon et al. 2012). PDAT can thus be responsible for the production of TAG in different ways, including the translocation of membrane lipids to TAG. An insertional mutant of PDAT in *C. reinhardtii* showed 25% less TAG accumulation showing the relevance of this pathway for TAG synthesis during nitrogen starvation (Boyle et al. 2012).

Another possible way to translocate fatty acids from membranes to TAG in lipid bodies is by lipases which liberate the fatty acids and thereby allowing translocation to TAG. For *C. reinhardtii* and *N. oceanica*, multiple lipases were upregulated upon nitrogen starvation (Miller et al. 2010; Li et al. 2014). The exact mechanism in which lipases are involved in translocation of fatty acids is, however, unknown.

EPA synthesis

EPA synthesis starts with the production of palmitic acid (C16:0) and stearic acid (C18:0) in FAS. EPA is synthesized by sequential desaturation and elongation steps performed by desaturases and elongases, respectively. The EPA biosynthesis pathway for *N. gaditana* has not been fully elucidated. However, based on the general EPA biosynthesis pathways proposed for *Nannochloropsis oceanica* and *Nannochloropsis* sp., there are multiple possible pathways (Fig. 2) (Schneider and Roessler 1994; Vieler et al. 2012). It is however importance to notice that the involvement of alternative pathways has not been shown in *N. gaditana*.

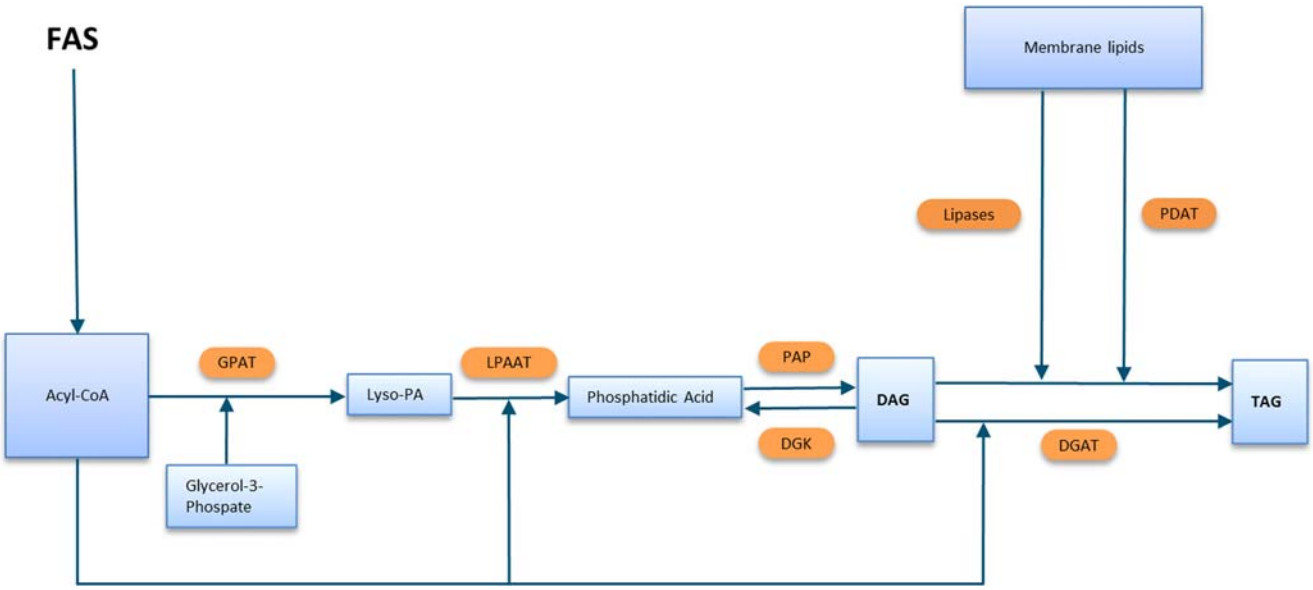

Fig. 1 Simplified TAG synthesis pathways of Nannochloropsis gaditana. *GPAT* glycerol-3-phosphate acyltransferase, *Lyso-PA* lysophosphatidic acid, *LPAAT* lysophosphatidic acid acyltransferase, *PAP* phosphatidic acid phosphatase, *DGK* diacylglycerol kinase, *DAG* diacylglycerol, *DGAT* diacylglycerol acyltransferase, *TAG* triacylglycerol and *PDAT* phospholipid:diacylglycerol acyltransferase. Adapted from (Radakovits et al. 2012; Ma et al. 2016; Banerjee et al. 2017)

