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An increase in the membrane lipids recycling by *PDAT* overexpression stimulates the accumulation of triacylglycerol in Nannochloropsis gaditana

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ABSTRACT

Oleaginous microalgae represent potential feedstocks for the sustainable production of lipids thanks to their ability to accumulate triacylglycerols (TAGs). TAG accumulation in several algal species is strongly induced under specific conditions such as nutrient deprivation and high light which, however, also negatively impact growth. Genetic modification of lipogenic pathways can potentially enhance TAG accumulation without negatively affecting growth, avoiding the trade-off between biomass and lipids productivity.

In this study, the phospholipid: diacylglycerol acyltransferase (PDAT), an enzyme involved in membrane lipid recycling, was overexpressed in the seawater alga *Nannochloropsis gaditana*. *PDAT* overexpression induced increased TAG content in actively growing algae cultures while no effects were observed in conditions naturally stimulating strong lipid accumulation such as high light and nitrogen starvation. The increase of TAG content was confirmed also in a strain cultivated in industrially relevant conditions even though *PDAT* overexpression, if too strong, the gene overexpression becomes detrimental for growth in the longer term. Results overall suggest that genetic modulation of the *PDAT* gene represents a promising strategy to increase microalgae lipids content by minimizing negative effects on biomass productivity.

KEYWORDS: algae, biodiesel, triacylglycerols, Nannochloropsis, lipids homeostasis, genetic engineering

1. INTRODUCTION

Global climate change linked to the accumulation of greenhouse gases is making extremely clear the necessity of finding new sustainable sources of energy and materials. Nowadays, most energy demand is satisfied by fossil fuels, causing anthropogenic emissions of carbon dioxide (CO_2) into the atmosphere. Developing sustainable alternatives to fossil fuels is thus essential to mitigate climate change without compromising energy availability and quality of life. Even though electric technologies for transportation are rapidly developing, it is expected that liquid fuels will remain the primary energy source in this sector in the next future (Oh et al., 2018). Liquid fuels are indeed extremely valuable for transportation because of their high energy density, which becomes essential for high-power transport vehicles, such as airplanes and trucks. This parameter includes high-capacity of energy storage, fast energy release, rapid charge, and it cannot be offered by renewable electricity with the present technology based on batteries (Liao et al., 2016).

A potential alternative to fossil fuels for transportation is biodiesel which can be produced from vegetal oils through a process of trans-esterification (Chisti, 2007). Microalgae have an interesting potential as feedstock for biofuel production avoiding any potential competition with crops cultivated for food production. These organisms have a large capacity of accumulating lipids, at least in a condition of nutrient starvation (Chisti, 2007; Malcata, 2011; Stephenson et al., 2011). It is worth noting that triacylglycerols (TAGs) produced from microalgae can find also many other applications beyond biofuels, including food (Vanthoor-Koopmans et al., 2013). TAGs enriched in polyunsaturated fatty acids (PUFAs), like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), are also highly requested by the pharmaceutical and food industry because of their beneficial effects on the cardiovascular system (Valenzuela et al., 2005; Ward and Singh, 2005; Xin et al., 2019).

Lipids are mainly accumulated in oil bodies as TAG, a glycerolipid composed of a 3-carbon glycerol backbone esterified with three fatty acids. In microalgae, TAGs are known to be synthesized via multiple pathways. The acyl-CoA-dependent pathway, also referred to as the Kennedy pathway, leads to the *de novo* formation of triacylglycerols in the endoplasmic reticulum (ER). In this pathway, fatty acids exported from the chloroplast are sequentially added to a molecule of glycerol-3-phosphate, forming a molecule of TAG. This TAG biosynthetic pathway is conserved among eukaryotes and it has been studied in yeast (Lardizabal et al., 2001; Liu et al., 2011; Sandager et al., 2002), mammals (Cases et al., 2001, 1998), plants (Bouvier-Navé et al., 2000; Hobbs et al., 1999; Xu et al., 2018) and some microalgae as well (Li-Beisson et al., 2015; Li et al., 2016; Niu et al., 2013).

Another pathway is the acyl-CoA independent pathway, mediated by phospholipid: diacylglycerol acyltransferase (PDAT), where acyl moieties from membrane phospholipid/glycolipid are diverted into diacylglycerol (DAG) forming TAG. In *Saccharomyces cerevisiae*, loss of function studies revealed that PDAT preferentially contributes to TAG synthesis during the exponential phase of cell growth (Oelkers et al., 2000). Moreover, PDAT was shown to partially compensate for the loss of DGAT accumulating storage lipids also during the stationary phase, when normally *de novo* synthesis is the prominent pathway (Oelkers et al., 2002; Sandager et al., 2002).

In *Arabidopsis thaliana*, two *PDAT* orthologs are found, among which *PDAT1* is the most studied (Fan et al., 2013; Mhaske et al., 2005; Ståhl et al., 2004; Zhang et al., 2009). Even though *pdat1* loss of function mutants did not show any significant impact on TAG accumulation (Mhaske et al., 2005), a clear demonstration of its role in TAG biosynthesis was provided when it was silenced by RNA interference in a *dgat1* mutant background (Zhang et al., 2009). Indeed, while *dgat1* null mutant showed a partial decrease of TAG content, the loss of both genes compromised TAG synthesis in both pollen and seeds, thereby indicating an overlapping role for *PDAT1* and *DGAT1* in seed oil accumulation. These results strongly suggest that PDAT and DGAT pathways cooperate for TAG synthesis in oil-storing tissues of *Arabidopsis*. A more recent study however revealed that PDAT plays a more crucial role in TAG biosynthesis than DGAT1 in developing leaves, diverting fatty acids from membrane lipids to TAGs (Fan et al., 2013).

Besides yeast and plants, a PDAT-mediated TAG synthesis was found also in microalgae. The model microalga *Chlamydomonas reinhardtii* harbours only one copy of *PDAT*, which contributes, as found in yeast, to TAG synthesis during the logarithmic phase under favourable growth conditions (Yoon et al., 2012), even though a loss of function study revealed that it contributed also to 25% of the total TAG accumulation under nitrogen-depleted conditions (Boyle et al., 2012). Similarly in another green alga, *Lobosphaera incisa*, PDAT was also found to be involved in the conversion of membrane lipids to TAG during nitrogen starvation (Liu et al., 2016).

All these results show the evolutionary conservation of PDAT function in TAG biosynthesis, although the extent of its contribution varies depending on the specific organism and growing conditions. This makes it particularly interesting to assess its role in an organism like *Nannochloropsis gaditana*, an oleaginous

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microalga that showed the ability to accumulate lipids. Being a secondary endosymbiont, *N. gaditana* is evolutionarily far from plants and green algae and is also characterized by peculiar properties of fatty acids and TAGs biosynthesis (Alboresi et al., 2016; Zienkiewicz et al., 2017). Therefore, investigating the role of *PDAT* in such organisms also enables to increase the knowledge about the biological diversity of lipid metabolism in photosynthetic organisms.

In this work, we showed that *PDAT* overexpression increases the lipid accumulation of *Nannochloropsis gaditana*. This phenotype is however dependent on the growing conditions, and it is evident only when TAG biosynthesis is not induced by other factors such as nutrient deprivation. When compared to wild-type, strains overexpressing *PDAT* showed an increase in lipid accumulation in fed-batch cultures without any impact on biomass productivity, underlining how *PDAT* represents an interesting target for genetic engineering aimed at manipulating the lipid content also in microalgae.

