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Omega-3 eicosatetraenoic acid production by molecular breeding of the mutant strain S14 derived from *Mortierella alpina* 1S-4

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We investigated the omega-3 eicosatetraenoic acid (ETA) production by molecular breeding of the oleaginous fungus *Mortierella alpina*, which can slightly accumulate ETA only when cultivated at a low temperature. The endogenous ω3-desaturase gene or the heterologous *Saprolegnia diclina* Δ17 (*sdd17m*) desaturase gene were overexpressed in *M. alpina* S14, a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4. *M. alpina* S14 transformants introduced with the endogenous ω3-desaturase gene showed ETA at 42.1% content in the total lipids that was 84.2-fold and 3.2-fold higher than that of the wild-type strain 1S-4 and host strain S14, respectively, when cultivated at 12°C. No accumulation of ETA was observed at 28°C. In contrast, transformants with the heterologous *sdd17m* gene showed 24.9% of the content of total lipids at 28°C. These results indicated that these *M. alpina* S14 transformants are promising strains for the production of ETA, which is hard to obtain from natural sources.

[**Key words:** Omega-3 polyunsaturated fatty acids; Lipid-producing fungus; *Mortierella alpina*; Rare fatty acid; Eicosatetraenoic acid]

Omega-3 polyunsaturated fatty acids (ω3-PUFAs) are found in natural sources. Each ω3-PUFA and its derivative have been reported to have some particular physiological function. For example, Eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 20:6ω3) now typically derived from a natural marine source and supplied sufficiently, have been known as precursors of the eicosanoids of signaling molecules including prostaglandins, thromboxanes, and leukotrienes (1). Recently, EPA and DHA have attracted much attention for the beneficial effects on human health in reducing the risk for sudden death caused by cardiac arrhythmias and all-cause mortality in patients with known coronary heart disease (2–4). In addition, there are some reports suggestive of effects on prevention of rheumatoid arthritis and asthma (5–7). Alpha-linolenic acid (ALA, 18:3ω3), which is contained in natural oils from higher plants such as linseed and soya, has been also reported to contribute to prevention several diseases, such as coronary heart disease and depressive illness (8,9). Furthermore, ω3-PUFA derivatives were also important as anti-inflammatory lipid mediators and candidates of anti-influenza agent (10,11). Therefore, the demand for ω3-PUFAs is rapidly increasing in the pharmaceutical, medical and nutritional fields.

EPA, DHA, and ALA, which can be prepared from natural sources, have been well studied because of their sufficient natural supply. On the other hand, there are few reports on the other ω3-PUFAs, such as stearidonic acid (SDA, 18:4ω3), docosapentaenoic acid (DPA, 22:5ω3), and eicosatetraenoic acid (ETA, 20:4ω3), because their supply has been limited to date. These minor ω3-PUFAs have been expected to possess beneficial function as precursors of bioactive substances as well as EPA and so on, therefore sufficient supply of such rare PUFAs has been required for elucidation of their physiological function. Recently, SDA has been reported to be accumulated in *Echium plantagineum* (12), and the development of its purification method is now under way. In addition, the SDA-accumulating soybean derived by the molecular breeding has been commoditized already (13). DPA also has been reported to be present in several natural oils produced by earless seal (14), *Cyanea capillata*, and *Mola mola* (15). Thus, sources of SDA and DPA are being developed, therefore research on their physiological function will be advancing in the near future.

On the other hand, ETA is hard to find in nature. In addition, there are only few reports of ETA production at a low level by overexpression of Δ6 desaturase gene in *E. plantagineum* (16), by mutation in *Mortierella alpina* (17), and molecular breeding in *Arabidopsis thaliana* (18). Although ETA has been expected to show beneficial effects on human health, the detailed bioactivity of ETA has remained almost unknown because of lack of its sources. Therefore establishment of a stable supply of ETA is needed for subsequent studies of physiological function of ETA

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followed by expansion of its applications in medical and pharmaceutical fields.

Mortierella alpina 1S-4, an oleaginous fungus, is known as an industrial strain that produces arachidonic acid (ARA, 20:4ω6) commercially (19). To date, we have reported considerable accumulation of EPA by overexpressing endogenous ω3-desaturase gene in *M. alpina* 1S-4 as a novel alternative source of ω3-PUFAs (20). We have also succeeded in the industrial production of various kinds of PUFAs by using mutants derived from *M. alpina* 1S-4 through chemical mutagenesis (21,22). *M. alpina* S14 is a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4, after treating the parental spores with a chemical mutagen (23) (Fig. 1). The strain S14 produces only a trace (about 1%) amount of ARA, and the ratio of dihomo-γ-linolenic acid (DGLA, 18:4ω6) to total fatty acids is markedly high, accounting for as much as 43% (24) and has been applied to the industrial production of DGLA (25). ETA can be biosynthesized by ω3- (e.g., similar to Δ17-) desaturation from DGLA; therefore, we hypothesized that this strain would be a good host strain for ETA production by expressing ω3- or Δ17-desaturase gene.