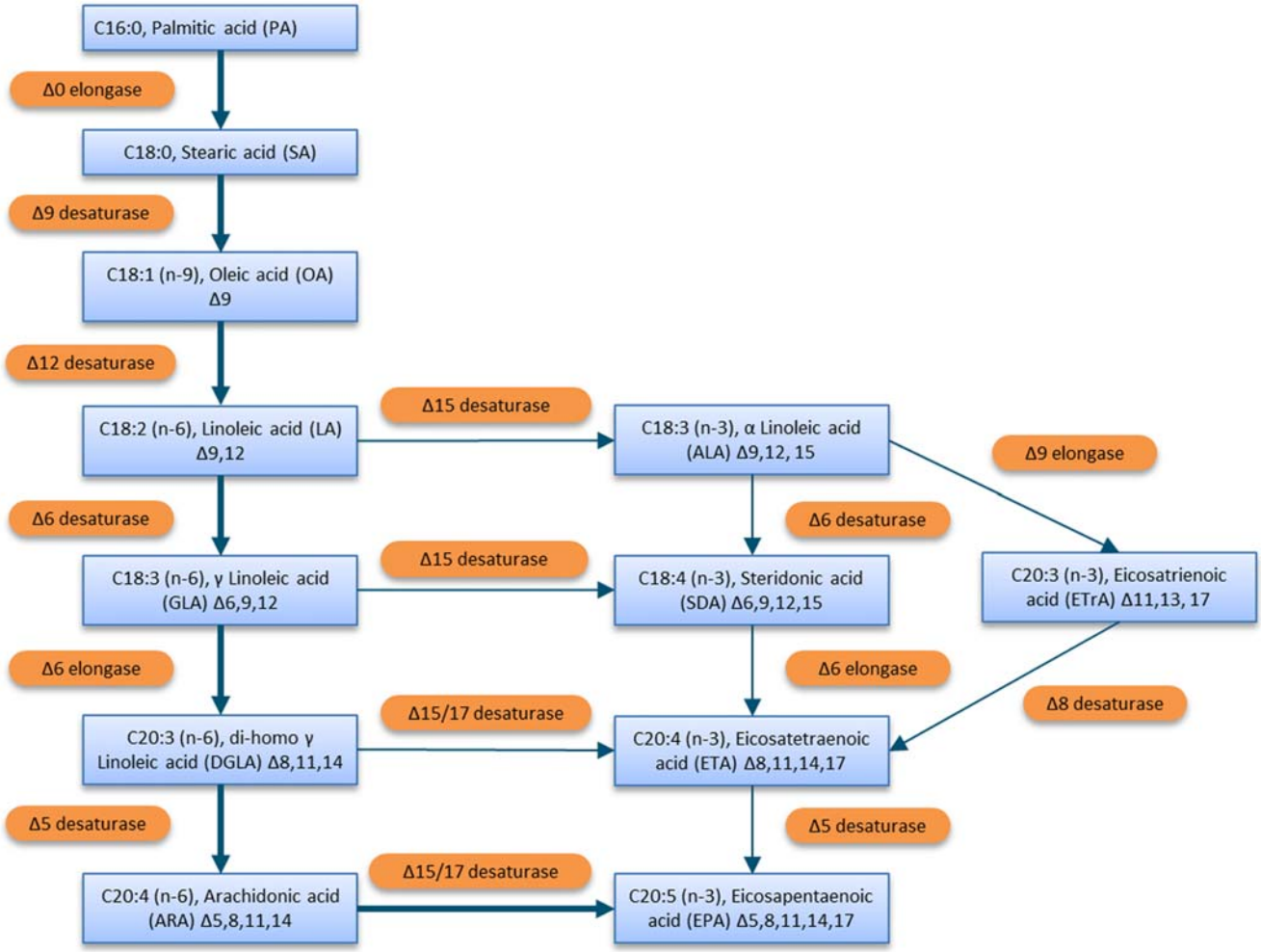

Fig. 2 General proposed EPA synthesis pathway for *Nannochloropsis* adapted from (Schneider and Roessler 1994; Vieler et al. 2012). The bold arrows show the most likely path used in *Nannochloropsis* sp. (Schneider and Roessler 1994). The alternative pathways have not yet been shown for *Nannochloropsis gaditana*

After the first desaturation step by Δ9 desaturase oleic acid (C18:1 n-9) is formed. The second desaturation step by Δ12 desaturase results in the formation of linoleic acid (C18:2 n-6). From linoleic acid, two main pathways can be used for EPA synthesis: the omega-6 and the omega-3 pathway. Previously, the omega-6 pathways were suggested to predominate in *Nannochloropsis* and is therefore shown with bold arrows (12) (Fig. 2). The EPA synthesized can be used in TAG or membrane lipids synthesis. One hypothesised role of TAG is to act as reservoir for plastid PUFA under stress conditions (Cohen et al. 2000; Khozin-Goldberg et al. 2005).

Aim

The aim of this research is to obtain more insight in the TAG and EPA production pathways in *N. gaditana* during nitrogen starvation by analysing transcriptomic changes in time. Previous research showed that upon nitrogen starvation, 23% of EPA present in TAG was intact translocated from the polar lipids in *N. gaditana,* 21% was de novo synthesized and 46% resulted from synthesis via carbon recycled within the cell with newly incorporated carbon (Janssen et al. 2019). In this research, we aim to get more insight in the translocation of fatty acid during nitrogen starvation and therefore studied the genes involved in the acyl-CoA dependent pathway and acyl-CoA independent pathway (PDAT and lipases). In addition, since EPA was also shown to be made de novo after nitrogen starvation, genes involved in the EPA synthesis were analysed. To be able to compare the transcriptome analysis data to the data from previous research where translocation was measured via ^{13}C labelling, the same experimental setup was used. *Nannochloropsis gaditana* was grown in two-phase batch cultivation where a growth phase was followed by a nitrogen starvation phase. Gene expression was analysed over a short (hours) and a longer time period (days), to be able to analyse the immediate transcriptional changes at the onset of nitrogen starvation and on a longer time period. The transcriptional expression levels during nitrogen starvation were

compared to nitrogen replete conditions. In addition to transcriptional expression levels, the fatty acid composition and content in the TAG and polar lipids were measured.

