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Research paper

Diacylglycerol acyltransferase 2 of *Mortierella alpina* with specificity on long-chain polyunsaturated fatty acids: A potential tool for reconstituting lipids with nutritional value

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ABSTRACT

Based on available genome sequences and bioinformatics tools, we searched for an uncharacterized open reading frame of *Mortierella alpina* (*MaDGAT2*) using diacylglycerol acyltransferase sequence (fungal DGAT type 2B) as a query. Functional characterization of the identified native and codon-optimized *M. alpina* genes were then performed by heterologous expression in *Saccharomyces cerevisiae* strain defective in synthesis of neutral lipid (NL). Lipid analysis of the yeast tranformant carrying *MaDGAT2* showed that the NL biosynthesis and lipid particle formation were restored by the gene complementation. Substrate specificity study of the fungal enzyme by fatty acid supplementation in the transformant cultures showed that it had a broad specificity on saturated and unsaturated fatty acid substrates for esterification into triacylglycerol (TAG). The *n-6* polyunsaturated fatty acids (PUFAs) with 18 and 20 carbon atoms, including linoleic acid, γ-linolenic acid, dihomo γ-linolenic and arachidonic acid could be incorporated into TAG fraction in the yeast cells. Interestingly, among *n-3* PUFAs tested, the MaDGAT2 enzyme preferred eicosapentaenoic acid (EPA) substrate as its highly proportional constituent found in TAG fraction. This study provides a potential genetic tool for reconstituting oils rich in long-chain PUFAs with nutritional value.

1. Introduction

Microbial lipids have been considered as alternatives to the traditional animal- and plant-derived sources. Oleaginous microorganisms are potential workhorses for sustainable production of diversified lipids with economic significance. Of them, functional lipids, particularly long-chain polyunsaturated fatty acids (LC-PUFAs) are of current interest due to their proven functions with benefits on human and animal health. Some LC-PUFAs are produced by exotic oleaginous species. However, lipid constituents in the natural sources might be not meet the market demand for specific purposes, particularly in nutraceutical and pharmaceutical applications. Apart from the lipid quality, the economic feasibility of microbial oil production is a major criterion for viable applications in several industrial sectors. The lipid titer and proportion of targeted PUFAs in total lipid (TL) are key parameters that have to be taken into account for the production of PUFA-rich oils. Strain improvement is an important upstream process for the production system, which should be rationally and systematically designed to complement with microbial chassis and bioprocessing.

In oleaginous microorganisms, the lipid storage mechanism plays biological roles in cell growth and development, particularly in nutrient-limiting condition. The biosynthesis of triacylglycerol (TAG), which is a major constituent of storage lipids usually found in the special organelle, termed lipid particle (LP), is associated with obese phenotypes. Diacylglycerol acyltransferase (DGAT), which catalyzes the final reaction in TAG synthesis, has been postulated to be a rate-limiting enzyme, controlling the lipid production (Czabany et al., 2007). As such, the genes encoding DGAT enzymes from various sources have been investigated, not only to gain better understanding in lipid accumulation process, but also to obtain a potential genetic material for cell

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; DGAT, diacylglycerol acyltransferase; DGLA, dihomo-γ -linolenic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; GLA, γ-linolenic acid; GAL1, β-galactosidase; HP-TLC, high performance-thin layer chromatography; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acid; LP, lipid particle; NL, neutral lipid; OA, oleic acid; PL, phospholipid; TAG, triacylglycerol; TL, total lipid

