



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

Jorijn H. Janssen¹ · Jacco Spoelder¹ · Jasper J. Koehorst² · Peter J. Schaap² · René H. Wijffels^{1,3} · Maria J. Barbosa¹ 

Received: 21 July 2019 / Revised and accepted: 15 December 2019
© The Author(s) 2020

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 $\Delta 9$ 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,

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s10811-019-02021-2>) contains supplementary material, which is available to authorized users.

✉ Maria J. Barbosa
maria.barbosa@wur.nl

¹ Bioprocess Engineering, AlgaePARC, Wageningen University and Research, P.O. Box 16, 6700 AA Wageningen, The Netherlands

² Laboratory of Systems and Synthetic Biology, Wageningen University and Research, Stippeneng 4, 6708 WE Wageningen, The Netherlands

³ Faculty of Biosciences and Aquaculture, Nord University, N-8049 Bodø, Norway

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*.

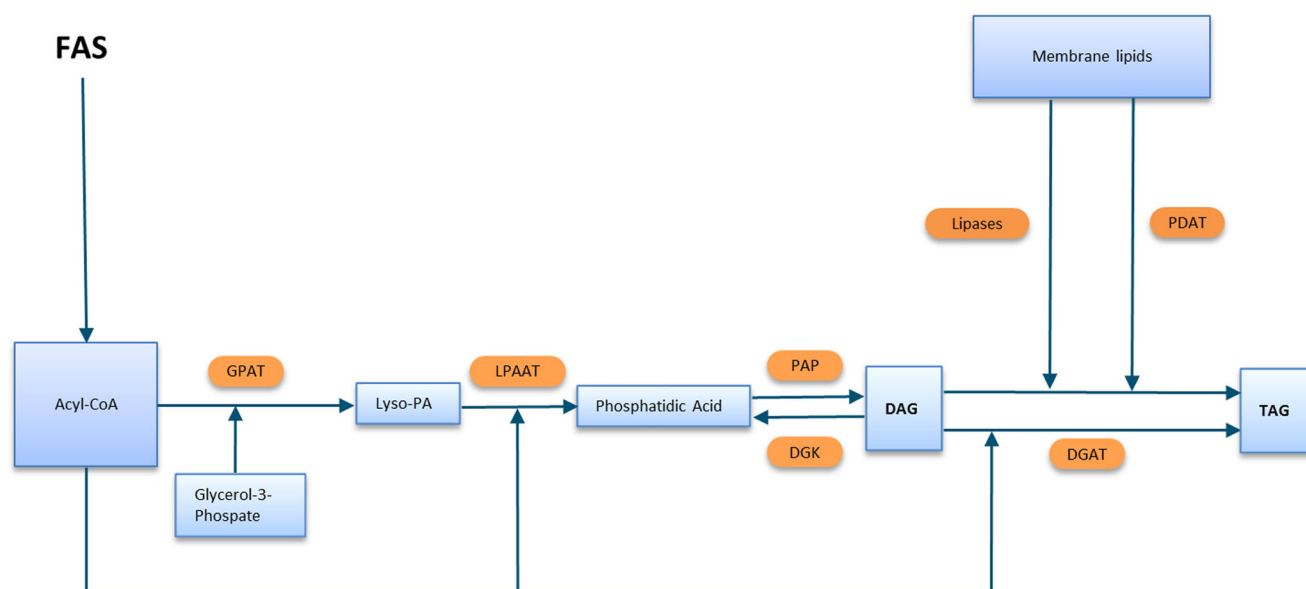


Fig. 1 Simplified TAG synthesis pathways of *Nannochloropsis gaditana*. *GPAT* glycerol-3-phosphate acyltransferase, *Lyso-PA* lysophosphatidic acid, *LPAAT* lysophosphatidic acid acyltransferase, *PAP* phosphatidic acid phosphatase, *DGK* diacylglycerol kinase, *DAG* diacylglycerol, *DGAT*

diacylglycerol acyltransferase, *TAG* triacylglycerol and *PDAT* phospholipid:diacylglycerol acyltransferase. Adapted from (Radakovits et al. 2012; Ma et al. 2016; Banerjee et al. 2017)