Materials and methods

Strain, cultivation medium and pre-cultivation

The microalga *Nannochloropsis gaditana* CCFM-01 was obtained from the Microalgae Collection of Fitoplancton Marino S.L. Pre-cultivation strategy and growth medium used were as described in Janssen et al. (2019).

Photobioreactor and experimental setup

Experiments were performed in an aseptic, heat-sterilized, flat-panel, airlift-loop photobioreactor (Labfors 5 Lux, Infors HT, Switzerland, 2010) with a reactor depth of 20.7 mm. The experimental setup was used as described in Janssen et al. (2019), with the exception that during the growth phase, the same experimental setup was used as during the nitrogen starvation phase so without gas recirculation. In short, during the growth and nitrogen starvation phase mixing was done by sparging 1 L min^{-1} of air mixed with 2% CO_2. The pH was controlled at 7.5 by on-demand addition of sulphuric acid (5% *v*/v), and the temperature was kept at 26 °C. During the growth phase, the light was increased daily to keep the outgoing light intensity at 30 μmol photons m^{-2} s^{-1} until it reached 636 μmol photons m^{-2} s^{-1} and kept constant onwards.

Offline measurements of the culture and lipid analysis

Biomass was measured by dry weight and cell concentration according to Janssen et al. (2019). The lipid analysis and TAG yield on light calculations were performed as described in Janssen et al. (2019). In brief, the lipids were extracted from the lyophilized biomass using chloroform-methanol after cell disrupting by beat beating. The TAG fraction was separated from the polar lipid fraction using SPE silica gel cartridges. The separated lipid fractions were methylated, and the fatty acid methyl esters (FAMEs) were analysed using gas chromatography.

RNA-sequencing

At 0, 0.1, 0.3, 0.8, 1, 2, 3, 5, 7 and 14 days biomass samples for RNA-sequencing were taken from the photobioreactor. The biomass samples were directly kept on ice and centrifuged for 5 min (4700 × *g*, 0 °C). The cell pellets were immediately frozen in liquid nitrogen and stored at − 20 °C. RNA was extracted using Maxwell 16 LEV simplyRNA Cells Kit following the standard protocol. Extracted RNA was tested for quality using an Experion RNA Analysis Kit (BIO-RAD). RNA samples were stored at − 80 °C and sequenced by Novogene (Illumina PE150).

The genomic annotation (GFF3) and corresponding genomic sequence (FASTA) of *N. gaditana* were converted into a semantic framework using SAPP according to the GBOL ontology (Koehorst et al. 2017; Van Dam et al. 2017). Each RNA-seq dataset was mapped using the transcriptomics module using STAR 2.5 as the read mapping software (Dobin et al. 2013). The expression for each gene for all conditions was converted into a data frame for differential analysis using DESeq2 (Love et al. 2014). Iteratively, differential expression for all comparisons between the different time points was analysed.

The Log_2fold change (LFC) for each time point in the starvation phase was calculated compared to the expression levels at the nitrogen replete phase. A principal component analysis (PCA) was performed to compare the biological replicates (S3 Fig). Most replicates of the same time point are closely aligned, showing good reproducibility. The samples of day 0.3 were less well aligned, but since only duplicates were available, both samples were included.

Genes were considered significant differentially expressed when the *p* value adjusted for false positives (padj) was below or equal to 0.05 and minimum of two for these time points were upregulated or downregulated LFC of 1.5 to 2, or at least one time point was upregulated or downregulated minimal LFC of 2 as described by Li et al. (2014).

Results and discussion

All results on biomass concentration and fatty acids are shown from the start of nitrogen starvation and are the average of two biological photobioreactor experiments with the error bars showing the absolute deviation between these two biological duplicates.

Biomass concentration

The biomass concentration increased from 0.92 ± 0.12 to 4.9 ± 0.36 g L^{-1} during 14 days of nitrogen starvation (Fig. 3A). The cell concentration increased from $2.43 \pm 0.15 \times 10^8$ to $8.23 \pm 0.86 \times 10^8$ cells mL^{-1} during this period (Fig. 3B), meaning that on average cells divided 1.5 times after nitrogen starvation. These growth results were similar to those previously obtained (Janssen et al. 2019) (S1 Fig).

Fatty acid accumulation

Fatty acid accumulation in TAG and polar lipid (PL) fractions was measured during nitrogen starvation.

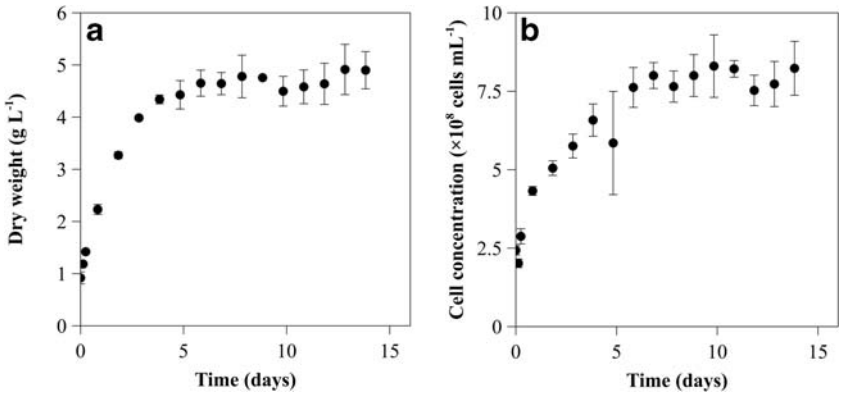

Fig. 3 Average dry weight concentration (g L^{-1}) (**A**) and average cell concentration (cells mL^{-1}) (**B**) from the moment of nitrogen starvation. The error bars show the absolute deviation between two biological photobioreactor experiments ($n = 2$)