2. MATERIAL AND METHODS

2.1 Microalgae cultivation.

Nannochloropsis gaditana (strain 849/5) from the Culture Collection of Algae and Protozoa (CCAP) is the WT strain. Cells were cultivated in sterile F/2 media with sea salts (32 g/l, Sigma Aldrich), 40 mM Tris–HCl (pH 8) and Guillard's (F/2) marine water enrichment solution (Sigma Aldrich). Cultures were generally kept in Erlenmeyer flasks with 100 µmol of photons m^{-2} s^{-1} of illumination and 100 rpm agitation at 22 ± 1 °C in a growth chamber, or in plates on the same F/2 medium supplemented by agar 10 g/L. Batch cultures experiments were performed using a Multicultivator MC 1000-OD system (Photon Systems Instruments, Czech Republic). Before starting the experiment, cultures were maintained in exponential phase for at least two weeks in flasks, diluting them every three or four days at a set initial concentration of OD_{750}= 0.4. These cultures were used to inoculate the Multicultivator MC 1000-OD system. The volume per tube was 80 mL and the starting OD_{680} was about 0.2. An air pump was used to aerate the culture and keep the cells in suspension and then OD_{680} was measured every hour. To acclimate the cultures to the new system, cells were diluted two times every four days. The light was provided continuously at the intensity of 100 µmol of photons m^{-2} s^{-1} using an array of white LEDs. After the period of acclimation, different conditions were tested. Control light (CL) and high light (HL) were set at 100 and 1000 µmol of photons m^{-2} s^{-1}, respectively. Nitrogen limitation (-N) was tested using a modified Guillard's (F/2) marine water enrichment solution without $NaNO_3$. Excess of nutrients (Nutr+) was performed using an F/2 medium enriched with nitrogen, phosphate, and iron sources (0.75 g/L $NaNO_3$, 0.05 g/L NaH_2PO_4 and 0.0063 g/L $FeCl_3 \cdot 6H_2O$ final concentrations).

A fed-batch system was exploited for the evaluation of productivity. Cultures were cultivated at 21 °C in 250 mL Drechsler bottles, at 400 µmol of photons m^{-2} s^{-1} constant light with an insufflation of air enriched with 5% CO_2 at 1 L/h. Cultures were diluted to the same cell concentration ($150 \cdot 10^6$ cell/mL) every 2-3 days. Cells were cultivated in an F/2 medium enriched with nitrogen, phosphate and iron sources (8.82 mM $NaNO_3$, 0.42 mM NaH_2PO_4 and 0.0063 g/L $FeCl_3 \cdot 6H_2O$ final concentrations) (Fattore et al., 2021).

2.2 Biomass productivity and cell duplication rate.

Biomass productivity was estimated by monitoring cultures' dry weight before and after the dilution. Cultures were filtered using 0.45 µm filters, dried at 60 °C for 24 h and weighed. Cell duplication rates were calculated as $([Cell]_F - [Cell]_I) / (t_F - t_I)$, where [Cell] is the cell concentration and t is the number of days.

2.3 Cloning of PDAT and transformation of N. gaditana genome by electroporation.

N. gaditana genomic DNA was extracted from cultures in the exponential phase, grown in Erlenmeyer flasks using F/2 liquid media. Cells were lysed using a Mini Bead Beater (Biospec Products) at 3500 RPM for

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20 s in the presence of glass beads (150–212 μm diameter). Genomic DNA was then purified using the EUROGOLD^{TM} Plant DNA Mini Kit (Euroclone), applying minor modifications. DNA concentration and purity were determined by 100 UV–VIS spectrophotometer (Cary Series, Agilent Technologies). *PDAT* genomic sequence (Supplementary Material) was amplified using the specific primers GGATCCATGCACATGGCATCGAAC (BamHI-ATG-PDAT) and CCCTTACAACTCGACATTCT (Stop-PDAT). The product was then subcloned in pJET1.2/blunt vector (Thermo Fisher). In parallel the exogenous terminator fcpA was sequenced from a vector already present in the lab using the specific primers TCTAGAACCTTCCTTAAAAATTTAAT (XbaI-fcpA for) and TCTAGAGAGCTCGAAAACTCATCCTGTGCCTT (XbaI-SacI-fcpA rev). The exogenous terminator fcpA was then subcloned downstream *PDAT* sequence exploiting XbaI restriction site. After that, PDAT-fcpA was subcloned in the expression vector LCF under the control of the strong promoter LDSP (Lipid Droplet Surface Protein), using BamHI and SacI as restriction sites. LCF harbours the blasticidin-S deaminase gene, conferring the resistance to zeocin. 5 μg of PvuII-linearized vector were used to transform the nuclear genome as described in (Perin et al., 2015). Transformants were selected in F/2 solid medium with zeocin 3.5 μg/mL and then screened for the presence of the *PDAT* transgene, exploiting the colony PCR protocol of (Cao et al., 2009) using templates obtained with the Chelex-100 method. Primers used for screening are XbaI-SacI-fcpA rev and PDAT RNA F reported in table S1.

2.4 In silico analysis.

Genomic DNA and cDNA were sequenced by BMR Genomics. PDAT-peptide sequences of *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Myrmeca incisa* and *Saccharomyces cerevisiae* were identified by performing a BLASTP and using NgPDAT as the query. Protein and nucleotide alignment were performed using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). Good-looking printouts of multiple aligned protein sequences have been created using BOXSHADE 3.21 https://embnet.vital-it.ch/software/BOX_form.html). Transmembrane domain (TMD), N- and C- terminal domains were predicted using InterPro (https://www.ebi.ac.uk/interpro/search/sequence/).

2.5 Total RNA extraction and cDNA preparation.

Total RNA was extracted from the *N. gaditana* batch cultures after 4 days of growth in Erlenmeyer flasks with F/2 media. Cells were lysed using liquid nitrogen and a Mini Bead Beater (Biospec Products) at 3500 RPM for 20 s in the presence of glass beads (150–212 μm diameter). Total RNA was thus purified using the TRI Reagent™ (Sigma Aldrich), applying minor modifications to the manufacturer's instruction. Total RNA concentration and purity were determined by 100 UV–VIS spectrophotometer (Cary Series, Agilent Technologies). The cDNA was prepared from 1 μg of total RNA-template with the Revert Aid Reverse Transcriptase cDNA kit (Thermo Fisher Scientific, Epson, UK). The cDNA was previously treated with the DNAse I kit (Sigma Aldrich). To confirm the efficient gDNA removal from the cDNA prep, the amplification of two flanking genes was performed. The absence of amplicon indicates the absence of gDNA.

2.6 RT-PCR and real-time PCR.

The cDNA was used as the template for the RT-PCR reactions, to detect the differences in expression levels between wild-type and *PDAT*-overexpressing lines. S-adenosyl-l-methionine synthase (SAM) was used as the housekeeping gene. The primers used are listed in Table S1. PCR was performed using 50 ng of cDNA and 25 cycles. Real-time PCRs were performed with the SYBR green (5xHotFire Evagreen qPCR mix, Solis Biodyne, Tartu, Estonia) method in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Data were normalized to the expression of S-adenosyl-l-methionine synthase (SAM (Rosic et al., 2011)) to take care of any cDNA quantity and quality variation. The cycling parameters were 95

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°C for 10 min, followed by 40 cycles at 95 °C for 30 s, annealing at 55° C for 30 s and extension at 72°C for 30 s. qPCR results were analysed using the ΔΔCt method using the Bio-Rad CFX Manager software Version 3.1 (Bio-Rad Laboratories). Three biological replicates of the experiments were performed, and all reactions were done as technical triplicates. Primer sequences are reported in Table S1.

2.7 Lipid quantification and fatty acid composition.

The neutral lipid content was determined by staining cells with Nile Red dye, as described in (Fattore et al., 2021).