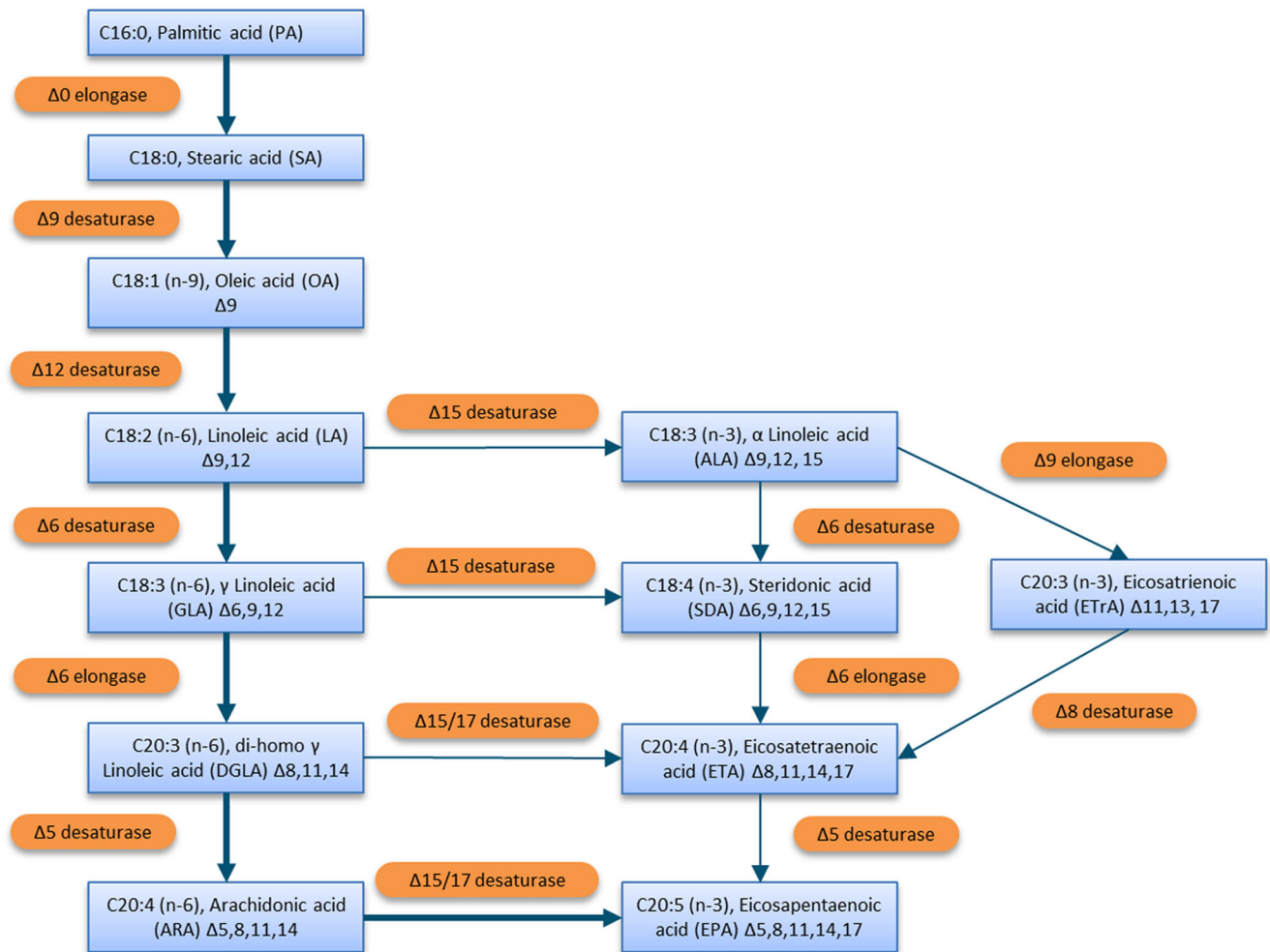


Fig. 2 General proposed EPA synthesis pathway for *Nannochloropsis* adapted from (Schneider and Roessler 1994; Vieler et al. 2012). The bold arrows show the most likely path used in *Nannochloropsis* sp. (Schneider

and Roessler 1994). The alternative pathways have not yet been shown for *Nannochloropsis gaditana*

After the first desaturation step by $\Delta 9$ desaturase oleic acid (C18:1 n-9) is formed. The second desaturation step by $\Delta 12$ 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 (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% CO₂. 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 Log₂fold 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.