The TAG content expressed per biomass increased from 0.07 ± 0.02 to 0.31 ± 0.003 g g_{dw}^{-1} during the 14 days of nitrogen starvation (Fig. 4A). The PL content expressed per biomass decreased from 0.09 ± 0.000 to 0.04 ± 0.001 g g_{dw}^{-1} after 14 days of nitrogen starvation (Fig. 4A). The PL mainly consist of lipid constituents like monogalactosyl diacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylglycerol (PG). These lipids species have been reported to decrease during nitrogen starvation in *N. gaditana* (Simionato et al. 2013), in accordance with our results of the decrease in PL.

The increase in TAG content and biomass concentration resulted in an increase in TAG concentration in the reactor from 0.07 ± 0.03 to 1.54 ± 0.13 g L^{-1} in 14 days of nitrogen starvation (Fig. 4B). In the same period, the PL concentration increased from 0.08 ± 0.01 to 0.20 ± 0.01 g L^{-1} (Fig. 4B). This shows that, although the PL content expressed per biomass decreased, the total amount increased and part of the PL were made de novo during nitrogen starvation. The maximal time-averaged TAG yield on light was 0.13 ± 0.01 g_{TAG} mol_{ph}^{-1} after 1 day of nitrogen starvation (0.16 ± 0.02 g_{TAG} mol_{ph}^{-1} after 2 days when not corrected for energy necessary for inoculum) (Fig. 4E). The EPA content present in the TAG lipid fraction expressed per dry weight increased, but decreased faster in the PL fraction resulting in an overall decrease in EPA content (Fig. 4C). The EPA concentration present in the TAG and PL fraction increased due to the increase in biomass concentration (Fig. 4D), resulting in a total increase in EPA concentration from 0.043 ± 0.005 to 0.174 ± 0.007 g L^{-1}.

Oleic acid (C18:1) content was very low at the start of the nitrogen starvation phase but increased in the TAG fraction from 0.002 ± 0.001 to 0.029 ± 0.000 g g_{dw}^{-1} during the 14 days of nitrogen starvation. In general, the largest changes in fatty acids content in TAG and PL, TAG yield and EPA content occurred within the first 3 days of nitrogen starvation. The fatty acids results were similar to previous results obtained under identical conditions (S2 Fig) (Janssen et al. 2019). Under these growth condition, EPA accumulated in TAG was partly made de novo during nitrogen starvation, as also shown here by the increase in EPA concentration in the reactor (Janssen et al. 2019). On the other hand, 23% of the EPA present in TAG was intact translocated from the polar lipid to the TAG during nitrogen starvation (Janssen et al. 2019). To study the involved TAG accumulation pathways, the expression levels of genes involved in acyl-CoA dependent and independent TAG synthesis pathways were analysed, next to the EPA synthesis pathway.

Differentially expressed genes

From the 10,486 genes annotated in the genome (Corteggiani Carpinelli et al. 2014) 10105 (96%) genes were identified in the RNA-sequencing data. From the identified genes, 6946 (68%) genes were significantly differential expressed based on the constraints set (Li et al. 2014). Within the differentially expressed genes 2908 (42%) were annotated as hypothetical or unknown protein.

TAG synthesis via acyl-CoA dependent pathway

Since *N. gaditana* accumulated TAG upon nitrogen starvation, the expression level of the genes involved in the acyl-CoA dependent TAG pathway was analysed (Fig. 5). The log_2fold change (LFC) during nitrogen starvation, with respect to nitrogen replete condition, was shown for different identified genes involved in this pathway.

The first enzyme involved in TAG synthesis via the acyl CoA-dependent pathway is glycerol-3-phosphate acyltransferase (GPAT) which produces lysophosphatidic acid from glycerol-3-phosphate and acyl-CoA. Three genes identified as GPAT were differentially expressed compared to nitrogen replete conditions (Corteggiani Carpinelli et al. 2014; Alboresi et al. 2016). Two genes (Naga_100019g49 and Naga_100562g3) were upregulated, and one was downregulated (Naga_100106g21) during nitrogen starvation (Fig. 5). Similar results were found for *N. oceanica* where one GPAT was upregulated and another one was downregulated (10). GPAT (Naga_100562g3) was

