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Metabolic engineering of long chain-polyunsaturated fatty acid biosynthetic pathway in oleaginous fungus for dihomo-gamma linolenic acid production

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ABSTRACT

Microbial lipids are promising alternative sources of long chain-polyunsaturated fatty acids (LC-PUFAs) for food, feed, nutraceutical and pharmaceutical sectors. Dihomo-γ-linolenic acid (C20:3$\Delta^{8,11,14}$; DGLA) is an important LC-PUFAs with anti-inflammatory and anti-proliferative effects. To generate a DGLA-producing strain, fatty acid reconstitution in *Aspergillus oryzae* was performed by metabolic engineering through co-expression of codon-optimized *Pythium* Δ^6-desaturase and Δ^6-elongase, which had high conversion rates of substrates to respective products as compared to the native enzymes. The Δ^6-desaturated and Δ^6-elongated products, γ-linolenic acid (C18:3$\Delta^{6,9,12}$; GLA) and DGLA, were accumulated in phospholipids rather than triacylglycerol. Interestingly, the manipulation of lipid quality in the oleaginous fungus did not affect growth and lipid phenotypes. This strategy might expand to development of the oleaginous fungal strain for producing other tailor-made oils with industrial applications.

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1. Introduction

Microbial lipids or single cell oils are thought to be promising alternatives for producing specialty and commodity oils. As such, there is a considerable interest in production of a variety of lipids with different properties via biological processes. LC-PUFAs, which are mostly derived from microorganisms, have been recognized as nutritionally important lipids with applications for food and feed industries. The 20-carbon *n*-6 PUFA, DGLA, is one of the most interesting PUFAs. Several studies have investigated the relationship between DGLA intake and health maintenance. Such an example given is anti-inflammatory effect due to its role as a direct precursor for biosynthesis of prostaglandin E1 (PGE1). This hormone-like eicosanoid compound also has anti-proliferative effects by inhibition of growth and differentiation of cancer cells (Wang et al., 2012). As a consequence, this fatty acid has been regarded as dietary supplement for treatment and prevention of proliferation and hyperplasia diseases. Furthermore, there has been a report of the safety of consumption of DGLA without adverse effect (Umeda-Sawada et al., 2006).

A set of membrane-bound enzymes, desaturases and elongases, is involved in DGLA biosynthesis (Fig. 1). The common monoene fatty acids found in eukaryotic microorganisms are palmitoleic acid (C16:1Δ^9; PA) and oleic acid (C18:1Δ^9; OA), which are the products catalyzed by Δ^9-desaturase enzyme. OA is further desaturated, in general, by Δ^{12}-desaturase yielding linoleic acid (C18:2$\Delta^{9,12}$; LA), which is a basic precursor for further formation of LC-PUFAs in *n*-3 and *n*-6 series. For DGLA synthesis, LA is desaturated by Δ^6-desaturase followed by elongation of the acyl chain as the function of Δ^6-elongase. Occurrence of DGLA is rare since it is an intermediate in biosynthesis of highly unsaturated fatty acids. The development of DGLA production has paid to certain fungi and algae, which are capable of the production of arachidonic acid (C20:4$\Delta^{5,8,11,14}$; ARA) through several approaches. Two main

Abbreviations: LC-PUFAs, long chain-polyunsaturated fatty acids; DGLA, dihomo-γ-linolenic acid; GLA, γ-linolenic acid; PGE1, prostaglandin E1; PA, palmitoleic acid; OA, oleic acid; LA, linoleic acid; ARA, arachidonic acid; *GAL1*, β-galactosidase promoter; PEG, polyethylene glycol; ALA, α-linolenic acid; STA, stearidonic acid; EPA, eicosapentaenoic acid; STC, sorbitol: Tris–HCl: $CaCl_2$ solution; TL, total lipids; TLC, thin-layer chromatography; NL, neutral lipids; FAMEs, fatty acid methyl esters; ETA, eicosatetraenoic acid; GFP, green fluorescent protein; *toxA*, *Pyrenophora tritici-repentis* necrosis toxon; *nos3*, *Agrobacterium tumefaciens* nopaline synthase; *DsRedExp*, *Discosome* sp. red expressed fluorescent protein; *gpdA*, *Aspergillus nidulans* glyceraldehyde-3-phosphate dehydrogenase promoter; TA, Gtriacylglycerol; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PL, phospholipids; DGAT, diacylglycerol acyltransferase.

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strategies have been implicated for enhancing the DGLA yield, including addition of enzyme inhibitors and chemical mutation targeted at Δ^5-desaturase reaction (Jareonkitmongkol et al., 1993). However, the drawbacks of such approaches have been reported (Kawashima et al., 2000; Abu-Ghosh et al., 2015). Addition of Δ^5-desaturase inhibitors might affect the microbial growth and cultivation process. Screening of Δ^5-desaturase defective mutants is laborious and time-consuming work, and the co-product (ARA) is still obtained. With the advance in modern technologies, engineering of target metabolic pathways is being used not only for PUFA production, but also for production development of other commodity and specialty oils (Liang and Jiang, 2013). To construct a DGLA-producing strain, the potent genetic materials and host system are required. Genes of choice are dependent on fatty acid substrates available in a host organism. For the *n*-6 pathway, two genes are required for DGLA biosynthesis including Δ^6-desaturase and Δ^6-elongase. However, the coding enzymes of various organisms have broad specificities on fatty acid substrates (Cinti et al., 1992 Zhang et al., 2004). Thus, the genes coding enzymes with high specificities on endogenous fatty acid substrates of a selected host system are of particular interest to eliminate undesired side-fatty acid products. Furthermore, several criteria of host strain should be taken into account for DGLA production. Apart from efficient expression system, other phenotypes should be considered, including growth rate, nutrient utilization and tolerance to stress conditions. In addition, lipid titer and productivity are important to bring down production expenditures. Among microorganisms, the oleaginous strains have potential in storage of intracellular lipids at high amounts in form of lipid droplets or lipid particles. The filamentous fungus, *Aspergillus oryzae*, renders such beneficial performances and thus has been categorized as oleaginous strain based on its obese feature (57% of lipids in dry biomass) (Vicente et al., 2010) and the existence of oleaginous gene sequences (Vorapreeda et al., 2013). With regard to endogenous fatty acids of *A. oryzae*, the diene fatty acid (LA) is predominant, which is a precursor for DGLA production.