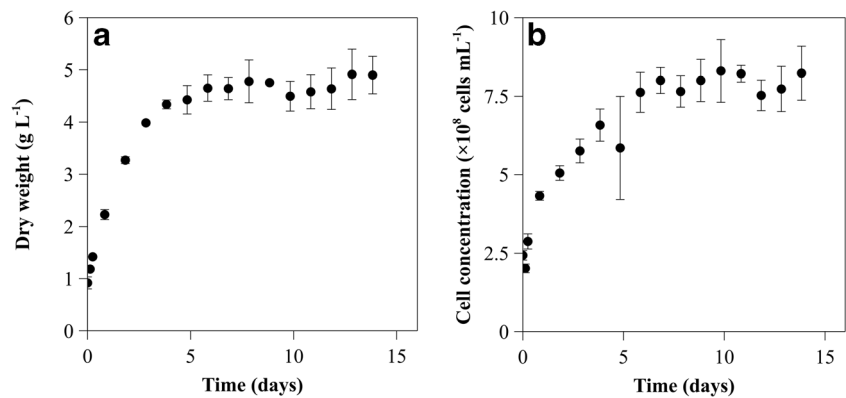
Biomass concentration

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 ± 0.15 × 10⁸ to 8.23 ± 0.86 × 10⁸ 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 $0.31 \pm 0.003 \text{ g g}_{\text{dw}}^{-1}$ during the 14 days of nitrogen starvation (Fig. 4A). The PL content expressed per biomass decreased from 0.09 ± 0.000 to $0.04 \pm 0.001 \text{ g g}_{\text{dw}}^{-1}$ 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 $1.54 \pm 0.13 \text{ g L}^{-1}$ in 14 days of nitrogen starvation (Fig. 4B). In the same period, the PL concentration increased from 0.08 ± 0.01 to $0.20 \pm 0.01 \text{ g L}^{-1}$ (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 $0.13 \pm 0.01 \text{ g}_{\text{TAG}} \text{ mol}_{\text{ph}}^{-1}$ after 1 day of nitrogen starvation ($0.16 \pm 0.02 \text{ g}_{\text{TAG}} \text{ mol}_{\text{ph}}^{-1}$ 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 $0.174 \pm 0.007 \text{ g L}^{-1}$.

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 $0.029 \pm 0.000 \text{ g g}_{\text{dw}}^{-1}$ 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_2 fold 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

