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Short communication

A type 2 diacylglycerol acyltransferase accelerates the triacylglycerol biosynthesis in heterokont oleaginous microalga *Nannochloropsis oceanica*

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ABSTRACT

Oleaginous microalgae have received a considerable attention as potential biofuel feedstock. However, lack of industry-suitable strain with lipid rich biomass limits its commercial applications. Targeted engineering of lipogenic pathways represents a promising strategy to enhance the efficacy of microalgal oil production. In this study, a type 2 diacylglycerol acyltransferase (DGAT), a rate-limiting enzyme in triacylglycerol (TAG) biosynthesis, was identified and overexpressed in heterokont oleaginous microalga *Nannochloropsis oceanica* for the first time. Overexpression of DGAT2 in *Nannochloropsis* increased the relative transcript abundance by 3.48-fold in engineered microalgae cells. TAG biosynthesis was subsequently accelerated by DGAT2 overexpression and neutral lipid content was significantly elevated by 69% in engineered microalgae. The fatty acid profile determined by GC–MS revealed that fatty acid composition was altered in engineered microalgae. Saturated fatty acids and polyunsaturated fatty acids were found to be increased whereas monounsaturated fatty acids content decreased. Furthermore, DGAT2 overexpression did not show negative impact on algal growth parameters. The present investigation showed that the identified DGAT2 would be a potential candidate for enhancing TAG biosynthesis and might facilitate the development of promising oleaginous strains with industrial potential.

1. Introduction

Incessant consumption of fossil fuels brings on global energy crisis and serious environmental concerns. Hence, the researchers have paid considerable attention to alternative renewable bioenergy. Microalgae has been considered as potential biofuel feedstock over terrestrial crops, because of their higher growth rate, CO_2 fixation, cultivable in wide range of water without encroaching farm land (Subramaniam et al., 2010) and capability of accumulating higher triacylglycerols (TAG) and oils that can be readily converted into biodiesel by transesterification (Chisti, 2007). However, lack of microalgal strains with higher TAG content and biomass is found to be one of the major obstacles in the economically viable fuel industry (Chisti, 2007). Metabolic engineering represents a promising strategy to enhance the microalgal TAG content by targeted engineering of metabolic pathways. Identification and manipulation of key genes that influence TAG biosynthesis are the critical determinants for engineering the microalgae for enhanced TAG production.

The assembly of TAG occurs in the ER where TAG can be synthesized by sequential acylation of glycerol starting with glycerol 3-phosphate. Diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzes the last and committed step in the TAG production, by transferring an acyl group from acyl-CoA to diacylglycerol (DAG) to form triacylglycerol. Previous studies showed that DGAT overexpression in plants resulted in elevated TAG accumulation (Andrianov et al., 2010; Bouvier-Nave et al., 2000). Overexpression of *Arabidopsis* DGAT in *Saccharomyces cerevisiae* resulted in increased DGAT activity, thereby increasing the TAG content (Jako et al., 2001). In addition, overexpression of DGAT2 in green microalga *Chlamydomonas reinhardtii* apparently increased the TAG content up to 9-fold (Hung et al., 2013). In algae, three types of DGAT have been characterized, and among them DGAT2 has been identified as the potent enzyme in TAG biosynthesis (Chungjatupornchai and Watcharawipas, 2015; Hung et al., 2013). Algal DGAT2s diverge from those of other eukaryotes by possessing multiple paralogs

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(Chen and Smith, 2012). The genome sequencing of *Nannochloropsis* revealed the existence of one form of DGAT1 and eleven forms of DGAT2s (Wang et al., 2014). DGAT1 and DGAT2 have been characterized in many different algal species, such as marine diatom *Phaeodactylum tricornutum* (Guiheneuf et al., 2011; Niu et al., 2013) and green microalga *C. reinhardtii* (La Russa et al., 2012). Especially, DGAT2 was more potent in TAG biosynthesis than DGAT1 in mice (Stone et al., 2004) and plants (Kroon et al., 2006), possibly due to its conserved orthologs in nature (Shockey et al., 2006) and its higher affinity towards the substrates (Chen and Smith, 2012). However, identification of DGAT2 in oleaginous microalga *Nannochloropsis oceanica* and elucidation of its functional role in microalgal TAG synthesis remain unclear.

Nannochloropsis species are unicellular photosynthetic heterokonts distributed in wide range of waters and ranging in size from 2 to 5 μm. *Nannochloropsis* has been of commercial interest because of its promising characteristics such as high TAG content, synthesis of economically valuable by-products and higher growth rate (Doan and Obbard, 2010; Wang et al., 2014). Our burgeoning knowledge of the microalgal genomes and metabolic pathways open up new opportunities to study and improve microalgae. Genetic manipulation to enhance lipid accumulation has been conducted in the model microalgal organisms such as *C. reinhardtii* (Mussgnug et al., 2007) and *P. tricornutum* (Xue et al., 2015). However, genetic engineering of oleaginous non-model microalgal species for enhanced lipid accumulation has been rarely achieved yet (Muto et al., 2015). It is of great interest to develop genetically improved strains of such microalga with high industrial potential. In this study, we demonstrate the identification, cloning and overexpression of a putative DGAT2 from *N. oceanica*. This report showed the promising role of DGAT2 in microalgal TAG biosynthesis and would also pave the way for metabolic engineering in photosynthetically driven cell factories for commercialization.