Very recently, we cloned Δ^6-desaturase and Δ^6-elongase genes from *Pythium* sp. BCC53698 (Jeennor et al., 2014, 2015). Heterologous expression of the codon-optimized Δ^6-desaturase in *Saccharomyces cerevisiae* showed that its conversion rate of LA substrate was higher than that of the native Δ^6-desaturase of *Pythium* sp. (GenBank accession no. KM609327). For the native Δ^6-elongase (GenBank accession no. KJ546459), it displayed a very high specificity on 18-carbon PUFAs containing Δ^6-double bond in their acyl chains. In this study, we used *A. oryzae* as a platform organism for production of the high-value lipid by engineering the fatty acid biosynthetic pathway. The codon optimization of *Pythium* Δ^6-elongase gene was carried out and the enzymatic function was assessed by heterologous expression in *S. cerevisiae*. Co-expression of the codon-optimized Δ^6-desaturase and Δ^6-elongase genes of *Pythium* sp. in *A. oryzae* was performed. Thus, comparative phenotypic analysis of cell growth, fatty acids and lipids of the wild-type and engineered strains of *A. oryzae* was performed.

2. Materials and methods

2.1. Microorganisms and cultivations

A. oryzae strain BCC14614, which was obtained from BIOTEC Culture Collection (BCC), Thailand, was cultivated on PDA (BD, USA) agar at 25 °C under normal light for 5–7 days. Spore inoculum was prepared by growing the fungal culture on polished rice at 25 °C. Then, spores were harvested by using 0.01% (v/v) Tween 80 to the culture and filtered through Miracloth (Merck, Germany). To cultivate the fungal strains of *A. oryzae* in broth medium, the spore suspension at the final concentration of 10^6 spores/ml was added to 50 ml of semi-synthetic medium, containing 4% glucose and 0.5% yeast extract. The fungal cultures were cultivated by shaking at 150 rpm, 22 °C.

S. cerevisiae strain DBY746 (*MAT*α, *his*3-Δ1, *leu*2-3, *leu*2-112, *ura*3-52, *trp*1-289), was used for studying substrate utilization of the codon-optimized Δ^6-elongase of *Pythium* sp. The yeast was grown either in a complete medium, YPD (1% bacto-yeast extract, 2% bacto-peptone and 2% glucose) or a selective medium, SD (0.67% bacto-yeast nitrogen base without amino acids and 2% glucose). Appropriate amino acids, L-tryptophane, L-histidine-HCl and L-leucine, were added to SD medium at concentrations of 20, 20 and 30 mg/l, respectively. For DNA assembly, *S. cerevisiae* strain INVSCI (*MAT*α, *his*3-Δ1, *leu2, trp1-289, ura3-52, MAT*, *his*3-Δ1, *leu2, trp1-289, ura3-52*) was used. The yeast cultures were incubated at 30 °C with shaking at 200 rpm.

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 as a recipient for propagation of recombinant plasmids. *E. coli* transformant was grown in Luria–Bertani medium (LB) containing 100 mg/l of ampicillin at 37 °C with shaking at 200 rpm.

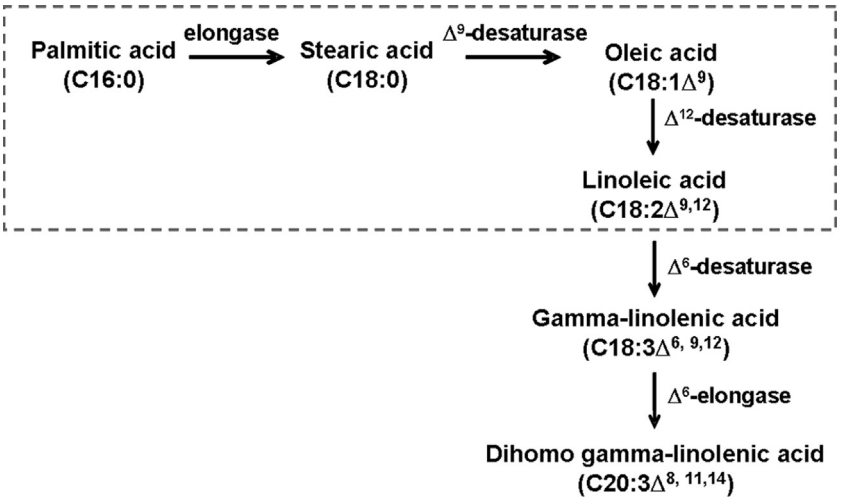

Fig. 1. Biosynthetic pathway of unsaturated fatty acids**.** Fatty acid composition of the wild type of *A. oryzae* is boxed. DGLA formation in the engineered strain of *A. oryzae* carrying the plasmid pAMDes6-MElo6 is shown.

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