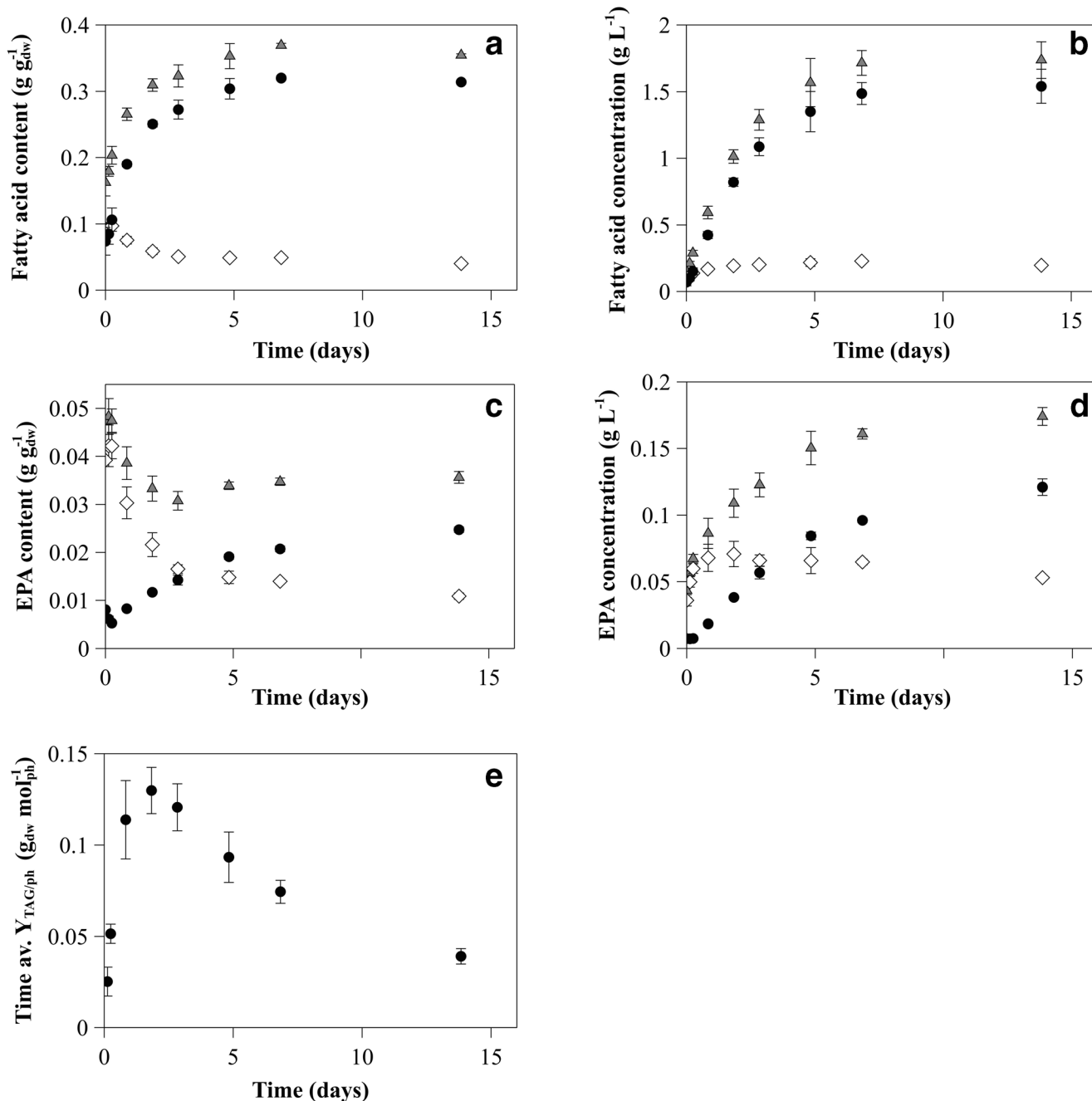


Fig. 4 Average fatty acid content (g g_{dw}⁻¹) (A) and fatty acid concentration (g L⁻¹) (B) in TAG (circles), PL (diamonds) and total lipids (triangles, TAG + PL). The average EPA content (g g_{dw}⁻¹) (C) and EPA concentration (g L⁻¹) (D) in TAG (circles), PL (diamonds) and total lipids (triangles, TAG + 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.

	t0	t0.1	t0.3	t0.8	t1.8	t2.8	t4.8	t6.8	t13.8	Accession	Annotation
GPAT											
GPAT										Naga_100106g21	glycerol-3-phosphate o-acyltransferase
GPAT										Naga_100019g49	calcium-dependent protein
GPAT										Naga_100562g3	hypothetical protein
LPAAT											
LPAAT										Naga_100002g46	lysocardiolipin acyltransferase 1
LPAAT										Naga_100015g9	1-acyl-sn-glycerol-3-phosphate acyltransferase
LPAAT										Naga_100904g1	1-acyl-sn-glycerol-3-phosphate acyltransferase
LPAAT										Naga_100007g86	1-acyl-sn-glycerol-3-phosphate acyltransferase
LPAAT										Naga_100501g5	acyl-lysophosphatidylglycerol acyltransferase 1
PAP											
PAP										Naga_100251g9	phosphoesterase pa-phosphatase related protein
PAP										Naga_100234g7	phosphoesterase pa-phosphatase related protein
DGK											
DGK										Naga_100017g47	diacylglycerol kinase
DGK										Naga_100007g24	conserved protein with dgk catalytic domain protein
DGAT											
DGAT2b										Naga_100010g31	mono- or diacylglycerol acyltransferase
DGAT2a										Naga_100343g3	diacylglycerol acyltransferase type 2
DGAT2g										Naga_100682g2	diacylglycerol acyltransferase
DGAT2e										Naga_100030g42	diacylglycerol o-acyltransferase 2
DGAT2k or PDAT										Naga_100004g173	Phospholipid-glycerol acyltransferase
DGAT2c										Naga_100251g8	2-acylglycerol o-acyltransferase 2
Log2Fold change											
	<-3	<-2	<-1	<-0.58	0	>0.58	>1	>2	>3		

Fig. 5 Gene expression log₂fold 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 Naga_100004g173). 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 tricorutum*, 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. tricorutum* 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 $\Delta 0$ elongation of palmitic acid. Several elongases have been identified as $\Delta 0$ elongases (Naga_100083g23, Naga_100162g5, Naga_100162g4, Naga_100004g102, Naga_100017g49 and Naga_100399g1) (Dolch et al. 2017).

