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Microbial production of dihomo-γ-linolenic acid by *Δ5-desaturase* gene-disruptants of *Mortierella alpina* 1S-4

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We constructed dihomo-γ-linolenic acid (DGLA)-producing strains with disruption of the *Δ5-desaturase* (*Δ5ds*) gene, which encodes a key enzyme catalyzing the bioconversion of DGLA to arachidonic acid (ARA), by efficient gene-targeting, using *Δlig4* strain of *Mortierella alpina* 1S-4 as the host. In previous study, we had already identified and disrupted the *lig4* gene encoding DNA ligase 4, which involves in non-homologous end joining, in *M. alpina* 1S-4, and the *Δlig4* strain had showed efficient gene-targeting. In this study, the uracil auxotroph of *Δlig4* strain was constructed, and then transformed for disruption of *Δ5ds*. The isolation of nine *Δ5ds*-disruptants out of 18 isolates indicated that the disruption efficiency was 50%. The ratio of DGLA among the total fatty acids of the *Δ5ds*-disruptants reached 40.1%; however, no ARA was detected. To our knowledge, this is the first study to report the construction of DGLA-producing transformants by using the efficient gene-targeting system in *M. alpina* 1S-4.

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[**Key words:** *Mortierella alpina*; Gene-targeting; *Δ5-desaturase*; Polyunsaturated fatty acid; Dihomo-γ-linolenic acid]

Dihomo-γ-linolenic acid (20:3ω6, 20:3^{8cis,11cis,14cis}, DGLA) is a precursor of physiologically active eicosanoids, including group 1 prostaglandins and thromboxanes. DGLA has attracted a great deal of interest because of its unique biological activities. For example, DGLA is used in combination with interferons for the enhancement of the anti-virus, anti-cancer, or anti-inflammatory effects (1), and in combination with prostaglandin E1 for the treatment of atopy of the skin and mucosa (2). A small amount of DGLA has been detected as a component of cellular lipids in fungi (3), algae (4), protozoa (5), and animals (6).

The oil-producing fungus *Mortierella alpina* 1S-4 is a producer of carbon 20 polyunsaturated fatty acids (PUFAs) such as arachidonic acid (20:4ω6, ARA) and eicosapentaenoic acid (20:5ω3, EPA), which are rich in triacylglycerols (7). Moreover, this fungus produces large amount of lipids. Therefore, it has been used as a model oleaginous microorganism for studying the biosynthesis and accumulation of lipids, including PUFAs (8–16). In previous studies, various PUFA-producing strains have been constructed by chemical mutation and/or molecular breeding (17,18). A *Δ5-desaturase* (*Δ5ds*, Fig. 1) gene-defective mutant (S14) was constructed by chemical mutation, and showed high DGLA production (19,20). However, some mutants and recombinants showed unfavorable characteristics with respect to mycelia growth, spore germination, and PUFA productivity, since the mutations and/or insertion of the plasmid vector might occur at random positions, thereby introducing defects in essential genes.

Recently, it was reported that disruption of *lig4*, which encodes DNA ligase 4, a key enzyme in non-homologous end joining, led to an improvement in gene-targeting efficiency through homologous recombination in various filamentous fungi. *Lig4*-disruptants have been used as hosts for various applications (21–25). We previously developed an efficient gene-targeting system through homologous recombination in *M. alpina* 1S-4 by disrupting *lig4* to avoid unfavorable gene mutations and random insertions (26).

In this study, we aimed to develop a highly efficient DGLA-producing strain that does not harbor unfavorable mutations and gene-defects from *M. alpina* 1S-4 by gene-targeting. We obtained a uracil auxotrophic (*ura5*$^-$) strain as a host strain from the efficient gene-targeting strain (*Δlig4::ura5*) (26), and attempted to construct a DGLA-producing strain by disrupting *Δ5ds*.

MATERIALS AND METHODS

Enzymes and chemicals Restriction enzymes and other DNA-modifying enzymes were obtained from Takara Bio (Shiga, Japan). All other chemicals were of the highest purity commercially available.

Strains, media, and growth conditions *Mortierella alpina* 1S-4 has been deposited in the Graduate School of Agriculture of Kyoto University (17). Czapek–Dox agar medium containing 0.05 mg/ml uracil, was used for sporulation of the uracil auxotrophic (*ura5*$^-$) strain, as described previously (27). Strains used in this research were listed in Table 1. Synthetic complete (SC) medium was used as a uracil-free synthetic medium to cultivate the transformants derived from the uracil auxotrophs at 28°C (27). GY medium (2% [w/vol] glucose and 1% yeast

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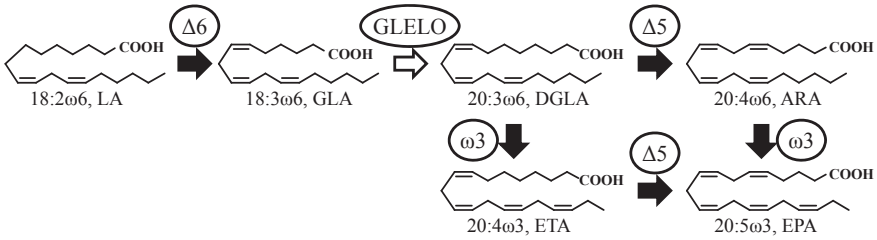

FIG. 1. Polyunsaturated fatty acid (PUFA) biosynthetic pathways in *M. alpina* 1S-4. ARA is synthesized by the desaturation of DGLA at the Δ5-position by Δ5-desaturase. LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomo-γ-linolenic acid; ARA, arachidonic acid; ETA, ω3-eicosatetraenoic acid; EPA, eicosapentaenoic acid; Δ6, Δ6-desaturase; GLELO, Δ6-elongase; Δ5, Δ5-desaturase; ω3, ω3-desaturase.

