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Inducible expression of *Haematococcus* oil globule protein in the diatom *Phaeodactylum tricornutum*: Association with lipid droplets and enhancement of TAG accumulation under nitrogen starvation

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ABSTRACT

Microalgae accumulate the storage lipids triacylglycerols (TAG) in lipid droplets (LDs) in response to environmental cues. Mechanistic insights into LD biogenesis in microalgal cells hold great promise for the manipulation of TAG production. In this report, we demonstrate the heterologous expression of a major LD protein of the green microalga *Haematococcus pluvialis* in the diatom *Phaeodactylum tricornutum*. The N-terminus of *Haematococcus* oil globule protein (HOGP) was fused with EGFP and expressed under the control of the strong light-inducible *fcp*A gene promoter and the novel nitrogen-starvation-inducible *DGAT1* gene promoter. Fatty acid feeding enabled fast monitoring of LDs formation under nitrogen-replete conditions and detection of a fluorescent EGFP-HOGP signal as ring-like structures adjacent to the periphery of LDs. During the prolonged nitrogen starvation, EGFP-HOGP was expressed only under the control of the *DGAT1* promoter and was similarly associated with LDs. Furthermore, expression of HOGP under nitrogen starvation enhanced TAG accumulation. The tools and approaches described in this report open the door for characterizing endogenous LD proteins in *P. tricornutum*. The results of this study indicate the significant biotechnological potential of such LD-associated protein genes in the metabolic engineering of microalgae for enhanced oil productivity.

1. Introduction

Numerous photosynthetic microalgae have the capacity to accumulate high concentrations of storage lipids triacylglycerols (TAG) in response to environmental stress conditions, and to nitrogen limitation or deprivation in particular [1–5]. The shortage in petroleum reserves combined with global warming and other environmental problems have necessitated the search for sustainable sources of renewable energy. The high oil (TAG) content of certain species makes them promising candidates for the production of biofuels, since even the most productive oil crops cannot compete with microalgae in terms of biofuel yields, growth rate, untapped water and non-arable land use [6]. Comprehensive understanding of TAG biosynthesis and lipid droplet (LD) biogenesis in microalgae should fuel genetic engineering approaches to modulate the oil-producing capacity of these organisms.

TAG biosynthesis is part of the primary cell metabolism generating carbon and energy-rich neutral lipid molecules that are ultimately sequestered and stored in specialized organelles, often called lipid droplets (LDs), lipid bodies, oil bodies or oil globules (OGs) in oil-seed plants [7]. While TAG biosynthesis and LD biogenesis in plant oil-storing tissues, yeast and mammalian adipose cells have been well studied, they are less understood in plant vegetative tissues, and apparently are even more multifaceted in diverse photosynthetic microalgae. Oil seed cells assemble the bulk of TAG in the endoplasmic reticulum (ER), followed by their sequestration into cytosolic OGs. In microalgal cells TAG biosynthesis and assembly can be also, at least partly, mediated by the chloroplast-localized pathway, which recruits chloroplast membrane lipids and enzymes [8–10]. The *de novo* TAG biosynthesis commences with the esterification of glycerol-3-phosphate and proceeds via four consecutive steps involving three acyltransferases—glycerol-3-phosphate acyltransferase, lyso-phosphatidic acid acyltransferase and acyl-CoA:diacylglycerol acyltransferase (DGAT). Genes encoding acyltransferases involved in this pathway, in particular encoding DGAT1 and DGAT2 isoforms, may show increased expression under stress conditions that facilitate or induce TAG production [11–13].

LDs are ubiquitous cellular structures composed of a neutral lipid core surrounded by a monolayer of amphipathic polar lipids and a variety of proteins. The major OG proteins in oilseeds — oleosins — are localized on the surface of OGs and play a role in preventing their fusion and ensuring seed germination via the binding of lipases for TAG degradation [14,15]. Novel LD-associated proteins involved in the packaging of TAG and stabilization of LDs have been recently discovered in plant oil-storing but non-seed tissues [16]. Unlike stable OGs in seeds, in many other cell types, dynamic LDs are functionally connected to a

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variety of other organelles and cellular processes, such as lipid homeostasis, lipid signaling, and stress response [7,17–21]. In microalgae, the dynamic nature of LDs is essential for growth resumption of the starved cells upon nutrient replenishment and exit from the quiescent state [22].

Until recently, relatively little was known about the biogenesis of LDs and the nature of their proteins in microalgae. Despite apparent progress [10,23–30], the structural proteins and enzymes involved in the biogenesis, maintenance, and degradation of algal oil-storing compartments in microalgae are still insufficiently studied. A number of proteomics studies have identified major lipid droplet proteins (MLDP) in green microalgae (Chlorophyta), such as *Chlamydomonas reinhardtii* [10,23,31], *Haematococcus pluvialis* [24] and *Dunaliella bardawil* [27,32]. The MLDPs of these microalgae show enhanced expression during nitrogen starvation in concert with TAG accumulation.

Despite structural variations, plant oleosins, animal LD-associated proteins of the PAT family [33,34] and major algal LD proteins may possess similar functions [25]. The knockout or silencing of oleosin-coding genes impacts the size and number of accumulated OGs in seeds [35,36]. The partial functional complementation of an oleosin-deficient phenotype of the Arabidopsis mutant *ole1* has been achieved by expression of the major LD surface protein (LDSP) of the oleaginous microalga *Nannochloropsis oceanica* [25]. The repression of *MLDP* gene expression, using an RNA-interference (RNAi) approach, led to increased LD size in *C. reinhardtii*, a phenomenon that strongly resembled the phenotype of the mouse *Btn1a1* (butyrophilin member A1) mutant and the *ole1* mutant of *Arabidopsis* [23]. Furthermore, RNAi-mediated silencing of *MLDP* impaired TAG breakdown and remobilization in nitrogen-starved cells of *C. reinhardtii* upon nitrogen replenishment [10].

So far, the impact of major LD proteins overexpression on TAG accumulation in microalgae has not been reported. We have previously identified a protein, referred to as the *Haematococcus* oil globule protein (HOGP) [24]. The remarkable ability of *H. pluvialis* to sequester large amounts of astaxanthin fatty acid esters and TAG within cytoplasmic LDs, in response to high light stress and nitrogen starvation, is associated with HOGP abundance, pinpointing the major role played by HOGP in LD biogenesis. Previous research lacked, however, direct experimental evidence of HOGP association with LDs and of its role in LD formation.

In this work, we overexpressed HOGP in the diatom *Phaeodactylum tricornutum*—an attractive microalgal model because of its short generation time, genome and transcriptome information [37–39], ease of genetic transformation [40], and availability of molecular tools required for functional genomics, such as gene silencing [41] and genome editing [42,43]. Therefore, this microalga is an emerging model for studying microalgal cellular and molecular biology and for applying molecular engineering approaches to biochemical pathways, such as lipid-biosynthesis pathways. Nitrogen starvation triggers TAG and LDs formation in *P. tricornutum* and induces a remarkable enhancement in the expression of the *DGAT1* gene [12].

Using *P. tricornutum* as a model, we overexpressed HOGP fused to an enhanced green fluorescent protein (EGFP) using two differently regulated *P. tricornutum* endogenous promoters: the light-regulated fucoxanthin chlorophyll binding protein A (*fcp*A) gene promoter and the nitrogen-starvation-inducible *DGAT1* promoter. We set to demonstrate whether the EGFP-HOGP localization signal is associated with LDs in diatom cells. By exploring a novel nitrogen-starvation-inducible *DGAT1* gene promoter, we also examined the impact of HOGP overexpression on TAG accumulation under nitrogen starvation.

