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RESEARCH PAPER

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Transcriptome-Based Analysis of *Euglena gracilis* Lipid Metabolic Pathways Under Light Stress

Wen-hui Zhang[1,2], Jinwei Gao[1,3], Wenli Zhou[1,*], Yeong Yik Sung[2]

[1] Tianjin Agricultural University, Tianjin Key Laboratory of Aqua-Ecology & Aquaculture, Fisheries College, Tianjin, China, 300384
[2] Universiti Malaysia Terengganu, Institute of Marine Biotechnology (IMB), 21030, Kuala Terengganu, Terengganu, Malaysia;
[3] Quality of Fishery Environment and Aquatic Products Supervision and Testing Center (Tianjin), Ministry of Agriculture and Village, 300221, Tianjin, China.

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Corresponding Author
Tel.: +8615122401092
E-mail: wlzhou69@126.com

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Abstract

To investigate lipid metabolism pathways and the genes encoding essential enzymes regulating these pathways, the present study was conducted to analyze the transcriptome of *Euglena gracilis* cultured under light and dark conditions for 5 days using the Illumina HiSeq 4000 sequencing platform. A total of 120,086 unigenes were obtained using Trinity assembly software, of which 48,031 were annotated. Through bioinformatics analysis, genes associated with *E. gracilis* lipid metabolism pathways were identified, and pathways for fatty acid biosynthesis, fatty acid degradation, and glycerolipid metabolism were preliminarily constructed. In addition, differences in gene expression under different culture conditions were compared. By regulating the activity of acetyl-CoA carboxylase, 3-ketoacyl-ACP synthase II, and phospholipid: diacylglycerol acyltransferase, enzymes associated with fatty acid and glycerolipid degradation may be inhibited or knocked out, thereby increasing fatty acid and glycerolipid content in *E. gracilis*. This study provides a basis for genetic modification of *E. gracilis* for construction of a high-quality oil-producing engineered strain.

Introduction

With globalization and the rapid development of the global economy, the energy crisis has become increasingly prominent (Molony, 2011). Massive exploitation and overdevelopment of traditional fossil fuels has led to an acute shortage of resources and supplies (Coyle & Simmons, 2014). Because of environmental degradation and petroleum depletion resulting from unreasonable energy consumption, biodiesel is receiving increasing attention. One source of biodiesel, namely the many species of microalgae, accumulate triacylglycerols (TAG) during growth and photosynthesis (Hu et al., 2010). After microalgae are used to prepare biodiesel, the residual biomass can be converted into valuable industrial materials (Krajčovič, Vesteg, & Schwartzbach, 2015). The lipids synthesized by microalgal cells constitute a highly promising source of raw material for biodiesel production. In the 1970s, the United States National Renewable Energy Laboratory led the pioneering Aquatic Species Program-Biodiesel from Algae (ASP) program. In the subsequent years (Savage, 2011; Ho, Chen, Lee, & Chang, 2010), microalgal biodiesel production has gradually become a global research focus (Scott et al., 2010; Yusuf, 2007).

Euglena gracilis is a single-celled eukaryotic species found in fresh water. It has a facultative mode of nutrition (Ahmadinejad, Dagan, & Martin, 2007), as it can produce nutrients not only through photosynthesis (Pulz & Gross, 2004), but also by ingesting organic substances such as beef extract, peptone, acetate, and ethanol through osmotic nutrition (Afiukwa & Ogbonna, 2017; Ogbonna, 2009). This “plant-animal duality” makes it rich in nutrients such as unsaturated fatty acids, polysaccharides, vitamins, and amino acids.

Light intensity not only directly affected the growth of microalgae, but also affected the pigment and ester contents of microalgae (Khozin-Goldberg, Bigogno, Shrestha, & Cohen, 2010; Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 2008b; Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 2008a). Solovchenko, Khozin-Goldberg, Didi-Cohen, Cohen, & Merzlyak, 's research (2008b) found that high light intensity was beneficial to increase the biomass of *Parietochloris incisa* and for accumulation of oil, but AA content was the highest under medium light intensity. The results of Zhang's study (Zhang, Cohen, Khozin-Goldberg, & Richmond, 2002) showed that higher intensity light conditions could rapidly promote the growth of *Parietochloris incisa*, but the AA content was not high. However, Sun, Hasan, Hobba, Nevalainen, & Te'o (2018) showed the highest accumulation of paramylose was the largest in *E. gracilis* under dark conditions.

Currently, methods such as genetic engineering, genetic manipulation, and metabolic regulation are used to construct engineered algal strains, purposefully regulate gene expression, obtain dominant algal strains, and induce accumulation of large amounts of fatty acids in microalgae by regulating essential enzymes in fatty acid biosynthesis (Rawisara, Supapon, & Kobkul, 2009). For example, heterologous expression of enzymes associated with TAG biosynthesis induces the accumulation of large amounts of TAG in *Chlorella* (Hsieh, Su, & Chien, 2012). However, not all essential enzymes can be overexpressed. High expression of 3-ketoacyl-acyl carrier protein synthase III (KASIII) in tobacco, for instance, increases palmitic acid content but reduces the rate of fatty acid synthesis (Dehesh, Tai, Edwards, Byrne, & Jaworski, 2001). For effective engineered regulation of fatty acid biosynthesis in *E. gracilis*, it is therefore necessary to first perform in-depth data-mining, build a core metabolic network, and analyze its regulatory mechanisms.

In this study, the content and composition of fatty acids in *E. gracilis* were analyzed under light stress conditions. The Illumina HiSeq 4000 sequencing platform was used for sequencing an *E. gracilis* transcriptome library, and a large amount of genetic data was obtained. Functional annotation and pathway analysis of the unigenes from the assembled sequences was performed. Through analysis of transcriptome data, genes essential for fatty acid biosynthesis, fatty acid degradation, and triacylglycerol metabolism in *E. gracilis* were studied, and their metabolic pathways constructed. Differential pathway-associated genes in the experimental group were analyzed with the goal of using genetic engineering technology to construct a high-quality engineered algal strain as a candidate and theoretical basis for large-scale, sustainable biodiesel production.

Materials and Methods

Experimental Materials

Euglena gracilis was provided by the Institute of Freshwater Aquatic Sciences at the Chinese Academy of Sciences. The culture medium was prepared using deionized water. Hutner's Modified Medium(HUT) (Table 1) was prepared at a pressure of 1.05 kg/cm^2 and sterilized at 121.3°C for 20 min before use. The culture temperature was 25±1°C, the illumination was 60 μmol/(m^2·s), and the light-dark ratio was 12 L:12 D (light group) and 0 L:24 D (dark group). Cultivation flasks (500 mL) were agitated several times each day to prevent microalgae from adhering to the walls or sedimenting.