Moreover, total lipids were extracted starting from lyophilized samples. The material was shredded as much as possible to have a powder easy to weigh and to facilitate lipids extraction. Samples were weighted (50-70 mg) in glass tubes. The protocol for Fatty Acid Methyl Esthers (FAME) extraction is the one used in (Perin et al., 2017) with minor modifications. As a difference, heptadecanoic acid (C17:0) was used as an internal standard. The sum of all FAME species normalized to mg of lyophilized biomass used for the extraction gave the quantification of lipids for the semi-continuous cultivation expressed as µg FAME/mg biomass.

The following abbreviations were used for the identified FA: C14:0= myristic acid; C16:0= palmitic acid; C16:1= palmitoleic acid; C18:0= stearic acid; C18:1(9)= oleic acid; C18:2(9,12)= linoleic acid; C18:3(6,9,12)= linolenic acid; C20:3(7,10,13)= eicosatriaenoic acid; 20:4(5,8,11,14)= arachidonic acid; 20:5(5,8,11,14,17)= eicosapentaenoic acid.

2.8 Pigment analysis.

Chlorophyll *a* and total carotenoids were extracted using a 1:1 biomass to solvent ratio of 100 % N, N-dimethylformamide (Sigma Aldrich), as reported in (Perin et al., 2015). Cells were collected after 4 or 9 days of growth at the end of the exponential phase. Pigments were extracted at 4 °C in the dark for at least 24h. In the semicontinuous system, pigments were extracted before each dilution of the culture.

2.9 Fluorescence analysis.

Chlorophyll fluorescence was determined *in vivo* using Dual PAM 100 from Waltz. The parameters Fv/Fm and Y(II) were calculated respectively as $(Fm-F_0)/F_m$ and (Fm'-F)/Fm', $qP \cdot F_0/F$ according to (Walz, 2006). For Fv/Fm measuring light was set at 42 µmol of photons m^{-2} s^{-1}, whereas saturating pulse was set at 6000 µmol of photons m^{-2} s^{-1} and lasted 600 ms. Y(II) was calculated exposing algae to a light intensity of 2000 µmol of photons m^{-2} s^{-1} for 8 minutes. All the measurements were performed after 20 minutes of adaptation to the dark.

3. RESULTS

3.1 In silico analysis of PDAT from Nannochloropsis gaditana

In the genome of *Nannochloropsis gaditana,* there is only one predicted isoform of phospholipid: diacylglycerol acyltransferase (PDAT, NgPDAT; Naga_100065g17; genomic DNA sequence in Supplementary Data S1) (Corteggiani Carpinelli et al., 2014). *PDAT* was previously shown to be upregulated when algae are cultivated under strong illumination, a condition that induced TAG accumulation (Alboresi et al., 2016), suggesting it could be a gene involved in lipid biosynthesis.

Genomic and cDNA NgPDAT were re-sequenced to confirm the protein-coding sequence (Figure S1). Comparison of NgPDAT protein sequence with the orthologs of *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Chlamydomonas reinhardtii* and *Lobosphaera incisa*, species where the function of the enzyme has already been demonstrated, showed approx. 30% of identity between all PDAT sequences (Figure 1A). The multiple alignment reveals that the most divergent region corresponds to the N-terminal cytosolic

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domain, whereas the C-terminal domain has a higher similarity. This is the protein region containing several amino acids known to be fundamental for the function of the enzyme (Pan et al., 2015), including a catalytic triad of Ser-His-Asp, a salt bridge Asp-Arg and a so-called lid region that includes a Trp residue with an important role in binding the substrate into the active site. The conservation of all these residues with a fundamental role in the activity as well as the presence of a transmembrane domain (Kim et al., 2011; Liu et al., 2016), suggests that NgPDAT is an integral membrane protein with a likely conserved enzymatic activity in fatty acid recycling as its homologs in other species (Figure 1B).

3.2 Isolation of N. gaditana strains overexpressing PDAT

PDAT genomic sequence was cloned into a *Nannochloropsis* transformation vector under the control of LDSP promoter and fcpA terminator (Figure S2A) and colonies were selected after transformation and growth in the presence of an antibiotic. Resistance and *PDAT* overexpression cassettes are likely inserted together during transformation and thus insertion in multiple copies or position effects should similarly affect both their expression levels. For this reason, a group of strains expressing more abundantly the antibiotic resistance should be more likely to include some over-expressing *PDAT* to higher levels as well. Following this hypothesis, 250 resistant strains were thus exposed to increasing concentrations of antibiotic identifying 12 strains showing higher tolerance to selection (Figure S2B). In all those 12 lines the integration of the transgene was confirmed by PCR (Figure S2B) and screened for higher transcript levels by semiquantitative PCR. Among these, two strains showed higher levels of transcript than wild-type – PDAT #11 and PDAT #38 (Figure S2C). The result was confirmed by real-time PCR, indicating they have 3.89 $\pm$ 1.25 and 6.89 $\pm$ 3.10 fold higher expression of *PDAT* than WT and thus were selected for phenotypical investigation (Figure 2).

3.3 Impact of PDAT on algae growth and lipid accumulation.

The two independent overexpressing lines identified were tested in batch cultures to assess if *PDAT* overexpression had any effect on growth and lipid content. To assess the possible impact of culture conditions on the phenotype, cells were cultivated in different nutrient availability and illumination intensity, factors known to affect both growth and lipid accumulation (Alboresi et al., 2016; Simionato et al., 2011). Cells cultivated in minimum media (F/2) and irradiated with 100 µmol of photons m^{-2} s^{-1} (CL, Figure 3A) were compared with others exposed to 1000 µmol of photons m^{-2} s^{-1} (High Light, HL, Figure 3B), or with a nitrogen depleted medium (-N, Figure 3C) or with increased nutrients (Nutr+, Figure 3D).

Different growing conditions showed clear effects on algae growth; however, PDAT #11 and PDAT #38 did not show any significant difference when compared to the parental line in all the tested conditions, as shown in Figure 3. Similar to WT, overexpressing strains showed slower growth in HL than CL, suggesting an inhibitory effect of intense illumination (Figure 3A-B) (Alboresi et al., 2016). Cells cultivated with increased nutrients were still in the exponential phase after 5 days of growth (Figure 3C), whereas, as expected, nitrogen deprivation drastically impaired cell duplication, causing a strong reduction of growth kinetics (Figure 3D).

In WT cells TAG accumulation, quantified using Nile Red staining that in *Nannochloropsis* was verified to correlate strongly with the accumulation of neutral lipids (Simionato et al., 2013), increased in HL and strongly enhanced under Nitrogen depletion while in Nutr+ its content decreased, all results consistent with previous data on the species (Figure 4, (Alboresi et al., 2016; Simionato et al., 2013)). *PDAT* overexpressing lines showed higher TAG accumulation by approx. 2-fold than the parental strain in both CL and Nutr+ conditions (Figure 4B, C). On the contrary, no significant difference was detectable in HL or N deprivation (Figure 4A, D). These results showed that the overexpression of *PDAT* increases TAG accumulation in *Nannochloropsis gaditana*, but only in conditions where these are not normally largely

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accumulated, such as control light and nutrient enrichment. On the contrary, when there is a metabolic induction of TAG accumulation, the overexpression of *PDAT* is not influential.