2. Materials and methods

2.1. Strain and culture conditions

Axenic culture of *P. tricornutum* strain 646 was obtained from the Culture Collection of Algae and Protozoa (CCAP) at the Scottish Marine Institute (SAMS Research Services Ltd., Oban, UK). *P. tricornutum* was cultivated in either 250 mL Erlenmeyer flasks (100 mL culture) or 96 well plates (200 μL culture), on reef-salt-enriched (RSE) medium in an incubator shaker (120 rpm) under enrichment with 200 mL min^{-1} CO_2, controlled temperature (18 °C) and illumination (50 μmol photon m^{-2} s^{-1}). Some experiments were performed at 25 °C and illumination of 100 μmol photon m^{-2} s^{-1}. The RSE medium was composed of 34 g L^{-1} reef salt (Seachem, Madison, GA) with the modifications described [12]. To induce nitrogen starvation, daily-diluted cultures were centrifuged (1200 *g* for 5 min), washed twice in and resuspended in a nitrogen-free RSE medium, lacking KNO_3. Samples for cell counting, RNA extraction for qRT-PCR analysis and protein extraction for western blot analysis were withdrawn periodically. The growth parameters [cell number, chlorophyll content and dry weight (DW)] were determined essentially as described in [44]; prior the DW determination, cells were washed with five volumes of ammonium formate (3%, *w*/*v*).

2.2. Plasmid design and construction

PCR amplifications were carried out with PhusionHot Start II DNA polymerase (Finnzymes, https://www.thermofisher.com). Primers were designed with the Primer Design tool for the In-Fusion HD Cloning Kit, and the ligation was made with the In-Fusion HD Cloning Kit (Clontech, http://www.clontech.com). The plasmids pPha-T1 (kindly provided by Prof. P. Kroth) and pPha-T1::EGFP [40] (kindly provided by Prof. A. Vardi), carrying the *fcp*A (fucoxanthin chlorophyll binding protein A) promoter region for the expression of recombinant genes, were used as the backbone for all constructed vectors. A full list of oligonucleotide primers used in this work can be found in Table S1.

The plasmids p*fcpA*::EGFP-HOGP and p*DGAT1*::EGFP-HOGP (Fig. S1) were constructed in several stages. The first step involved creating p*DGAT1*::EGFP, by digesting pPha-T1::EGFP with *Nde*I and *EcoR*I to remove the *fcp*A promoter. The *DGAT1* (GI:325,073,417) promoter sequence, 2150 bp upstream of ATG, was obtained from JGI (http://genome.jgi-psf.org/Phatr2/Phatr2.home.html), amplified from *P. tricornutum* DNA and ligated into the final plasmids. PCR amplification was carried out with a forward primer (1) containing a *Nde*I restriction site, and a reverse primer (2) with an *EcoR*I restriction site (Table S1, underlined). The second step involved cutting p*DGAT1*::EGFP with *Stu*I and *Sph*I to remove *EGFP*, followed by insertion of the *EGFP* (without stop codon) and *HOGP* genes simultaneously. These were produced as follows: *EGFP* was amplified with a forward primer (3) containing a *Stu*I restriction site, and a reverse primer (4); *HOGP* was amplified using a forward primer (5) and a reverse primer (6), containing a *Sph*I restriction site. To build p*fcpA*::EGFP-HOGP, pPha-T1::EGFP was digested with *Stu*I + *Xba*I to remove EGFP, followed by re-ligation of EGFP fused to HOGP, and these were amplified using p*DGAT1*::EGFP-HOGP as a template with a forward primer (7) containing a *Stu*I restriction site, and reverse primer (8) containing an *Xba*I restriction site.

2.3. Diatom transformation

Transformation of *P. tricornutum* was carried out as described in [40] with minor modifications. Prior to transformation, *P. tricornutum* cells grown as described above were harvested at the mid-logarithmic phase. Approximately 2.5–5 × 10^8 cell mL^{-1} were plated onto the RSE agar plate (1.5%). One mg gold microcarriers (0.6 μm diameter particle size, Bio-Rad, Hercules, CA) were coated with 5 μg of plasmid DNA in the presence of 2.5 M $CaCl_2$ and 0.1 M spermidine. Vectors were introduced into *P. tricornutum* by microparticle bombardment using a Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). The bombardment was performed at 1350 psi under a negative pressure of 27 in. mercury with a target distance of 6 cm. Bombarded cells on agar plates were illuminated with white light (40 μmol photon m^{-2} s^{-1}) at 18 °C for 24 h; cells were recovered by resuspension in liquid RSE

and were replated onto a solid medium containing 100 μg mL^{-1} zeocin (~1×10^7 cells per plate). The plates were placed under constant illumination (75 μmol photon m^{-2} s^{-1}) at 22–25 °C for 4–6 weeks to allow transgenic colonies to appear.

2.4. Colony screening

After 4–6 weeks, screening for positive colonies was performed in 96-well plates (50,063, Edge BioSystems, https://www.edgebio.com). The transformants, exhibiting EGFP fluorescence in association with LDs, were initially selected among the colonies that survived selection pressure. To each well, 200 μL RSE (with nitrogen source) was added. After 2 weeks of growth in an incubator shaker as described above, each well was supplemented with palmitoleic acid (C16:1, Nu-Check Prep, www.nu-chekprep.com) at a final concentration of 100 μM (from a stock of 100 mg mL^{-1} in DMSO). Cell cultures were incubated under these conditions for at least 16 h, and then observed by fluorescence microscopy.

2.5. DNA and RNA isolation, and cDNA synthesis

DNA was isolated from *P. tricornutum* as described in [45]. Prior to total RNA isolation, the cells were harvested by centrifugation at 1000 g for 5 min, washed twice with 20 mM HEPES and 150 mM NaCl (pH 7), flash-frozen in liquid nitrogen, and stored at −80 °C until further use. Total RNA was isolated using the ZR Plant RNA MiniPrep Kit (Zymo Research, https://www.zymoresearch.com) following the manufacturer's instructions. Total RNA samples were treated with RapidOut DNA Removal Kit (Thermo Scientific, http://www.thermoscientific.com) according to the manufacturer's protocol, before being used for cDNA synthesis. Total RNA concentration was measured by a NanoDrop 1000 Spectrophotometer (DE, USA), and RNA integrity was estimated by formaldehyde gel electrophoresis. cDNA was prepared from 500 ng of total RNA template in a total volume of 10 μL with a blend of random hexamers and anchored oligo-dT (3:1, *v*/*v*) by the Verso cDNA Kit (Thermo Scientific).

2.6. Gene expression analysis

Gene expression was analyzed by qRT-PCR using triplicate reactions for each sample of two independent RNA isolations with a gene-specific primer pair and SsoFast EvaGreen Supermix (Bio-Rad; www.bio-rad.com) in a CFX96 Real-Time System (Bio-Rad). The amplification reaction was run at: 95 °C for 30 s, and 40 cycles of 95 °C for 5 s, and 55 °C for 5 s. A melting curve was obtained for each pair of primers to confirm that a single, specific product was produced in each reaction.

qRT-PCR primer pairs were designed for all relevant genes (Table S2) with PrimerQuest (http://eu.idtdna.com/PrimerQuest). Primer pairs were validated with five serial fivefold dilutions of cDNA samples and primers. Standard curves were plotted to test for the linearity of the response. The primer pairs and primer concentrations with reaction efficiencies of 100 ± 10% were chosen for the qRT-PCR analysis of relative gene expression.