Experimental Methods

Analysis of Fatty Acid Composition

For methyl esterification, samples were added to a 15 mL centrifuge tube and 2 mL of 2% sodium hydroxide in methanol was added. The mixture was flushed until oil droplets disappeared. Subsequently, 3 mL of 14% boron trifluoride in methanol was added, and the mixture was boiled for 30 min. An appropriate amount of isooctane solution was added, the condenser was removed, and 20 mL of saturated sodium chloride solution was added. One to two milliliters of the upper layer of the solution was collected by pipetting, the sample was dehydrated by addition of anhydrous sodium sulfate, and the sample was injected (Chen & Johns, 1996).

The analysis was performed using an Agilent 7890A gas chromatograph-mass spectrometer equipped with a CNW CD-2560 chromatography column with a size of 100 m × 0.25 mm × 0.20 μm. The operating parameters were: inlet temperature, 250°C; detector type, FID; detection temperature, 260°C; injection volume, 1 μL;

Table 1. Huter's Modified Medium(HUT)

Component	Amount
KH_2PO_4	0.02 g
$MgSO_4·7H_2O$	0.025 g
Yeast extract	0.4 g
Sodium acetate	0.4 g
peptone	0.6 g
Deionized water	1000 mL

split ratio, 10:1; carrier gas flow rate, 0.5 mL·min-1. The temperature program used was 130°C for 5 min, after which the temperature was increased to 240°C at a rate of a 4°C per min. The column was then maintained at 240°C for 30 min.

Preparation of Sequencing Samples

After 5 days of culture, *E. gracilis* was centrifuged at 4°C (2000 r/min, 10 min), and the supernatant was discarded. The sample was immediately frozen in liquid nitrogen (−196℃) until RNA isolation (Zhao W, et al. 2017). Total RNA was extracted using a Total RNA Extractor (Trizol, Sangon Biotech) according to the manufacturer's instructions. The quality of RNA samples was verified by NovogeneBioinformatics Technology Co., Ltd. (Beijing, China). The RNA was then enriched using magnetic beads containing oligo(dT). Fragmentation buffer was added to break the mRNA into short fragments, which served as templates to synthesize single-stranded cDNA using random hexamers. Buffer, dNTPs, DNA polymerase I, and RNase H were then added for the synthesis of double-stranded cDNA, which was later purified using AMPure XP beads. The purified double-stranded cDNA was first subjected to end-repair, A-tailing, and adapter ligation, followed by size selection of the fragments using AMPure XP beads. Finally, polymerase chain reaction (PCR) amplification was performed, the products were purified using AMPure XP beads and a final library was obtained.

Preliminary quantification of the constructed library was performed using Qubit2.0. The library was diluted to 1.5 ng/μL, and the insert size of the library was determined using the Agilent 2100 system. After the expected insert size was obtained, accurate quantification of the effective concentration of the library was performed using quantitative PCR (valid effective concentration > 2 nM) to ensure the quality of the library. After controlling for quality, the library was subjected to sequencing.

Data Processing and De Novo Assembly

The raw reads obtained from sequencing were preprocessed. Bases with linkers, low-quality bases, and uncertain bases were discarded to yield clean reads (The base number of Qphred <= 20 was more than 50% of the reads). Trinity (Grabherr et al., 2011) was used for assembly of the clean reads, yielding unigenes for the samples.

Functional Annotation and Metabolic Pathway Analysis

The unigenes obtained from assembly were compared to the Nr, Nt, Pfam (http://pfam.sanger.ac.uk/), KOG/COG (http://www.ncbi.nlm.nih.gov/COG/), SWISS-PROT (http://www.ebi.ac.uk/uniprot/), KEGG (http:///www.genome.jp/kegg/), and GO (http://www.geneontology.org/) databases. The EC numbers of the KEGG-annotated unigenes involved in the metabolic pathway were recorded.

Finally, the PKFM values in fatty acid biosynthesis, lipid degradation, and glycerolipid metabolism pathways for each unigene under different conditions were calculated to compare the expression levels of metabolic pathway genes.

Results

Change in *E. gracilis* Fatty Acid Content Under Light Stress

Table 2 lists the fatty acid content of *E. gracilis* under light and dark culture conditions. The fatty acid carbon chain composition of *E. gracilis* was between C12-C22 in the light group and mainly contained methyl oleate (C18:1ω9C), trans-linoleic acid (C18:2ω6T), and α-linoleic acid (C18:3ω3), which accounted for over 60% of all fatty acids. There were 19 fatty acid components in the light group, with the remainder comprising of 5

Table 2. Fatty acid content and composition of *E. gracilis*

Type of fatty acid	Light group (g/100 g)	Dark group (g/100 g)	Type of fatty acid	Light group (g/100 g)	Dark group (g/100 g)
C8:0	—	0.002 ± 0.000	C17:1	0.002 ± 0.000	0.011 ± 0.001
C10:0	—	0.031 ± 0.001	C18:1ω9C	0.300 ± 0.021	0.170 ± 0.001
C11:0	—	0.003 ± 0.000	C18:1ω9T	0.006 ± 0.001	0.002 ± 0.000
C12:0	0.051 ± 0.002	0.125 ± 0.001	C22:1ω9	—	0.0002 ± 0.000
C13:0	—	0.046 ± 0.001	C24:1	—	0.0001 ± 0.000
C14:0	0.016 ± 0.001	0.347 ± 0.001	C18:2ω6C	0.111 ± 0.018	0.065 ± 0.001
C15:0	0.002 ± 0.000	0.040 ± 0.001	C18:2ω6T	0.273 ± 0005	—
C16:0	0.051 ± 0.002	0.324 ± 0.003	C18:3ω3	0.513 ± 0.003	0.021 ± 0.001
C17:0	—	0.014 ± 0.001	C18:3ω6	0.002 ± 0.000	0.006 ± 0.000
C18:0	0.007 ± 0.001	0.061 ± 0.001	C20:2	0.009 ± 0.002	0.102 ± 0.001
C20:0	—	0.002 ± 0.000	C20:3ω3	0.025 ± 0.002	0.035 ± 0.001
C24:0	—	0.013 ± 0.000	C20:3ω6	—	0.053 ± 0.001
C14:1	—	0.051 ± 0.000	C20:4ω6	0.010 ± 0.000	0.175 ± 0.001
C15:1	0.055 ± 0.005	0.001 ± 0.000	C20:5ω3	0.085 ± 0.002	0.175 ± 0.002
C16:1	0.013 ± 0.001	0.147 ± 0.001	C22:6ω3	0.045 ± 0.003	0.023 ± 0.001

types of SFA, 5 types of MUFA, and 9 types of PUFA. The 5 types of saturated fatty acids mainly are lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitic acid (C16:0), and octadecanoic acid (C18:0). Compared with the dark group, they had reduced content of (C8:0), (C10:0), (C11:0), (C13:0), (C17:0), (C20:0) and (C24:0).