3.4 Evaluation of lipid accumulation and biomass productivity in a lab-scale photobioreactor

The above-described lipid phenotype is potentially interesting for lipid production at an industrial scale since it could increase TAG accumulation in *Nannochloropsis gaditana* cultures in conditions of active growth, where TAG accumulation is normally not strongly induced. To verify this point *PDAT* overexpressing strains were cultivated in lab-scale photobioreactors in conditions simulating industrial cultivation (Perin et al., 2017). Cultures were run in semi-continuous mode (see Material and Methods for details), an operational strategy largely employed at industrial-scale and that allows a shorter downtime than batch processes while ensuring culture stability and productivity over prolonged periods (Benvenuti et al., 2015). Cultures were set up to work at high biomass concentration, around 1 g/L, to simulate the growth conditions achieved in industrial cultivation systems (Benvenuti et al., 2015). Nutrients (nitrogen, phosphate, iron) were all provided in excess, as well as the CO_2 supply, to avoid the influence of these potentially limiting factors on performances.

Surprisingly, in these conditions, only one of the two lines was able to maintain a stable growth (Figure 5). Indeed, one of the overexpressing strains (#38) stopped growing after approx. 10 days of semicontinuous culture (Figure 5), despite showing no detectable growth defect in batch cultures nor in the first days of semicontinuous cultivation. This defect was confirmed by starting the culture multiple independent times in the photobioreactor, with the same result (two examples are shown in figure 5). This phenotype was also confirmed with a third overexpressing line that similarly showed an unstable growth under semi-continuous cultures (Figure S4).

This growth inhibition is correlated with a loss of photosynthetic efficiency, with PSII quantum yield (Fv/Fm) that decreased progressively. On the contrary, PDAT #11 was instead able to grow in a semicontinuous mode for over 8 weeks showing a constant growth rate. PDAT#11 also did not show any significant difference in the content of chlorophyll (Chl) and Carotenoid (Car) per cell nor in photosynthetic activity (Table 1), not even if cells are exposed to excess illumination (Figure S3).

Table 1 Pigment composition and photosystem II efficiency (Fv/Fm) of PDAT-overexpressing line and WT. Pigment content of WT and PDAT #11 was assessed at every dilution of the cultures run in semi-continuous mode. Chl content and Car content are reported (n = 15). Photosystem II quantum yield quantified from Fv/Fm, of WT and PDAT #11 run under semi-continuous mode. No differences were observed (n = 18).

	WT	**PDAT #11**
Chl a content (pg/cell)	0.08 ± 0.02	0.09 ± 0.02
Car content (pg/cell)	0.03 ± 0.01	0.02 ± 0.01
Fv/Fm	0.56 ± 0.02	0.54 ± 0.02

In PBR cultures, the cell duplication rate in PDAT#11 was 19% lower than WT (Figure 6A) but showed similar biomass productivity (Figure 6B). PDAT #11 cells were larger (figure 6C) and accumulated more TAG accumulation on a per cell basis (Figure S5). When normalized to the biomass content, the increase in TAG accumulation was smaller but confirmed the effect of *PDAT* overexpression also in a lab-scale photobioreactor (Figure 6D).

A comparison of WT and PDAT #11 fatty acid profiles showed no major differences with only a minor increase in oleic acid (C18:1) in PDAT #11 (Figure 7), suggesting that the modification had no major effect on FA biosynthesis but rather on the distribution of FA in lipids classes.

4. DISCUSSION

4.1 PDAT is a potential target gene to stimulate TAG accumulation under optimal growth conditions

The biosynthesis of triacylglycerols in microalgae can follow two pathways: the Kennedy pathway, where TAGs are *de novo* formed by the sequential addition of three fatty acids to glycerol-3-phosphate, and the acyl-CoA independent pathway where membrane fatty acids are recycled into TAGs. The latter pathway is catalysed by PDAT, an enzyme found in photosynthetic organisms and yeasts, but not in animals. PDAT role has been functionally characterized in some plants and microalgae, demonstrating its involvement in fatty acid recycling and triacylglycerol synthesis, and also showing that the overexpression of this gene can successfully increase the accumulation of TAGs (Boyle et al., 2012; Fan et al., 2013; Kim et al., 2011; Liu et al., 2016; Pan et al., 2013; Wang et al., 2013). Sequence similarity suggests a conserved enzymatic activity for the PDAT *Nannochloropsis gaditana* (figure 1). Transcriptomic analysis showed that *PDAT* is upregulated in conditions triggering lipid accumulation (Alboresi et al., 2016; Janssen et al., 2020), suggesting that the increase in *PDAT* expression could enhance lipid accumulation.

In this work, we demonstrated that the overexpression of *PDAT* induces the accumulation of TAGs in *N. gaditana*, with a two-fold accumulation of TAGs in control light and with increased nutrients (Figure 4 A, D). On the other hand, no effect on TAG content was observed in overexpressing lines compared to WT both in nitrogen deprivation and high light (Figure 4B, C), where TAG accumulation is already strongly induced (Alboresi et al., 2016; Pal et al., 2011; Sforza et al., 2012; Simionato et al., 2013; Teo et al., 2014). Most likely, in those conditions algal metabolism has already been altered for enhanced TAG biosynthesis and in these conditions, the *PDAT* overexpression has no visible impact.

This phenotype can be also explained by considering that stressful conditions such as HL and nutrient limitation are known to inhibit the synthesis of polar lipids like galactolipids (Simionato et al., 2013), the likely substrates of PDAT. It can thus be expected that in those conditions, *PDAT* overexpression shows no visible effect. This is different from what was observed by targeting *DGAT*, the main gene involved in the Kennedy pathway, whose overexpression was shown to induce a 129% increase of TAG content also under nitrogen limitation in *Nannochloropsis* (Li et al., 2016). The substrates of TAG *de novo* synthesis, such as glycerol-3-phosphate and free fatty acids, likely remain available in those conditions (Alboresi et al., 2016; Dong et al., 2013; Driver et al., 2017; Fukuda et al., 2018), enabling an increase of TAG biosynthesis with a *DGAT* overexpression.

On the other hand, PDAT overexpression during optimal growth conditions when the synthesis of polar lipids is more active can likely increase the flux of their recycling to TAGs, improving their accumulation. Similar observations were found also in yeast and *Chlamydomonas*, where *PDAT* knock-out and knock-down studies caused a less TAG content during the logarithmic phase of growth under favourable conditions (Oelkers et al., 2000; Yoon et al., 2012). In *Arabidopsis,* PDAT was shown to play a major role in developing leaves, with a less extent during senescence (Fan et al., 2013). Taken together, all these results highlight the evolutionary conservation of PDAT function during rapid cell growth and membrane proliferation.

These results are potentially interesting from the perspective of exploiting modified strains to improve lipid productivity for cultivation on a large scale. To achieve this objective ideally TAG accumulation should be obtained in conditions where cells are also growing optimally. In several algal species, large lipid accumulation can be induced by controlling growing conditions, with nutrient deprivation well known to have a major effect. While this method is very effective at the lab scale, however, fine control of nutrient supply is complex to implement at a large scale. Furthermore, conditions inducing lipid accumulation also inhibit biomass productivity with no positive impact on overall lipid yield. Strains capable of accumulating more lipids while maintaining optimal growth are thus highly valuable for practical applications. This work showed in an industrially relevant environment that *PDAT* overexpression can result in a higher lipid

production than WT also when algae are cultivated in a lab-scale photobioreactor. This was obtained while having no negative impact on biomass productivity or FA composition. The cell duplication rate is reduced by 19% but this is compensated by an increase in lipid accumulation (Figure 6, Figure S5). Consistent with this maintained biomass productivity, the photosynthetic analysis revealed no differences in the PSII quantum yield and pigment composition in the overexpressing line, suggesting that there is no major impact on the photosynthetic complexes' composition and activity (Table 1).

4.2 PDAT overexpression redirects photosynthates to TAG biosynthesis with potential effects on lipids homeostasis

Results obtained suggest that *PDAT* overexpression causes an alteration of carbon flux toward TAG synthesis, likely mediated by enhanced membrane lipid recycling (Figure 8), thus increasing the turnover rate of membrane lipids. However, membranes are also essential cellular components and their homeostasis is essential for growth duplication.