In this study, we aimed to evaluate ETA production using expression of the endogenous ω3-desaturase gene and the heterologous Δ17-desaturase gene in *M. alpina* S14.

MATERIALS AND METHODS

Strain, media, and growth conditions *M. alpina* S14, a Δ5-desaturation activity-defective mutant derived from *M. alpina* 1S-4 (24), was maintained on Potato Dextrose Agar medium (Difco, MI, USA), and the uracil auxotrophs (*ura5*$^-$ strain) derived from *M. alpina* S14 on the PDA medium containing uracil (0.05 mg/mL). GY medium consisting of 2% (w/v) glucose and 1% yeast extract was used for fatty acid composition analysis and for extracting genomic DNA. Czapek-Dox minimal medium consisting of 3% sucrose, 0.2% $NaNO_3$, 0.1% KH_2PO_4, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot 7H_2O$, pH 6.0 was used for the sporulation of the fungi. SC medium was used as a uracil-free synthetic medium, which contained 5 g of Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco), 1.7 g of $(NH_4)_2SO_4$, 20 g of glucose, 20 mg of adenine, 30 mg of tyrosine, 1 mg of methionine, 2 mg of arginine, 2 mg of histidine, 4 mg of lysine, 4 mg of tryptophan, 5 mg of threonine, 6 mg of isoleucine, 6 mg of leucine, and 6 mg of phenylalanine per liter. Solid media contained 1.5% agar. *Escherichia coli* DH5α cells were used for DNA manipulation and cultivated at 37°C with vigorous shaking at 300 rpm.

Isolation of uracil auxotrophs of *M. alpina* S14 Isolation of uracil auxotrophs was performed as described previously (26). Mutant S14 was incubated on Czapek-Dox agar medium at 28°C for 1 month, and allowed to sporulate at 12°C for 1 month. Spores of S14 were harvested from the surface of Czapek-Dox (2.6×10^8 spores/225 cm^2); 2.6×10^7 spores were spread on a GY agar medium containing 1.0 mg/mL of 5-fluoroorotic acid (5-FOA) and 0.05 mg/mL of uracil. By means of 5-FOA positive selection, uracil auxotrophs that acquired 5-FOA resistance could be isolated.

Fatty acid analysis All strains were inoculated in GY medium and then the culture was carried out at 12°C or 28°C with reciprocal shaking (120 strokes/min) for a desired period. The mycelia were harvested by suction filtration and dried at 120°C. The dried cells were weighed and transmethylated with 10% methanolic HCl and dichloromethane at 55°C for 2 h, containing 0.2 mg of *n*-tricosanoic acid as an internal standard. The resultant fatty acid methylesters were extracted with *n*-hexane, concentrated and then analyzed using gas chromatography.

Isolation of the *ura5* genomic gene of uracil auxotrophs of S14 The *ura5* genomic gene was amplified using forward primer ura5upF (5′-TTTCTGATGTGTCTCCCACC-3′) and reverse primer ura5downR (5′-TTCCAACAGAACCTTCCCTCG-3′) with uracil auxotrophic S14 genomic DNA as the template. A 700-bp PCR product was cloned into the pUC118 vector using Reagent Set for Mighty Cloning Kit (Takara, Shiga, Japan).

DNA sequencing Nucleotide sequences were identified using the dideoxy chain termination method by means of a CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter Inc., CA, USA) and an automated sequencer DNA Analysis System CEQ 8000 (Beckman Coulter Inc.). The sequence data were analyzed in the Genetyx-Windows software, ver. 11.0.3 (Software Development, Japan).

Construction of a transformation vector for *M. alpina* S14 *ura5*$^-$ strain Transformation vectors pSDura5ω3 and pSDura5ω3×2 were constructed by the modification of pSDura5 z (27,28). The ω3-desaturase gene was amplified using a forward primer, w3F2PciI (5′-GGGAATATTAAGCTTACATGTCCCC-3′) and a reverse primer, w3R2BamHI (5′-GCCGGATCCAAATTGTTAATGCTTG-3′) at 56°C with *M. alpina* 1S-4 cDNA as a template. The 2 primers contained a *Pci*I and a *Bam*HI site, respectively (underlined). About 1.3-kb of PCR product was ligated to the pT7 Blue T-Vector (Novagen, Darmstadt, Germany), resulting in construction of a plasmid named pT7ω3. We checked its sequence. The ω3-desaturase gene was digested with *Pci*I and *Bam*HI, followed by ligation into pBlueshtp treated with *Nco*I and *Bam*HI to construct pBluesω3 (29). The ω3-desaturase expression unit including a promoter and a terminator was cut out by *Eco*RI from pBluesω3 and ligated into pSDura5 digested with the same enzyme to generate pSDura5ω3 and pSDura5ω3×2 (Fig. 2). The latter plasmid possessed two ω3-desaturase expression units.