The fatty acid carbon chain composition of *E. gracilis* in the dark group was between C8-C22. The dark group contained 29 fatty acid components, mainly myristic acid (C14:0), palmitic acid (C16:0), oleic acid (C18:1ω9C), arachidonic acid (C20:4ω6), and eicosapentaenoic acid (EPA) (C20: 5ω3), which accounted for over 58% of the total content, with the remainder composed of 12 types of SFA, 8 types of MUFA, and 9 types of PUFA.

Although the composition of fatty acid carbon chains in the light and dark groups was similar, the differences in fatty acid content and their relative proportions were very obvious. The total fatty acid content in the light and dark groups was 1.576 g/100 g and 2.042 g/100 g, respectively. Figure 1 shows that the fatty acid content was higher in the dark than in the light group. The dark group also had the highest proportion of saturated fatty acids, at 49.29%. The light group had the highest proportion of polyunsaturated fatty acids, at 68.08%, while the proportion of SFA was only 8.05%.

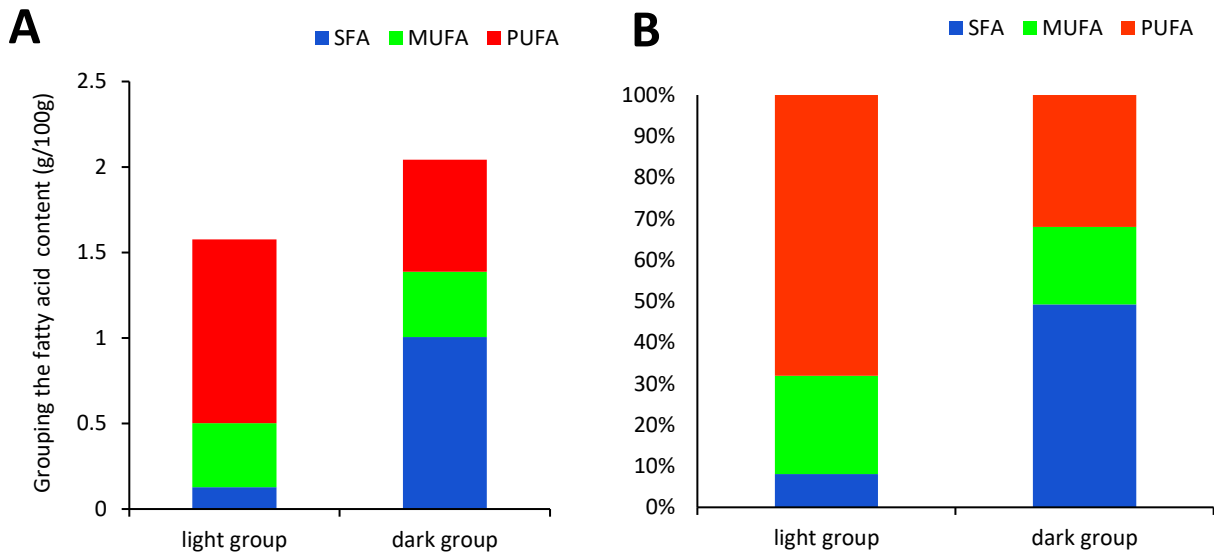

Figure 1. Fatty acid content and composition of *E. gracilis* grown under conditions of light or darkness. **A:** Fatty acid content grouped according to mass, **B:** Fatty acids grouped by percentage composition.

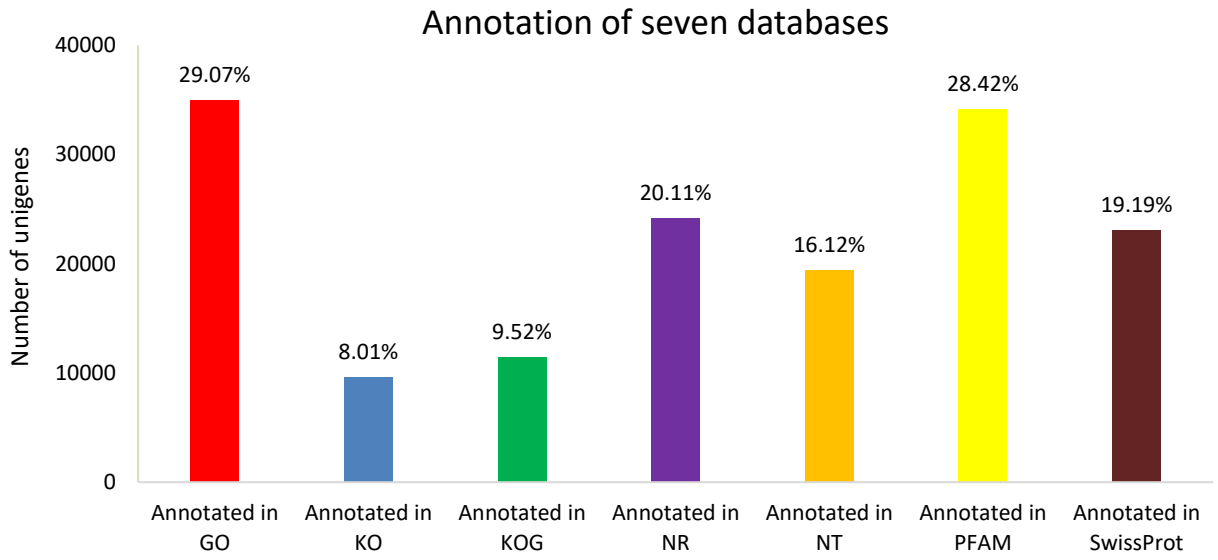

Figure 2. Annotation results for *E. gracilis* unigenes according to seven major databases

Transcriptome Sequencing Data and De Novo Assembly

To understand the genes and metabolic pathways of enzymes associated with lipid synthesis in *E. gracilis*, algae in the light and dark groups were cultured for 5 days and then RNA was extracted. Illumina HiSeq 4000 paired-end sequencing was performed and 290,820,100 raw reads were obtained. After processing, 277,749,398 clean reads were obtained for de novo assembly, which accounted for 95.51% of raw reads. The clean reads were assembled using Trinity, and 297,648 transcripts and 120,086 unigenes were obtained, with an average length of 636 bp.

Functional Annotation

The unigenes obtained were subjected to functional annotation using seven major databases, with the results shown in Figure 2. The GO database had the most annotated unigenes at 34,915, accounting for 29.07% of all unigenes, followed by the PFAM database, with annotations for 28.42%, KOG database, with annotations of 11436 transcripts (accounting for 9.52%), and the KEGG database, with annotations of 9630 transcripts, accounting for 8.01%.

Metabolic Pathway Annotation and Lipid Metabolic Pathway Construction

One hundred twenty-eight KEGG pathways were annotated from 9630 unigenes, including pathways for fatty acid biosynthesis, fatty acid degradation, glycerolipid metabolism, starch, and sucrose metabolism.