2.7. Western blot analysis of transgene protein expression

For the western blot analysis, 20 mL of cultured cells, were harvested by centrifugation at 3000 *g* for 5 min, and resuspended in 20 mM HEPES containing 150 mM NaCl (pH 7.0). Glass beads (2.5 mm in diameter) were added and the cells were supplemented with a SIGMA FAST protease inhibitor cocktail (S8820 Sigma–Aldrich http://www.sigmaaldrich.com) and 10 mM Tris–HCl (pH 7.5). The cells were broken by beating three times sequentially for 1 min at a time with cooling on ice for 1 min between repetitions. Proteins were extracted in × 3 electrophoresis separating buffer (195 mM Tris–HCl, 15% SDS (*w*/*v*), 45% glycerol (*w*/*v*), 6% mercaptoethanol (*v*/*v*), pH 6.8); cell lysates were incubated for 1 h at room temperature. Protein concentration was determined by a Bradford reagent (Bio-Rad), and a final protein concentration in lysates was adjusted to 1 μg μL^{-1}. Total proteins (20 μg per lane) were resolved by SDS-PAGE, and blotted onto a 0.2-μm nitrocellulose membrane (Bio-Rad). To visualize the proteins on the SDS gels, a GelCode Blue Stain Reagent (Thermo Scientific) was used. For visualization of proteins on the nitrocellulose membrane, Ponceau S (Sigma-Aldrich) was used. EGFP was detected by incubation with Anti-Tag(CGY)FP antibodies (Evrogen, Moscow, Russia; http://www.evrogen.com) at a dilution of 1:5000, and with goat anti-rabbit alkaline phosphatase conjugated to horseradish peroxidase (Bio-Rad) at a dilution of 1:3000. The membrane was developed by an enhanced chemiluminescence EZ-ECL Kit (Biological Industries, Beit Haemek, Israel) detection system according to the manufacturer's instructions and documented with a MicroChemi imager (DNR Bio-Imaging 16 Systems Ltd., Jerusalem, Israel).

2.8. Fluorescence microscopy

Basic colonies screening and microscopic observations were performed by differential interference contrast (DIC) and fluorescence microscopy with a Zeiss Axio Imager A2 fluorescence microscope (Carl Zeiss MicroImaging Inc., Göttingen, Germany) equipped with an AxioCam digital camera. Images were acquired with ZEN imaging software; filter sets 38 HE and 16 were used for visualization and documentation of EGFP and chlorophyll autofluorescence, respectively. A fluorescent probe for the detection of intracellular neutral lipids Nile Red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one; Sigma-Aldrich) was used to visualize LD formation. Ten μL-aliquots of *P. tricornutum* cell cultures were stained with 1 μL Nile Red solution in DMSO (final concentration 50 μg/mL), incubated for 3 min at room temperature, and observed by fluorescence microscopy with the band path (BP) filter set 16 (Carl Zeiss MicroImaging Inc.), allowing maximum excitation at 485/20 nm and long path (LP) emission above 515 nm.

A Zeiss confocal microscope (LSM 510 Meta) was used to capture *in vivo* localizations of EGFP signal; a 488-nm laser was used for the excitation of both EGFP and chlorophyll *a*, and the emitted fluorescent light was split using a NFT565 beam splitter and detected simultaneously in two channels with BP500-550 and LP650 filters, respectively.

2.9. Cultivation of P. tricornutum transformant lines for induction of TAG production under nitrogen starvation

Lipid and fatty acid analyses were performed on the nitrogen-depleted cultures cultivated in 100-mL Erlenmeyer flasks as described above. The pre-cultures were grown in a complete RSE medium under a light intensity of 100 μmol photon m^{-2} s^{-1} at 25 °C with periodical dilution to a chlorophyll concentration of 10 mg L^{-1}. To induce nitrogen starvation, cells were pelleted by centrifugation, washed three times and reconstituted in nitrogen-free RSE medium to the initial chlorophyll content of 10 mg L^{-1} (~1×10^7 cell mL^{-1}).

2.10. Lipid extraction and fatty acid analysis

Prior to lipid extraction, *P. tricornutum* biomass samples, freeze–dried and kept at −20 °C, were treated for 10 min with hot isopropanol at 80 °C to prevent lipase activity. Following centrifugation, isopropanol was collected, and lipids were extracted from the remaining cell pellet according to the method of Bligh and Dyer [46] and combined with isopropanol. TAG was isolated from the total lipid extract by TLC on Silica Gel 60 plates (Merck, http://www.merck.com/index.html) using a solvent system of petroleum ether:diethyl ether:acetic acid (70:30:1, *v*/*v*/*v*).

Fatty acid analysis by GC-FID was performed on dried biomass samples, and on TAG recovered from TLC plates by extraction with chloroform:methanol (1:1, *v*/*v*). Prior to the direct transmethylation of dried biomass, 5 mL of culture was pelleted by centrifugation, and the

cell pellets were dried for 45 min at room temperature in a SpeedVac concentrator (Thermo Fisher Scientific, https://www.thermofisher.com) or overnight in a vacuum freeze-dryer. Dried samples were incubated in dry methanol containing 2% (*v*/*v*) H_2SO_4 at 80 °C for 1.5 h under an argon atmosphere and continuous stirring. For total fatty acid content quantification, pentaenoic acid (C15:0; Sigma-Aldrich https://www.sigmaaldrich.com) was added as an internal standard and ash-free dry weight was taken into account. Fatty acid methyl esters (FAMEs), were analyzed on a Trace GC Ultra (Thermo, Italy) equipped with a flame ionization detector (FID) and a programmed temperature vaporizing (PTV) injector as described in [44].

2.11. Statistical analyses

Statistical analyses were carried out using JMP version 10 (SAS Institute, Cary, NC). Analysis of variance (ANOVA) was used to identify overall significant differences between treatments. When significant differences were found, mean separations were calculated by Tukey post hoc test. The significance level was $P \leq 0.05$.

3. Results

3.1. EGFP-HOGP signal decorates LDs in P. tricornutum

To overexpress the EGFP-HOGP fusion protein in *P. tricornutum*, we performed a comparative analysis of two gene promoters (Fig. 1; Fig. S1) under two distinct assay conditions. We used the promoter region of the *P. tricornutum DGAT1* containing 2150 bp upstream of the ATG triplet (chr_2:1,105,000–1,107,149; http://genome.jgi.doe.gov/Phatr2/Phatr2.home.html). The *DGAT1* gene expression is up-regulated upon the transfer of *P. tricornutum* to a medium lacking nitrogen [12]. The strong light-regulated *fcp*A promoter has been widely explored for expression studies in *P. tricornutum* under nutrient-replete conditions [40] and was used for comparison. The integration of *EGFP-HOGP* fusion into the *P. tricornutum* genome was confirmed by on-colony PCR on the zeocin-resistant colonies using specific primers (Fig. S2).

Next, to screen for the EGFP-HOGP signal localization in the cells of the transgenic lines, we induced LD formation in nutrient-replete TAG-deficient cells by free fatty acid loading (Figs. 2 and 3). Fatty acid feeding is commonly used in lipid research to assess cellular localization of the putative LD-associated proteins (see for example, [17,47]. Lipid droplets formed as a result of FA feeding in the cells of WT and transgenic lines were observed by differential interference contrast (DIC) microscopy and by fluorescence microscopy after staining with the lipophilic dye Nile Red (Fig. 3). This approach enabled the fast and robust selection of transformant lines expressing EGFP-HOGP in association with LDs and the detection of fluoresce signal driven by both promoters in nutrient-replete cells.

The uptake of the exogenously added fatty acid and the concurrent time-dependent TAG formation was evident from the TLC and GC-FID analyses of neutral lipids (Fig. S4). Palmitoleate constituted 85–90% of total fatty acids in the TAG produced and in the free fatty acid (FFA) pool after the supplementation (Table S3). After washing the cells and resuspension in a fresh nutrient-replete medium, TAG was depleted within 24 h (Fig. S4), along with the disappearance of LDs and growth recovery (Figs. S5 and S6).

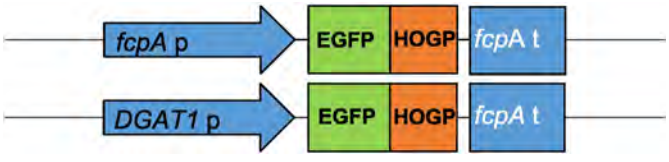

Fig. 1. Schematic presentation of vector constructs for the expression of the EGFP-HOGP fusion protein in *P. tricornutum*. Top: under control of the *fcpA* promoter; bottom: under control of the *DGAT1* promoter.