It is thus interesting to observe that among the lines tested #38, showing the stronger *PDAT* overexpression also showed negative effects in long-term cultures (Figure 5), impairing growth. These growth defects are visible only in photobioreactors, where CO_2 and nutrients were provided in excess, thus strongly stimulating growth and cell duplication, likely increasing the demand for polar lipids. Also, it is interesting to observe that these negative effects are not immediately observed, but only after several culture dilutions, suggesting that cells have some reserves capable of compensating for some biosynthesis defects. After several generations, however, these reserves are depleted, disrupting membrane homeostasis. The observation that the different effect observed in the various strains correlates with *PDAT* expression levels suggests that any genetic modification must be precisely optimized to find the best trade-off between induction of TAG accumulation and growth capacity.

5. CONCLUSIONS

Results presented in this work demonstrate that modulation of *PDAT* can be exploited to stimulate TAG accumulation under favourable growth conditions, thus allowing to development of algae strains where optimal growth is combined with a larger accumulation of lipids. A strain overexpressing *PDAT* showed increased TAG content and unaltered biomass productivity, also when cultivated in industrially relevant conditions in lab scale photobioreactors, demonstrating the possibility of improving algae lipid productivity by genetic engineering. Excessive overexpression, however, can result in negative effects that are observable only in the longer term.

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Authors' contributions N.F. performed most experiments and analysed the data. A.B. contributed to transformation and strain characterization. F.B., S.B. and MEM performed the fatty acid analysis. TM designed and supervised the project. NF and TM wrote the paper. All authors revised the text

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Figure legends

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A

	NgPDAT	LiPDAT	CrPDAT	ScPDAT	AtPDAT1	AtPDAT2
NgPDAT	100.00	36.42	31.29	31.68	34.61	32.92
LiPDAT		100.00	37.96	30.29	32.74	33.33
CrPDAT			100.00	29.70	30.91	30.02
ScPDAT				100.00	33.05	34.22
AtPDAT1					100.00	59.38
AtPDAT2						100.00

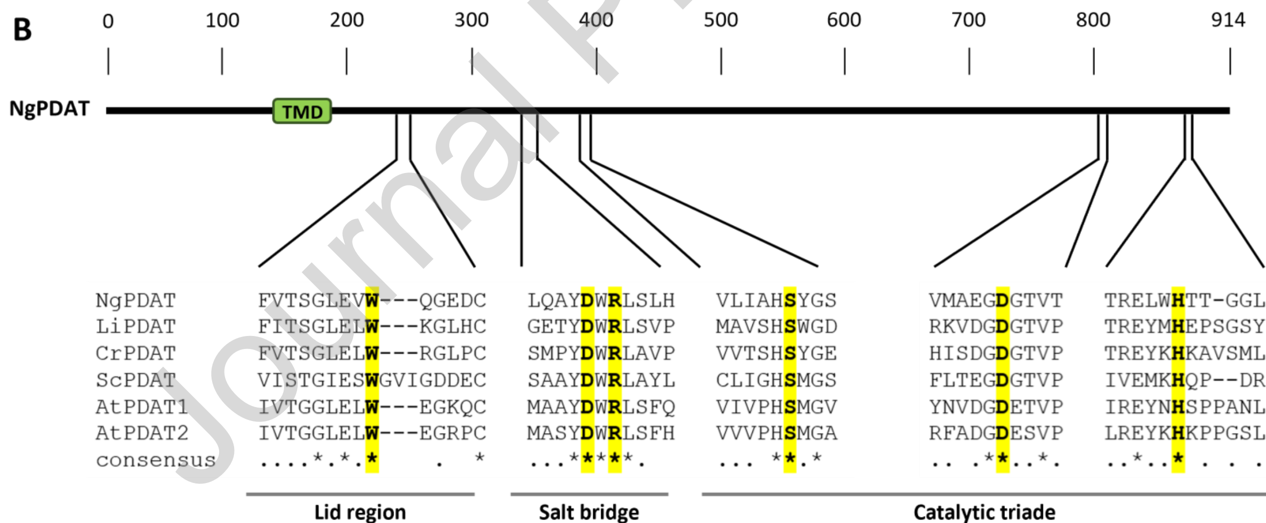

Figure 1. Comparison of peptide sequence of NgPDAT with putative orthologs. A) Multiple alignment was performed using the peptide sequences of *N. gaditana* (NgPDAT), *C. reinhardtii* (CrPDAT), *L. incisa* (LiPDAT), *A. thaliana* (AtPDAT1 and AtPDAT2) and *S. cerevisiae* (ScPDAT). The alignment was achieved using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle/). B) Conservation of specific amino acid in NgPDAT: a Trp in Lid region, the Asp-Arg salt bridge and the Ser-His-Asp catalytic triad. All the conserved amino acid residues are highlighted in yellow. The green box shows the presence of a conserved transmembrane domain (adapted from Pan et al. 2015). NgPDAT: EWM21809.1; LiPDAT: ANH71127.1; CrPDAT: AFB73928.1; ScPDAT: NP_014405.1; AtPDAT1: NP_196868.1; AtPDAT2: NP_190069.1.

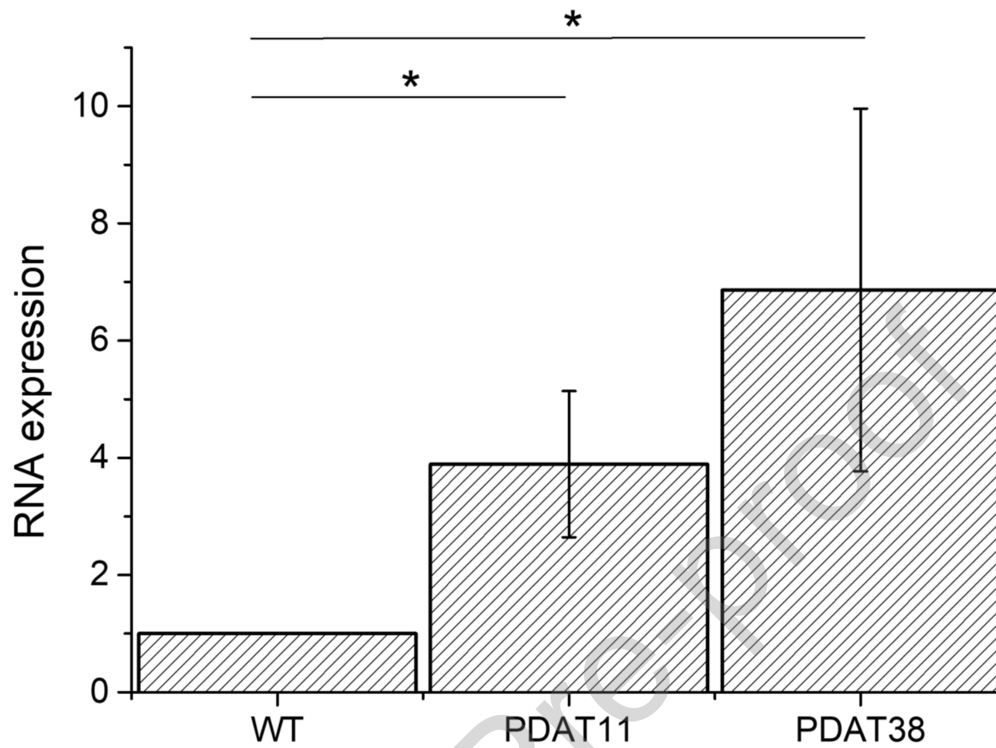

Figure 2. Verification of RNA expression levels in *PDAT*-overexpressing lines. Real-time PCR on WT, PDAT#11 and PDAT#38; expression analysis has been performed with the ΔΔCt method. RNA expression is represented as a relative increase with respect to the WT. Mean values ± SD are indicated. The asterisks evidence that the increase of expression is statistically significant. The data are normalized on WT signal ($n = 4$, One-way ANOVA $p < 0.01$).