Fatty Acid Biosynthesis

The *E. gracilis* transcriptome contains transcripts of enzymes involved in fatty acid biosynthesis, as shown in Table 3. Based on the functional annotation of the *E. gracilis* transcriptome, a free fatty acid biosynthesis pathway was constructed in conjunction with the KEGG annotation results (Figure 3). Fatty acid synthesis occurs in the cytosol, and requires the transport of acetyl-CoA from the mitochondria to the vacuole, after which acetyl-CoA carboxylase (EC: 6.4.1.2 6.3.4.14) catalyzes the reaction of acetyl-CoA and carbonate to form malonyl-CoA, which is transformed into malonyl-ACP under the action of malonyl-CoA:acyl carrier protein transacylase (EC: 2.3.1.39). Acetyl-CoA carboxylase (EC: 6.4.1.2 6.3.4.14) is the rate-limiting enzyme in this pathway and is essential for de novo lipid synthesis and the synthesis of various fatty acids.

Figure 3B shows the fatty acid carbon chain extension reaction, where malonyl-ACP serves as a carbon donor, adding two carbon-synthesized butyryl-ACPs to the carbon skeleton in the condensation reaction, 3-ketoacyl-ACP synthase II (EC: 2.3.1.179), 3-ketoacyl ACP reductase (EC: 1.1.1.100), 3-hydroxyacyl-ACP dehydratase (EC: 4.2.1.59), and enoyl-ACP reductase catalyze fatty acid condensation, reduction, dehydration, and re-reduction processes, respectively. 3-ketoacyl-ACP synthase II (EC: 2.3.1.179) catalyzes the initial condensation reaction and is an essential enzyme in the carbon chain extension process.

Under the catalysis by 3-ketoacyl-ACP synthase II (EC: 2.3.1.179), malonyl-ACP is converted into β-ketoacyl-ACP (3-oxooctadecanoyl-ACP), which is reduced under catalysis by 3-ketoacyl-ACP reductase (EC: 1.1.1.100), dehydrated under catalysis by 3-hydroxyacyl-ACP dehydratase (EC: 4.2.1.59), and reduced under catalysis of enoyl ACP reductase (EC: 1.3.1.9 1.3.1.10) into a saturated fatty acid-ACP. At the same time, saturated fatty acid-ACP is produced under the catalysis by 3-ketoacyl-ACP synthase II (EC: 2.3.1.179), after which δ-9 acyl-ACP desaturase (EC: 1.14.19.2) introduces a double bond into acyl-ACP, forming an unsaturated fatty acid-ACP. The saturated and unsaturated fatty acid-ACPs are finally converted into saturated and unsaturated fatty acids, respectively, under the action of oleoyl-ACP hydrolase (EC: 3.1.2.14).

Fatty Acid Degradation

The *E. gracilis* transcriptome contains transcripts of enzymes involved in fatty acid catabolism pathways, as shown in Table 4. Under the catalysis by acyl-CoA

Table 3. Enzymes identified in the fatty acid biosynthesis in *E. gracilis*

KO name	Name	Unigene number	EC number
K01897	long-chain acyl-CoA synthetase	27	6.2.1.3
K11262	acetyl-CoA carboxylase / biotin carboxylase 1	10	6.4.1.2 6.3.4.14
K00059	3-oxoacyl-[acyl-carrier protein] reductase	9	1.1.1.100
K03921	acyl-[acyl-carrier-protein] desaturase	7	1.14.19.2
K09458	3-oxoacyl-[acyl-carrier-protein] synthase II	6	2.3.1.179
K10781	fatty acyl-ACP thioesterase B	3	3.1.2.14 3.1.2.21
K00645	—	3	2.3.1.39
K02372	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	2	4.2.1.59
K00208	enoyl-[acyl-carrier protein] reductase I	2	1.3.1.9 1.3.1.10
K01961	acetyl-CoA carboxylase, biotin carboxylase subunit	1	6.4.1.2 6.3.4.14
K10782	fatty acyl-ACP thioesterase A	1	3.1.2.14

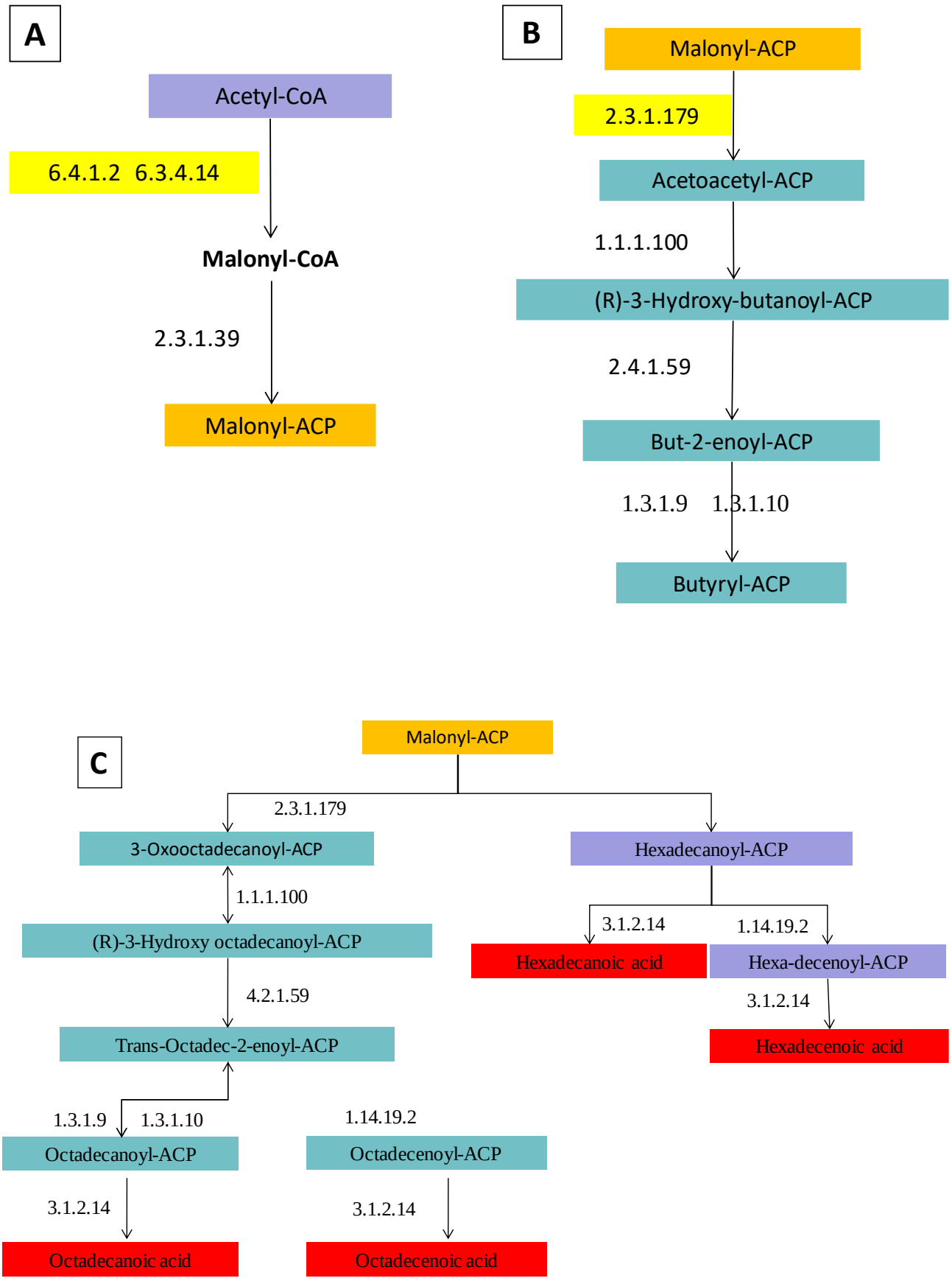

Figure 3. Fatty acid biosynthesis pathway reconstructed on the basis of de novo assembly and annotation of the *E. gracilis* transcriptome. **A:** Biosynthesis of malonyl-ACP, **B:** Biosynthesis of butyryl-ACP, **C:** Biosynthesis of fatty acids.