2. Materials and methods

2.1. Microalgal strain and culture conditions

Nannochloropsis oceanica CCAP 849/10 (formerly CCMP1779) was procured from NCMA (National Center for Marine Algae and Microbiota, USA). The algal cells were cultivated in f/2 liquid medium or f/2 agar plates at 25 ± 1 °C in an artificial climate incubator provided with cool-white fluorescence light of 200 μmol photons m^{-2} s^{-1} under a photoperiod of 15/9 h light/dark (Ningbo, China).

2.2. Cloning of DGAT2 and transformation of nannochloropsis by electroporation

Genomic DNA was extracted from *N. oceanica* using DNA isolation kit (Omega, China) following the manufacturer's instruction. The extracted DNA was PCR amplified employing the gene specific primers (N01f 5′-ACAATTACAATCCAGTGGTACCATGTTGCTGGCGTCGTCTC-3′ and N02r 5′-GTCCTTGTAGTCCAGGTGTGTGACGATGCGAAGCGTCTTGTGC-3′). The PCR product was cloned into a transformation vector pNa03 under the control of Hsp20 promoter, and then electroporated into microalgae cells using a Gene Pulser Xcell electroporation system (Bio-Rad) following the protocol reported by Zhang and Hu (2014) (Zhang and Hu, 2014) with modifications. Briefly, *N. oceanica* cells in logarithmic phase were harvested by centrifugation at 4000*g* for 5 min at 4 °C. Pelleted cells were washed twice with 375 mM sorbitol and resuspended in 0.2 mL of 375 mM sorbitol. Five μg of linearized plasmid and 3 μg of salmon sperm DNA (heat denatured at 95 °C for 1 min) were added and transferred to 2 mm cuvettes. After incubation on ice for 10 min, electroporation was performed by employing the following parameters: 2.2 kV, capacitance at 50 μF, resistance at 600 ohm. The electroporated cells were transferred into 5 mL of f/2 media and incubated in dark for 24 h. Thereafter, the algal cells were harvested by centrifugation and spread on f/2 agar medium supplemented with 5 μg/mL zeocin. The grown transformed cells were picked by tips and inoculated into the f/2 liquid media supplemented with 5 μg/mL zeocin. Cells were grown to stationary growth phase and employed for further experiments.

2.3. Molecular characterization of transformed microalgae

The *DGAT2* gene integration into host genome was confirmed by single cell PCR analysis using the primers flanking the inserted *DGAT2* in the vector: Na01 (5′- ACAATTACTATTTACAATTACAATCCA-3′) and Na02 (5′- AAACCAAAGCGGAGTGACTGCAAC-3′). Two transformed lines with same phenotype were randomly selected and subjected for further analysis. The relative transcript abundance was quantified by real-time quantitative PCR (qPCR) using a SYBR green qPCR SuperMix (Invitrogen, USA) on ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA). Total RNA was isolated from both the transformed and wild type (WT) cells using RNA/DNA/Protein isolation kit (Omega) and the first strand cDNA was synthesized using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer's specification. The qPCR were carried out in 96-well plates in a reaction volume of 20 μL following the manufacturer's instruction (Applied Biosystems, USA). The transcript level was calculated by standard-curve and normalized against β-actin gene as an internal control. The primers used for *DGAT2* were DGAT-F1 (5′-GTGCCTGTGATCCCTGTATA-3′) and DGAT-F2 (5′-CCCCTGTTACTGACCTTGAG-3′).

Total protein extraction from cell and protein concentration estimation methods were reported previously (Niu et al., 2013). Protein fractions were resolved on 10% SDS-PAGE and subsequently electrotransferred onto polyvinylidene fluoride membrane. The membrane was blocked with nonfat milk for 2 h at 4 °C and incubated with primary anti-flag antibody (1:5000, Sigma-Aldrich, USA), followed by incubation with HRP-conjugated goat anti-rabbit antibody (1:3000, CST, USA). The anti- β-actin was used as reference (1:5000). Membrane development was performed using chemiluminescent system.

2.4. Fatty acid analysis

Lipids were extracted from engineered and wild type cells and fatty acid composition was analyzed as FAMEs by Gas Chromatography–Mass Spectrometry (GC–MS) as described previously (Peng et al., 2014; Yang et al., 2013). N-nonadecyl ester (10 mg/mL) was used as an internal standard. All the samples were analyzed in triplicate by GC–MS employing the following conditions GC conditions were as follows: initial column temperature was set as 60 °C for 1 min, ramped at 10 °C/min to 160 °C and to a final temperature of 250 °C at 2.5 °C/min. The injector temperature was set at 280 °C and the samples (1 μL) were injected in a splitless mode. Fatty acids were analyzed using the equipped NBS spectrum library. The integrated peak areas were determined and normalized to obtain the relative content of fatty acid composition.

2.5. Neutral lipid content determination and cell morphological observation

The microalgal neutral lipid content was determined by Nile red staining method as per the protocol reported by Doan and Obbard (2010). To 300 μL of algal culture, 75 μL of glycerol (0.5 g/mL) and