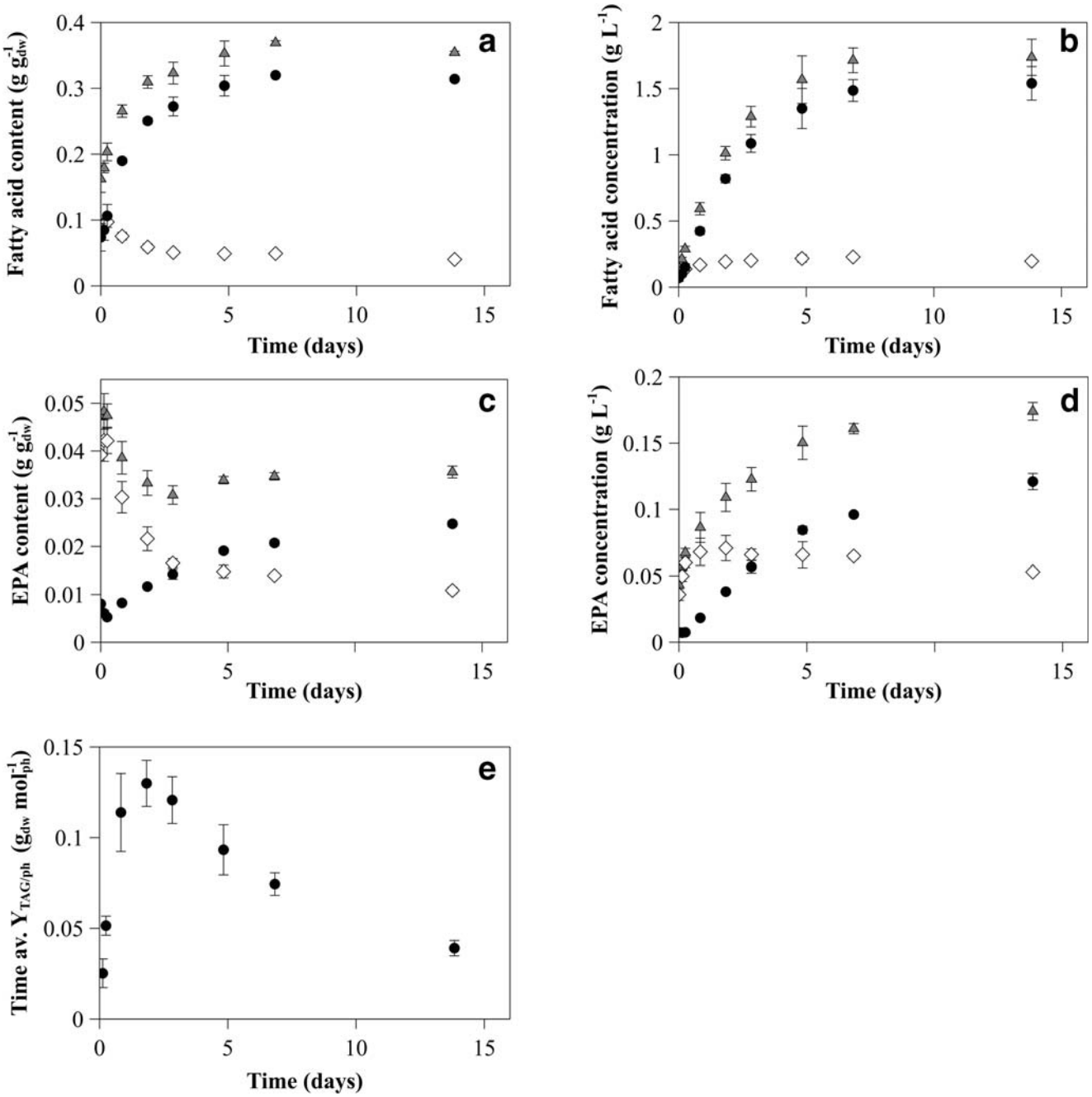

Fig. 4 Average fatty acid content (g g_{dw}^{-1}) (**A**) and fatty acid concentration (g L^{-1}) (**B**) in TAG (circles), PL (diamonds) and total lipids (triangles, TAG + PL). The average EPA content (g g_{dw}^{-1}) (**C**) and EPA concentration (g L^{-1}) (**D**) in TAG (circles), PL (diamonds) and total lipids (triangles, TAG + PL). The average time-averaged TAG yield on light (g_{TAG} mol_{ph}^{-1}) including correction for energy necessary for inoculum production (**E**). Time zero corresponds to the start of nitrogen starvation. The error bars show the absolute deviation between two biological photobioreactor experiments ($n = 2$)

upregulated twofold from 0.8 day onward, and GPAT (Naga_100106g21) was downregulated 1.5 fold at 0.8 day and decreased to 2.4 fold from 1.8 days onward.

The next step in TAG synthesis is the formation of phosphatidic acid by lysophosphatidic acid acyltransferase (LPAAT). Five identified LPAAT genes (7,21) were differentially expressed (Naga_100002g46, Naga_100015g9, Naga_100904g1, Naga_100007g86 and Naga_100501g5). LPAAT (Naga_100015g9) was upregulated 1.5 to 2.6-fold consistently from 0.1 day onward, and another LPAAT gene (Naga_100002g46) was upregulated from day 1.8 onward 2 to 2.5-fold. On the other hand, LPAAT (Naga_100501g5) was 1.5-fold downregulated at 0.3 day and from 3 days onward twofold downregulated.

Fig. 5 Gene expression $\log_2$fold change compared to nitrogen replete conditions over time of the identified genes involved in TAG synthesis via the Kennedy pathway. *GPAT* glycerol-3-phosphate acyltransferase, *LPAAT* lysophosphatidic acid acyltransferase, *PAP* phosphatidic acid phosphatase, *DGK* diacylglycerol kinase and *DGAT* diacylglycerol acyltransferase

The following step in the TAG synthesis is the formation of DAG by phosphatidic acid phosphatase (PAP). One of two PAP genes (Naga_100234g7) was twofold upregulated at the start of nitrogen starvation and 1.5-fold at days 7 and 14. The second PAP gene (Naga_100251g9) was downregulated 1.5-fold at 0.3, 7 and 14 days of nitrogen starvation. Similar results have been reported in *N. oceanica* where one putative PAP gene was downregulated and four other PAP genes were upregulated in the first 48 h of nitrogen starvation (Li et al. 2014).