synthetase (EC: 6.2.1.3), fatty acids are converted into hexa-decanoyl-CoA, which then enters the β-oxidation pathway. Two carbons are removed from hexa-decanoyl-CoA through dehydrogenation, hydration, reduction, and thiolysis by ester acyl-CoA oxidase (EC: 1.3.3.6), enoyl-CoA hydratase (EC: 4.2.1.17), 3-hydroxyalkyl-CoA dehydrogenase (EC: 4.2.1.17 1.1.1.35 1.1.1.211), and acetyl-CoA acyltransferase 1 (EC: 2.3. 1.16), respectively, forming acetyl-CoA, which enters the tricarboxylic acid cycle, eventually being degraded into H_2O and CO_2 (Figure 4).

Glycerolipid Metabolism

The *E. gracilis* transcriptome contains transcripts of enzymes involved in Glycerolipid metabolism, as shown in Table 5. Triacylglycerol synthesis and degradation pathways were constructed based on KEGG cluster analysis of *E. gracilis* (Figure 5A). Glycerol kinase (EC: 2.7.1.30) catalyzes the conversion of glycerol into sn-glycerol-3-phosphate which, along with acyl-CoA, is a precursor for triacylglycerol synthesis. Glycerol-3-phosphate O-acyltransferase (EC: 2.3.1.15) catalyzes

Table 4. Enzymes involved in fatty acid degradation in *E. gracilis*

KO name	Name	Unigene number	EC number
K00128	aldehyde dehydrogenase (NAD+)	29	1.2.1.3
K01897	long-chain acyl-CoA synthetase	27	6.2.1.3
K00626	acetyl-CoA C-acetyltransferase	9	2.3.1.9
K10527	enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	7	4.2.1.17 1.1.1.35 1.1.1.211
K00121	S-(hydroxymethyl)glutathione dehydrogenase / alcohol dehydrogenase	4	1.1.1.284 1.1.1.1
K07513	acetyl-CoA acyltransferase 1	4	2.3.1.16
K18857	alcohol dehydrogenase class-P	3	1.1.1.1
K00232	acyl-CoA oxidase	3	1.3.3.6
K07511	enoyl-CoA hydratase	3	4.2.1.17
K00249	acyl-CoA dehydrogenase	2	1.3.8.7
K14085	aldehyde dehydrogenase family 7 member A1	1	1.2.1.31 1.2.1.8 1.2.1.3

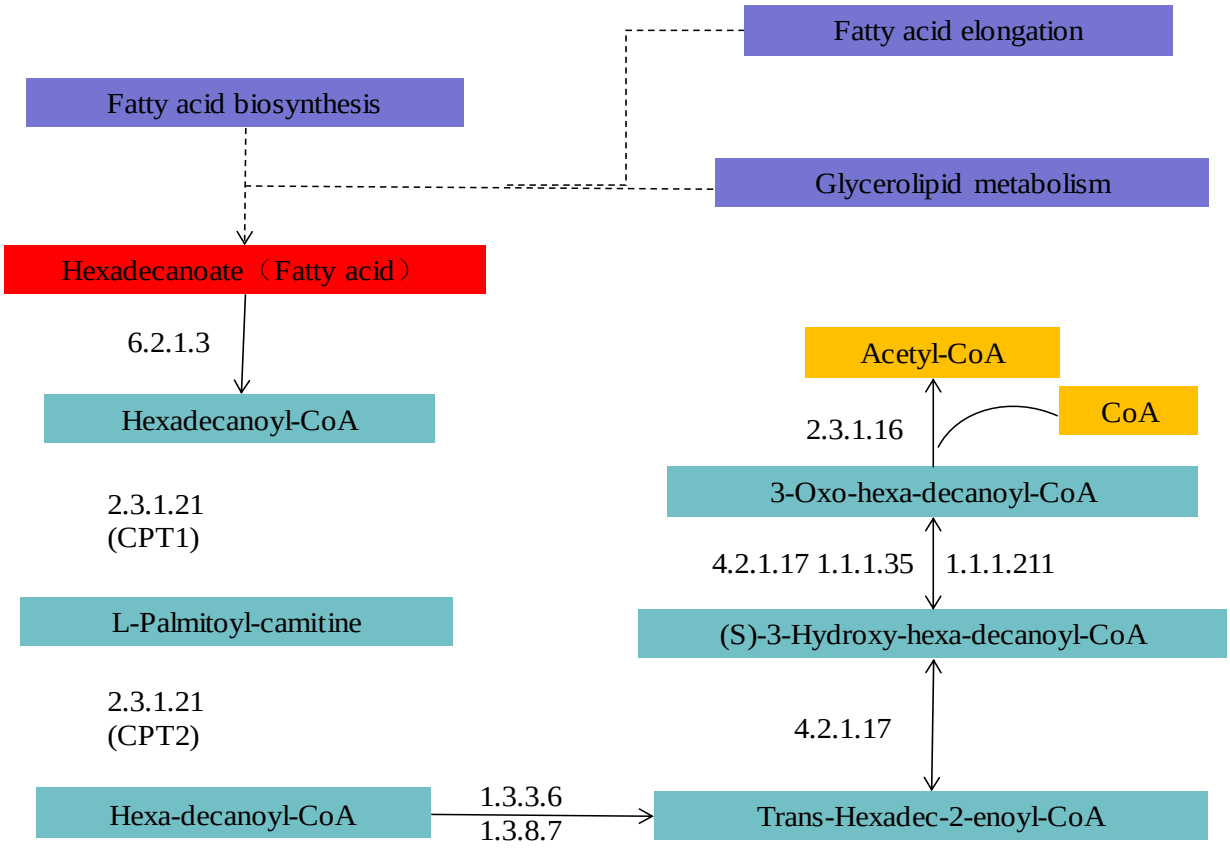

Figure 4. Fatty acid degradation pathway reconstructed on the de basis of de novo assembly and annotation of the *E. gracilis.* transcriptome

esterification of the 1-carbon, forming 1-acyl-sn-glycerol-3-phosphate. 1-acyl-sn-glycero-3-phosphate acyltransferase catalyzes the esterification of the 2-carbon, forming 1,2-diacyl-sn-glycerol-3-phosphate. Phosphatidate phosphatase (EC: 3.1.3.4) then catalyzes phosphorylation of 1,2-diacyl-sn-glycerol-3-phosphate into diacylglycerol. Phospholipid:diacylglycerol acyltransferase (EC: 2.3.1.158) catalyzes the esterification reaction that follows to form triacylglycerol. Phospholipid:diacylglycerol acyltransferase (EC: 2.3.1.158) is the rate-limiting enzyme for the synthesis of stored lipids, and is an essential enzyme for triacylglycerol synthesis.