The $\Delta 9$ -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). $\Delta 9$ -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 $\Delta 9$ -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

	t0	t0.1	t0.3	t0.8	t1.8	t2.8	t4.8	t6.8	t13.8	Accession
PDAT										
PDAT (lecithin:cholesterol acyltransferase)										Naga_100065g17
PDAT or DGAT2k (Phospholipid-glycerol acyltransferase)										Naga_100004g173
Lipases										
Acyl transferase/acyl hydrolase/lysophospholipase										Naga_100040g9
Acyl transferase/acyl hydrolase/lysophospholipase										Naga_100012g87
Acyl transferase/acyl hydrolase/lysophospholipase										Naga_100343g4
Acyl transferase/acyl hydrolase/lysophospholipase										Naga_100055g28
Acyl transferase/acyl hydrolase/lysophospholipase										Naga_100156g4
Calcium-independent phospholipase a2-gamm										Naga_100251g1
Calcium-independent phospholipase a2-gamma										Naga_100090g11
Esterase lipase										Naga_100016g85
Esterase lipase thioesterase family protein										Naga_100147g12
Gdsl esterase lipase										Naga_100183g2
Gdsl lipase acylhydrolase family protein										Naga_100099g8
Group xv phospholipase a2										Naga_100053g23
Hormone-sensitive lipase										Naga_100150g1
Lipase										Naga_100241g4
Lipase (putative)										Naga_100889g1
Lipase										Naga_100011g88
Lipase (family)										Naga_100046g19
Lipase (family)										Naga_100016g73
Lipase (family)										Naga_100718g2
Lipase-like										Naga_100530g1
Lipase (domain)										Naga_100012g35
Lipase, class 3										Naga_100101g8
Lipase, class 3										Naga_100008g63
Lipase, class 3										Naga_100171g1
Lipase, class 3										Naga_100529g6
Lipase, class 3										Naga_100043g24
Lipase, class 3										Naga_100104g14
Lipase, class 3										Naga_100271g1
Lipase, class 3										Naga_100426g5
TAG lipase										Naga_100057g25
TAG lipase										Naga_100013g62
TAG lipase										Naga_100045g8
TAG lipase- cholesterol esterase										Naga_101607g1
Lysophospholipase										Naga_100017g25
Lysophospholipase 1										Naga_100436g1
Lysophospholipase-like 1										Naga_100017g37
Phospholipase A2										Naga_100247g4
Phospholipase B										Naga_100439g1
Phospholipase d										Naga_100454g3
Phospholipase membrane-associated										Naga_100007g77
sn1-specific diacylglycerol lipase alpha-like protein										Naga_100020g21
Log2Fold change										
	<-3	<-2	<-1	<-0.58	0	>0.58	>1	>2	>3	

Fig. 6 Gene expression Log₂fold 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 ($\Delta 9$ -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 $\Delta 12$ -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 $\Delta 12$ -desaturase (Naga_100092g4) was upregulated during the first 2 days of nitrogen starvation and downregulated afterwards. Another identified $\Delta 12$ -desaturase (Naga_100092g5), which

	t0	t0.1	t0.3	t0.8	t1.8	t2.8	t4.8	t6.8	t13.8	Accession	Annotation
$\Delta 0$ elongase										Naga_100083g23	fatty-acyl
$\Delta 0$ elongase										Naga_100162g5	fatty-acyl elongase
$\Delta 0$ elongase										Naga_100162g4	fatty-acyl elongase
$\Delta 0$ elongase										Naga_100017g49	elongation of very long chain fatty acids protein 6
$\Delta 0$ elongase										Naga_100004g102	fatty-acyl
$\Delta 0$ elongase										Naga_100399g1	fatty-acyl elongase
$\Delta 6$ elongase										Naga_100003g8	elongation of very long chain fatty acids
$\Delta 9$ FAD										Naga_100027g27	stearyl- desaturase 5
$\Delta 9$ FAD										Naga_100013g52	Fatty acid desaturase type 2
FAD										Naga_100115g11	fatty acid desaturase domain protein
$\Delta 12$ FAD / $\omega 3$ FAD										Naga_100092g4	fatty acid desaturase
$\Delta 12$ FAD or MGD										Naga_100092g5	monogalactosyldiacylglycerol synthase
$\Delta 6$ FAD										Naga_100061g21	delta-6 fatty acid desaturase
$\Delta 5$ FAD										Naga_100273g7	delta 5 fatty acid desaturase
$\Delta 5$ FAD										Naga_100042g12	Cytochrome b5, heme-binding site
$\Delta 3$ FAD										Naga_100063g13	Kua-ubiquitin conjugating enzyme hybrid, localisation
$\Delta 4$ FAD										Naga_101483g1	delta-4 fatty acid desaturase
FAD										Naga_100608g3	fatty-acyl
FAD										Naga_100427g6	fatty acid desaturase
FAD										Naga_102739g1	fatty acid desaturase
FAD										Naga_100017g62	Fatty acid hydroxylase
Log2Fold change											
	<-3	<-2	<-1	<-0.58	0	>0.58	>1	>2	>3		