As depicted in Fig. 2, when inspected 24 h after feeding, LDs were visible both in the cells of WT and transformants, however, only the lines transformed with both constructs featured distinct decoration of LDs with a bright fluorescent signal. EGFP fluorescence signal seemed to be evenly distributed over the surface of LDs. Using a strong *fcpA* gene promoter (line C8) prompted the swift marking of newly formed LDs in nutrient-replete cells already after 4 h of feeding (Fig. S4). In contrast, in the cells transformed with the plasmid construct harboring the *DGAT1* promoter (line C3), a visible EGFP signal, predominantly associated with the periphery of LDs, was detected later (Fig. 2). Such cellular localization was clearly distinguishable from the cytoplasmic EGFP signal distribution in the *P. tricornutum* cells transformed with the original plasmid which does not carry any targeting signal [40] (Fig. S7). No EGFP signal was detected in the EGFP-HOGP-transformed but non-supplemented cells or in the cells after FFA removal and TAG depletion (Figs. S4 and S5). These results supported our previous assumption that HOGP is a LD-associated protein [24], and indicated that N-terminal fusion of EGFP to this protein does not interfere with its association with LDs in a heterologous host. Interestingly, the repeated attempts to express EGFP fused to the C-terminus of HOGP were unsuccessful, implying that the C-terminus of HOGP could be important for its association with LDs.

An important finding of this part was that the cloned fragment of the *P. tricornutum DGAT1* promoter was efficient at expressing genes under conditions associated with intensive acyl group esterification and deposition into LDs in nitrogen-replete cells. The utility of using the promoter of photosynthetic gene under such conditions was also demonstrated. It should be also noted that, while the EGFP-coding sequence in our constructs was originally engineered to employ codons similar to those preferred by *P. tricornutum* (for details, see [40]), no codon-optimization was required to express HOGP in *P. tricornutum*.

3.2. Expression of EGFP-HOGP driven by the inducible DGAT1 promoter directs EGFP signal toward LDs under nitrogen starvation

Using the FA-feeding approach, both promoters proved to be active in driving expression of the fusion protein in nutrient-replete cells. However, under the stressful conditions of nitrogen starvation, which triggers TAG biosynthesis from the endogenously produced acyl groups, hinders cell division, and imposes drastic alterations to photosynthetic apparatus and down-regulation of photosynthesis-relates genes [48], transformed lines obtained using the promoter of photosynthetic gene (the *pfcp*A- bearing construct) did not reveal any EGFP fluorescence. In contrast, cells expressing the fusion protein under the control of the *DGAT1* promoter, which is induced under nitrogen starvation [12], revealed a bright green fluorescent signal adjacent to the LDs after 7 days of nitrogen starvation (Fig. 4). As depicted in Fig. 5, LDs were stained with the fluorescent dye Nile Red in N-starved cells, but we could not manage to capture both fluorescent signals simultaneously. Additional indication for the association of HOGP with LDs was obtained by application of hypotonic shock to the nitrogen-starved cells of line C3 (Fig. S8). After this treatment, cells were largely disrupted while the EGFP-HOGP signal at a great extent remained associated with the released LDs.

3.3. Regulation of EGFP expression in transgenic lines

Microscopic observations showed that EGFP-HOGP under the control of the *fcp*A promoter was insufficiently expressed under nitrogen starvation to be detected. To assess the regulation of EGFP-HOGP expression in the course of nitrogen starvation, its expression was quantified in one p*fcp*A::EGFP-HOGP transformant (C8) and in two different p*DGAT*1::EGFP-HOGP expressing lines (C3 and C18) by immunostaining of western blots using a polyclonal anti-GFP antibody (Fig. 6A). The p*fcp*A::EGFP-HOGP transformant strain (C8) revealed a significant signal of expected size for EGFP-HOGP (~ 60 kDa) before the onset of

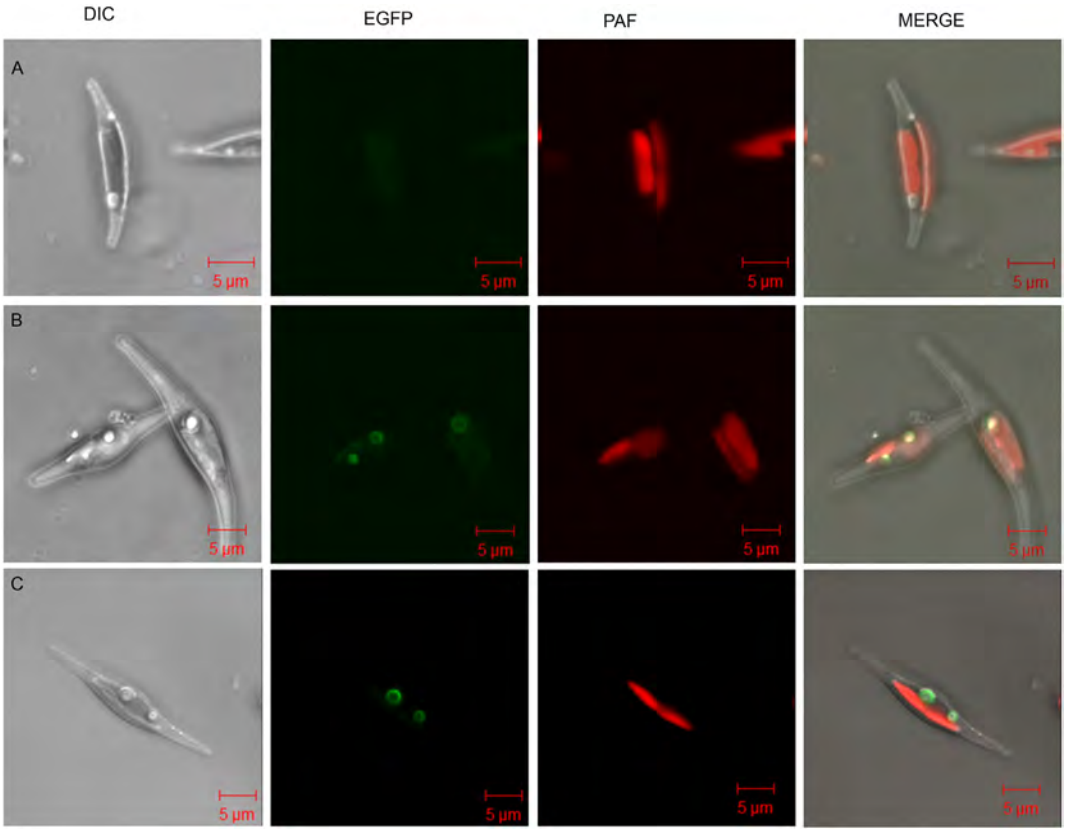

Fig. 2. EGFP-HOGP signal associates with lipid droplets induced by palmitoleic acid (16:1) administration in the nutrient-replete *P. tricornutum* transformant cells. Confocal micrographs of WT and transformant cells (lines C8 and C3) supplemented with palmitoleic acid (100 μM) for 24 h. Fluorescent signal was observed upon expression using the *fcp*A promoter and the *DGAT1* promoter. DIC: differential interference contrast; EGFP: EGFP-HOGP fusion protein fluorescence; PAF: photosynthetic apparatus fluorescence; merge: overlay. (A) Wild type cells. (B) Cells transformed with p*fcp*A::EGFP-HOGP (line C8). (C) Cells transformed with p*DGAT1*::EGFP-HOGP (line C3).

nitrogen starvation, but no signal was detected neither after 3 or 7 days of nitrogen starvation. In contrast, strain C18 revealed an immunologically positive band only after 7 days of nitrogen starvation; remarkably, strain C3 was already showing a strong signal for EGFP-HOGP on western blots 3 days after the onset of nitrogen starvation, as well as detectable EGFP fluorescence under the fluorescent microscope (not shown).

