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Comprehensive analysis of triacylglycerol lipases in the oleaginous diatom *Fistulifera solaris* JPCC DA0580 with transcriptomics under lipid degradation

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Microalgal biofuels are desirable alternatives for traditional liquid fuels, but further improvements of productivity are necessary for microalgal biofuels to be economically feasible. As strategies for improving lipid productivity, repression of lipid degradation has a great potential because the lipid accumulation level is determined by a balance between lipid synthesis and degradation. However, studies of lipid degradation and its primary functioning enzyme triacylglycerol (TAG) lipases in microalgae are currently limited. In this study, we report the comprehensive analysis of TAG lipases in the oleaginous diatom *Fistulifera solaris* JPCC DA0580. First, we confirmed that TAGs in the lipid accumulation organelles, oil bodies, were degraded when the cells were transferred from the nutrient depleted conditions to nutrient replete condition. Further analysis revealed that, after eicosapentaenoic acid (C20:5*n*-3)-containing TAGs are were degraded, its molecular skeletons were likely to be recycled to produce glycolipids and phospholipids for chloroplast regeneration and cell growth, respectively. Next, we searched putative TAG lipase genes from the draft genome sequence of *F. solaris*, and discovered 42 candidates based on the amino acid sequence homology analysis. Subsequent transcriptome analysis revealed that 16 of the 42 lipase genes were up-regulated during lipid degradation. Among the up-regulated lipases, a number of enzymes were predicted to localize in endoplasmic reticulum which is closely associated to the lipid accumulation organelles, oil bodies. Our study provided new insights of lipid degradation in oleaginous microalgae, and putative TAG lipases which could be candidates for metabolic engineering in future study to improve microalgal lipid productivity.

[**Key words:** Oleaginous diatom; *Fistulifera solaris* JPCC DA0580; Lipid degradation; Triacylglycerol lipase; Transcriptome; Fatty acid composition]

Microalgal biofuel production has attracted much attention during the last two decades due to the increasing demand for alternative liquid fuels to reduce the carbon dioxide emission. Various eukaryotic microalgal species accumulate abundant neutral lipids, which are mainly in the form of triacylglycerol (TAG) and stored within intracellular organelle called oil bodies, after entering to the stationary phase or under stress conditions (1,2). These neutral lipids could be a source of biofuel, converted into fatty acid methyl esters (FAMEs) by transesterification. Thus, lipid-accumulating microalgae are considered to be a potential feedstock for biodiesel fuel production (3).

Studies in those eukaryotic microalgae have been focusing on the strain screening, biofuel production process development and optimization, elucidation of lipid accumulating mechanism in terms of the genetic and transcriptomic regulation (4,5), and enhancement of lipid productivity and fuel quality through metabolic engineering (6,7). In order to enhance lipid productivity, there are two engineering strategies; overexpression of lipid biosynthesis enzymes, and suppression of enzymes for degradation. Recent studies of genetic engineering for improving lipid productivity have mainly focused on the enhancement of the lipid biosynthesis pathways. Previous attempts of enhancing the lipid biosynthesis include overexpression of the genes encoding TAG-synthesizing enzymes to directly reinforce the responsible pathway (8–10). These studies succeeded to enhance the lipid production, while some of these studies also induced the decrease in the growth. On the other hand, studies of lipid degradation in microalgae are obviously limited. If enzymes responsible for lipid degradation are identified, they must be attractive targets of metabolic engineering to enhance lipid production by repressing their expression.

The initial step of lipid degradation is a catabolic reaction of TAG, in which TAG is degraded into diacylglycerol (DAG) and free fatty acids by enzyme TAG lipases [EC 3.1.1.3]. Although several TAG lipases have been identified in model organisms, such as *Arabidopsis thaliana* (11) and *Saccharomyces cerevisiae* (12,13), only two TAG lipases have been functionally characterized in microalgae. The first microalgal TAG lipase was identified in model centric diatom *Thalassiosira pseudonana* by identifying the down-regulated genes with lipase domains under the lipid accumulation condition. (14). This lipase (*T. pseudonana* genome version 3 protein ID 264297; Thaps3_264297) was reported as a homolog of α/β hydrolase domain-containing protein CGI-58 (comparative gene identifier 58 (15)), and thus it is referred to as CGI-58-like lipase. Repression of the CGI-58-like lipase in *T. pseudonana* resulted in increase in the

