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Heterologous production of free dihomo-γ-linolenic acid by *Aspergillus oryzae* and its extracellular release via surfactant supplementation

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Free dihomo-γ-linolenic acid (DGLA) and its desaturated form, free arachidonic acid (ARA) are polyunsaturated free fatty acids (FFAs). They are useful raw materials to produce eicosanoid pharmaceuticals. In this study, we aimed at their production by the oleaginous filamentous fungus *Aspergillus oryzae* via metabolic engineering. Three genes encoding enzymes involved in the synthesis of DGLA and ARA, were isolated from the filamentous fungus *Mortierella alpina* that produces ARA in a triacylglycerol form. These genes were concatenated to promoters and terminators of highly expressed genes of *A. oryzae*, and the concatenated DNA fragments were further concatenated with each other to generate a single DNA fragment in the form of a biosynthetic gene cluster. By homologous recombination, the resulting DNA fragment was integrated to the chromosome of the *A. oryzae* acyl-CoA synthetase gene disruptant whose FFA productivity was enhanced at 9.2-fold more than the wild-type strain. The DNA-integrated disruptant produced free DGLA but did not produce free ARA. Thus, focusing on free DGLA, after removal of the gene for converting DGLA to ARA, the constructed strain produced free DGLA at 145 mg/l for 5 d. Also, by supplementing Triton X-100 surfactant at 1% to the culture, over 80% of free DGLA was released from cells without inhibiting the growth. Consequently, the constructed strain will be useful for attempting production of free DGLA-derived eicosanoids because it bypasses excision of free DGLA from triacylglycerols by lipase. To our knowledge, this is the first report on microbial production of free DGLA and its extracellular release.

[**Key words:** Free dihomo-γ-linolenic acid; *Aspergillus oryzae*; Metabolic engineering; Surfactant; Extracellular release]

Several microbial fatty acids are useful as pharmaceuticals, dietary supplements, and their raw materials. Specially, polyunsaturated fatty acids such as eicosanoids, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) have various bioactivities, and some of them have been utilized as pharmaceuticals and dietary supplements. For example, the eicosanoid prostaglandin E1 inhibits platelet aggregation and vasodilation, and has thus been used as a pharmaceutical in patients of ischemic or myocardial reperfusion injury (1). Prostaglandin E1 is synthesized *in vivo* from free dihomo-γ-linolenic acid (C20:3$\Delta^{8,11,14}$; DGLA) by a cyclooxygenase (2). Another eicosanoid, prostaglandin F2α, and its derivatives are utilized as pharmaceuticals in glaucoma (3). Prostaglandin F2α is synthesized from free arachidonic acid (C20:4$\Delta^{5,8,11,14}$; ARA) by the coordinated reactions of cyclooxygenase and prostaglandin F2α synthase (4). Regarding DHA and EPA, they might have benefits in normal brain aging, and are thus used as dietary supplements (5).

Microbial production of DGLA and ARA has been studied in filamentous fungi because these are useful as both dietary supplements and as raw materials of pharmaceuticals (6,7). DGLA is a precursor of ARA, and conversion of DGLA to ARA is catalyzed by Δ5-desaturase. With regard to ARA, the filamentous fungus *Mortierella alpina* was isolated as a high-producer of ARA, and has been used for ARA production in the fermentation industry (8). As for DGLA, its production has been studied in this *M. alpina* strain by screening of Δ5-desaturase gene mutants from a natural mutant library (9) or by knocking out the gene using genetic engineering techniques (10). Moreover, in the filamentous fungus, *Aspergillus oryzae*, DGLA production has been studied by heterologous expression of both Δ6-desaturase and elongase genes derived from *Pythium* sp. (11). However, these studies on fatty acid production involved their accumulation in the cytosol in the form of triacylglycerol (TG). From this form, free DGLA and free ARA molecules must be generated by excising fatty acid side chains of the TG using lipase, before their conversion to eicosanoids. Therefore, if ARA and DGLA were to be produced in an FFA form using metabolically engineered microorganisms, eicosanoids could be produced more efficiently.

A. oryzae is safe and industrially relevant because of non-toxin-production and high innate enzyme productivity. *A. oryzae* is also one of the oleaginous fungi containing lipids at around 60% of dry

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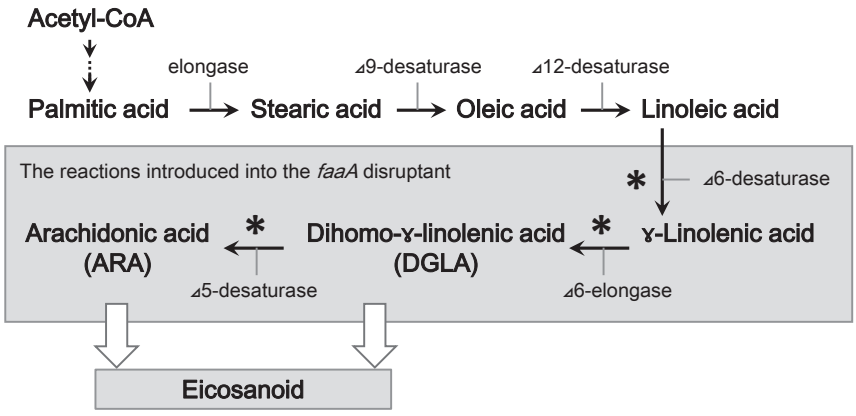


FIG. 1. Schematic illustration of free DGLA and free ARA production leading to eicosanoid production in *A. oryzae*. Three genes marked by asterisk from *M. alpina* NBRC8568 were introduced into the *A. oryzae ΔfaaA* strain whose free fatty acid productivity was increased by 9.2 fold compared to that by the wild-type strain.

cell weight (12). Here, we studied increasing production of FFAs in *A. oryzae* by metabolic engineering. By disrupting the *faaA* gene encoding acyl-CoA synthetase, FFA productivity was increased by 9.2 fold (0.54 mmol/g of dried hyphae) compared to that by the parental wild-type control strain (13), and 2.7 g FFAs was produced per liter of culture over 120 h. These FFAs were confirmed to include palmitic acid (C16:0; PA), stearic acid (C18:0; SA), oleic acid (C18:1Δ^9; OA), and linoleic acid (C18:2$\Delta^{9,12}$; LA). Among them, LA is the most polyunsaturated form, with two double carbon–carbon bonds. These FFAs can also be released with high efficiency from the cells of *A. oryzae* to the culture medium by supplementation with a non-ionic surfactant (14). The aforementioned results have shown that *A. oryzae* is a highly productive microorganism of FFA.