To further clarify the mechanism governing the expression of the fusion protein under the two promoters, we quantified relative gene expression levels by real-time qPCR using *HOGP*-specific primers and *CHC* (PHATRDRAFT_54801) as a house-keeping gene (Table S1). The results showed that the *fcp*A promoter is very rapidly and efficiently shut down under nitrogen starvation, with essentially no detectable mRNA remaining after 3 days (Fig. 6B). In contrast, expression levels in strain C18 (p*DGAT1*::EGFP-HOGP) were near zero under nutrient-replete conditions, but increased substantially after 3 and 7 days of starvation. However, even at these time points, the relative expression remained far below the *fcp*A-promoter-driven expression under nitrogen-replete conditions. In strain C3 (p*DGAT1*::EGFP-HOGP), *HOGP* mRNA levels at time 0 were already substantial, and increased only slightly after 3 and 7 days of starvation. Nevertheless, the fusion protein became visible by fluorescence microscopy and immunostaining after 3 days of starvation, while no protein was detected on day 0, in the absence of LDs. The combined results of these analyses suggested that strong post-transcriptional mechanisms, rather than mere transcriptional control, might control fusion protein accumulation. The strong difference between strains C3 and C18 might indicate that the insertion location of the construct provides additional regulatory signals for gene expression. Another important factor affecting gene expression to note is the stability of transcripts under nitrogen stress.

3.4. Overexpression of HOGP leads to enhanced TFA and TAG production and does not impact fatty acid composition of TAG under nitrogen starvation

Seven days after nitrogen starvation, one to two mature LDs are visible in *P. tricornutum* cells on average (Fig. 5). Those are formed by fusion and enlargement of smaller and more scattered LDs that are abundant in the cells at the earlier time-points. We compared the diameter of LDs in wild type and transformed (C3) nitrogen-starved cells (7 days), using the built-in tool-kit of a ZEN imaging software (http://www.zeiss.com/microscopy). However, we did not observe significant differences between the cell lines in the average diameter of LDs (2.8 ± 0.68 μm in wild type compared to 2.9 ± 0.57 in C3, $n = 100$). Perhaps more close inspection in the course of LD formation may reveal differences in LD diameter and size distribution.

Two clones expressing EGFP-HOGP were chosen from 25 positive clones as representatives to assess the effect on TAG accumulation. To determine whether the HOGP expression impacts TAG deposition in the LDs of *P. tricornutum* under nitrogen starvation, the contents of TFA and TAG were quantified in the biomass of the wild type and transformant strains C3 and C18 after 3 and 7 days of nitrogen starvation. Changes in the chlorophyll and cell biomass culture contents during 7 days of nitrogen starvation are shown in Fig. 7. A gradual decline in the Chl culture content occurred concomitantly with the increase in the biomass upon the transfer of the cells to nitrogen-free medium. There were no significant differences determined between the strains in these parameters. Cell number doubled during the first 3 days and remained unaltered in all examined cultures (not shown). Our results are consistent with a well-documented phenomenon of continued biomass production and initial cell division in microalgae cultures under

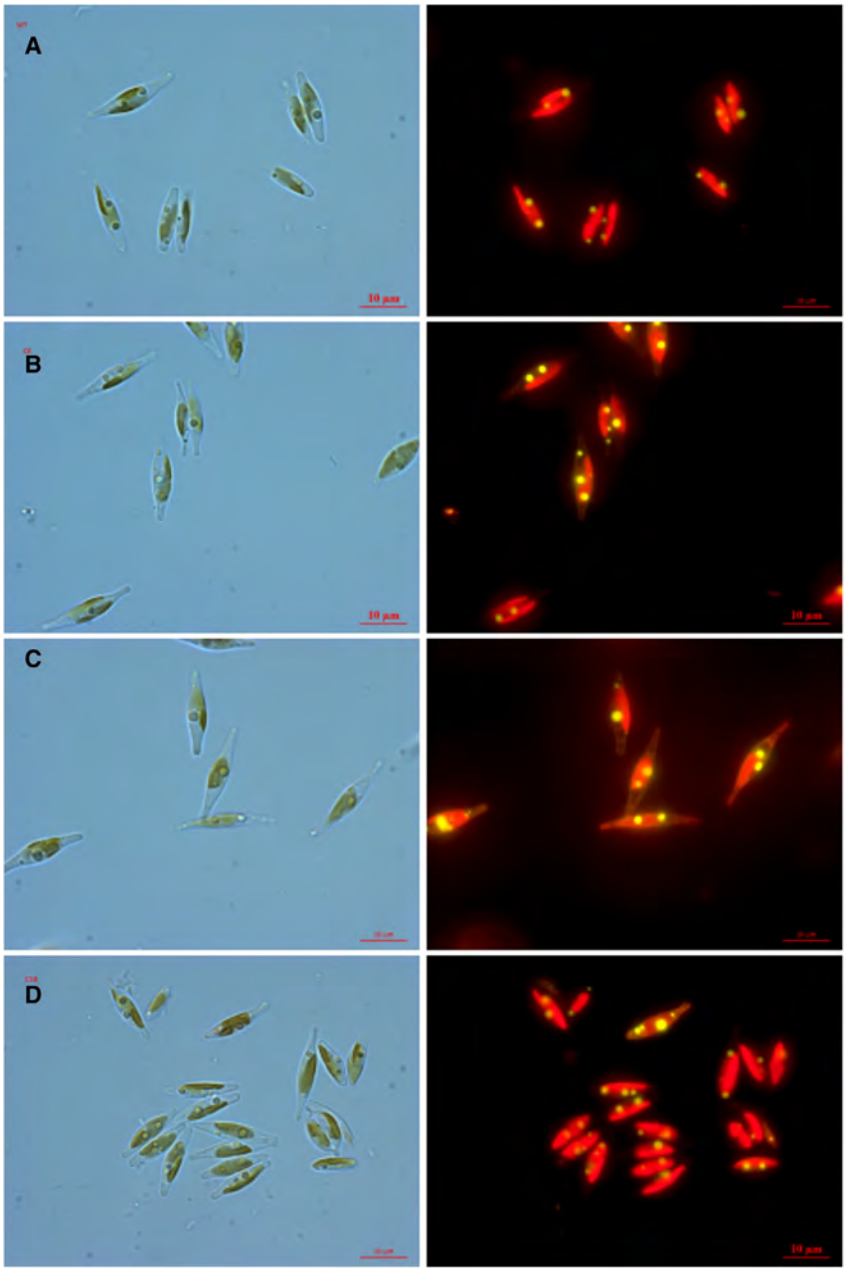

Fig. 3. Lipid droplets visualization in fatty-acid loaded cells by Nile Red staining. (A) Wild type cells. (B) Cells transformed with p*fcp*A::EGFP-HOGP (line C8). (C) Cells transformed with p*DGAT1*::EGFP-HOGP (line C3). (D) Cells transformed with p*DGAT1*::EGFP-HOGP (line C18). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nitrogen starvation despite a gradual dismantling of chloroplast and endomembrane systems [12,48,49]. Ongoing biomass production continues to be driven by photosynthesis and recycling of internal cellular reserves (including nitrogenous compounds) toward production of carbon-rich storage products, such as carbohydrates and/or TAG. Cell biomass enrichment in storage lipids manifested itself by an increase in TFA content to *ca.* 25% (WT) and 34–35% (C3 and C18) of DW after 7d of nitrogen starvation (Fig. 8). Both transformant lines revealed significantly higher TFA contents compared to the wild type: strains C3 and C18 had 25–30% higher TFA content (expressed as % of DW and per culture volume unit) than the wild type after 3 days and 7 days of starvation (Fig. 8A and B), while biomass accumulation was not altered (Fig. 7B). TAG was isolated from total lipids by TLC and quantified using GC-FID. In line with the increased TFA content of biomass, the transformant strains had elevated TAG contents (% of DW) after 7 days of starvation [29.6 ± 0.09 (C3); 29.4 ± 0.39 (C18) as compared to 24.5 ± 0.70 in the wild type, student test, $P < 0.001$] (Fig. 8C). The results of these analyses showed a slight but significant effect of HOGP overexpression on TFA content and TAG enrichment of the biomass of transgenic lines compared to the wild type, in particular 7 days after the transfer to nitrogen-depleted medium. However, overexpression of EGFP-HOGP did not alter the fatty acid composition of the TAG (Fig. 8D), suggesting that our manipulations did not affect either the activity or the acyl preference of TAG-assembly enzymes. The major acyl groups accumulating in TAG under nitrogen starvation were palmitate (>30% of TFA) and palmitoleate (>40% of TFA), whereas the proportions of the long-chain polyunsaturated fatty acid (LC-PUFA) eicosapentaenoic acid (EPA) amounted to less than 5% percent.