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TAG accumulation, and intriguingly, without growth inhibition (14). Second a TAG lipase was identified in another model pennate diatom *Phaeodactylum tricornutum* based on sequence homology search for TAG lipase in terrestrial plant *A. thaliana* (16). This lipase (coding protein locus tag: PHATRDRAFT_1971) is referred to as Tgl1. This lipase family is common in many organisms, and many lipases previously discovered, including lipase Tgl3p, Tgl4p and Tgl5p in *S. cerevisiae* (12,13,17); SDP1 in *A. thaliana* (11); and also adipose triglyceride lipase (ATGL) in human (18) are categorized in this lipase group. Suppression of Tgl1 expression in *P. tricornutum* also contributed to enhancement of lipid accumulation (16). However, previous study in *T. pseudonana* employed an indirect strategy to discover the TAG lipase, in which the lipase genes down-regulated during the lipid accumulation were identified based on transcriptome analysis. Another study in *P. tricornutum* did not directly use the comprehensive transcriptome data, but employed sequence homology search for lipase finding. Therefore, none of studies have performed comprehensive transcriptome analysis of TAG lipases under lipid degradation conditions, which are hypothesized to be up-regulated.

Previously, we have discovered an oleaginous marine pennate diatom *Fistulifera solaris* JPCC DA0580, which has one of the highest lipid accumulating ability in microalgae (~65%, w/w), and high growth rate in large-scale outdoor cultivation systems (19–21). Genetic manipulation technique for this diatom has already been established (22), and thus this diatom shows great promise for improving its oleaginous phenotype by metabolic engineering (23,24). We recently determined its whole genome sequence by next-generation sequencing technology, and identified the major metabolic pathways (25). Lipid metabolism has also been analyzed in multi-omics studies include transcriptomics (25), proteomics (26) and lipidomics (27–29) to obtain the mechanistic insight of its oleaginous phenotype. However, the TAG lipases have not been investigated yet. If it is possible to estimate TAG lipases in the oleaginous diatom *F. solaris*, they must be attracting knockdown targets for enhancement of lipid productivity.

Here, we report the TAG lipases in oleaginous diatom *F. solaris* based on comprehensive transcriptome analysis. First we identified the condition in which *F. solaris* massively degraded the neutral lipids in the oil bodies. Lipid content and fatty acid composition analysis implied that DAG skeletons derived from TAG degradation were likely to be used for production of polar lipids, including glycolipids and phospholipids, during the lipid degradation. Then, TAG lipases were searched from the genome information of *F. solaris* based on the sequence homology. Subsequent transcriptome analysis under the lipid degradation condition revealed that some of the putative lipase genes were expressed in high level and up-regulated during lipid degradation. The results of our study provide the insight of lipid degradation in oleaginous microalga which could provide the novel targets for metabolic engineering to improve the lipid production.

MATERIALS AND METHODS

Identification and sequence analysis of putative TAG lipase genes Genome information of *F. solaris* is available online (GenBank: BDSP00000000.1). BlastP search was performed with BLAST+ v2.2.31 (30) against collected amino acid sequences from following database; gene entries at National Center for Biotechnology Information (NCBI), Kyoto Encyclopedia of Genes and Genomes (KEGG) and Evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG) with predicted TAG lipase function in diatoms *P. tricornutum*, *T. pseudonana*, green alga *Chlamydomonas reinhardtii*, terrestrial plant *A. thaliana* and yeast *S. cerevisiae*; and functionally characterized TAG lipase in previous studies (11–14,16). Every hits with an e-value $<10^{-5}$ were identified as putative TAG lipases. Sequence analysis and lipase motif confirmation were performed with InterPro (31) and the MEME FIMO v4.12.0 (32), respectively. For predicting subcellular localization, SignalP v4.1 (33), TargetP v1.1 (34), Mitoprot v1.101 (35), HECTAR v1.3 (36), and TMHMM v2.0 (37) were used comprehensively, as described previously (38).

Strain and culture conditions In this study, 10f medium (26) dissolved in artificial seawater (ASW) was selected as the nutrient-replete condition. ASW without any nutrient supplementation was selected as the nutrient-depleted condition. *F. solaris* was cultured under the nutrient-replete condition with the initial cell concentration of 1.0×10^6 cells ml^{-1} for 72 h in flat-shaped flasks (500 ml of media in 1.5 l of flasks). The cultured cells were collected by centrifugation at 8500*g* for 10 min at 25°C. The collected cells were then transferred to the nutrient-depleted condition by re-suspending the cells in ASW, and incubated for another 96 h. After that, the cells were finally transferred back to the nutrient-replete condition. Cell concentration at each time point was determined through cell counting by using hemocytometer (Sun Lead Glass Company, Tokyo, Japan). The cultures were bubbled with 2% of CO_2 at the flow rate of 0.8 l l^{-1} min^{-1} (the volume of gas per the volume of media per minute; vvm). The temperature was maintained at 25 ± 1°C, and continuous illumination was applied at 200 μmol photons m^{-2} s^{-1}.