If genes involved in the conversion of LA to DGLA and ARA were expressed in the *A. oryzae faaA* disruptant, DGLA and ARA could be produced in an FFA form. Based on this expectation, three enzyme genes involved in the synthesis of DGLA and ARA were isolated from an *M. alpina* strain, and introduced into the *faaA* disruptant for heterologous expression, resulting in production of free DGLA, but no free ARA. Thus, free DGLA production and its extracellular release were studied.

MATERIALS AND METHODS

Fungal strains and culture media We used an *A. oryzae* wild-type RIB40 strain distributed by the National Research Institute of Brewing (NRIB, Hiroshima, Japan) and its derivative *ΔfaaA* strain lacking *pyrG* (RIB40 *ΔligD::ptrA ΔniaD::niaD of Aspergillus nidulans ΔpyrG::sC* of *A. nidulans ΔfaaA*) that was constructed in our previous study (15). Each strain was maintained on Czapek–Dox (CD) agar medium with and without 5 mM uridine and 10 mM uracil at 30°C. The CD agar medium contained 2% glucose (16), whereas the glucose concentration was increased to 10% in the liquid medium. Cultures were prepared by inoculating 2.5×10^7 spores of each *A. oryzae* strain to 50 mL of CD liquid medium with or without Triton X-100 in a 250-mL flask, followed by incubation at 30°C, with shaking at 200 rpm for 120 h. The *A. nidulans* A851 strain was obtained from the Fungal Genetics Stock Center (FGSC) (Kansas City, MO, USA), and used to amplify *pyrG* as a selection marker. The A851 strain was maintained on potato dextrose agar medium at 30°C. The *M. alpina* NBRC8568 strain was distributed by the National Institute of Technology and Evaluation (NITE, Chiba, Japan). The NBRC8568 strain was maintained on CD agar medium containing 10 mM sodium glutamate at 25°C. In order to extract total RNA for preparing cDNA of Δ6-desaturase, Δ6-elongase, and Δ5-desaturase genes involved in DGLA and ARA synthesis, the NBRC8568 strain was cultured at 25°C at 200 rpm for 96 h in 200 mL of ARA-producing liquid medium (glucose 20 g, yeast extract 5 g, KNO_3 10 g, KH_2PO_4 1 g, $MgSO_4 \cdot 7H_2O$ 0.25 g, pH 6.5) (17).

Construction of the *A. oryzae faaA* disruptant expressing *M. alpina* Δ6-desaturase, Δ6-elongase, and Δ5-desaturase genes The metabolic pathway of FFA synthesis in *A. oryzae*, and the plausible usage of the constructed strain after introduction of the genes involved in the synthesis of free DGLA and free ARA are illustrated in Fig. 1. Because the coding sequences of the genes to be introduced were unknown in the NBRC8568 strain, their cDNA fragments were amplified. Total RNA was extracted from NBRC8568 cells, and then total cDNA was synthesized from total RNA using PrimeScript II 1st strand cDNA Synthesis Kit (Takara, Shiga, Japan). All the primers used in this study are listed in Table S1. The cDNA fragments encoding Δ6-desaturase, Δ6-elongase, and Δ5-desaturase were amplified from total cDNA by KOD-PLUS using the test_fwd and test_rev primers that were designed to be most common among the identified sequences of *Mortierella* sp. registered at the NCBI database. The promoters and terminators of *tef1*, *enoA*, and *pgkA* genes that are constitutively highly expressed in *A. oryzae* (18–20) were used for expression of these three genes. The DNA fragments of promoters and terminators of *tef1*, *enoA*, and *pgkA*; *pyrG* selection marker; and Cre/mutant *lox* marker-recycling gene unit; were amplified by KOD-PLUS using the RIB40 chromosomal DNA; the A851 chromosomal DNA; and pAAAXG-Cre (21) as templates, respectively. The amplified DNA fragments were purified by gel extraction using Wizard SV gel and PCR Clean-Up system (Promega, Madison, WI, USA). Two or three purified DNA fragments were then concatenated and cloned to *Bam*HI-digested pUC19 using the In-Fusion HD cloning kit (Takara) (Figs. S1A–S1D). Alternatively, DNA fragments of the front side and rear side of the *sC* promoter were subjected to phosphorylation of their blunt ends, and the phosphorylated DNA fragment was then ligated with dephosphorylated *Sma*I-digested pUC19 using a DNA ligation kit <Mighty Mix> (Takara) (Figs. S1E and S1F). The In-Fusion reaction mixture or ligation reaction mixture was used for transformation of *Escherichia coli* Stellar competent cells (Takara), followed by a clone check with PCR. Positive clones were subjected to sequence check by the Sanger sequencing.

Concatenation of respective DNA fragments cloned into pUC19 was performed with the OGAB method (22) using *Bacillus subtilis*. A 15.1 kb long DNA fragment named ARA1 (Fig. S2) was constructed with the six cloned DNA fragments concatenated consecutively and cloned into the pGETS118-t0-AarI plasmid (23) using the OGAB method.

The resulting pGETS118-t0-AarI-ARA1 was extracted from *B. subtilis*, and transferred into *E. coli* DH5α cells by transformation. The plasmid was prepared a lot from the *E. coli* cells by a large scale culture, and then digested with *Pme*I and *Swa*I to generate the ARA1 DNA fragment. The ARA1 fragment was separated from other DNA fragments by 0.4% agarose gel electrophoresis (Fig. S3A), followed by gel extraction using Zymoclean Large Fragment DNA Recovery Kit (Zymo Research, Irvine, CA, USA). One microgram of the gel-extracted ARA1 fragment (Fig. S3B) was used for transformation of the *A. oryzae faaA* disruptant. Transformation was performed as described (16). Transformants were selected on CD agar, and were confirmed by PCR with primers cU (Ao_sC) and cL (Ao_sC), and by Southern hybridization.