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 *Nochloris oleoabundans*, $\Delta 12$ -desaturase was downregulated during nitrogen starvation (Rismani-Yazdi et al. 2012; Valledor et al. 2014). The same gene responsible for $\Delta 12$ desaturation was suggested also being responsible for the $\omega 3$ desaturation of arachidonic acid (ARA) to eicosapentaenoic acid (EPA) (Dolch et al. 2017).

The next step in the $\omega 6$ pathway is performed by $\Delta 6$ -desaturase (Naga_100061g21) which was upregulated at 0.3 day and downregulated from day 5 onwards. The $\Delta 6$ -desaturase isolated from *N. oculata* was able to desaturate linoleic acid and α -linolenic acid, producing γ -linoleic acid and stearidonic acid, so both $\omega 3$ and $\omega 6$ precursors could be used (Ma et al. 2011). The following step, using $\Delta 6$ elongase (Naga_100003g8), was also downregulated. The identified $\Delta 5$ 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 $\Delta 5$ desaturase was shown to increase PUFA synthesis in *P. tricornutum* (Peng et al. 2014). The last desaturation step uses $\omega 3$ desaturase or $\Delta 17$ -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 $\Delta 9$ 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.

Author contributions Conceptualization: JJ RW MB. Formal analysis: JJ JS JK PS. Funding acquisition: RW MB. Investigation: JJ JS JK. Methodology: JJ RW MB. Project administration: JJ RW MB. Resources: RW. Software: JK PS. Supervision: RW MB. Validation: JJ MB. Visualization: JJ. Writing – original draft: JJ. Writing – review & editing: JJ JS JK PS RW MB.

Funding information This project has received funding from the European Union's Seventh Framework Programme for research, technological development and demonstration under grant agreement no 613588.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit <http://creativecommons.org/licenses/by/4.0/>.