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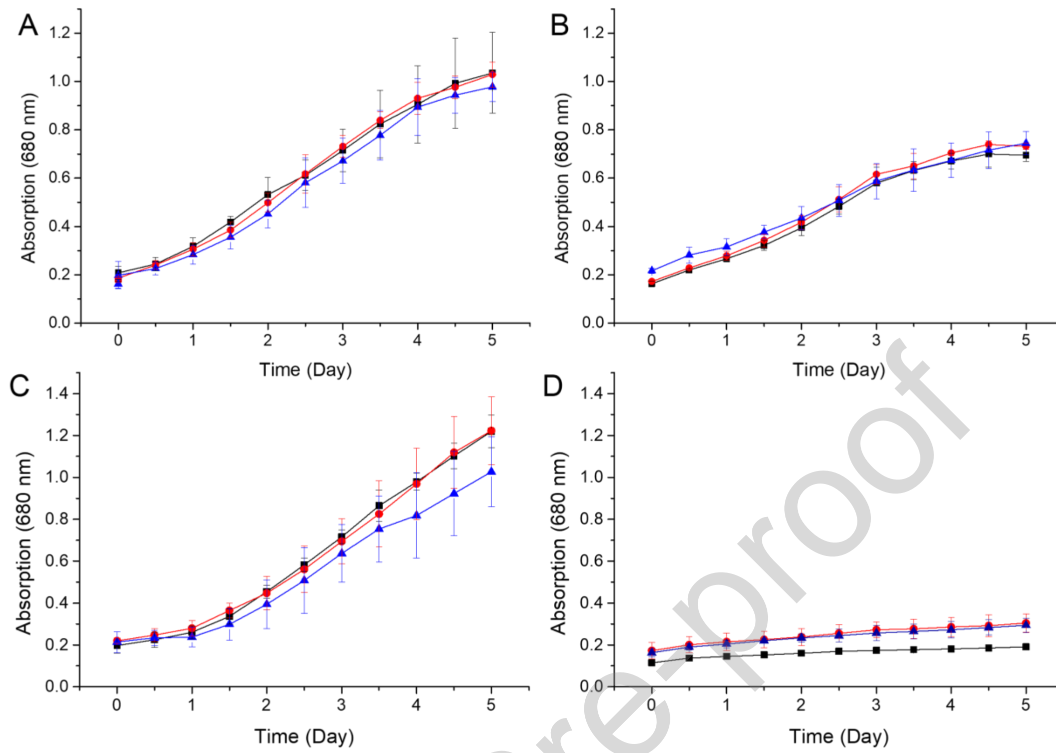

Figure 3. Growth curve *PDAT*-overexpressing lines and wild type. Growth curve of *Nannochloropsis gaditana* cells cultivated for 5 days with CL (100 µmol of photons m^{-2} s^{-1}; A), HL (1000 µmol of photons m^{-2} s^{-1}; B), increased nutrients (Nutr+.; C) and nitrogen deprivation (-N; D). WT, PDAT #11 and PDAT #38 are represented in black, red and blue, respectively (n=3 for CL, HL and Nutr+; n=2 for -N). Optical density at day 0 is 0.2, which corresponds to approx. $9 \cdot 10^6$ cells/mL.

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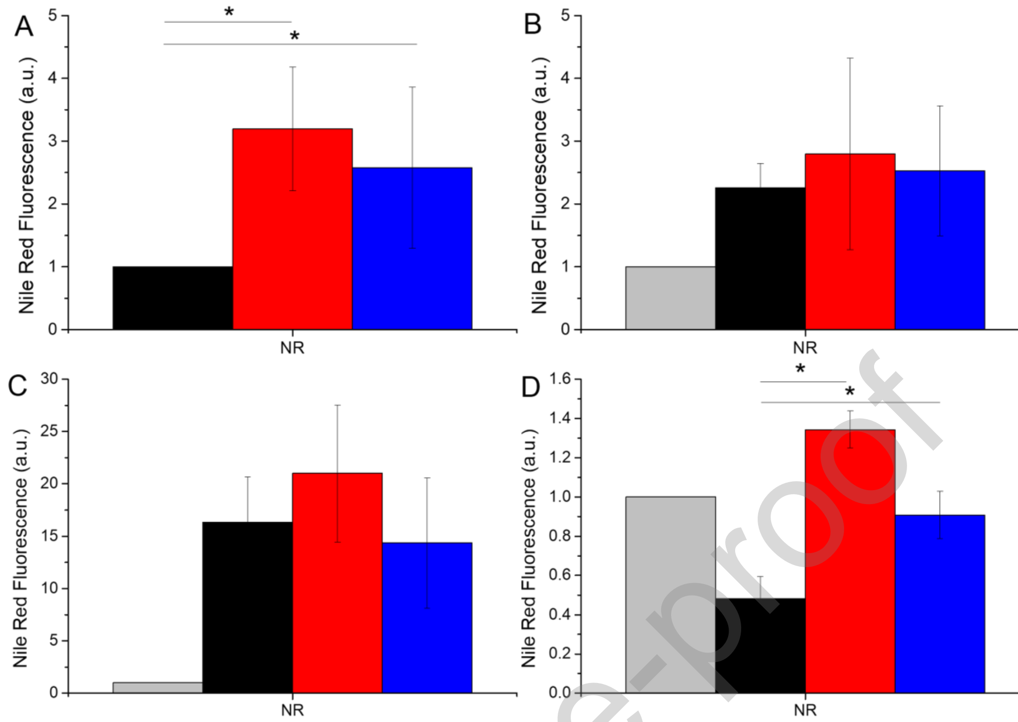

Figure 4. Lipid fluorescence analysis of *PDAT*-overexpressing lines and wild type. Lipid fluorescence analysis was performed using Nile Red staining after 5 days of growth with CL (100 µmol of photons m^{-2} s^{-1}; A), HL (1000 µmol of photons m^{-2} s^{-1}; B), nitrogen deprivation (-N; C) and increased nutrients (Nutr+; D). WT, PDAT #11 and PDAT #38 are represented in black, red and blue, respectively. Grey boxes represent WT lipid fluorescence of A condition, to make comparisons in each different condition. All data are normalized to WT in the control light to check the relative increase. Averages $\pm$ SD are represented (n = 3). Asterisks indicate statistical differences with WT PLUS (One-way ANOVA, $p < 0.05$).