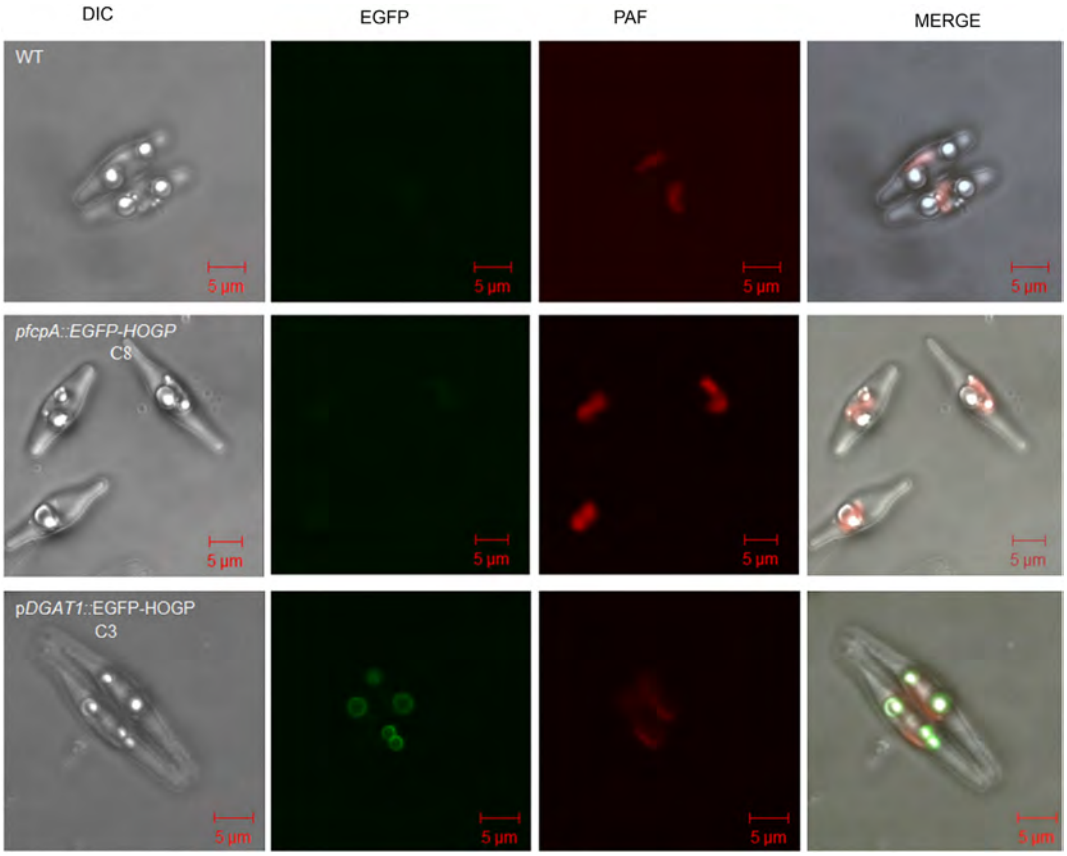

Fig. 4. *In vivo* localization of EGFP-HOGP expressed in *P. tricornutum* under the control of *DGAT1* promoter after 7 days of nitrogen starvation. Confocal micrographs of WT and p*fcpA*-EGFP::HOGP transformant strain (C8) and p*DGAT1*-EGFP-HOGP transformant strain (C3). Only the latter reveals a clear EGFP signal localized to the LDs. DIC: differential interference contrast; EGFP: GFP HOGP fusion protein fluorescence; PAF: photosynthetic apparatus fluorescence; merge: overlay.

4. Discussion

Despite growing interest in LD biogenesis in microalgae, the basic molecular tools to explore this mechanism and the biological function of LD-associated proteins are still limited. Recombinant expression of proteins under nutrient stresses requires the development of adequate tools, such as promoters that are stably active under such conditions. In this work, we demonstrated the significance of overexpressing genes that encode LD-associated proteins in microalgal cells driven by suitable endogenous promoters. The *PtDGAT1* expression is induced under nitrogen starvation [12]. Using its promoter region, we were able to express EGFP-HOGP and locate the fluorescence signal in the close proximity to LDs. We demonstrate for the first time the utility of an endogenous *DGAT1* promoter to drive transgene expression under prolonged nitrogen-starvation. In contrast, the *fcp*A promoter of a photosynthetic gene seemed to be active under nitrogen-replete conditions and at the very early stages of nitrogen starvation. Using it as a reference promoter, the *EGFP-HOGP* transcript and the fused protein fluorescence signal were undetectable under prolonged nitrogen starvation.

Our protein-similarity searches in the *P. tricornutum* genome did not identify proteins with significant similarity to previously identified or annotated major lipid droplet proteins of green algae or eustigmatophytes. Furthermore, the *P. tricornutum* genome has no homologues of the plant and animal LD proteins, such as oleosins and perilipins. It seems likely that LD biogenesis in the diatom *P. tricornutum* does not involve protein analogues of the previously characterized major LDs proteins, involved either in stabilization or maintenance of LDs. This premise is in agreement with the results of the first recent proteomics study of LD-associated proteins in *P. tricornutum* [50]. In this report, we overexpressed a major LD protein from the green alga *H. pluvialis* in *P. tricornutum* to test the *DGAT1* promoter and to assess HOGP cellular localization in heterologous system.

Our findings on the cellular distribution of EGFP-HOGP signal in *P. tricornutum* cells are in line with the immunofluorescence localization data on MLDP in *C. reinhardtii* cells as ring-like structures surrounding LDs. However, in *C. reinhardtii,* a certain sub-fraction of MLDP was also seen as reticular structures outside the LDs, probably in the ER [10]. Indeed, the earlier study [26] showed the preponderant MLDP localization to certain ER sub-domains in close proximity to LDs, but not directly on the surface of LDs. On the other hand, immuno-gold labelling studies in *D. bardawil* revealed MLDP localization on the LD periphery [32]. We also used an alternative approach for investigating the association of EGFP-HOGP signal with LDs and could show that the fluorescent signal remained bound to LDs even after disruption of the cells by hypotonic shock (Fig. S8). Hence, based on the results of two assay conditions, triggering LDs formation, it is thus conceivable that HOGP is a LD-associated scaffolding protein [24]. Admittedly, in order to affirm that HOGP is localized to the surface of LDs or in ER subdomains adjacent to LDs (or in both locations) further investigations using high resolution electron microscopy [19], and immuno-gold labelling [32] are required in both native and heterologous systems.