Construction of the *A. oryzae faaA* disruptant expressing *M. alpina* Δ6-desaturase and Δ6-elongase genes Firstly, the *pyrG* selection marker was removed by inducing the Cre/mutant *lox* system in the ARA1-introduced *faaA* disruptant. The strain was cultured on CD agar containing 2% xylose as the carbon source. In the presence of xylose, Cre protein expression is induced by the promoter of an *A. oryzae* xylanase gene *xynG2* and the terminator of an *A. oryzae* alpha-glucosidase gene *agdA* (21). The spores formed were then transferred to CD agar containing 2% xylose, 5 mM uridine, 10 mM uracil, and 1 mg/mL 5-fluoroorotic acid (5-FOA) for screening of the *pyrG*-defective strain. The ARA1-introduced *faaA* disruptant lacking *pyrG* was confirmed by PCR with primers AosC_5UTR_cU and Ptef_rev (Fig. S4).

Next, to remove the expression unit of Δ5-desaturase from the ARA1-introduced *faaA* disruptant lacking *pyrG*, the DNA fragment for removal (Fig. S5) was constructed by fusion PCR. The ARA1-introduced *faaA* disruptant lacking *pyrG* was transformed with the constructed DNA fragment. After single spore isolation, the Δ5-desaturase-defective ARA1-introduced *faaA* disruptant was confirmed by PCR with primers Teno_fwd and AosC_5UTR_rev2, and by Southern hybridization. The constructed strain was named DGLA1 that was a *faaA* disruptant expressing the *M. alpina* Δ6-desaturase and Δ6-elongase genes.

Phylogenetic analyses Phylogenetic analyses were performed as described for the ketosynthase domains of predicted polyketide synthases (24). The amino acid sequences of the Δ6-desaturase and Δ5-desaturase derived from *M. alpina* strains were aligned using Clustal W. The genealogy was inferred by maximum-likelihood (ML) analyses using MEGA software package version 6 (25). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). In addition, a more robust approach was used to infer phylogenetic relationships by performing a randomized bootstrap ML analysis using MEGA version 6, setting the bootstrap analysis to 1000 runs. The Dayhoff mutation data matrix was used to analyze the alignment. The ML trees were obtained using the Nearest-Neighbor-Interchange algorithm, in which the initial trees were obtained automatically.

Southern hybridization Aliquots (3 μg) of genomic DNA were digested with each restriction enzyme (*Hind*III and *Nco*I), fractionated on 0.6% agarose gel, and transferred onto a Nitran SuperCharge membrane (GE Healthcare, Piscataway, NJ, USA). Hybridization and signal detection were performed with a digoxigenin (DIG) system according to the manufacturer's instructions (GE Healthcare). Briefly, DIG-labeled probes (approximately 500–800 bases long) were prepared using a PCR DIG Probe Synthesis Kit (Roche Applied Science, Mannheim, Germany) using primers SBU and SBL (Table S1), followed by agarose gel extraction. The probes were hybridized to the membrane-bound DNA fragments, and chemiluminescence signals were detected from the anti-DIG Fab-fragment-alkaline phosphatase conjugate (Roche Applied Science) using the CDP-*Star* Detection Reagent (GE Healthcare).

Composition analysis of intracellular and extracellular FFAs To analyze the composition of intracellular FFAs, a 50 mg sample of lyophilized *A. oryzae* hyphae was transferred to a 2 mL screw-cap microtube, and 300 mg of 0.5 mm glass beads (GB-05, TOMY, Tokyo, Japan), 500 μL of Milli-Q water, and 500 μL of chloroform were added. The hyphae were disrupted using the MS-100 (TOMY) at 5500 rpm for 60 s. To analyze the composition of extracellular FFAs, 5 mL of *A. oryzae* culture supernatant was transferred to a 50 mL conical tube. After 5 mL of chloroform was added, the mixture was agitated by vigorous shaking. After centrifugation at 14,000 ×*g* (for 2 mL microtube) or 3000 ×*g* (for 50 mL conical tube) at room temperature for 10 min, a hole was made in the bottom of the tube with a pin, followed by recovery of the chloroform fraction into a 1.5 mL microtube. A 150 μL aliquot of the chloroform fraction was transferred to a 10 mL screw-cap glass centrifuge tube. The transferred chloroform fractions containing the intracellular lipids were evaporated with a centrifugal vacuum concentrator. The resulting precipitates were dissolved in 300 μL of ethanol. The FFA molecules in each lipid sample were specifically labeled with 2-nitrophenylhydrazine at the carboxyl groups using a free fatty acid analysis kit from YMC (Kyoto, Japan). After the reaction was completed, the labeled FFAs were extracted into 5 mL of hexane and the hexane fraction was evaporated with a centrifugal vacuum concentrator. The labeled FFA precipitates were dissolved in 300 μL of methanol and stored at 4°C prior to measurement by high performance liquid chromatography (HPLC) using Chromaster (Hitachi, Ibaraki, Japan). Similarly to aforementioned samples, 100 μL of each FFA standard solution at 3 stepwise concentrations (1.67, 3.33, and 5 mM) dissolved in ethanol was used to construct standard curves with linear regression. Six FFA standards [PA, SA, OA, LA, γ-linolenic acid (C18:3$\Delta^{6,9,12}$; GLA), and DGLA] were used. The labeling reaction and successive treatments were performed by the same protocol as those for sample preparation. Ten microliters of each labeled FFA sample or standard dissolved in methanol was applied to HPLC equipped with a YMC-Pack FA column (YMC; 0.46 × 25 cm) using an acetonitrile-water (85:15) mixture as the mobile phase, at a flow rate of 0.7 mL/min and a column temperature of 35°C. The labeled FFAs were detected by monitoring absorbance at 400 nm. The FFA composition and quantification of each sample were calculated based on the peak area using the respective labeled FFA standard curves.

Composition analysis of fatty acids contained in intracellular TG Sample preparation and subsequent composition analysis using HPLC were basically performed by the same protocol as that of intracellular FFA. One exception is that an ester-type fatty acid analysis kit (YMC) was used instead of a free fatty acid analysis kit. Because the ester-type kit assays both fatty acids derived from FFA and TG, fatty acid amount in TG was calculated by subtracting the FFA-derived amount that was assayed using a free fatty acid analysis kit.