The reverse reaction, from DAG to phosphatidic acid, is catalysed by diacylglycerol kinase (DGK). Both identified DGK genes (Naga_100017g47 and Naga_100007g24) were upregulated. Upregulation of DGK was also found in the nitrogen-starved green microalgae *Micractinium pusillum* (Li et al. 2012).

The last step in TAG synthesis is the conversion of DAG into TAG by diacylglycerol acyltransferase (DGAT). This enzyme converts DAG to TAG via an acyl-CoA dependent acylation. There are two types of DGAT genes, DGAT-1 and DGAT-2. One identified DGAT-1 enzyme (Naga_101968g1) was not significantly differently expressed. On the other hand, six of the identified DGAT-2 genes were significantly differentially expressed (Naga_100343g3, Naga_100010g31, Naga_100251g8, Naga_100030g42, Naga_100682g2 and Naga100004g173). One of these genes (Naga_100004g173) was annotated as PDAT by (Corteggiani Carpinelli et al. 2014) and annotated as DGAT2k by Alboresi et al. (2016). This gene was downregulated approximately twofold from 0.8 days of nitrogen starvation onwards. In another research, none of the predicted DGAT genes in *N. gaditana* were differentially expressed upon 3 or 6 days of nitrogen starvation (Corteggiani Carpinelli et al. 2014). In *N. oceanica*, however, seven putative DGAT genes were upregulated, and six others were downregulated under nitrogen starvation (Li et al. 2014). For *C. reinhardtii*, two of five DGAT genes were upregulated under nitrogen starvation and were suggested to be involved in TAG synthesis (Boyle et al. 2012). Overexpression of a DGAT-2 from *C. reinhardtii* in *Nannochloropsis* showed increased TAG accumulation under phosphorus starvation (Iwai et al. 2015).

In general, different copies of genes with same function involved in acyl-CoA dependent TAG pathway in *N. gaditana* showed contradictory results with one being upregulated and the other one being downregulated. This might be dependent on different localization of the enzymes in the cell. This complex regulation of the different expression levels for genes with the same functions makes it difficult to draw concrete conclusions regarding regulation of TAG production pathway.

TAG synthesis via acyl-CoA independent pathway

Translocation of intact EPA from the polar lipids to TAG takes place under the conditions used in this research (Janssen et al.

2019). In order to unravel the cellular mechanisms responsible for translocation, genes involved in acyl-CoA independent pathways were analysed. Enzymes possibly involved in this pathway include phospholipid:diacylglycerol acyltransferase (PDAT) and lipases (Fig. 6).

In the sequenced genome, one PDAT gene was annotated (Nga02737) (Radakovits et al. 2012) which had the most comparable sequence to Naga_100065g17 which was, however, annotated as lecithin:cholesterol acyltransferase (Corteggiani Carpinelli et al. 2014). This gene was also described as putative PDAT by Dolch et al. (2017) and as PDAT by Alboresi et al. (2016). Another gene (Naga_100004g173) was annotated by Corteggiani Carpinelli et al. (2014) as PDAT but (Alboresi et al. 2016) annotated this gene as DGAT2k. The PDAT gene (Naga_100065g17) was, however, upregulated upon nitrogen starvation and increased up to twofold upon nitrogen starvation from 2 days onward. This was similar to what was found for *N. oceanica* where PDAT was approximately 50% upregulated after 2 days of nitrogen starvation (Li et al. 2014). In *Phaeodactylum tricornutum*, the expression of PDAT also increased under nitrogen starvation (Mus et al. 2013). This is also in accordance with the measured translocation of EPA from the first day of nitrogen starvation onward (Janssen et al. 2019). As PDAT was upregulated from the start of nitrogen starvation, translocation of membrane lipids might be a result of this enzyme. A knock out of PDAT in *C. reinhardtii* showed a decrease of 25% of TAG at 2 days of nitrogen starvation, confirming a role during nitrogen starvation. PDAT only increased under nitrogen starved conditions showing its role during stress conditions (Boyle et al. 2012). The PDAT or DGAT2k gene (Naga_10004g173) was downregulated from 1 day of nitrogen starvation onwards up to twofold. In contrast to our result, no differential expression of PDAT (Naga_100004g173) in *N. gaditana* was found upon nitrogen starvation in Corteggiani Carpinelli et al. (2014). PDAT from *C. reinhardtii* was shown to have a broad substrate specificity (Yoon et al. 2012). The translocation was more specific for EPA than for other fatty acids in *N. gaditana* (Janssen et al. 2019). Therefore, it would be interesting to study the substrate specificity of PDAT for EPA.

Fatty acids can also be liberated from membrane lipids, like phospholipids and galactolipids, by lipases and used for TAG synthesis. The differentially expressed lipase genes are shown in Fig. 6.

Several types of identified lipases showed differential expression. Several phospholipases and lysophospholipases were upregulated, suggesting a role during nitrogen starvation. The largest upregulation was shown in phospholipase B (Naga_100439g1). In *P. tricornutum* and *N. oceanica* phospholipases were also upregulated during nitrogen starvation (Li et al. 2014; Mus et al. 2013). Other phospholipases were, however, clearly downregulated (e.g. Naga_100454g3 and Naga_100247g4). For *C. reinhardtii*, it was shown that a galactoglycerolipid lipase was required for TAG accumulation under nitrogen starvation (X. Li et al. 2012). No specific galactoglycerolipid lipase was annotated in *N. gaditana*.

