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Identification of the triacylglycerol lipase in the chloroplast envelope of the diatom *Phaeodactylum tricornutum*

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ABSTRACT

Triacylglycerol (TAG) accumulation mechanisms under stress have been intensively investigated in recent years in microalgae and efforts aiming to increase TAG synthesis have been made through gene engineering. However, only a few studies report the contribution of lipid breakdown to TAG accumulation in microalgae. In this study, a putative TAG lipase OmTGL in *P. tricornutum* was identified based on conserved domain and phylogenetic analysis. OmTGL is different from those known lipases and localized in the third outermost plastid membrane. *OmTGL* mRNA levels exhibited the consistent decrease during TAG accumulation and knockdown of *OmTGL* considerably enhanced neutral lipid content detected by Nile Red assay at the stationary growth phase, and TAGs detected by thin layer chromatography (TLC) increased by 1.2–1.4 folds in the *OmTGL* RNAi lines at day 6. Although C20:5ω3 fatty acid in the total lipid did not change in *OmTGL* mutants compared with the wild-type, its content in TAGs increased by 68–70%, indicating that TAG enriched with C20:5 was degraded under nitrogen stress and the degraded fatty acids and diacylglycerol (DAG) might feed into the biosynthesis of plastid membrane lipids. Therefore, the lipase is important for maintaining lipid homeostasis in plastid, and a slight slower growth and decreased glycolipids and phospholipids have also been found in the mutants. This study shows that this lipase is a good target for regulating not only TAG accumulation but also long-chain polyunsaturated fatty acids (LC-PUFA) flux to TAGs.

1. Introduction

Diatoms are a group of unicellular eukaryotic oleaginous algae, which account for over 20% of the primary productivity on Earth [1]. As the model of diatoms, *Phaeodactylum tricornutum* has drawn a significant attention and is widely used in fields of sustainable new energy research due to its fast growth, simple fatty acid profile [2], available sequenced genome [3] and genetic tools [4–8]. In this diatom, the end-products of photosynthesis are partly stored in the form of triacylglycerol (TAG), which accounts for 20–30% of the dry cell weight [9]. In some special physiological conditions such as nitrogen starvation, cells can accumulate more lipids [9–12]. It has been well documented that TAG accumulation in *P. tricornutum* under nitrogen stress is a consequence of re-allocation of carbon skeletons mainly from the intermediates of the tricarboxylic acid cycle [12,13]. In recent years, researchers have targeted genes associated with carbon and nitrogen metabolism or lipid biosynthesis to increase TAG accumulation in this diatom [13–18]. So far, only a few studies report the contribution of lipid degradation to TAG accumulation in diatoms and other algae [19,20].

Lipases (E.C 3.1.1.3) are conventionally defined as TAG hydrolases (or TAG lipase). With broad substrate specificity, they are widespread in animals, plants and microorganisms. Although a diverse array of genetically distinct lipases are found in nature, most of lipases are characterized by a common alpha/beta hydrolase fold and a conserved catalytic triad [21,22] consisting of a serine nucleophile, a histidine base, and an acid residue (aspartate or glutamate) with the serine being part of the consensus sequence GxSxG [23]. Some of SGNH-hydrolases belonging to GDSL family also show TAG hydrolase activity *in vitro* [24,25]. This enzyme subfamily is defined by four invariant residues Ser-Gly-Asn-His in four conserved blocks I, II, III and V, respectively, and catalytic serine is located within the GDSL motif [26]. TAG lipases Tgl3p, Tgl4p and Tgl5p play a critical role in oil breakdown in *Saccharomyces cerevisiae*, and they are localized at lipid particles. Deletion of *TGL3* or *TGL4* leads to an increased cellular level of TAG in *S. cerevisiae* [27,28]. SDP1, a homolog of Tgl3p, Tgl4p and Tgl5p, in *Arabidopsis thaliana* is identified to be associated with storage oil breakdown in germinating seeds [29]. A SDP1-patatin like lipase Tgl1 is also identified in *P. tricornutum*, and knockdown of *tgl1* increases TAG content by almost two-folds [19]. Thaps3_264297, homologous to

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Table 1
PCR primers used in this study.

Primer	Sequence (5'→3')
vector construction	
omtgfp-fw	CGgaattcATGCGTTTTTGGAAGCATG
omtgfp-rv	GGggtaccTCCTCCTCCTCCTCCCCTAGGTCCCTTCACCGCAGTGGACAC
mrfp-fw	GCtctagaGGACCTAGGGGAGGAGGAGGAGGAATGGCCTCCTCCGAGGAC
mrfp-rv	CCCaagcttTTAGGCGCCGGTGGAGTG
omp85-fw	GGgaattcATGAAGACGTCTTCACTTG
omp85-rv	GCtctagaAAAATCAAAATCCGCGCCG
omti-fw	CGgaattcCTTTGCTGGTACCGACGATT
omti-rv1	GCtctagaAACGAATGGCCGGTAGTAAA
omti-rv2	GCtctagaCAAAGTCACGCCAGTACGAA
qPCR	
qH4-fw	AGGTCCTTCGCGACAATATC
qH4-rv	ACGGAATCACGAATGACGTT
qomt-fw	GCTTGCCCAAGCTGATCTAC
qomt-rv	GTCCTTCCTCCACATCTCCA

*Restriction sites are underlined, linker fragment between two genes in the box.

human Comparative Gene Identification-58 (CGI-58), exhibits TAG lipase activity in the diatom *Thalassiosira pseudonana*, and knockdown of this gene increases lipid yields by 2–4 folds without compromising growth [30]. These findings suggest that lipases might act as a good target for regulating TAG accumulation.