Quantitative reverse transcription polymerase chain reaction To determine transcript levels using quantitative reverse transcription polymerase chain reaction (qRT-PCR), the ARA1-introduced *faaA* disruptant was cultured at 30°C at 200 rpm for 48 h in the same inoculum and culture medium as the case of FFA composition analysis. RNA extraction and the subsequent qRT-PCR were performed as described previously (15).

RESULTS

Isolation of the genes for Δ6-desaturase, Δ6-elongase, and Δ5-desaturase from *M. alpina* NBRC8568 The NBRC8568 strain cultured in the ARA producing condition was confirmed to produce ARA in a TG form in our preliminary experiment. However, the genomic sequence is unknown, and thus coding sequences of the genes of Δ6-desaturase, Δ6-elongase, and Δ5-desaturase of NBRC8568 were attempted to be isolated. They were predicted to be 1–1.5 kb with reference to those identified in other *Mortierella* sp. strains and registered at the NCBI database. Primers for amplifying their coding sequences from NBRC8568 were designed by selecting the most conserved nucleotides at the 18–20 bp translation initiation and termination sites. Using the primers, DNA fragments were amplified specifically from the total cDNA of NBRC8568 at the predicted length of each gene (Fig. 2A). Thus, DNA fragments of the coding sequence for these three genes were considered to be obtained successfully from NBRC8568. They were cloned into pUC19 to compose expression units, and three positive clones for each gene were sequenced to determine their coding sequence. The DNA sequences showed high homology with the identified coding sequences of these enzyme genes in other *Mortierella* strains (26–28). Thus, the DNA fragments amplified from NBRC8568 and cloned into pUC19 for expression in *A. oryzae* were confirmed to encode Δ6-desaturase, Δ6-elongase, and Δ5-desaturase. The coding sequences confirmed are denoted in Figs. S6A–S6C. Based on these sequences, phylogenetic analysis was done for Δ6-desaturase (Fig. 2B) and Δ5-desaturase (Fig. 2C). As shown in Fig. 2B, the NBRC8568 Δ6-desaturase gene used in this study corresponds to the *des6I* gene of *M. alpina* 1S-4.

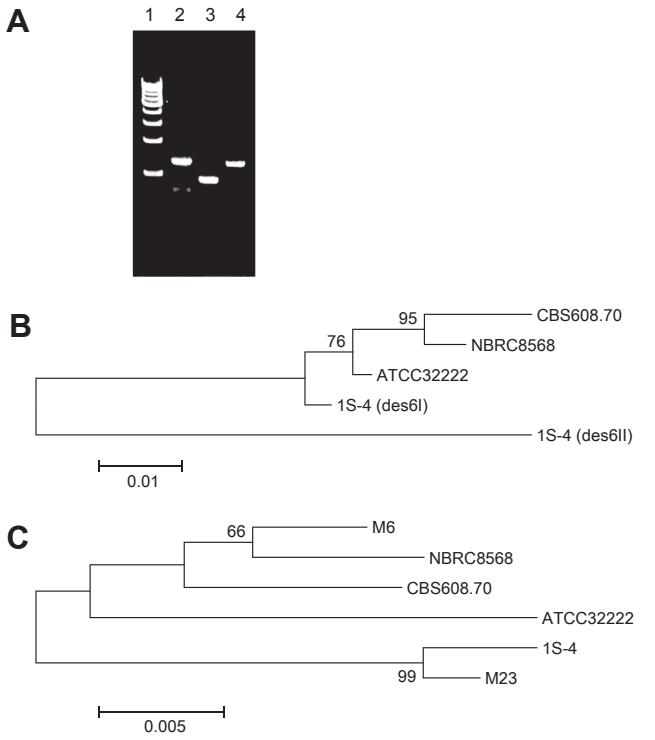


FIG. 2. The Δ6-desaturase, Δ6-elongase, and Δ5-desaturase genes of *M. alpina* NBRC8568. cDNA of each gene is amplified using the primers designed to be most common among these *M. alpina* strains (A). Genealogy of Δ6-desaturase (B) and Δ5-desaturase (C), inferred by maximum likelihood analysis of the whole protein region of several *M. alpina* strains is shown. The derivation of each gene is written at the right side of each tree. The 1S-4 strain has two Δ6-desaturase genes, des6I and des6II, and thus they are distinguished to denote the name (B). Numbers close to the branches indicate the percentage bootstrap support for each clade (numbers below 50% are not shown).

Investigation into possibility of heterologous polyunsaturated FFA production in *A. oryzae* It was unknown what varieties of polyunsaturated fatty acids could be synthesized in an FFA form in *A. oryzae*. Thus, to investigate the

possibility, the Δ6-desaturase, Δ6-elongase, and Δ5-desaturase genes isolated from *M. alpina* were heterologously expressed at one time *in A. oryzae* using the long DNA construction technology named OGAB (22). As described in Materials and methods, the ARA1 DNA fragment harboring expression units for these three genes, a *pyrG* selection marker, and a Cre/mutant *lox* gene unit, was constructed using the OGAB technology. Then, it was introduced into the *A. oryzae faaA* disruptant by transformation, and the *faaA* disruptant expressing these three genes were constructed. By Southern hybridization, the ARA1 DNA was confirmed to be site-specifically integrated to only the *sC* locus on the *A. oryzae faaA* disruptant's chromosome by homologous recombination (Fig. 3A and C). The *sC* locus was chosen for the integration site because the *sC* was expressed constitutively high in the wild-type strain as well as was lacking the function by a point mutation in the *faaA* disruptant.

The fatty acid composition of FFA and TG of the ARA1-introduced *faaA* disruptant was then assayed (Table 1). After culture in CD liquid medium for 120 h, the intracellular lipids containing FFA and TG were extracted. They were selectively labeled, and applied to HPLC as described in Materials and methods. The resulting chromatogram of FFA is shown in Fig. 4. Consequently, free DGLA and free GLA were produced, whereas free ARA was not produced. Similarly, the fatty acid composition of FFA and TG of RIB40 was assayed (Table 1). As a result, ARA was also not contained in TG of the ARA1-introduced *faaA* disruptant. In RIB40, only four fatty acids (PA, SA, OA, and LA) were observed as FFA and TG, and content ratio of FFA in total was 9.5%. On the other hand, the ratio increased to 72.4% in the ARA1-introduced *faaA* disruptant, probably due to increase of FFA productivity by the *faaA* disruption. Commonly to both strains, saturated fatty acids, PA and SA, existed most and second most, respectively. They were more than 60% in total fatty acids of FFA and TG.