Other lipases annotated in the genome were lipase class 3, which are lipases targeting TAG. Several lipases class 3 are upregulated upon nitrogen starvation (Naga_100008g63, Naga_100171g1, Naga_100529g6, Naga_100043g24 and Naga_100104g14). Two other lipases class 3 were downregulated (Naga_100271g1 and Naga_100426g5). The upregulation suggests increased TAG degradation, what may decrease TAG production. Therefore, blocking this degradation by inhibiting these lipases might be a strategy to increase TAG production. The classification of lipases with different substrate specificity is, however, challenging based on sequences (Miller et al. 2010). In *C. reinhardtii*, TAG lipases were also upregulated, and it was suggested that these might play a role in releasing fatty acids from membrane lipids for TAG synthesis (Boyle et al. 2012). More research on the specificity of the different lipases is necessary to elucidate the specific enzymes involved. Because both PDAT and lipases gene copies were upregulated, we cannot distinguish which of these enzymes or if both were responsible for the translocation of EPA into TAG during nitrogen starvation.

Eicosapentaenoic acid (EPA) synthesis

To study the EPA synthesis during nitrogen starvation, the transcriptomic regulation of several elongases and desaturases involved in the EPA synthesis pathway were measured over time (Fig. 7). The end products of de novo fatty acids synthesis: palmitic acid, stearic acid or oleic acid produced by the fatty acid synthesis complex, are the substrates for EPA synthesis.

The first step in the EPA synthesis is the Δ0 elongation of palmitic acid. Several elongases have been identified as Δ0 elongases (Naga_100083g23, Naga_100162g5, Naga_100162g4, Naga_100004g102, Naga_100017g49 and Naga_100399g1) (Dolch et al. 2017).

The Δ9-desaturases were the only identified desaturases which was clearly upregulated (Naga_100027g27, Naga_100013g52 and Naga_100115g11) (Ajjawi et al. 2017; Dolch et al. 2017). Δ9-desaturase was also shown to be upregulated in *N. oceanica* and *C. reinhardtii* during nitrogen starvation (Miller et al. 2010; Li et al. 2014; Valledor et al. 2014). This upregulation was suggested to have a role in prevention of excess reactive oxygen species (Li et al. 2014). During the nitrogen starvation oleic acid (C18:1) increased from in the TAG fraction from 0.002 ± 0.001 to 0.029 ± 0.000 g g_{dw}^{-1} during the 14 days of nitrogen starvation. The increased expression of Δ9-desaturase correlates with the increase in oleic acid (C18:1). The increase in oleic acid upon nitrogen starvation was also reported for different *Nannochloropsis* species (Rodolfi et al. 2009; Simionato