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optimization in a selected strain (Xue et al., 2006). The DGAT enzymes are integral membrane proteins that reside in the endoplasmic reticulum (ER). They catalyze the transfer of an acyl group from acyl-coenzyme-A to the *sn*-3 position of diacylglycerol (DAG), yielding TAG. Three DGAT families have been reported, including the ER-localized DGAT1 and DGAT2, and the soluble cytosolic DGAT3. These enzymes do not share substantial sequence homology together, and thus have distinct physiological roles in lipid metabolisms of living cells (Turchetto-Zolet et al., 2011). So far, the functional role of the DGAT3 has not been clarified (Rani et al., 2010; Saha et al., 2006), whereas DGAT1 and DGAT2 are mainly responsible for the TAG biosynthesis (Turchetto-Zolet et al., 2011). However, the DGAT1 and DGAT2 have unrelated functions in TAG biosynthesis (Liu et al., 2012). It has been reported that the DGAT2 enzymes have higher affinity to substrates than the DGAT1 enzymes (Yen et al., 2008). Although the role of DGAT2 in enhancement of lipid content has been well known (Andrianov et al., 2010; Bouvier-Nave et al., 2000; Greer et al., 2015; Jako et al., 2001), reports of the enzymatic activity in terms of substrate specificity are still rare (Liu et al., 2012). In diatom *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, and microalga *Ostreococcus tauri*, the DGAT2 enzymes preferred either medium-chain or long-chain unsaturated fatty acids for esterification into storage lipids (Gong et al., 2013). Overexpression of DGAT2 with specificity for *n*-3 LC-PUFA substrate in *P. tricornutum* led to 35% increase of neutral lipid content, and 76% increase of eicosapentaenoic acid (EPA; C20:5 *n*-3) proportion in TL (Niu et al., 2013). In fungi, expression study of *Mortierella ramanniana* DGAT2 showed an enhanced activity towards medium-chain fatty acyl-CoAs, especially myristoyl-CoA (Lardizabal et al., 2001). So far, the DGAT2 enzyme specific to LC-PUFAs has not been reported in fungi even though some of them are able to synthesize and accumulate LC-PUFA as a major proportion in intracellular lipids. Certain oleaginous fungus, *Mortierella alpina* is a promising strain for production of *n-6* PUFAs having 18- and 20-carbon atoms (γ-linolenic acid; GLA, dihomo-γ-linolenic acid; DGLA and arachidonic acid; ARA) (Jang et al., 2005; Sakuradani et al., 2013), referring that it might contain either potent genes or regulatory mechanisms specially involved in lipid over-production.

This study aims to identify a gene coding for DGAT2 with preference on LC-PUFA substrates, and thus the LC-PUFA producing strain as a genetic source is of our target. Using available genome data, we identified and functionally characterized the putative *DGAT2* gene of *M. alpina* by heterologous expression in the mutant strain of *Saccharomyces cerevisiae*, which is unable to synthesize neutral lipids. Its substrate specificity and preference were also investigated.

2. Materials and methods

2.1. Strains and cultivations

H1246 mutant strain of *S. cerevisiae* defective in TAG and sterol biosyntheses (QM) (*MATα*, *are1-Δ::HIS3*, *are2-Δ::LEU2*, *dga1-Δ::KanMX4*, *lro1-Δ::TRP1*, *ADE2*), which acyl-CoA:diacylglycerol acyltransferase (*DGA1*), lecithin:cholesterol acyltransferase (*LRO1*), acyl-CoA:sterol acyltransferases (*ARE1* and *ARE2*) genes were disrupted (Sandager et al., 2002), was used as a recipient in this work. The H1246 strain was previously generated from the parental strain SCY62 (*MATα*, *ADE2*) (Thomas and Rothstein, 1989). Yeast cells were grown in a complete medium, YPD (1% (w/v) bacto-yeast extract, 2% (w/v) bacto-peptone and 2% (w/v) glucose) at 30 °C. For transformant selection, a minimal medium lacking uracil (SD), which consisted of 0.67% (w/v) yeast nitrogenous base without amino acid and 2% (w/v) glucose, was used, and appropriate amino acids were added according to complete supplement mixture of Bio 101, Inc.

