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Understanding lipid metabolism in high-lipid-producing *Chlorella vulgaris* mutants at the genome-wide level

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ABSTRACT

In this study, physical and chemical mutagenesis methods were applied to enhance lipid productivity in *Chlorella vulgaris*. Then, *de novo* RNA-seq was performed to observe lipid metabolism changes at the genome-wide level. Characterization of two mutants, UV-715 and EMS-25, showed marked increases in lipid contents, *i.e.*, 42% and 45%, respectively. In addition, the biomass productivity of the UV-715 cells was 9% higher than that of wild-type cells. Furthermore, gas chromatography-mass spectrophotometry analysis showed that both mutants have higher fatty acid methyl ester (FAME) contents than wild-type cells. To understand the effect of mutations that caused yield changes in UV-715 and EMS-25 cells at a genome-wide level, we carried out *de novo* RNA-seq. As expected, the transcriptional levels of the lipid biosynthesis genes were up-regulated, while the transcriptional levels of genes involved in lipid catabolism were down-regulated. Surprisingly, the transcriptional levels of the genes involved in nitrate assimilation and detoxification of reactive oxygen species (ROS) were significantly increased in the mutants. The genome-wide analysis results highlight the importance of nitrate metabolism and detoxification of ROS for high biomass and lipid productivity.

1. Introduction

Chlorella vulgaris is a fast-growing freshwater alga that can grow in harsh conditions [1,2]. Triacyglycerols can reach up to 58% of the total lipid content in *C. vulgaris* [3]. These lipids include palmitic acid C16:0, stearic acid C18:0, palmitoleic acid C16:1 and oleic acid C18:1, which can be used for biodiesel production [4]. In addition, this alga is an extremely versatile photosynthetic organism and produces some valuable pigments, such as β-carotene, astaxanthin, and canthaxanthin, and some nutritional polyunsaturated fatty acids (PUFAs), such as linoleic acid C18:2, linolenic acid C18:3 and eicosapentaenoic acid C20:5 [5]. In some countries, *Chlorella* extracts are consumed as a dietary food supplement due to their pigment composition, which possess immune-modulating and anticancer properties [6–8]. Collectively, these features make *C. vulgaris* an ideal model organism to study lipid and pigment biosynthesis.

Although several studies have indicated that production of biodiesel from microalgae is promising, large-scale production is not still economically feasible. Some barriers should be overcome regarding low oil content and biomass productivity [9,10]. Therefore, studies were carried out on different microalgae, including *C. vulgaris*, to improve their lipid content and the growth rate through mutagenesis. In one study, *C. vulgaris* was exposed to ultraviolet light (UV) to generate mutants that have enhanced lipid contents. The result was a mutant cell with a 26.9% increase in lipid content, but there was a significant reduction (11.8%) in the biomass [11]. Liu et al. obtained a mutant with 21.97% and 5.6% increases in lipid content and biomass, respectively [12]. In another study, mutagenesis of *Nannochloropsis oceanica* resulted in a mutant with a 19% increase in biomass and a 28% increase in lipid productivity [13]. In spite of these studies and promising improvements achieved in the lipid content and biomass of microalgae, there is still a lack of understanding of the lipid metabolism on a genome-wide level. Therefore, in this study, we employed random mutagenesis followed by *de novo* RNA-seq to understand overall lipid metabolism in *Chlorella vulgaris* SAG 211-12 strain (hereafter abbreviated *C. vulgaris*).

C. vulgaris was subjected to UV irradiation and ethyl methanesulfonate (EMS), and two mutants (UV-715 and EMS-25) were selected based on the highest lipid productivity. Further characterization of the

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selected mutants revealed that UV-715 and EMS-25 displayed a high lipid contents of 42% and 45%, respectively. Gas chromatography-mass spectrophotometry (GC–MS) analysis showed that EMS-25 and UV-715 contained 39% and 32% more fatty acid methyl ester (FAME) than the wild-type, respectively. To understand how transcriptional responses changed to accumulate more lipids in the mutant cells at a genome-wide level, we performed *de novo* RNA-seq on both wild-type and mutant cells. RNA-seq results revealed that the transcript abundance of genes involved in nitrate assimilation, lipid biosynthesis and detoxification of reactive oxygen species, such as nitrate reductase, siroheme, aldehyde dehydrogenase B4 and superoxide dismutase, were significantly increased in the mutant cells. In contrast, the expression of some genes that participate in amino acid synthesis and lipid catabolism, such as serine acetyltransferase and arachidonate 5-lipoxygenase, were down-regulated. The observed differential gene expression in mutants will help us identify the candidate target genes involved in cell growth and lipid metabolism with improved lipid yields.

2. Materials and methods

2.1. Microalgae strain and cultivation

The parent wild-type of *Chlorella vulgaris* (SAG 211-12) was obtained from the collection of algal cultures at the University of Göttingen, Germany. The inoculum was grown in Bold's Basal medium consisting of the following (per liter): 0.25 g $NaNO_3$, 0.075 g $MgSO_4{\cdot}7H2O$, 0.075 g K_2HPO_4, 0.175 g KH_2PO_4, 0.025 g NaCl, 0.025 g $CaCl_2{\cdot}2H_2O$, 8.82 mg $ZnSO_4{\cdot}7H_2O$, 0.44 mg $MnCl_2{\cdot}4H_2O$, 0.71 mg MoO_3, 1.57 mg $CuSO_4{\cdot}5H_2O$, 0.49 mg $CoCl_2{\cdot}6H_2O$, 11.42 mg H_3BO_3, 50 mg EDTA, 31 mg KOH, and 4.98 mg $FeSO_4{\cdot}7H_2O$. The cultures were maintained at 25 °C under continuous 4 Klux cool white LED lights throughout this study.