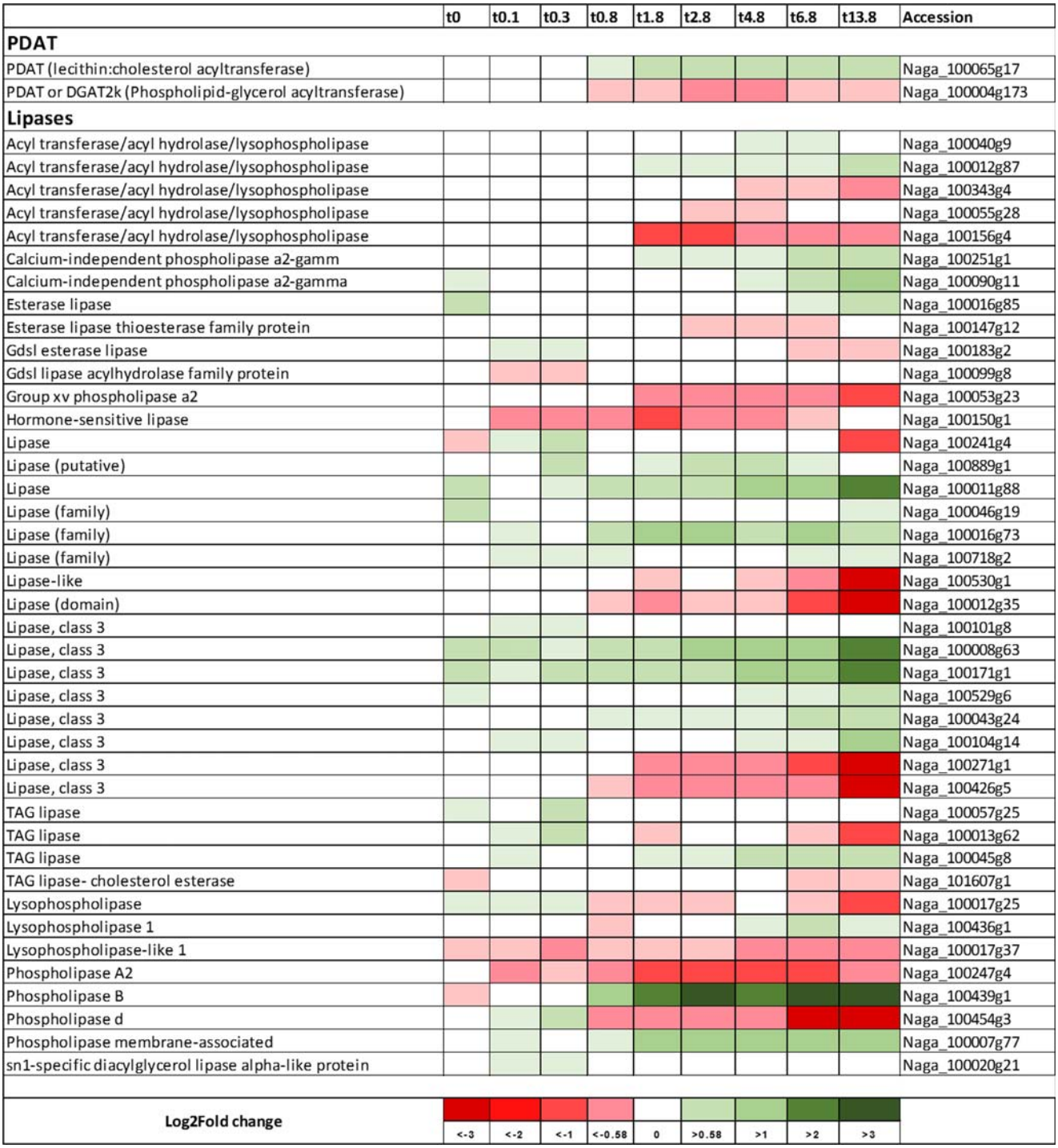

Fig. 6 Gene expression Log_2fold change compared to nitrogen replete conditions over time of the identified genes which might be involved in TAG synthesis via the acyl-CoA independent pathway. *PDAT* phospholipid:diacylglycerol acyltransferase

et al. 2013; Xiao et al. 2013). Silencing stearoyl-ACP desaturase (Δ9-desaturase), which converts stearic acid into oleic acid, resulted in an increased stearic acid content in TAG (de Jaeger et al. 2017). The abundance of stearoyl-ACP desaturase proteins was decreased under initial and long term nitrogen depletion in *N. oceanica* (Dong et al. 2013). The increase in oleic acid suggest that the next desaturation step with Δ12-desaturase might be rate limiting for EPA synthesis. By overexpression of this gene, linoleic acid was shown to be further converted to PUFAs resulting in increased arachidonic acid in TAG (Kaye et al. 2015). In the present study, the Δ12-desaturase (Naga_100092g4) was upregulated during the first 2 days of nitrogen starvation and downregulated afterwards. Another identified Δ12-desaturase (Naga_100092g5), which

Fig. 7 Log twofold change in expression levels of genes involved in EPA synthesis pathway (elongases and desaturases) over time compared to nitrogen replete condition

was also annotated as monogalactosyldiacylglycerol synthase (MGD), was downregulated from day 2 onwards. In *C. reinhardtii* and *Neochloris oleoabundans*, Δ12-desaturase was downregulated during nitrogen starvation (Rismani-Yazdi et al. 2012; Valledor et al. 2014). The same gene responsible for Δ12 desaturation was suggested also being responsible for the ω3 desaturation of arachidonic acid (ARA) to eicosapentaenoic acid (EPA) (Dolch et al. 2017).

The next step in the ω6 pathway is performed by Δ6-desaturase (Naga_100061g21) which was upregulated at 0.3 day and downregulated from day 5 onwards. The Δ6-desaturase isolated from *N. oculata* was able to desaturate linoleic acid and α-linolenic acid, producing γ-linoleic acid and stearidonic acid, so both ω3 and ω6 precursors could be used (Ma et al. 2011). The following step, using Δ6 elongase (Naga_100003g8), was also downregulated. The identified Δ5 desaturases (Naga_100273g7 and Naga_100042g12) were downregulated up to fourfold after 14 days of nitrogen starvation. This desaturase was also shown to decrease up to 6.6-fold in *C. reinhardtii* (Boyle et al. 2012). Overexpression of Δ5 desaturase was shown to increase PUFA synthesis in *P. tricornutum* (Peng et al. 2014). The last desaturation step uses ω3 desaturase or Δ17-desaturase.

Multiple gene copies of the same enzymes indicate possible different localization. Different expression levels make it difficult to identify the exact pathways resulting in TAG accumulation. Moreover, enzymes with multiple possible functions make it difficult to determine which function they actually perform in the pathways. Different genes with the same function might operate in different locations. Since a large part (42%) of the differently expressed genes is annotated as hypothetical or unknown proteins, it is possible that important alternative enzymes involved in the studied pathway are unknown. Therefore, improvement of the annotation would help to identify more genes or additional copies of genes involved in the studied pathways. Furthermore, more research on the specificity of the enzymes like PDAT or the lipases would improve our understanding of the pathways and substrates involved.

Transcriptome analysis only gives information about the transcriptional regulation of genes and not on its translation into proteins. Therefore, proteomic analysis of the discussed pathways would increase our understanding of the active pathways.

Conclusions

Transcriptional expression levels of genes involved in TAG and EPA synthesis showed a complex regulation. Multiple copies of the genes were identified and might indicate different localization in the cell. Some of these gene copies involved

in the acyl-CoA dependent pathway for de novo TAG synthesis were upregulated (e.g. GPAT and DGAT). PDAT gene and multiple lipases were upregulated suggesting a role during nitrogen starvation. These genes were identified as possible candidates to be responsible for the translocation of EPA from membrane lipid to TAG. The EPA synthesis pathway showed upregulation of Δ9 fatty acid desaturase what yielded an increase in oleic acid. The other identified desaturases and elongases involved in this pathway were downregulated during nitrogen starvation. Improvements in annotation and enzyme specificity will help understanding the pathways involved in TAG synthesis during nitrogen starvation.

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