Free DGLA production by the *faaA* disruptant expressing Δ6-desaturase and Δ6-elongase genes Because free DGLA production was confirmed possible in *A. oryzae*, it was focused next. Free DGLA is a precursor of the 1-series of prostaglandins such as prostaglandin E1. Therefore, similar to free ARA, it is expected to be used as a raw material for synthesizing eicosanoid pharmaceutical agents by microorganisms. The strain producing free DGLA as the final product in FFA synthesis was attempted to be constructed. For the construction, the expression unit for the Δ5-desaturase gene was removed from the ARA1-introduced *faaA* disruptant as described in Materials and methods. Resultantly, the DGLA1 strain was constructed. The construction was confirmed by Southern hybridization, and the positive clone was

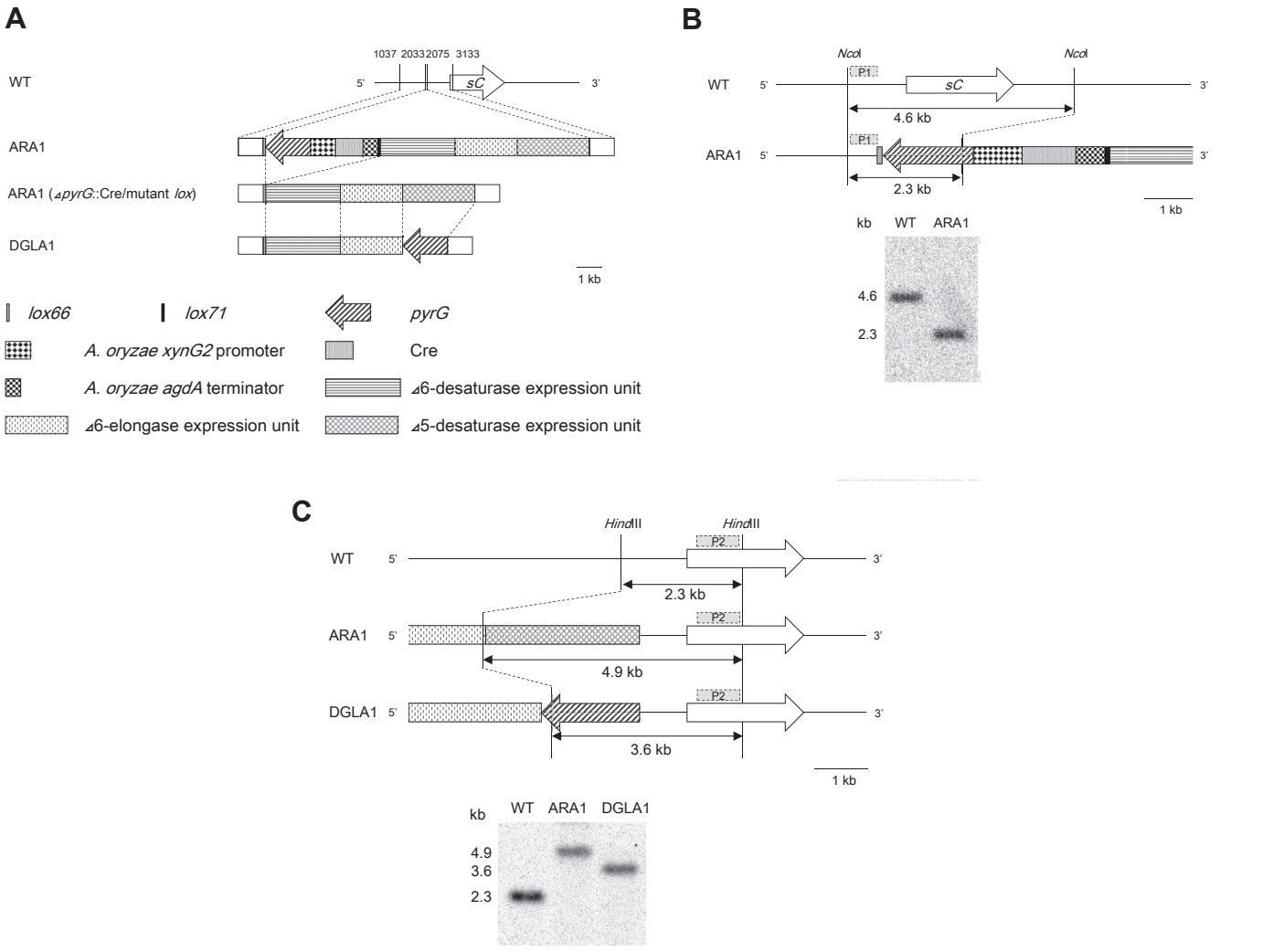


FIG. 3. Construction of the DGLA1 strain and the confirmation. Stepwise changes in the *sC* promoter region throughout the strain construction process are shown (A). Construction of the ARA1-introduced *faaA* disruptant (B, C) and the derivative DGLA1 strain (C) was confirmed by Southern hybridization. The location where each probe hybridizes is shown as P1 and P2.

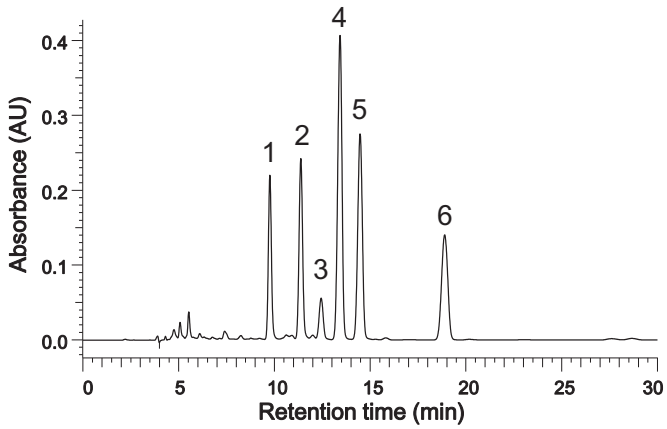
TABLE 1. Productivity of fatty acids in the wild-type RIB40, ARA1-introduced *faaA* disruptant, and DGLA1 strains.