References

- Ajjawi I, Verruto J, Aquí M, Soriaga LB, Coppersmith J, Kwok K, Peach L, Orchard E, Kalb R, Xu W, Carlson TJ, Francis K, Königsfeld K, Bartalis J, Schultz A, Lambert W, Schwartz AS, Brown R, Moellering ER (2017) Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator. *Nat Biotechnol* 35:647–652
- Alboresi A, Perin G, Vitulo N, Diretto G, Block MA, Jouhet J, Meneghesso A, Valle G, Giuliano G, Maréchal E, Morosinotto T (2016) Light remodels lipid biosynthesis in *Nannochloropsis gaditana* by modulating carbon partitioning between organelles. *Plant Physiol* 171:2468–2482
- Banerjee A, Maiti SK, Guria C, Banerjee C (2017) Metabolic pathways for lipid synthesis under nitrogen stress in *Chlamydomonas* and *Nannochloropsis*. *Biotech Lett* 39:1–11
- Boyle NR, Page MD, Liu B, Blaby IK, Casero D, Kropat J, Cokus SJ, Hong-Hermesdorf A, Shaw J, Karpowicz SJ, Gallaher SD, Johnson S, Benning C, Pellegrini M, Grossman A, Merchant SS (2012) Three acyltransferases and nitrogen-responsive regulator are implicated in nitrogen starvation-induced triacylglycerol accumulation in *Chlamydomonas*. *J Biol Chem* 287:15811–15825
- Cohen Z, Khozin-Goldberg I, Adlerstein D, Bigogno C (2000) The role of triacylglycerol as a reservoir of polyunsaturated fatty acids for the rapid production of chloroplastic lipids in certain microalgae. *Biochem Soc Trans* 28:740–743
- Corteggiani Carpinelli E, Telatin A, Vitulo N, Forcato C, D'Angelo M, Schiavon R, Vezzi A, Giacometti GM, Morosinotto T, Valle G (2014) Chromosome scale genome assembly and transcriptome profiling of *Nannochloropsis gaditana* in nitrogen depletion. *Mol Plant* 7:323–335
- de Jaeger L, Springer J, Wolbert EJH, Martens DE, Eggink G, Wijffels RH (2017) Gene silencing of stearoyl-ACP desaturase enhances the stearic acid content in *Chlamydomonas reinhardtii*. *Bioresour Technol* 245:1616–1626
- Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29:15–21
- Dolch L-J, Rak C, Perin G, Tourcier G, Broughton R, Leterrier M, Morosinotto T, Tellier F, Faure J-D, Falconet D, Jouhet J, Sayanova O, Beaudoin F, Maréchal E (2017) A palmitic acid elongase affects eicosapentaenoic acid and plastidial monogalactosyldiacylglycerol levels in *Nannochloropsis*. *Plant Physiol* 173:742–759
- Dong H-P, Williams E, Wang D, Xie Z-X, Hsia R, Jenck A, Halden R, Li J, Chen F, Place AR (2013) Responses of *Nannochloropsis oceanica* IMET1 to long-term nitrogen starvation and recovery. *Plant Physiol* 162:1110–1126
- Iwai M, Hori K, Sasaki-Sekimoto Y, Shimojima M, Ohta H (2015) Manipulation of oil synthesis in *Nannochloropsis* strain NIES-2145 with a phosphorus starvation-inducible promoter from *Chlamydomonas reinhardtii*. *Front Microbiol* 6:912
- Janssen JH, Lamers PP, De Vos RCH, Wijffels RH, Barbosa MJ (2019) Translocation and *de novo* synthesis of eicosapentaenoic acid (EPA) during nitrogen starvation in *Nannochloropsis gaditana*. *Algal Res* 37:138–144
- Kaye Y, Grundman O, Leu S, Zarka A, Zorin B, Didi-Cohen S, Khozin-Goldberg I, Boussiba S (2015) Metabolic engineering toward enhanced LC-PUFA biosynthesis in *Nannochloropsis oceanica*: overexpression of endogenous $\delta 12$ desaturase driven by stress-inducible promoter leads to enhanced deposition of polyunsaturated fatty acids in TAG. *Algal Res* 11:387–398
- Khozin-Goldberg I, Shrestha P, Cohen Z (2005) Mobilization of arachidonyl moieties from triacylglycerols into chloroplastic lipids following recovery from nitrogen starvation of the microalga *Parietochloris incisa*. *Biochim Biophys Acta* 1738:63–71
- Koehorst JJ, van Dam JCJ, Saccenti E, Martins dos Santos VAP, Suarez-Diez M, Schaap PJ (2017) SAPP: functional genome annotation and analysis through a semantic framework using FAIR principles. *Bioinformatics* 34:1–3
- Li X, Moellering ER, Liu B, Johnny C, Fedewa M, Sears BB, Kuo M-H, Benning C (2012) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in *Chlamydomonas reinhardtii*. *Plant Cell* 24:4670–4686
- Li Y, Fei X, Deng X (2012) Novel molecular insights into nitrogen starvation-induced triacylglycerols accumulation revealed by differential gene expression analysis in green algae *Micractinium pusillum*. *Biomass Bioenergy* 42:199–211
- Li J, Han D, Wang D, Ning K, Jia J, Wei L, Jing X, Huang S, Chen J, Li Y, Hu Q, Xu J (2014) Choreography of transcriptomes and lipidomes of *Nannochloropsis* reveals the mechanisms of oil synthesis in microalgae. *Plant Cell* 26:1645–1665
- Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:1–21
- Ma X, Yu J, Zhu B, Pan K, Pan J, Yang G (2011) Cloning and characterization of a delta-6 desaturase encoding gene from *Nannochloropsis oculata*. *Chin J Oceanol Limnol* 29:290–296