Escherichia coli strain DH5α (*supE*44, *ΔlacU*169, (Φ80, *lacZΔ*M15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*1, *relA*1) was used for plasmid propagation. It was grown in Luria-Bertani medium (LB) containing 100 mg/l of ampicillin at 37 °C with shaking at 200 rpm.

2.2. Characterization of the putative DGAT2 sequence of the ARA-producing fungus

Using the DGAT type 2 B of *M. ramanniana* var. *angulispora* sequence (GenBank accession no. AF391090) as a query, putative *DGAT2* gene sequences of the ARA-producing strain, *M. alpina* ATCC 32222, were retrieved from Whole Genome Shotgun Contigs (WGS) database (http://www.ncbi.nlm.nih.gov/) by BlastN search (https://blast.ncbi.nlm.nih.gov/Blast.cgi/). The conserved amino acid sequences of DGAT2 enzymes were characterized using ClustalW alignments. Transmembrane domains were predicted by the TMHMM algorithm (Krough et al., 2001) and Phobius (Kall et al., 2004). A phylogenetic tree of the targeted sequences and others belonging to DGAT family was generated by the neighbor-joining method using MEGA6 software (Tamura et al., 2013). The rate of replication was calculated from bootstrap tests (1000 replicates).

2.3. Gene synthesis and codon optimization of the putative DGAT2 of M. alpina

Using a service of Genscript (Piscataway, U.S.A.), the DNA fragments coding for native (MaDGAT2) and codon-optimized (mMaDGAT2) enzymes of *M. alpina* were synthesized, which were ligated into the pUC57 plasmid, generating pUC-mMaDGAT2 and pUC-MaDGAT2, respectively. The coding sequence of the targeted gene was optimized based on the general rule of RNA stability and the codon usage of the fungal system using the OptimumGene™ algorithm. The sequences of *MaDGAT2* and *mMaDGAT2* have been deposited in GenBank and assigned the accession no. of KY828121 and KY859195, respectively.

2.4. Heterologous expression of the M. alpina DGAT2 gene in S. cerevisiae mutant

Full-length cDNA fragments of *MaDGAT2* and *mMaDGAT*2 were amplified by PCR using pUC-MaDGAT2 and pUC-mMaDGAT2 as templates, respectively. A high fidelity *Taq* polymerase (Invitrogen, CA) was used for the amplification. Oligonucleotide primers for PCR contained appropriate restriction sites (underlined letters) to facilitate subsequent cloning (Table 1). The amplified products were subcloned into pYES2 expression vector (Invitrogen, CA) downstream of the *β*-galactosidase (*GAL1*) promoter, generating PyMaDGAT2 and PymMaDGAT2 plasmids and then sequenced. The constructed plasmids and the empty vector (pYES2) were individually transformed to the *S. cerevisiae* strain H1246 by the PEG/lithium acetate method following the manufacturer's protocol (Invitrogen). Transformed cells were selected on a uracil-deficient agar medium. Selected yeast transformants were grown in SD broth containing 2% (w/v) raffinose at 30 °C for 48 h. Subsequently, the gene expression was induced by addition of 2% (w/v) galactose into the cultures. Cells were harvested by centrifugation, washed twice with 0.1% (v/v) TritonX 100 solution, dried and then used for lipid analysis.

Table 1
Oligonucleotide primers used for amplifying the native (*MaDGAT2*) and codon-optimized (*mMaDGAT2*) genes.

Primer name	Oligonucleotide sequence (5′-3′)	Strand[a]
MaDGAT2-*Hind*III	CCCAAGCTTATGGCCATCTTTGCCCCCA	+
MaDGAT2-*Kpn*I	GGGGTACCCTATTCAATGATCTGGAGC	−
mMaDGAT2-*Bam*HI	CGCGGATCCATGGCAATTTTCGCGCCTA	+
mMaDGAT2-*Xba*I	GCTCTAGATCATTCGATAATTTGCAGC	−

[a] Sense and anti-sense strands are denoted by plus (+) and minus (−) symbols, respectively.