The triacylglycerol degradation pathway is shown in Figure 5B. In this process, triacylglycerol is deacylated, forming diacylglycerol, and fatty acids are re-degraded. Triacylglycerol and diacylglycerol are converted into diacylglycerol and free fatty acids, respectively, by triacylglycerol lipase (EC: 3.1.1.3).

Preliminary Analysis of Differential Gene Expression in Lipid Metabolism-Associated Pathways

The expression levels of fatty acid biosynthesis pathway genes were compared using FPKM (expected number of fragments per kilobase of transcript sequence per million base pairs sequenced) values (Table 6). It was found that, among the 71 unigenes involved in fatty acid biosynthesis metabolic pathways, there were 10 differentially-expressed genes, of which 5 were upregulated and 5 were downregulated. All changes in differentially expressed genes were no more than 2-fold. The essential and rate-limiting enzyme in the fatty acid biosynthesis pathway (EC: 6.4.1.2 6.3.4.14) was upregulated 1.69-fold under dark conditions.

There were 92 unigenes annotated to fatty acid degradation pathways, including 12 differentially-expressed genes (Table 7), of which, 2 were upregulated, whereas 10 were downregulated. The downregulated genes included acyl-CoA synthetase (EC: 6.2.1.3) and acyl -CoA dehydrogenase (EC: 1.3.8.7). Downregulation indicates that fatty acid degradation pathways are inhibited under dark conditions.

A total of 81 unigenes were annotated to triacylglycerol metabolic pathways, including 13 differentially-expressed genes, of which 4 genes were upregulated and 9 genes were downregulated under dark conditions; all changes in differentially expressed genes were within 2-fold (Table 8). The upregulated genes arylamine N-acetyltransferase (EC: 2.3.1.5) and phosphatidate phosphatase (EC: 3.1.3.4) are mainly involved in the triacylglycerol synthesis pathway, indicating that *E. gracilis* tends toward triacylglycerol accumulation under dark conditions.

Table 5. Enzymes identified in glycerolipid metabolism in *E. gracilis*

KO name	Name	Unigene number	EC number
K01897	long-chain acyl-CoA synthetase	27	6.2.1.3
K11262	acetyl-CoA carboxylase / biotin carboxylase 1	10	6.4.1.2 6.3.4.14
K00059	3-oxoacyl-[acyl-carrier protein] reductase	9	1.1.1.100
K03921	acyl-[acyl-carrier-protein] desaturase	7	1.14.19.2
K09458	3-oxoacyl-[acyl-carrier-protein] synthase II	6	2.3.1.179
K10781	fatty acyl-ACP thioesterase B	3	3.1.2.14 3.1.2.21
K00645	—	3	2.3.1.39
K02372	3-hydroxyacyl-[acyl-carrier-protein] dehydratase	2	4.2.1.59
K00208	enoyl-[acyl-carrier protein] reductase I	2	1.3.1.9 1.3.1.10
K01961	acetyl-CoA carboxylase, biotin carboxylase subunit	1	6.4.1.2 6.3.4.14
K10782	fatty acyl-ACP thioesterase A	1	3.1.2.14

Table 6. Differentially expressed genes involved in the fatty acid biosynthesis pathway in *E. gracilis*

Unigene ID	KO id	EC	FPKM (light group)	FPKM (dark group)	Extent of change
TRINITY_DN30844_c0_g1	K00059	1.1.1.100	75.21	45.92	-1.67
TRINITY_DN33990_c0_g2	K03921	1.14.19.2	269.65	212.14	-1.27
TRINITY_DN37603_c4_g1	K09458	2.3.1.179	389.38	333.78	-1.17
TRINITY_DN33947_c0_g2	K01897	6.2.1.3	152.98	135.07	-1.13
TRINITY_DN38551_c1_g2	K01897	6.2.1.3	355.52	349.17	-1.02
TRINITY_DN40360_c1_g1	K11262	6.4.1.2 6.3.4.14	61.31	109.97	+1.79
TRINITY_DN41561_c0_g2	K11262	6.4.1.2 6.3.4.14	107.92	176.76	+1.64
TRINITY_DN35843_c0_g1	K00059	1.1.1.100	91.56	134.91	+1.47
TRINITY_DN39253_c0_g1	K01897	6.2.1.3	644.83	819.77	+1.27
TRINITY_DN33890_c1_g2	K01897	6.2.1.3	191.73	244.26	+1.27

Discussion

Effect of Light Exposure on Fatty Acid Content in *E. gracilis*

Light is one of the most important factors affecting the growth of unicellular algae and the composition of their metabolites. Light can participate in signal transduction through a series of ferredoxin and thioredoxin proteins, stimulating acetyl-CoA carboxylase (ACCase), and regulating fatty acid synthesis (Kozaki, Kamada, Iguchi, & Sasaki, 2000). In general, the lipid content of *E. gracilis* is much lower than that of oleogenic microalgal species such as *Chlorella* (30-57% lipid) (Wang, Rischer, Eriksen, & Wiebe, 2013) and *Nannochloropsis* (26-42% lipid) (Millán-Oropeza & Fernández-Linares, 2016), but *E. gracilis* contains a high percentage of unsaturated fatty acids (Wang, Seppänen-Laakso, Rischer, & Wiebe, 2018). In this study, 14 unsaturated fatty acids were found in the light group, whereas 29 were found in the dark group.