Transcriptome analysis Cell samples were collected before switching back from the nutrient-depleted condition to the nutrient-replete condition as 0 h sample. Subsequently, samples were also collected at 5, 10 and 15 h after switching to the nutrient-replete condition. Cells were harvested by centrifuged at 8000*g* for 10 min. Pellets were frozen at −80°C and then grinded in liquid nitrogen and suspended in Plant RNA Isolation Reagent (Life Technologies, Carlsbad, CA, USA). Total RNA extraction was performed as described previously (25). After extraction, cDNA libraries for RNA sequencing were constructed by TruSeq RNA Sample Preparation Kit v2, and then sequenced by Illumina Hiseq 2000 to generate single reads of 100 bases in length. Sequenced reads data were mapped onto the *F. solaris* genome (GenBank accession no.: GCA_002217885.1) using the TopHat software (v2.0.14) (39) and reads per kilobase per million (RPKM) values were computed by the Genedata Expressionist software v9.1.1a (Genedata AG, Basel, Switzerland). For comparing the time course RPKM, the genes are defined as either upregulated or downregulated if the RPKM values were differentiated two-fold or greater than the 0 h. In transcriptome analysis, n = 1.

Confocal imaging to measure the volume of the intracellular organelles Microalgal cells stained with BODIPY 505/515 were observed under a confocal microscope (FV1000, Olympus Corp., Tokyo, Japan). The oil body volume was determined by the fluorescent signal of BODIPY, and chloroplast volume was determined by the signal of its autofluorescence as previously reported (40). Briefly, the z-stack images of cells were obtained with a 100 oil-immersion objective. Three-dimensional reconstructions of confocal images were generated using Volocity 5.0 image analysis software (Perkin Elmer, Waltham, MA, USA), and fluorescent signal volumes were measured using intensity-based thresholds.

Lipid extraction and fractionation Cell samples were collected before switching back from the nutrient-depleted condition to the nutrient-replete condition as 0 h sample. Subsequently, samples were also collected at 3, 6, 12, 18, 24 and 48 h after switching to the nutrient-replete condition. Two-step chloroform/methanol extraction was used for total lipid extraction as described previously (27). Briefly, 30 mg of the lyophilized microalgal cells were suspended in 5 ml of chloroform/methanol (20:1 v/v) and disrupted by 20 min of sonication. Phase separation was induced by the addition of 1 ml of H_2O. After centrifugation at 800*g* for 10 min, the lower phase was collected. The aqueous material was re-extracted with 5 ml chloroform/methanol (2:1, v/v). The lower organic phase was combined with the previously collected one. The crude lipid extracts were dried using argon gas and stored at −30°C before further analyses.

The total lipids were then fractionated into neutral lipids (NLs), glycolipids (GLs), and phospholipids (PLs) according to previously reported method (27), with minor modifications. The Supelclean LC-Si SPE Tubes (bed weight 100 mg, volume 1 ml, Sigma–Aldrich, Tokyo, Japan) were conditioned with 4 ml of chloroform. The crude lipid extracts were dissolved in 200 μl of chloroform and loaded into the column. NLs were eluted with 4 ml of chloroform; GLs were eluted with 4 ml of acetone/methanol (9:1 v/v), and PLs were eluted with 4 ml of methanol. Each lipid fraction was collected into a glass vial and dried using argon gas. The dried weight of fractionated lipids was then measured before further analyses. The total lipids dried weight was the sum of dried weight of NL, GL and PL fractions.

Transesterification and gas chromatography-mass spectrometry Transesterification was conducted according to our previously reported method (27). Dried NLs, GLs and PLs were transesterified by heating with 5 ml of 1.25 M HCl-methanol at 100°C for 1 h (for NLs, the incubation time was expanded to 90 min because NLs were not dissolved well in the HCl-methanol). After methanolysis, the FAMEs were extracted 3 times with 5 ml of *n*-hexane. Shimadzu GCMS QP2010 with a capillary FAMEWAX column (30 m, 0.25 mm ID, 0.25 μm, Shimadzu Co., Kyoto, Japan) were used for FAME identification and quantification under the following conditions: interface temperature, 240°C; injection-port temperature, 240°C; ion-source temperature, 260°C; and helium gas pressure, 80 kPa. Oven temperatures were programmed at 140°C for 5 min, then to 240°C at 4°C min^{-1} and holding at 240°C for 10 min. The Supelco 37 Component FAME Mix was used as the standards for identification of each FAME compounds and construction of standard calibration curve.

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