- Ma XN, Chen T-P, Yang B, Liu J, Chen F (2016) Lipid production from *Nannochloropsis*. *Mar Drugs* 14:E61
- Miller R, Wu G, Deshpande RR, Vieler A, Gartner K, Li X, Moellering ER, Zauner S, Cornish AJ, Liu B, Bullard B, Sears BB, Kuo M-H, Hegg EL, Shachar-Hill Y, Shiu S-H, Benning C (2010) Changes in transcript abundance in *Chlamydomonas reinhardtii* following nitrogen deprivation predict diversion of metabolism. *Plant Physiol* 154:1737–1752
- Mus F, Toussaint J-P, Cooksey KE, Fields MW, Gerlach R, Peyton BM, Carlson RP (2013) Physiological and molecular analysis of carbon source supplementation and pH stress-induced lipid accumulation in the marine diatom *Phaeodactylum tricorutum*. *Appl Microbiol Biotechnol* 97:3625–3642
- Peng K-T, Zheng C-N, Xue J, Chen X-Y, Yang W-D, Liu J-S, Bai W, Li H-Y (2014) Delta 5 fatty acid desaturase upregulates the synthesis of polyunsaturated fatty acids in the marine diatom *Phaeodactylum tricorutum*. *J Agric Food Chem* 62:8773–8776
- Radakovits R, Jinkerson RE, Fuerstenberg SI, Tae H, Settlege RE, Boore JL, Posewitz MC (2012) Draft genome sequence and genetic transformation of the oleaginous alga *Nannochloropsis gaditana*. *Nat Commun* 3:686
- Rismani-Yazdi H, Haznedaroglu BZ, Hsin C, Peccia J (2012) Transcriptomic analysis of the oleaginous microalga *Neochloris oleoabundans* reveals metabolic insights into triacylglyceride accumulation. *Biotechnol Biofuels* 5:74
- Rodolfi L, Zittelli GC, Bassi N, Padovani G, Biondi N, Bonini G, Tredici MR (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol Bioeng* 102:100–112
- Schneider JC, Roessler P (1994) Radiolabeling studies of lipids and fatty acids in *Nannochloropsis* (Eustigmatophyceae), an oleaginous marine alga. *J Phycol* 30:594–598
- Schwartz AS, Brown R, Ajjawi I, McCarren J, Atila S, Bauman N, Richardson TH (2018) Complete genome sequence of the model oleaginous alga *Nannochloropsis gaditana* CCMP1894. *Genome Announc* 6:e01448–e01417
- Simionato D, Block MA, La Rocca N, Jouhet J, Maréchal E, Finazzi G, Morosinotto T (2013) The response of *Nannochloropsis gaditana* to nitrogen starvation includes de novo biosynthesis of triacylglycerols, a decrease of chloroplast galactolipids, and reorganization of the photosynthetic apparatus. *Eukaryot Cell* 12:665–676
- Valledor L, Furuhashi T, Recuenco-Muñoz L, Wienkoop S, Weckwerth W (2014) System-level network analysis of nitrogen starvation and recovery in *Chlamydomonas reinhardtii* reveals potential new targets for increased lipid accumulation. *Biotechnol Biofuels* 7:171
- Van Dam JCJ, Koehorst JJ, Vik JO, Schaap PJ, Suarez-diez M (2017) Interoperable genome annotation with GBOL, an extendable infrastructure for functional data mining. *bioRxiv* 1–9
- Vieler A, Wu G, Tsai CH, Bullard B, Cornish AJ, Harvey C, Reza IB, Thornburg C, Achawanantakun R, Buehl CJ, Campbell MS, Cavalier D, Childs KL, Clark TJ, Deshpande R, Erickson E, Armenia Ferguson A, Handee W, Kong Q, Li X, Liu B, Lundback S, Peng C, Roston RL, Sanjaya Simpson JP, TerBush A, Warakanont J, Zäuner S, Farre EM, Hegg EL, Jiang N, Kuo MH, Lu Y, Niyogi KK, Ohlrogge J, Osteryoung KW, Shachar-Hill Y, Sears BB, Sun Y, Takahashi H, Yandell M, Shiu SH, Benning C (2012) Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CCMP1779. *PLoS Genet* 8:e1003064
- Wang D, Ning K, Li J, Hu J, Han D, Wang H, Zeng X, Jing X, Zhou Q, Su X, Chang X, Wang A, Wang W, Jia J, Wei L, Xin Y, Qiao Y, Huang R, Chen J, Han B, Yoon K, Hill RT, Zohar Y, Chen F, Hu Q, Xu J (2014) *Nannochloropsis* genomes reveal evolution of microalgal oleaginous traits. *PLoS Genet* 10:e1004094
- Xiao Y, Zhang J, Cui J, Feng Y, Cui Q (2013) Metabolic profiles of *Nannochloropsis oceanica* IMET1 under nitrogen-deficiency stress. *Bioresour Technol* 130:731–738
- Yoon K, Han D, Li Y, Sommerfeld M, Hu Q (2012) Phospholipid:diacylglycerol acyltransferase is a multifunctional enzyme involved in membrane lipid turnover and degradation while synthesizing triacylglycerol in the unicellular green microalga *Chlamydomonas reinhardtii*. *Plant Cell* 24:3708–3724

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.