In the present study, the total amount of fatty acids in the dark group was 1.3-fold that in the light group. The dark group showed 7.9-fold higher accumulation of SFA than in the light group, whereas PUFA content was 1.6-fold lower that in the light group. Dark conditions favored the accumulation of EPA (C20:5ω3) and ARA (C:20:4ω6), which were 2.06-fold and 17.5-fold higher, respectively, than in the light group. A study by Lee and Tan (1988) showed that the EPA content of *Porphyridium cruentum* increased as light intensity increased, which is contrary to the results of the present study. However, Cao, Sun, & Mai (2010) found that ARA and PUFA content in green algae decreased as light intensity increased, consistent with the results of the present study. The observation that light leads to

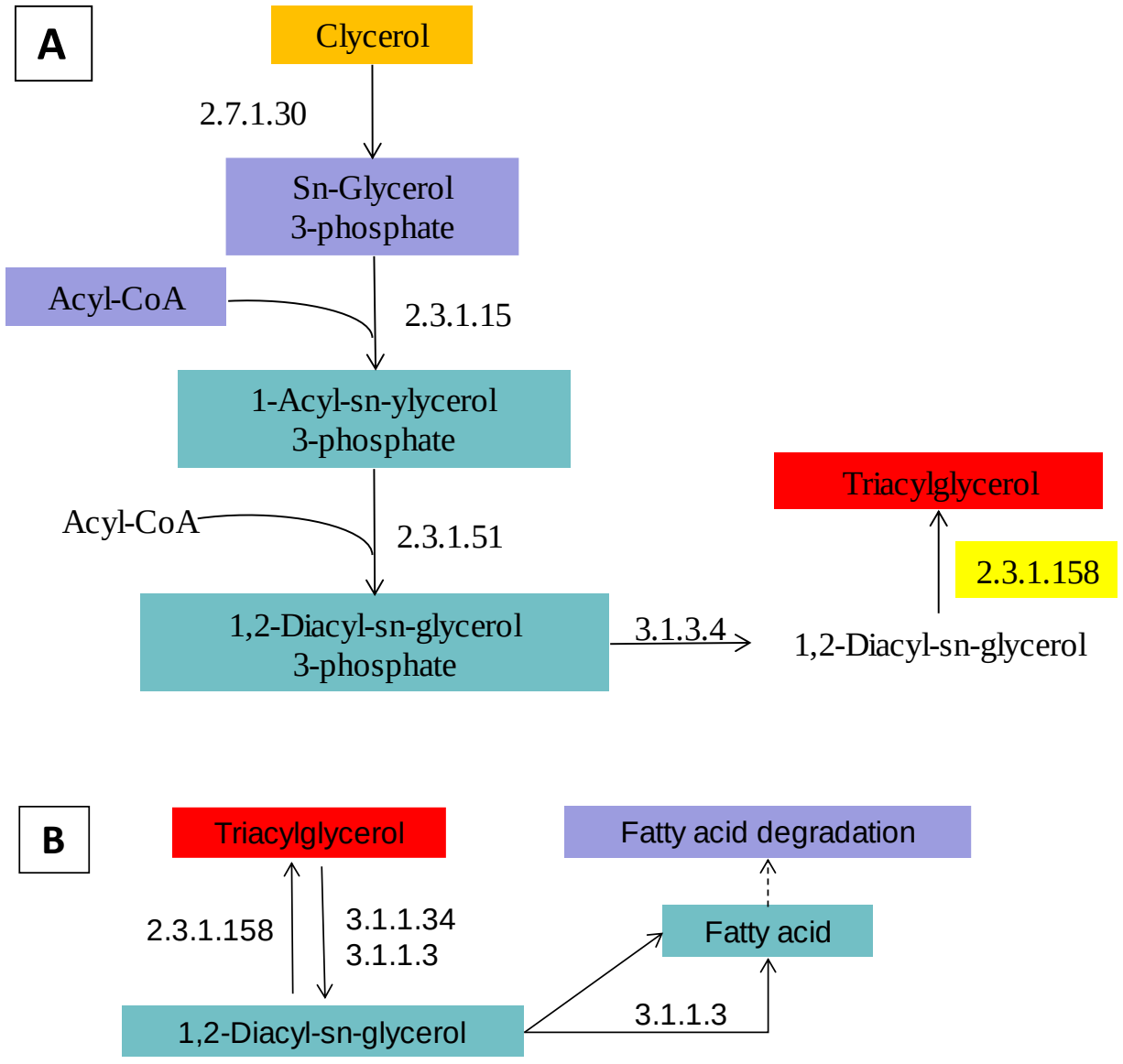

Figure 5. Glycerolipid metabolism pathway reconstructed on the basis of de novo assembly and annotation of *E. gracilis* transcriptome. **A:** Glycerolipid biosynthesis, **B:** Glycerolipid catabolism.

different changes in fatty acid composition and content in microalgae could be species-related, a result of different types of desaturase present in the cells, or different effects of light intensity on enzymatic activity (Renaud et al., 1991). Another possibility is that the changes are a be the result of differences in light intensity used in the various experiments.

Analysis of Transcriptome Data

With the continued development of second-generation sequencing technologies, transcriptome sequencing has been applied in various fields. Currently, transcriptome sequencing has been used to study *Dunaliella tertiolecta* (Rismani-Yazdi, Haznedaroglu, Hsin, & Peccia, 2012), *Rhodomonas* (Li et al., 2016), *Chlorella minutissima* (Yu, Yang, & Lin, 2016), *Thalassiosira pseudonana* (Armbrust et al., 2004), and several other species. In addition, transcriptome data have been used to analyze genes coding for enzymes essential for some metabolic processes. In the present study, high-throughput sequencing technology was used to sequence the transcriptome of *E. gracilis*. After sequencing and assembly, 120,086 unigenes were obtained. A total of 48,031 unigenes were annotated using seven databases with an annotation rate of 39.99%. This relatively low rate may be related to the relative lack of genetic sequence data for *Euglena* in public databases. It may also be that the unannotated genes represent genes unique to *E. gracilis* (Chen, Li, Xiao, & Liu, 2015).

Analysis of Lipid-Associated Metabolic Pathways
Lipid Biosynthesis and Degradation Pathways

The unigenes were compared with those from the KEGG database, and 71 unigenes involved in fatty acid biosynthesis pathways, and 92 unigenes from catabolic pathways were screened. Using the resultant data, the fatty acid biosynthesis and degradation pathways of *E. gracilis* were constructed, and differential gene expression (FPKM) between the light and dark groups was analyzed.

Acetyl CoA carboxylase (ACCase, EC: 6.4.1.2 6.3.4.14) and 3-ketoacyl-ACP synthase II (EC: 2.3.1.179) are essential enzymes in the fatty acid biosynthesis pathway. Acetyl CoA carboxylase (ACCase, EC: 6.4.1.2) is the rate-limiting enzyme, and is essential for de novo lipid synthesis, and the synthesis of various fatty acids. ACCase is composed of a biotin carboxylase subunit, a biotin carboxyl carrier protein (BC-CP), and the α-CT and β-CT4 subunits of the two BCCP carboxyltransferases. Acetyl-CoA carboxylase (EC: 6.4.1.2 6.3.4.14) expression was upregulated 1.69-fold under dark conditions. 3-

Table 7. Differentially expressed genes involved in the fatty acid degradation pathway in *E. gracilis*