At NCBI 49 putative lipase sequences are listed for *P. tricornutum* [19], we further identified that there were at least 18 lipase-encoding genes in the genome by performing a search in *P. tricornutum* annotation 3 (Phatr3) at Ensembl. Except 2 SDP1-patatin-like lipase genes (*tgl1* and *Phatr3_45518*) and a homolog of *CGI-58* (*Phatr3_54974*), there were 15 lipases containing fungal lipase-like domain. Among the 15 lipases, 5 of them were predicted to be located in the plastid and *Phatr3_J37711* (*OmTGL*) contained transmembrane helices. In consideration of the importance of plastid and the membrane structure in photosynthetic organisms, localization and phylogenetic evolution of OmTGL were analyzed, and the RNA interference (RNAi) knockdown strains were generated to interpret the role of the gene in lipid metabolism in the study. Our results strongly suggest that OmTGL does not belong to any of the proven lipase families and it plays a critical role in plastid lipid homeostasis.

2. Materials and methods

2.1. Growth conditions

Cells of *Phaeodactylum tricornutum* Bohlin clone Pt1 8.6 (CCMP2561) from culture collection of the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (Bigelow Laboratory for Ocean Sciences, USA) and transformants were grown in artificial seawater enriched with f/2 (nitrate concentration was reduced to 500 μM) [31] at 22 °C under continuous illumination of 70 μmol photons $m^{-2} s^{-1}$ on a shaking table with continuous shaking at 60 rpm. The initial cell density for batch cultures is 2.5×10^5 mL^{-1}, and cell proliferation was determined by counting the cell number using a Malassez chamber (0.01 μL for a rectangle). Sampling was performed every 2 days for determining nitrate concentration in the culture using spectrophotometry at 220 nm [32].

2.2. Phylogenetic analysis, vector construction and transformation

At Ensembl the coding sequence of *Phatr3_J37711* (*OmTGL*) is 1332 bp in length, encoding a protein of 444 amino acids. The full-length cDNA of this gene was further verified based on expressed sequence tag libraries and 5'-RACE. For the phylogenetic analysis, the majority of lipase protein families were incorporated into a maximum-likelihood tree. For the localization analysis of OmTGL, the ORF of this gene was amplified by PCR using the *omtgfp-fw* and *omtgfp-rv* primers from the *P. tricornutum* cDNA and inserted between the *Eco*RI and *Kpn*I sites (reside immediately upstream of the *egfp* sequence) of plasmid pPhaT1-eGFP [5] to obtain the pPhaT1-OmTGL-eGFP expression vector. *mRFP* gene was amplified from plasmid pGWB754 (GenBank accession number AB608308) using the sense primer *mrfp-fw* with *Xba*I site and the antisense primer *mrfp-rv* with *Hind*III site. The PCR product was digested and cloned into the *Xba*I-*Hind*III sites of pPha-T1 to generate the pPhaT1-mRFP vector. *P. tricornutum Omp85* (*ptOmp85*) was amplified using the *omp85-fw* (containing *Eco*RI site) and *omp85-rv* (containing *Xba*I site) primers, and then the PCR product was digested and ligated in front of the *mRFP* gene in plasmid pPhaT1-mRFP to obtain plasmid pPhaT1-Omp85-mRFP.

For the knockdown of *OmTGL*, inverted repeat constructs were generated. A 225-bp fragment (corresponding to the *OmTGL* gene sequence from 189 to 413 bp) and a 404-bp fragment (corresponding to the *OmTGL* gene sequence from 189 to 592 bp) were amplified from the cDNA, respectively, with the primer pairs *omti_fw* and *omti_rv1*, and *omti_fw* and *omti_rv2*. These two fragments were digested with *Eco*RI and *Xba*I and ligated in sense and antisense orientations to the *Eco*RI site of the linearized phir-PtGUS vector [6]. Primers used for vector construction are shown in Table 1.

The expression vectors were introduced into *P. tricornutum* by electroporation and transformants were screened by checking the integration of the genes according to Zhang & Hu [5]. For the co-localization studies, two linearized plasmids pPhaT1-OmTGL-eGFP and pPhaT1-Omp85-mRFP were co-transformed into *P. tricornutum*. Fluorescence of eGFP and plastid autofluorescence were excited at 488 nm, and were detected with two photomultiplier tubes at 500–520 nm and 625–720 nm respectively using a Leica TCS SP8 laser scanning confocal microscope. mRFP fluorescence was excited at 552 nm and detected at 580–600 nm.

2.3. Quantitative real-time PCR (qRT-PCR)

To compare the relative quantity of *OmTGL* mRNA levels in wild-type and the RNAi silenced lines, cells grown for 4, 6 and 10 d were harvested by centrifugation (3000 rpm, 15 min) and used for RNA

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