2.2. Mutagenesis and colony screening

In the mid-exponential growth phase when the concentration was approximately 1×10^7 cells·mL^{-1}, 10 mL of the sample was taken and centrifuged at 12000 rpm for 5 min. After washing the pellets with distilled water for two times, the cells were resuspended in 250 μL of distilled water, and 1×10^8 cells of WT were spread on solid Bold's basal media (solidified with 1% agar) and then exposed to UV irradiation (254 nm) with an intensity of 2.9×10^{-2} W/cm^2 (measured by UVX Digital Radiometer (UVP, USA)) for 0.5 to 10 min at a distance of 15 cm. The plates were kept in the dark for 24 h to prevent light-induced repair. EMS treatment was carried out as descried in [14,15] with some modifications. In brief, 5×10^7 cells of *C. vulgaris* were treated with 25 mM EMS for 1 h, agitated gently under dark conditions in distilled water, washed 3 times with culture media and then plated on solid media.

All the plates were then cultivated under the conditions described in the previous section for 2 weeks. In the first step of screening, single colonies on plates were selected and transferred individually to 2 mL liquid Bold's basal medium. After 10 days, the cell densities were determined at OD_{680} nm, and the neutral lipid amount was determined using Nile red (NR; Sigma-Aldrich, USA) as described in [16]. First, a stock solution (500 μg mL^{-1}) of NR in dimethyl sulfoxide (DMSO) was prepared. Then, a working solution was prepared by dissolving 100 μL of the stock solution in 50 mL of 50% DMSO in distilled water. For staining, 150 μL of the working solution was mixed with 150 μL of samples with known cell concentrations in a 96-well plate. The plate was shaken at 150 rpm for 10 min at room temperature. Then, fluorescence measurements were carried out using a Fluoroskan Ascent microplate reader (Thermo Scientific, USA) at maximum excitation and emission spectra of 544 and 590 nm, respectively. We used a triolein (Sigma–Aldrich, USA) standard for absolute quantification of neutral lipids.

In the second step of screening, colonies that exhibited high lipid productivity (both lipid content and growth rate) were selected and inoculated in 100 mL flasks. Samples were taken at different time intervals. Cell densities were determined spectrophotometrically at 680 nm. The dry cell weight (DCW) was determined by correlating OD_{680} with the dry weight by the following equation: DCW (g dry cell/L) = $OD_{680} \times 0.198$, $R^2 = 0.988$ and the neutral lipid was measured by NR. Finally, two colonies that had higher lipid productivity and FAME content than WT were selected for detailed characterization.

2.3. FAME analysis by GC–MS

After harvesting, cells were disrupted with an ultrasonic homogenizer (Bandelin Sonoplus, Germany) at 90% power (three 30-second cycles) and then dried at 65 °C for 24 h. For direct transesterification, methanol with 2% HCl was used during the course of reaction for 2 h at 90 °C. Then, the samples were cooled to room temperature, and fatty acid methyl esters (FAMEs) were separated by adding distilled water and hexane.

Next, 1 μL of FAME extract from each sample was injected into a 7890-B Agilent GC equipped with a 5977A Agilent MSD detector and DB-23 column (60 m × 0.25 mm × 0.25 μm film thickness). The split ratio was adjusted to 50, and the injector temperature was 250 °C. Helium at 50 °C and 180 kPa (33 cm/S) was used as the carrier gas. The column temperature was increased from 50 °C to 175 °C at 25 °C per min and then from 175 °C to 235 °C at 4 °C per min, and the detector temperature was 250 °C. Quantification and identification of FAME was performed by using a C4–C24 FAME mix standard solution (Sigma-Aldrich, USA).

2.4. Preparation of cDNA libraries for RNA-seq

Total RNA was extracted from 15 mL cell culture, cultivated for 14 days using TRIzol reagent (Invitrogen, USA) as described in our previous works [17,18]. Briefly, after quality and quantity measurement using 2100 BioAnalyzer (Agilent, USA), extracted RNA was treated with RNase-free DNase I (Thermo Scientific, USA) at a concentration of 1 U/μg to remove residual genomic DNA. Then, RNA-seq library preparation was performed using TruSeq mRNA Sample Preparation Kit (Illumina, USA) as described in [19]. Briefly, mRNAs were purified from the 1 μg of total RNA using oligo (dT) magnetic beads and then were fragmented. After that, the cleaved short RNA fragments were used for first-strand cDNA synthesis using first strand synthesis mix, and the second strand was synthesized using second strand marking master mix. The double strand cDNAs were purified with AMPure XP beads (Beckman Coulter, USA) and eluted with resuspension buffer followed by 3′end adenine nucleotide addition. Finally, sequencing adaptors were ligated to the fragments and cDNA fragments were enriched by PCR amplification. Enriched cDNA libraries were used for cluster generation and sequencing. 75 × 2 paired-end sequencing of three cDNA libraries (WT, UV-715 and EMS-25) with two biological replicates were performed using the Illumina MiSeq sequencing platform (Illumina, USA).

2.5. De novo transcriptome assembly and DEG analysis

All sequence data were at 2 × 75 bp length. The high quality reads were saved in fastq files and deposited in the GEO database at NCBI with the accession number: GSE95708 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnitsgcotfwjdyr&acc=GSE95708). The fluorescent images were processed into sequences, and base calling and quality value calculation were performed using the Illumina data processing pipeline (v1.5). Following the initial quality evaluation of the raw reads using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) (v0.11.5), the following steps were performed for sequencing quality controls using Trimmomatic (v0.35) tool [20]: (1)

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