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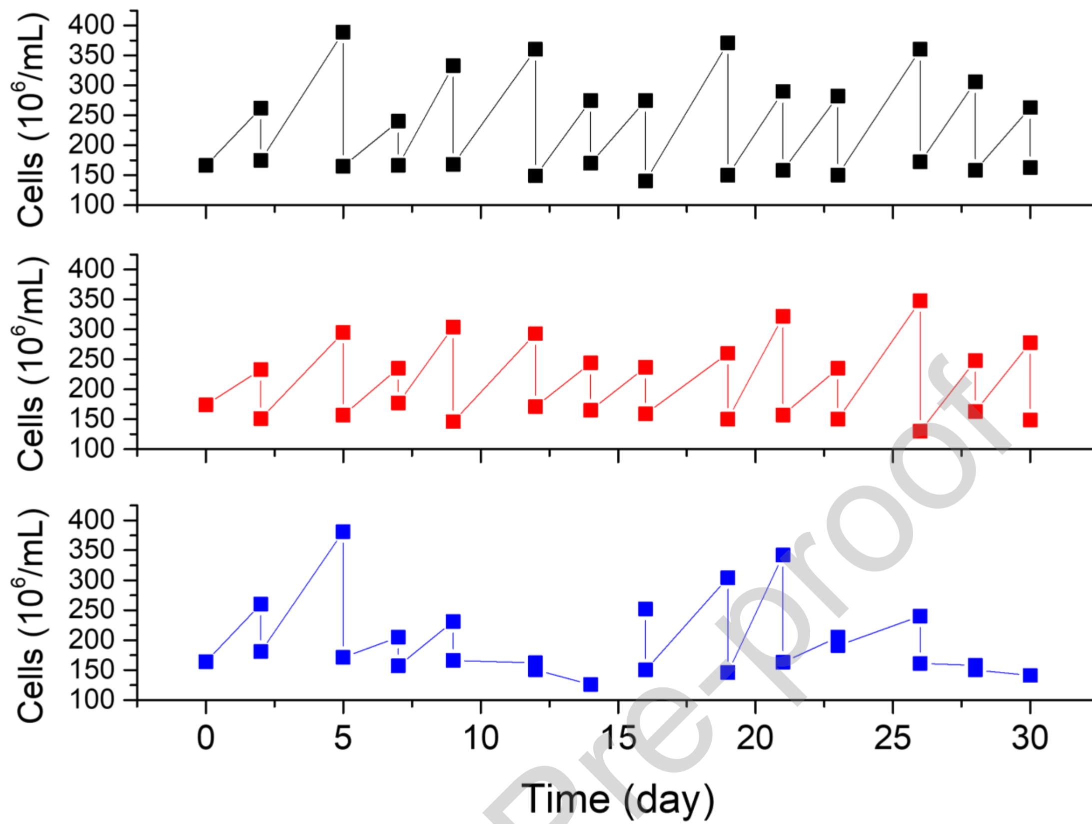

Figure 5. Semicontinuous growth of *PDAT*-overexpressing lines. Example of data of cells cultivated in semicontinuous mode. Cultures were diluted back to a set cell concentration (150 · 10^6 cells/mL) every two or three days. WT, PDAT #11 and PDAT #38 are represented in black, red and blue respectively.

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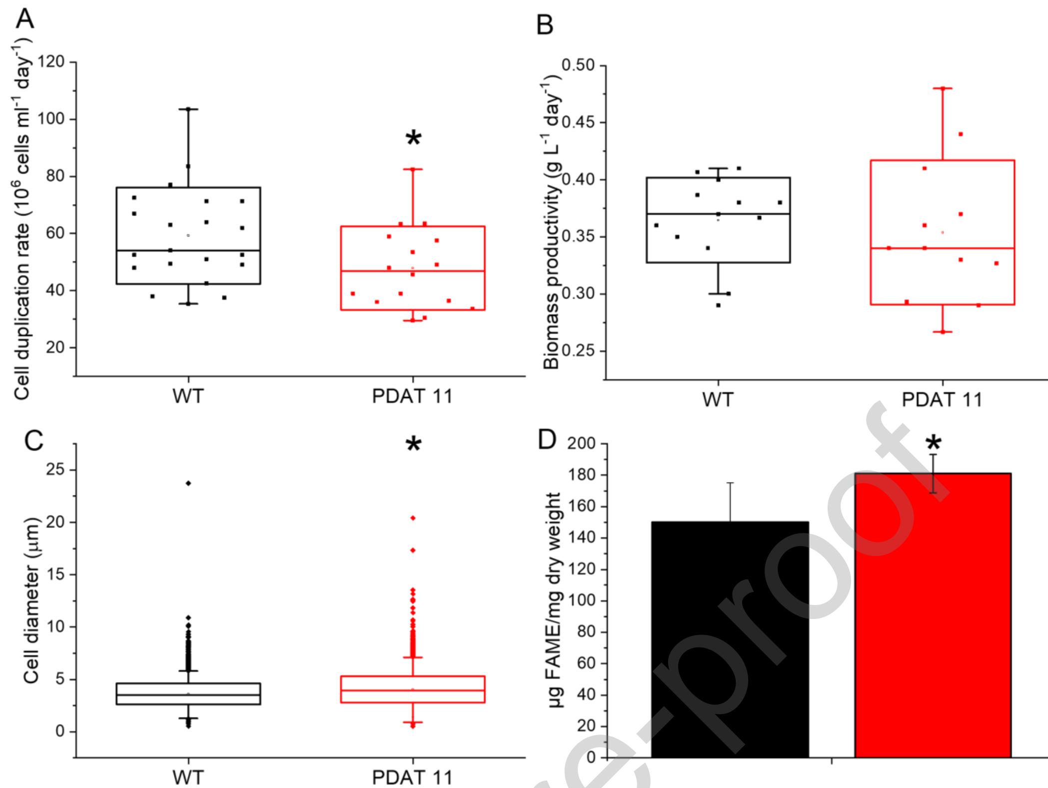

Figure 6. Effect of *PDAT* overexpression on cell duplication, biomass productivity, cell dimension, lipid accumulation. Cell duplication (A) and biomass productivity (B) were calculated from initial and final values before and after each culture dilution. All original data are shown as squares (n > 12). C) Before each dilution cell diameter was estimated (n > 40). D) Lipid accumulation was quantified by Total Fatty Acid Methyl Esthers (FAME) extraction expressed as µg FAME/mg dry weight. WT and PDAT #11 are always represented in black and red, respectively. Statistically significant differences with WT are marked with an asterisk (One-way ANOVA, $p < 0.05$, n > 16 for A), C); n ≥ 4 for D)). Averages are shown as white squares while boxes represent median and 25-75 percentiles in figures A, B, and C, whereas averages ± SD are represented in figure D. All data were obtained from cells cultivated in lab-scale photobioreactors.

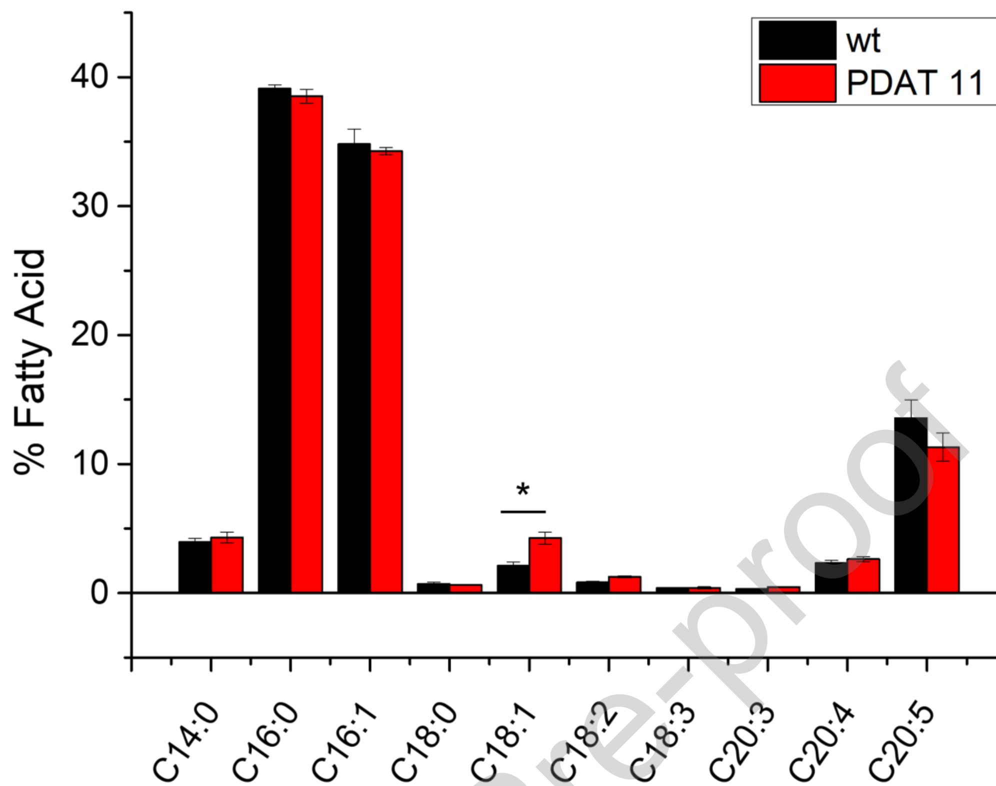

Figure 7. Total fatty acid profile of WT and PDAT #11 overexpressing line. The material from dilutions of the lab-scale photobioreactor was analysed through GC. WT and PDAT #11 are always represented in black and red, respectively. Averages ± SD are represented (n = 3). The only statistically significant difference is for C18:1 (One-way ANOVA, $p < 0.05$).