Strain	Type	Fatty acid productivity [mmol (fatty acids)/g (dried hyphae)]					
		PA	SA	OA	LA	GLA	DGLA
RIB40	FFA	0.016 ± 0.006	0.002 ± 0	0.004 ± 0.002	0.011 ± 0.007	N.D.[a]	N.D.
	TG	0.126 ± 0.013	0.064 ± 0.007	0.055 ± 0.004	0.070 ± 0.007	N.D.	N.D.
ARA1	FFA	0.332 ± 0.04	0.183 ± 0.016	0.051 ± 0.008	0.045 ± 0.006	0.039 ± 0.005	0.049 ± 0.007
	TG	0.103 ± 0.016	0.069 ± 0.003	0.038 ± 0.002	0.022 ± 0.002	0.018 ± 0.001	0.016 ± 0.004
DGLA1	FFA	0.304 ± 0.002	0.169 ± 0.004	0.053 ± 0.003	0.043 ± 0.001	0.032 ± 0	0.056 ± 0.001
	TG	0.114 ± 0.046	0.058 ± 0.022	0.045 ± 0.013	0.023 ± 0.006	0.018 ± 0.006	0.017 ± 0.008

[a] Not detected.

probed (Fig. 3A and C). FFAs and fatty acids in TG produced by the DGLA1 strain were then analyzed by HPLC. Liquid culture for 120 h in CD medium was found to produce 0.056 mmol of free DGLA per g of dried hyphae (Table 1), which accounted for 8.5% of the total FFAs produced and was slightly more than the ARA1-introduced *faaA* disruptant (0.049 mmol/g). However, content ratio of FFA was 70.5% and similar between these two strains. The yield of free DGLA in the DGLA1 strain was 145 mg per liter.

Extracellular release of free DGLA by Triton X-100 from metabolically engineered *A. oryzae* In our previous study, non-ionic surfactants like Triton X-100 was found to cause extracellular release of FFAs from *A. oryzae* cells (14). Considering industrial use of the FFAs produced, this finding is considered attractive in the point of removing labor-intensive process of cell disruption. The release of FFAs was found only for the ones originally produced in *A. oryzae*, such as PA, SA, OA, and SA. Thus, to confirm the release for free DGLA, Triton X-100 was supplemented to the CD medium at a final concentration of 1% at the time of spore inoculation of the DGLA1 strain. After culture, both amounts and varieties of FFAs contained in each culture supernatant and cell were assayed by HPLC. As a result, over 80% of free DGLA and free GLA molecules produced were released extracellularly from *A. oryzae* by 1% Triton X-100 (Table 2). Thus, effect of Triton X-100 on the release of FFAs was confirmed even for these polyunsaturated FFAs. The productivity of free DGLA under 1% Triton X-100 was 0.083 mmol/g of dried hyphae, which was 1.5-fold more than the one under 0% Triton X-100. However, the dried hyphae decreased under 1% Triton X-100 (329 mg) from those under 0% Triton X-100 (422 mg). Thus, the total free DGLA yield per liter of culture was only 1.2-fold more under 1% Triton X-100, and was almost the same as the one under 0% Triton X-100.



Peak No. : Retention time: FFA

1 : 9.8 : γ-Linolenic acid (GLA)
2 : 11.4 : Linoleic acid (LA)
3 : 12.4 : Dihomo-γ-linolenic acid (DGLA)
4 : 13.4 : Palmitic acid (PA)
5 : 14.5 : Oleic acid (OA)
6 : 18.4 : Stearic acid (SA)

FIG. 4. Analysis of FFAs produced by the ARA1-introduced *faaA* disruptant. The composition of FFAs produced was analyzed by HPLC. The chromatogram and information of FFAs detected are shown.

DISCUSSION

In this study, three genes encoding enzymes involved in ARA synthesis from LA were isolated from *M. alpina*. These were heterologously expressed in an *A. oryzae faaA* disruptant. Resultantly, including the originally produced FFAs (PA, SA, OA, and LA), only production of free DGLA and free GLA could be observed for the first time in metabolic engineered microorganisms. The yield of free DGLA was 145 mg/L, which was about the one-sixth of the *M. alpina* mutant that highly produced DGLA by knocking out the Δ5-desaturase gene (10). As for the DGLA production system by *A. oryzae* reported previously (11), the yield was not described. Thus, it is difficult to compare the yield. However, DGLA was produced in the form of TG in both previous studies. Thus, the DGLA production system constructed in *A. oryzae* in this study seems superior to the one of previous cases in the point of supplying free DGLA as a direct feedstock of eicosanoids. In addition, because content ratio of FFA drastically increased in the DGLA1 strain from 9.5% of RIB40 to 70.5% by the *faaA* disruption (Table 1), it is considered reasonable to continuously focus on enhancing free DGLA productivity. As for free GLA production that was also attained in this study, it is a precursor of free DGLA as a feedstock of eicosanoids. Thus, from the point of view of industrial use, production of free GLA is considered less meaningful than that of free DGLA.

As for the research on microbial FFA production performed so far, saturated FFAs and mono- or di-unsaturated FFAs are produced (29). It means that both elongation and desaturation of fatty acyl chain has been attempted in the microbial FFA production. In this study, we could successfully certify the polyunsaturated FFA production by applying the genes of these functions to *A. oryzae*. The finding will be useful for attempting polyunsaturated FFA production in other microorganisms.

FFAs are reported to interfere with the cell membrane and interrupt normal hyphae growth (30,31). We have succeeded in enhancing the productivity of FFAs in *A. oryzae* by metabolic engineering, which did not result in any interruption of cell membrane and hyphal growth (13,15). However, free ARA is likely exclusive as the production was not confirmed in this study even though three genes involved in ARA synthesis were introduced into *A. oryzae*. Because the three genes introduced into the *faaA* disruptant were all derived from the *M. alpina* NBRC8568 strain, it will not be plausible that Δ5-desaturase alone does not function in *A. oryzae*. In fact, we tested the Δ5-desaturase gene expression on the following three points; (i) the gene sequence itself, (ii) its expression level, and (iii) mutations. However, in terms of point i, the Δ5-desaturase gene introduced from the NBRC8568 strain was considered correct due to its high homology with the identified Δ5-desaturase gene of the *M. alpina* 1S-4 strain (10). In terms of point ii, the gene expression level of Δ5-desaturase was measured by qRT-

TABLE 2. Productivity and distribution of FFAs produced by the DGLA1 strain.