Taken together, our results indicate that LD structures are sufficiently conserved between green algae and diatoms to allow for association with LDs and possibly for mutually exchangeable functional complementation. Our findings on the cellular localization of a green algal LD protein in *P. tricornutum* further extend a number of previous observations on the ability of ectopically expressed structural LD proteins to be targeted to LDs in various host cells. For example, plant oleosins and a few mammalian perilipin family members were targeted to LDs when expressed in yeast [51–53]. Likewise, a rapeseed oleosin was

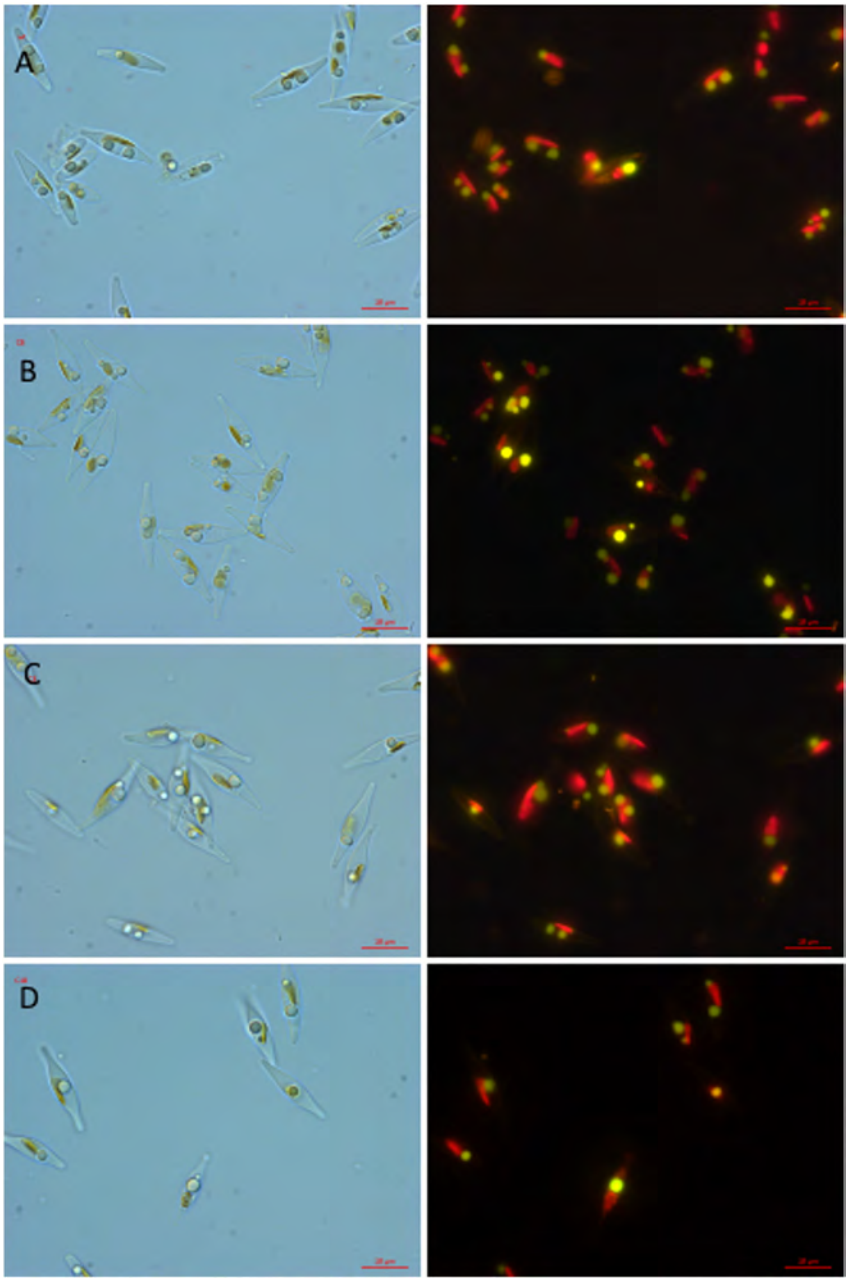

Fig. 5. Lipid droplets visualization in the nitrogen-starved cells by Nile Red staining. (A) Wild type cells. (B) Cells transformed with p*fcp*A::EGFP-HOGP (line C8). (C) Cells transformed with p*DGAT1*::EGFP-HOGP (line C3). (D) Cells transformed with p*DGAT1*::EGFP-HOGP (line C18). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expressed in mammalian cells and retained the capacity to bind to LDs [54]. The major LDSP of the heterokont microalga *N. oceanica* partially complemented an oleosin-deficient phenotype *in the mutant of Arabidopsis thaliana* [25]. Our findings thus further imply that the major LD structural proteins of evolutionarily distinct organisms can be directed to the LDs of heterologous hosts, presumably owing to their intrinsic hydropathic and topological properties.

Feeding with FFA has been widely employed in studies of LD biogenesis in mammalian and yeast cells, but rarely in microalgae, especially for monitoring LD formation. Administration of palmitoleic acid promptly induced LD formation in *P. tricornutum*. The primary advantage of this assay is the rapid screen for transformant colonies expressing FP-tagged LD-associated proteins. High contents of FFA are toxic to the cells and must therefore be isolated and sequestered within LDs to avoid harmful effects on cell membranes and organelles. Specifically, in *P. tricornutum*, supplementation with different FAs and in particular its major LC-PUFA, EPA, led to photosynthetic pigment degradation and chloroplast membrane deterioration, and stimulated the release of volatile compounds implicated in cell defense in diatoms [55]. In that earlier study, administration of 16:1 exerted a less toxic effect, did not promote intensive volatile chemical production and even decreased the release of hexanal. Based on our observations, we can now explain this phenomenon by *P. tricornutum*'s efficient sequestration of palmitoleic acid – the major acyl group component of TAG – into LDs. Fatty-acid-loaded cells efficiently degraded LDs and consumed TAG after removing the supplement of palmitoleic acid without a deleterious effect on growth resumption.

Nitrogen starvation is the best studied and widely employed trigger for induction of storage lipid production in microalgae and leads to progressive accumulation of TAG within LDs [48,56,57]. Under our

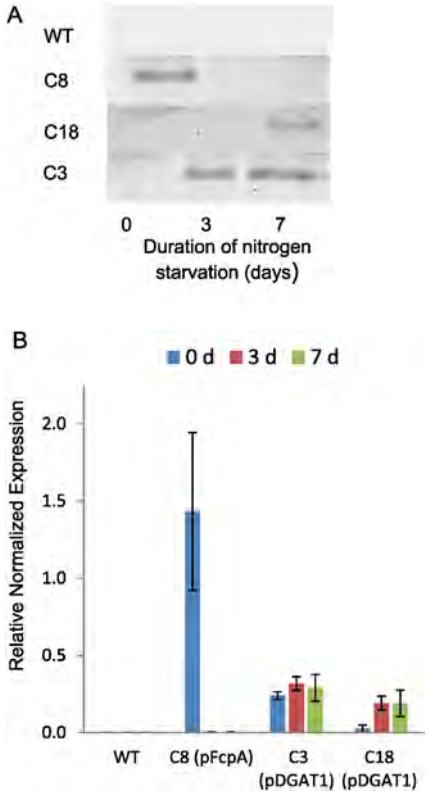

Fig. 6. Western blot analysis of EGFP-HOGP fusion protein and expression level of the *HOGP* mRNA in transformant strains in the course of LD formation under nitrogen starvation. (A) EGFP-HOGP accumulation in *P. tricornutum* transformant lines at the onset (0) and after 3 and 7 days of nitrogen starvation upon expression under control of the *fcp*A promoter (line C8), and under control of the *DGAT1* promoter in two transformant lines C3 and C18. The wild type strain was used as a negative control. (B) Expression levels of *EGFP-HOGP* mRNA at the onset (0) and after 3 and 7 days of nitrogen starvation under control of the *fcp*A promoter (line C8) and under control of the *DGAT1* promoter in two transformant lines, C3 and C18. The wild type strain was used as a negative control.