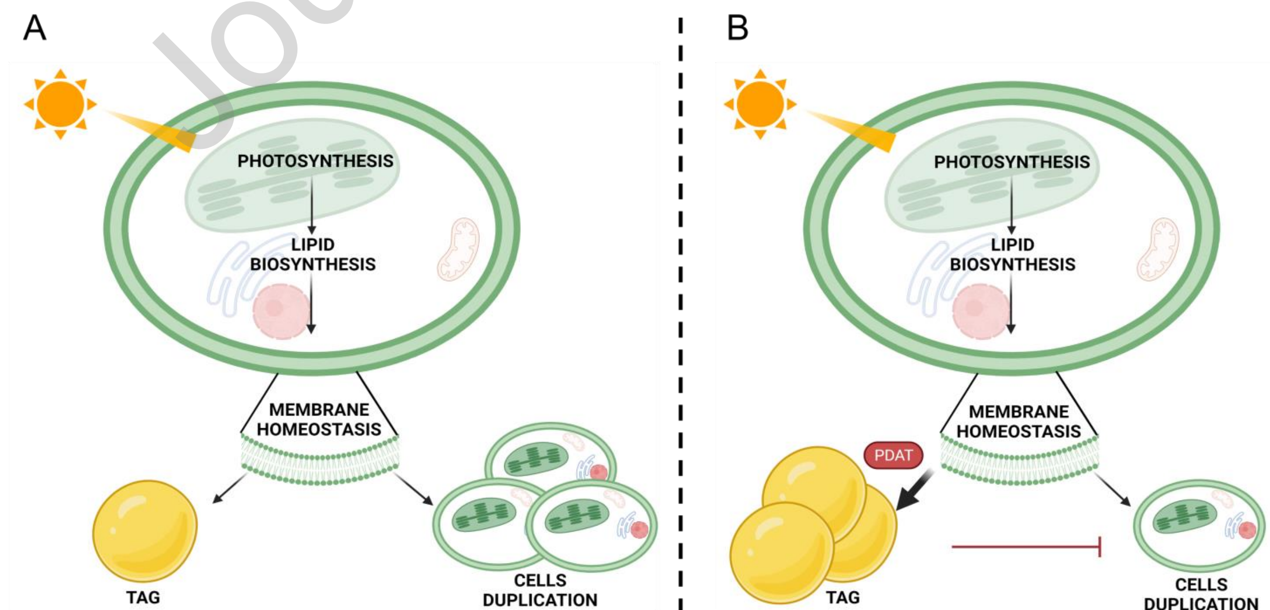

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Figure 8. Effect of *PDAT* overexpression on cell metabolism. Schematic representation of cell metabolism in WT (A) and *PDAT*-overexpressing line (B). In the parental line, photosynthates are mainly exploited for cell duplication during optimal growth conditions. PDAT overproduction, however, enhances the diversion of membrane fatty acids into TAGs, the content of which is increased. To maintain membrane homeostasis, part of photosynthates is reallocated toward lipid synthesis, at the expense of cell duplication. Created with BioRender.com.

Supplementary

Table S1. Primer sequences used in RT-PCR and real-time experiments.

Name	Sequence
PDAT RNA F	GGCAGTTCAACTCCCTCAAG
PDAT RNA R	CCGCGCATAGATAGAAGACC
Q SAM F	CACCCTTTGTCGGTCTTTGT
Q SAM R	GGGCCGTTTCAAATTCAGGT
PDAT RT_2R	CATCCGGCTTACCATTGTCG
PDAT RT_2F	CGGTCCACCTGTGAAGGATT

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Figure S1. *PDAT* cDNA sequence. A) Comparison of *PDAT* coding sequence annotated in www.nannochloropsis.org (target) and the sequence obtained by cDNA amplification (query). The picture shows that the nucleotide sequence of the intron 3 underlined by the red line is instead part of the cDNA. B) Schematic representation of PDAT genomic sequence with intron/exon alternation, and cDNA sequence with the retention of intron 3.

Figure S2. Verification of *PDAT*-expression vector in *N. gaditana* genome and verification of expression levels in *PDAT*-expressing lines. A) *PDAT* genomic sequence (orange) was cloned under the control of a constitutive strong promoter (LDSP, grey) and upstream of an exogenous terminator (fcpA, red). Black arrows indicate the primer used for the PCR colony. B) PCR to verify the integration of the vector in the genome, using a forward primer inside the genomic sequence of *PDAT* and a reverse primer at the level of

the exogenous terminator, to discriminate transgenic lines from wild-type. An amplicon of ~714 bp was obtained for all the transgenic lines but not in wild type. S-adenosyl-l-methionine synthase (SAM) was used as a positive control (marker: GeneRuler 1 kb DNA Ladder – Thermo Scientific). C) RT-PCR performed on WT and four *PDAT*-expressing lines selected with a high concentration of antibiotic. Two transgenic lines (PDAT #11 and PDAT#38) have a more intense band than the parental line. S-adenosyl-l-methionine synthase (SAM) was used as a housekeeping gene as reference.

Figure S3. Evaluation of photosynthetic functionality in *PDAT*-overexpressing line. The effective quantum yield of PSII (Y(II)) of WT and PSAT #11 (black and red respectively) was calculated by exposing cells to 8 minutes of light (2000 µmol of photons m^{-2} s^{-1}) (n = 7).

Figure S4. Semicontinuous growth of 3rd *PDAT*-overexpressing line. As in figure 5, cells were cultivated in semicontinuous mode. Cultures were diluted back to a set cell concentration ($150 \cdot 10^6$ cells/mL) every two or three days. WT, PDAT #55 are shown in black and green respectively.

Figure S5. Lipid quantification of *PDAT*-overexpressing lines and wild type in semi-continuous mode. Lipid quantification has been represented in figure 6D as µg FAME/mg dry weight. We also verified lipid content by Nile Red staining (A) and normalizing the µg FAME on mln cells instead of dry weight (B).

Data S1. Genomic DNA sequence of PDAT (Naga_100065g17).

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CRediT authorship contribution statement

TM and NF designed the study; NF, AB, FB, SB and MEM Collected and assembled data; NF, FB, and TM analyzed the data; NF and TM wrote the paper; All authors critically revised the manuscript.

Declaration of Competing Interest

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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HIGHLIGHTS

- • PDAT was overexpressed in *Nannochloropsis gaditana*
- • Overexpression induced accumulating of triacylglycerols
- • Increased lipids accumulation was confirmed in long term photobioreactors cultures
- • Strong overexpression showed negative impact on growth, only visible on long term

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