Triton X-100 (%)	Localization	FFA productivity [mmol (FFA)/g (dried hyphae)]					
		PA	SA	OA	LA	GLA	DGLA
0	Cell	0.304 ± 0.002	0.169 ± 0.004	0.053 ± 0.003	0.043 ± 0.001	0.032 ± 0	0.056 ± 0.001
	Culture supernatant	N.D.[a]	N.D.	N.D.	N.D.	N.D.	N.D.
1	Cell	0.065 ± 0.003	0.022 ± 0.001	0.016 ± 0.001	0.011 ± 0.001	0.012 ± 0.001	0.012 ± 0.001
	Culture supernatant	0.474 ± 0.036	0.048 ± 0.014	0.064 ± 0.005	0.065 ± 0.006	0.052 ± 0.005	0.071 ± 0.005

[a] Not detected.

PCR, but all three introduced genes were confirmed to be expressed at high levels due to the constitutive gene promoters (Table S2). As for point iii, no mutation was detected by Sanger sequencing. Thus, the Δ5-desaturase of *M. alpina* would not function properly in *A. oryzae* for unknown reasons.

Alternatively, we considered the converse situation that Δ5-desaturase of *M. alpina* does function in *A. oryzae*. As the first possibility, in mammals, prostaglandin H2 is synthesized from free ARA by cyclooxygenase, and eicosanoids such as a variety of prostaglandins are then synthesized from prostaglandin H2 by various prostaglandin synthases. In fungi, several dioxygenases are reported to show homology with the mammalian cyclooxygenase, and thus demonstrate the same functions in prostaglandin synthesis (32). The eicosanoid synthesis process could be similar between mammals and fungi, and genes homologous to dioxygenase and cyclooxygenase could be present in the unmodified *A. oryzae* genome. If so, free ARA would be synthesized properly in the ARA1-introduced *faaA* disruptant, but would promptly be converted to other kinds of metabolites, resulting in negative ARA detection. As the second possibility, ARA would have been produced, whereas it would specifically have been incorporated to membrane lipid and thus was not detected in FFA and TG. However, further analysis is required to elucidate this point.

Hereafter, for free DGLA production, introduction of additional metabolic engineering will contribute to increasing productivity. Because palmitic acid and stearic acid are predominant in the fatty acid composition of the DGLA1 strain (Table 1), overexpression of Δ9-and Δ12-desaturase genes that originally exist in *A. oryzae* would increase free DGLA productivity. In addition, overexpression of cytochrome *b* is reported to increase desaturation in *Saccharomyces cerevisiae* (33). Overexpression of the gene in the DGLA1 strain might also increase the DGLA productivity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jbiosc.2018.09.013.