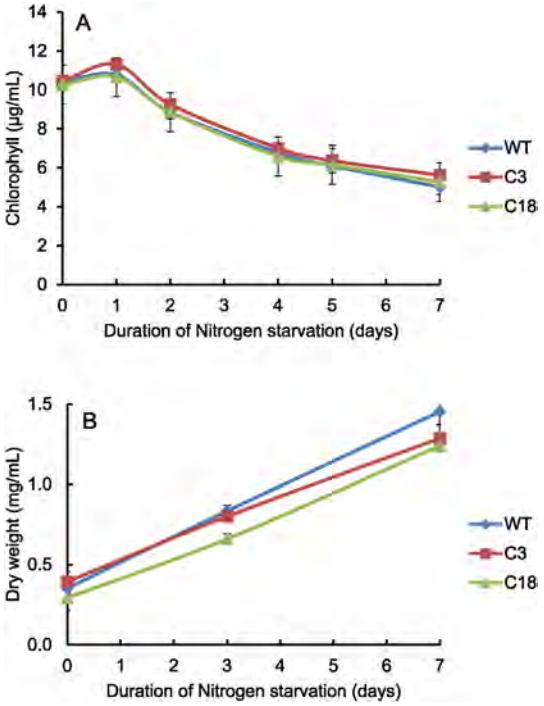

Fig. 7. Changes in chlorophyll (A) and cell biomass (B) concentration in the cultures of WT and transformant lines (C3 and C18) under nitrogen starvation. Results are shown as the mean ± standard deviation ($n = 3$).

experimental conditions, 7d after the transfer to nitrogen-free medium, TAG content in the biomass of *P. tricornutum* lines amounted for about 25–30% of DW. A comparative analysis of TFA and TAG contents revealed that in the two chosen transgene lines, expressing HOGP under the control of p*DGAT1*, lipid productivities were 20–30% higher than in wild type. The fact that overexpression of HOGP in *P. tricornutum* enhanced TAG (oil) productivity further argues for the biotechnological relevance of this protein in regulating LD biogenesis and modulating TAG accumulation under oil-inducing conditions. Our findings are consistent with the results of previous reports on the enhancement of storage lipid capacity by recombinant expression of LD proteins in different cell types e.g. [58,59].Whether HOGP overexpression facilitates the biosynthesis and deposition of TAG or interferes with TAG degradation by lipases, as described for perilipin A overexpression [60], remains to be determined. As reported by Trentacoste et al. [61], downregulation of lipase activity in the diatom *Thalassiosira pseudonana* resulted in enhanced TAG content under nutrient-replete conditions. Likewise, knock-down of *Tgl1* encoding the major lipase activity enhanced the content of TAG in *P. tricornutum* [62]. It is thus conceivable that HOGP association with LDs mediates their increased stability and storage capacity, for example by reducing accessibility to lipases whose activities reduce oil accumulation.

There is circumstantial evidence that argues for a central structural role of MLDP in LD biogenesis in *C. reinhardtii* [10]. It is assumed that MLDP recruits specific proteins and membrane lipids and hence plays a critical role in LD formation and stabilization. It is tempting to speculate that HOGP plays a similar role in *H. pluvialis*. In *C. reinhardtii*, MLDP interacts with tubulin as was corroborated by means of co-immuno-precipitation [10]. In *H. pluvialis*, the intracellular migration of LDs to the cell periphery in response to high light exposure appeared to involve the interaction with actin [63].What is the nature of HOGP interactions with cytoskeleton elements in heterologous host remains to be elucidated.

Our findings on the enhancement of TFA production and TAG content in *P. tricornutum* upon overexpression of HOGP are consistent with the above proposition, regarding the major role played by MLDP in green algal cells in LD biogenesis. We do expect that the achieved effect might be further enhanced by the additional impact of acyltransferase overexpression, which may result in synergistically increased TAG production when associating with LD surface. Overexpression of acyltransferases participating in the reactions of TAG assembly has been shown to enhance TAG formation in microalgae when adequate gene promoters tuned for certain cultivation conditions are utilized [9, 64]. It can therefore be expected that the efforts presented here will assist both in advancing our understanding of microalgal cell biology related to LD biogenesis, and in providing further insight into the biotechnologically important question of enhancing oil production by microalgae. Nevertheless, significant and various productivity improvements will have to be achieved to justify higher lipid strain development and outdoors cultivation costs.

Author contributions

Z.S. performed and designed most of the experiments, analyzed the data and wrote the paper. S.L. and I.K.G. designed the research, performed some of the experiments, analyzed the data and wrote the paper. S.D.C. performed some of the physiological experiments and the lipid analyses. A.Z. analyzed the data and wrote the paper. S.B. supervised the project and wrote the paper. All authors commented on the results and the manuscript.

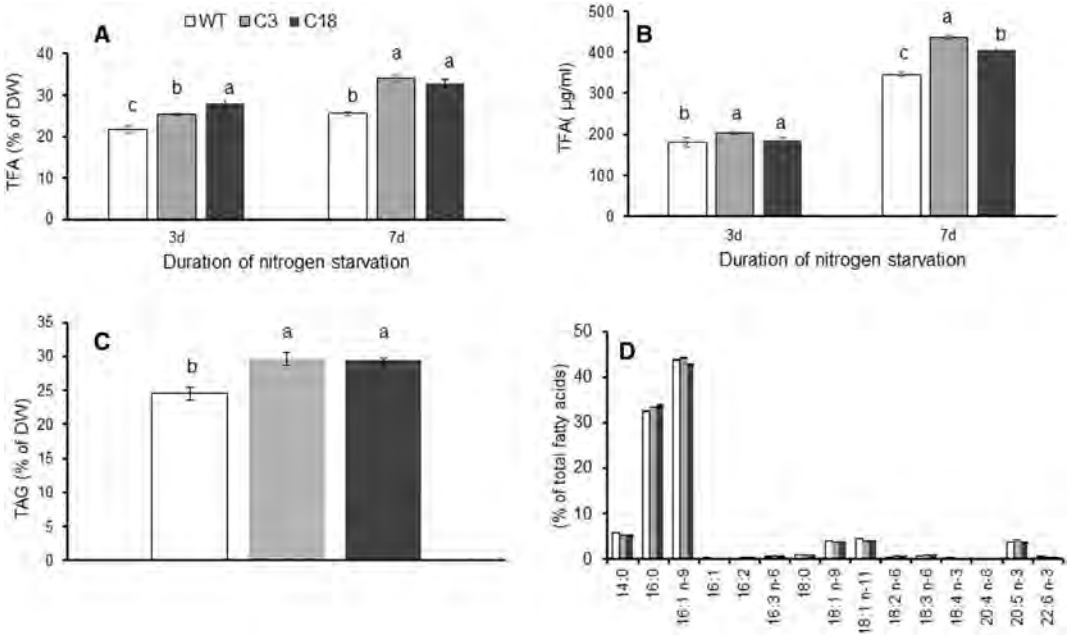

Fig. 8. Effect of EGFP-HOGP overexpression on total fatty acid production and fatty acid composition under nitrogen starvation. (A) Total fatty acid content (% of dry weight) in wild type, and in two transformant lines C3 and C18, transformed with the p*DGAT1*::EGFP-HOGP construct, after 3 and 7 days of nitrogen starvation. a, b and c indicate a significant difference between wild type and transformants (C3 and C18) according to the Tukey post hoc test with $p < 0.0001$. Results are shown as the mean ± standard deviation ($n = 6$). (B) Total fatty acid culture content (µg/mL) in wild type and in transformant lines C3 and C18 after 3 and 7 days of nitrogen starvation. The differences observed between wild type and transformants (C3 and C18) are significant according to the Tukey post hoc test with $p < 0.0001$. Results are shown as the mean ± standard deviation ($n = 6$). (C) Accumulation of TAG in wild type and transformant lines C3 and C18 after 7 days of nitrogen starvation. Analysis of TAG content was performed from pooled samples of three biological replicates and analyzed in triple technical replicates. The differences observed between wild type and transformants (C3 and C18) are significant according to the Tukey post hoc test with $p < 0.0001$. (D) Fatty acid composition of TAG in wild type and in transformants lines C3 and C18 after 7 days of nitrogen starvation. Results are shown as the mean ± standard deviation ($n = 3$).

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Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2016.07.002.

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