Saprolegnia diclina Δ17 desaturase gene (*sdd17m*) was synthesized with optimized codon usage to reflect the codon bias of *M. alpina* 1S-4 (obtained from the Kazusa database; http://www.kazusa.or.jp/codon/), with additional *Spe*I and *Bam*HI restriction enzyme sites at the 5′ and 3′ ends, respectively. The *sdd17m* expression cassette, with the *SSA2* promoter (30) and *SdhB* terminator (19), was generated by fusion PCR with *Xba*I and *Nhe*I restriction sites at the 5′ and 3′ ends of the cassette, respectively. This cassette was then digested with *Xba*I and *Nhe*I and ligated into pBIG35ZhSSA2psdd17m, which had been digested with same restriction enzymes (Fig. 2).

Transformation of *M. alpina* by microprojectile bombardment A spore suspension from the *M. alpina* S14 *ura5*$^-$ strain was freshly prepared from cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/mL uracil; the suspension was filtered through Miracloth (Calbiochem) and spread on a uracil-free SC medium. A PDS-1000/He Particle Delivery System (Bio-Rad Laboratories Inc., CA, USA) was used for the transformation. Tungsten particles (0.4 μm in diameter) coated with pSDura5, pSDura5ω3 and pSDura5ω3×2 were prepared according to the manufacturer's manual. Bombardment was performed in the plate placed on the device under a helium pressure of 1100 psi (7580 kPa). After the bombardment, the plate was incubated at 28°C (3–6 days) (28).

Transformation of *M. alpina* using the ATMT method The spore suspension from the *M. alpina* S14 *ura5*$^-$ strain was prepared as mentioned at the description of microprojectile bombardment method.

Transformation of the *M. alpina* S14 *ura5*$^-$ strain with pBIG35ZhSSA2psdd17m was performed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method described previously (20) with slight modification. Initially, *A. tumefaciens* C58C1 was transformed with each vector via electroporation as

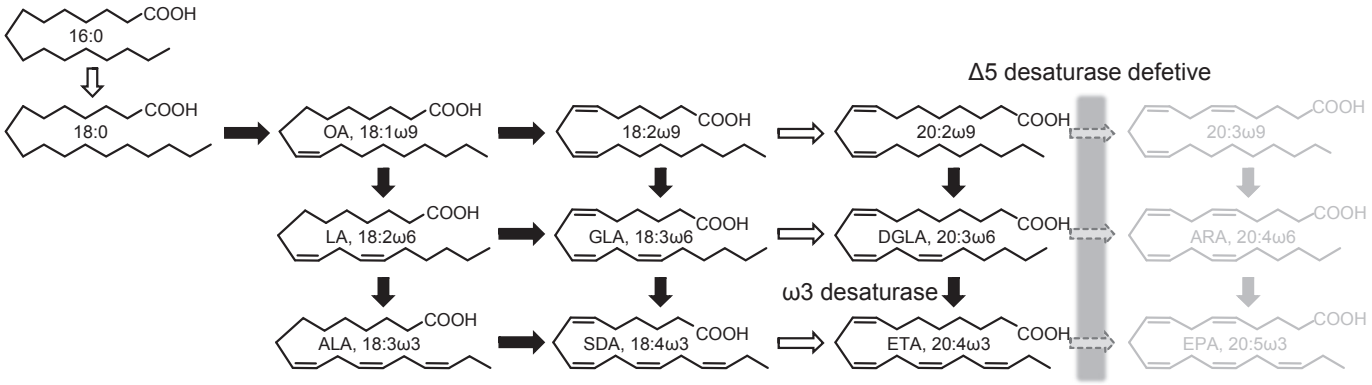

FIG. 1. A biosynthetic pathway of polyunsaturated fatty acids (PUFA) in the mutant strain S14 derived from *Mortierella alpina* 1S-4. OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; ALA, α-linolenic acid; SDA, stearidonic acid; ETA, ω3 eicosatetraenoic acid; EPA, ω3 eicosapentaenoic acid.