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References

1. **Zhu, H., Xu, X., Ding, Y., Zhou, L., and Huang, J.:** Effects of prostaglandin E1 on reperfusion injury patients: a meta-analysis of randomized controlled trials, Medicine (Baltim.), **96**, e6591 (2017).
2. **Levin, G., Duffin, K. L., Obukowicz, M. G., Hummert, S. L., Fujiwara, H., Needleman, P., and Raz, A.:** Differential metabolism of dihomo-γ-linolenic acid and arachidonic acid by cyclo-oxygenase-1 and cyclo-oxygenase-2: implications for cellular synthesis of prostaglandin E1 and prostaglandin E2, Biochem. J., **365**, 489–496 (2002).
3. **Choi, Y. M., Diehl, J., and Levins, P. C.:** Promising alternative clinical uses of prostaglandin F2α analogs: beyond the eyelashes, J. Am. Acad. Dermatol., **72**, 712–716 (2015).
4. **Simmons, D. L., Botting, R. M., and Hla, T.:** Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition, Pharmacol. Rev., **56**, 387–437 (2004).
5. **Dyall, S. C.:** Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA, Front. Aging Neurosci., **7**, 52 (2015).
6. **Davis-Bruno, K. and Tassinari, M. S.:** Essential fatty acid supplementation of DHA and ARA and effects on neurodevelopment across animal species: a review of the literature, Birth Defects Res. B Dev. Reprod. Toxicol., **92**, 240–250 (2011).
7. **Wang, X., Lin, H., and Gu, Y.:** Multiple roles of dihomo-γ-linolenic acid against proliferation diseases, Lipids Health Dis., **11**, 25 (2012).
8. **Sakuradani, E. and Shimizu, S.:** Single cell oil production by *Mortierella alpina*, J. Biotechnol., **144**, 31–36 (2009).
9. **Sakuradani, E., Ando, A., Ogawa, J., and Shimizu, S.:** Improved production of various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding, Appl. Microbiol. Biotechnol., **84**, 1–10 (2009).
10. **Kikukawa, H., Sakuradani, E., Ando, A., Okuda, T., Shimizu, S., and Ogawa, J.:** Microbial production of dihomo-γ-linolenic acid by Δ5-desaturase gene-disruptants of *Mortierella alpina* 1S-4, J. Biosci. Bioeng., **122**, 22–26 (2016).
11. **Chutrakul, C., Jeennor, S., Panchanawaporn, S., Cheawchanlertfa, P., Suttiwattanakul, S., Veerana, M., and Laoteng, K.:** Metabolic engineering of long chain-polyunsaturated fatty acid biosynthetic pathway in oleaginous fungus for dihomo-gamma linolenic acid production, J. Biotechnol., **218**, 85–93 (2016).
12. **Meng, X., Yang, J., Xu, X., Zhang, L., Nie, Q., and Xian, M.:** Biodiesel production from oleaginous microorganisms, Renew. Energy, **34**, 1–5 (2009).
13. **Tamano, K., Bruno, K. S., Koike, H., Ishii, T., Miura, A., Umemura, M., Culley, D. E., Baker, S. E., and Machida, M.:** Increased production of free fatty acids in *Aspergillus oryzae* by disruption of a predicted acyl-CoA synthetase gene, Appl. Microbiol. Biotechnol., **99**, 3103–3113 (2015).
14. **Tamano, K., Miura, A., Koike, H., Kamisaka, Y., Umemura, M., and Machida, M.:** High-efficiency extracellular release of free fatty acids from *Aspergillus oryzae* using non-ionic surfactants, J. Biotechnol., **248**, 9–14 (2017).
15. **Tamano, K. and Miura, A.:** Further increased production of free fatty acids by overexpressing a predicted transketolase gene of the pentose phosphate pathway in *Aspergillus oryzae faaA* disruptant, Biosci. Biotechnol. Biochem., **80**, 1829–1835 (2016).
16. **Tamano, K., Satoh, Y., Ishii, T., Terabayashi, Y., Ohtaki, S., Sano, M., Takahashi, T., Koyama, Y., Mizutani, O., Abe, K., and Machida, M.:** The β-1,3-exoglucanase gene *exgA* (*exg1*) of *Aspergillus oryzae* is required to catabolize extracellular glucan, and is induced in growth on a solid surface, Biosci. Biotechnol. Biochem., **71**, 926–934 (2007).
17. **Wang, L., Chen, W., Feng, Y., Ren, Y., Gu, Z., Chen, H., Wang, H., Thomas, M. J., Zhang, B., Berquin, I. M., and other 16 authors:** Genome characterization of the oleaginous fungus *Mortierella alpina*, PLoS One, **6**, e28319 (2011).
18. **Kitamoto, N., Matsui, J., Kawai, Y., Kato, A., Yoshino, S., Ohmiya, K., and Tsukagoshi, N.:** Utilization of the TEF1-alpha gene (TEF1) promoter for expression of polygalacturonase genes, *pgaA* and *pgaB*, in *Aspergillus oryzae*, Appl. Microbiol. Biotechnol., **50**, 85–92 (1998).
19. **Nakajima, K., Kunihiro, S., Sano, M., Zhang, Y., Eto, S., Chang, Y. C., Suzuki, T., Jigami, Y., and Machida, M.:** Comprehensive cloning and expression analysis of glycolytic genes from the filamentous fungus, *Aspergillus oryzae*, Curr. Genet., **37**, 322–327 (2000).
20. **Maeda, H., Sano, M., Maruyama, Y., Tanno, T., Akao, T., Totsuka, Y., Endo, M., Sakurada, R., Yamagata, Y., Machida, M., and other 6 authors:** Transcriptional analysis of genes for energy catabolism and hydrolytic enzymes in the filamentous fungus *Aspergillus oryzae* using cDNA microarrays and expressed sequence tags, Appl. Microbiol. Biotechnol., **65**, 74–83 (2004).
21. **Zhang, S., Ban, A., Ebara, N., Mizutani, O., Tanaka, M., Shintani, T., and Gomi, K.:** Self-excising Cre/mutant *lox* marker recycling system for multiple gene integrations and consecutive gene deletions in *Aspergillus oryzae*, J. Biosci. Bioeng., **123**, 403–411 (2017).
22. **Tsuge, K., Matsui, K., and Itaya, M.:** One step assembly of multiple DNA fragments with a designed order and orientation in *Bacillus subtilis* plasmid, Nucleic Acids Res., **31**, e133 (2003).
23. **Tsuge, K., Sato, Y., Kobayashi, Y., Gondo, M., Hasebe, M., Togashi, T., Tomita, M., and Itaya, M.:** Method of preparing an equimolar DNA mixture for one-step DNA assembly of over 50 fragments, Sci. Rep., **5**, 10655 (2015).
24. **Baker, S. E., Perrone, G., Richardson, N. M., Gallo, A., and Kubicek, C. P.:** Phylogenomic analysis of polyketide synthase-encoding genes in *Trichoderma*, Microbiology, **158**, 147–154 (2012).

25. **Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S.:** MEGA6: molecular evolutionary genetics analysis version 6.0, Mol. Biol. Evol., **30**, 2725–2729 (2013).
26. **Michaelson, L. V., Lazarus, C. M., Griffiths, G., Napier, J. A., and Stobart, A. K.:** Isolation of a Δ^5-fatty acid desaturase gene from *Mortierella alpina*, J. Biol. Chem., **273**, 19055–19059 (1998).
27. **Huang, Y. S., Chaudhary, S., Thurmond, J. M., Bobik, E. G., Jr., Yuan, L., Chan, G. M., Kirchner, S. J., Mukerji, P., and Knutzon, D. S.:** Cloning of Δ12- and Δ6-desaturases from *Mortierella alpina* and recombinant production of γ-linolenic acid in *Saccharomyces cerevisiae*, Lipids, **34**, 649–659 (1999).
28. **Parker-Barnes, J. M., Das, T., Bobik, E., Leonard, A. E., Thurmond, J. M., Chaung, L. T., Huang, Y. S., and Mukerji, P.:** Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acids, Proc. Natl. Acad. Sci. USA, **97**, 8284–8289 (2000).
29. **Lennen, R. M. and Pfleger, B. F.:** Engineering *Escherichia coli* to synthesize free fatty acids, Trends Biotechnol., **30**, 659–667 (2012).
30. **Avis, T. J. and Bélanger, R. R.:** Specificity and mode of action of the antifungal fatty acid *cis*-9-heptadecenoic acid produced by *Pseudozyma flocculosa*, Appl. Environ. Microbiol., **67**, 956–960 (2001).
31. **Desbois, A. P. and Smith, V. J.:** Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential, Appl. Microbiol. Biotechnol., **85**, 1629–1642 (2010).
32. **Tsitsigiannis, D. I., Bok, J. W., Andes, D., Nielsen, K. F., Frisvad, J. C., and Keller, N. P.:** *Aspergillus* cyclooxygenase-like enzymes are associated with prostaglandin production and virulence, Infect. Immun., **73**, 4548–4559 (2005).
33. **Yazawa, H., Iwahashi, H., Kamisaka, Y., Kimura, K., and Uemura, H.:** Improvement of polyunsaturated fatty acids synthesis by the coexpression of CYB5 with desaturase genes in *Saccharomyces cerevisiae*, Appl. Microbiol. Biotechnol., **87**, 2185–2193 (2010).