Unigene ID	EC	FPKM (light group)	FPKM (dark group)	Extent of change
TRINITY_DN29885_c2_g2	2.3.1.9	2033.84	1450.84	-1.41
TRINITY_DN31507_c0_g2	1.2.1.3	59.99	45.48	-1.32
TRINITY_DN30652_c3_g2	1.2.1.3	15890.99	13009.2	-1.22
TRINITY_DN31275_c0_g2	1.3.8.7	229.63	187.75	-1.22
TRINITY_DN38249_c0_g2	2.3.1.9	334.01	277.25	-1.20
TRINITY_DN33947_c0_g2	6.2.1.3	152.98	135.07	-1.13
TRINITY_DN40906_c0_g3	1.1.1.284 1.1.1.1	146.34	141.65	-1.03
TRINITY_DN38551_c1_g2	6.2.1.3	355.52	349.17	-1.02
TRINITY_DN53848_c0_g1	1.2.1.3	3.6	0	—
TRINITY_DN8615_c0_g1	1.2.1.3	2.56	0	—
TRINITY_DN39253_c0_g1	6.2.1.3	644.83	819.77	+1.27
TRINITY_DN33890_c1_g2	1.2.1.3	191.73	244.26	+1.27

Table 8. Differentially expressed genes involved in the glycerolipid metabolism pathway in *E. gracilis*

Unigene ID	KO id	EC	FPKM (light group)	FPKM (dark group)	Extent of change
TRINITY_DN35317_c0_g1	K07407	3.2.1.22	100.73	53.7	-1.88
TRINITY_DN34620_c1_g1	K14457	2.3.1.22	70.23	50.62	-1.39
TRINITY_DN31507_c0_g2	K00128	1.2.1.3	59.99	45.48	-1.32
TRINITY_DN35334_c2_g3	K13513	2.3.1.-2.3.1.51	98.71	79.98	-1.23
TRINITY_DN30652_c3_g2	K00128	1.2.1.3	15890.99	13009.2	-1.22
TRINITY_DN38913_c0_g2	K00901	2.7.1.107	70.7	58.96	-1.20
TRINITY_DN36567_c1_g1	K00655	2.3.1.51	327.99	211.77	-1.54
TRINITY_DN53848_c0_g1	K00128	1.2.1.3	3.6	0	—
TRINITY_DN8615_c0_g1	K00128	1.2.1.3	2.56	0	—
TRINITY_DN33712_c0_g1	K00630	2.3.1.15	50.14	74.16	+1.47
TRINITY_DN38411_c2_g5	K15728	3.1.3.4	24.32	32.11	+1.32
TRINITY_DN40234_c1_g1	K06119	2.4.1.-	49.66	64.59	+1.30
TRINITY_DN31198_c0_g1	K15728	3.1.3.4	39.12	42.94	+1.10

ketoacyl-ACP synthase II (EC: 2.3.1.179) catalyzes the condensation reaction of a 16-carbon chain to an 18-carbon chain and is an essential enzyme in the condensation reaction. While no 3-ketoacyl-ACP synthase I was found in the present study, this transcript was found in *Eustigmatos cf. polyphem* (Wan et al., 2012), *Dunaliella tertiolecta* (Rismani-Yazdi, Haznedaroglu, Bibby, & Peccia, 2011), and *Neochloris oleoabundans* (Rismani-Yazdi, Haznedaroglu, Hsin, & Peccia, 2012).

Under dark conditions, the rate-limiting enzyme in the fatty acid biosynthesis pathway, acetyl-CoA carboxylase (ACCase, EC: 6.4.1.2 6.3.4.14), was upregulated, while acyl-CoA synthetase (EC: 6.2.1.3) and acyl-CoA dehydrogenase (EC: 1.3.8.7) in the fatty acid degradation metabolic pathway was downregulated. This indicated that the increase in fatty acid content was due to the promotion of the fatty acid biosynthesis pathway and inhibition of the fatty acid degradation pathway, consistent with the increase in fatty acid content in dark conditions in the present study.

Glycerolipid Metabolic Pathways

A triacylglycerol metabolic pathway for *E. gracilis* was constructed using 81 unigenes. In addition, differential gene expression (FPKM) of these genes in the light and dark groups was analyzed.

In the present study, phospholipids acted as donor groups in triacylglycerol synthesis catalyzed by phospholipid:diacylglycerol acyltransferase (EC:2.3.1.258). This triacylglycerol synthesis pathway exists in some plants and yeast (Anders et al., 2000). A transcript encoding a phospholipid:diacylglycerol acyltransferase (EC: 2.3.1.258) was also found in the transcripts of *Eustigmatos cf. polyphem* (Wan et al., 2012) and *Dunaliella tertiolecta* (Rismani-Yazdi, Haznedaroglu, Bibby, & Peccia, 2011). In addition to these pathways, another triacylglycerol synthesis pathway exists, in which 1,2-diacylglycerol is acylated at the 3-carbon to form triacylglycerol under the catalysis by diacylglycerol acyltransferase (EC: 2.3.1.20). However, no transcripts encoding this enzyme were found, and whether this pathway exists in *E. gracilis* remains to be determined.

Acetyl-CoA carboxylase (ACCase, EC: 6.4.1.2 6.3.4.14) and 3-ketoacyl-acylase ACP synthase II (EC: 2.3.1.179) are essential enzymes in fatty acid synthesis. Phospholipid: diacylglycerol acyltransferase (EC: 2.3.1.258) is a rate-limiting enzyme in the synthesis of stored lipids, and is essential for triacylglycerol synthesis. Regulation of its activity can increase the content and composition of fatty acids and triacylglycerols in *E. gracilis* (Padham et al., 2007; Kroon, Wenxue, Simon, & Slabas, 2006; Wendel, Lewin, & Coleman, 2009). Inhibition or knockout of enzymes associated with fatty acid and triacylglycerol degradation through genetic engineering could also increase fatty acid and triacylglycerol content in *E. gracilis*. In this regard, the most common genetic modification used to date is overexpression of ACCase (Roessler, Bleibaum, Thompson, & Ohlrogge, 2010). In addition, modification of essential enzymes regulating the condensation reaction in fatty acid synthesis pathways (Verwoert, Linden, Walsh, Nijkamp, & Stuitje, 1995) and blockade of β-oxidation (Radakovits, Jinkerson, Darzins, & Posewitz, 2010) are also effective means of promoting fatty acid accumulation.

Conclusion

In the present study, transcripts encoding enzymes related to fatty acid synthesis in the *E. gracilis* transcriptome were identified, and the pathways they are involved in were described. We studied lipid metabolism in *Euglena gracilis* under conditions of light stress using transcriptome analysis to characterize the enzymes and pathways involved. We also analyzed the lipid composition and content under different light conditions using gas chromatography. We found that by regulating the activity of acetyl-CoA carboxylase, 3-ketoacyl-ACP synthase II, and phospholipid:diacylglycero acyltransferase, enzymes associated with fatty acid and glycerolipid degradation may be inhibited or knocked out, thereby increasing fatty acid and glycerolipid content and composition in *E. gracilis*. Our findings can provide a clear guide for genetic engineering of *E. gracilis* and modification of its lipid metabolism pathways. Additionally, our study provides a basis for genetic modification of *E. gracilis* for the construction of high-quality oil-producing engineered strains.

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