TABLE 1. *M. alpina* 1S-4 strains used in this study.

Strain	Parental strain	Genotype	Reference
M. alpina 1S-4	–	Wild-type	7
Uracil auxotroph of wild-type	Wild-type	*ura5*$^-$	7
Δ5ds-defective mutant S14	Wild-type	*Δ5ds*$^-$	20
Δlig4	Uracil auxotroph of wild-type	*ura5*$^-$ Δ*lig4::ura5*	26
Uracil auxotroph of *Δlig4*	*Δlig4*	*ura5*$^-$ Δ*lig4::ura5*$^-$	This study
Δ5ds-dispurtants	Uracil auxotroph of *Δlig4*	*ura5*$^-$ Δ*lig4::ura5*$^-$ Δ*Δ5ds::ura5*	This study

extract) was used for fatty acid analysis and extraction of genomic DNA. GY agar plates containing 0.1% 5-fluoroorotic acid (5-FOA) were used to obtain uracil auxotrophs from the *lig4* gene-disrupted (*Δlig4::ura5*) strain (26). Uracil-free SC agar plates containing 0.03% Nile blue A (Sigma–Aldrich, St. Louis, MO, USA) and GY agar plates containing 10 μg/ml carboxin and 0.03% Nile blue A were used to select transformants. *Escherichia coli* strain DH5α was used for DNA manipulation and was grown on LB agar plates containing 50 μg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was used to transform the *M. alpina* 1S-4 *ura5*$^-$ strain. LB-Mg agar medium, minimal medium, and induction medium were used for transformation, cultivation, and infection of *A. tumefaciens*, respectively (28). All solid media were made with 2.0% agar.

Isolation of uracil auxotrophs of the *Δlig4::ura5* strain from *M. alpina* 1S-4 Uracil auxotrophs were isolated as described previously (29). The *Δlig4* strain from *M. alpina* 1S-4 was incubated on Czapek–Dox agar medium at 28°C for a month, and allowed to sporulate at 12°C for a month. Spores of the *Δlig4::ura5* strain were harvested from the surface of Czapek–Dox medium (2.6×10^8 spores/225 cm^2); 2.6×10^7 spores were spread on GY agar medium containing 5-FOA (1.0 mg/ml) and uracil (0.05 mg/ml).

Identification of a mutation site on *ura5* integrated in the *lig4* genomic gene locus The *lig4* gene locus containing the *ura5* gene marker cassette was amplified with Lig4 up F and Lig4 down R primers (Table 2), using genomic DNA isolated from the uracil auxotroph of the *lig4* gene-disrupted strain as a template. The PCR product was sequenced with a Beckman–Coulter CEQ8000 system using Hispro stop F, ura5 300 F, ura5 400 R, and SdhBt R primers (Table 2).

Genomic DNA preparation *Mortierella alpina* strains were cultivated in 100 ml of GY liquid medium at 28°C for 5 days with shaking at 100 rpm. Their genomic DNA was prepared as described previously (26).

Construction of the plasmid vector for *Δ5ds* gene-targeting A T-DNA binary vector for *Δ5ds* gene-targeting was constructed by modifying the *M. alpina* 1S-4 transformation vector pBIG35Zh (28). The *Δ5ds* front fragment (1.2-kb) and *Δ5ds* rear fragment (2.4-kb) were amplified with primer sets, Δ5 F1 *Apa*I and Δ5 R1 *Apa*I, and Δ5 F2 *Xba*I and Δ5 R2 *Xba*I (Table 2), by using the genomic DNA of *M. alpina* 1S-4 as a template. The *Δ5ds* rear fragment was digested with *Xba*I and ligated into pBIG35Zh digested with same restriction enzyme. Then, the *Δ5ds* front fragment was digested with *Apa*I and ligated into the resulting plasmid vectors digested with same restriction enzyme and designated pBIG35ZΔD5 (Fig. 2). The sequences between the left and right borders were integrated into the genomic DNA of spores of *M. alpina* 1S-4 through *A. tumefaciens*.

Transformation of the *Δlig4* strain of *M. alpina* 1S-4 Spores of the uracil auxotroph of the *Δlig4* strain was freshly prepared from cultures growing on Czapek–Dox agar medium supplemented with 0.05 mg/ml uracil, and the suspension was filtered through Miracloth (Calbiochem) (27).

Transformation of the *M. alpina* strains were performed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method, as described previously (26).

Fatty acid analysis Fatty acid productivity and composition of the transformants were analyzed as described previously (26). The mycelia of the *Δlig4* uracil auxotroph strain used as a host strain, the *Δ5ds* gene-defective mutant S14 (20), and the *Δ5ds*-disruptants were inoculated into 10 ml of GY liquid medium and cultivated at 28°C with reciprocal shaking at 300 rpm for 7 days. After cultivation, the fungal strains were harvested by filtration and used for fatty acid analysis. All experiments were performed in triplicate, and the average of three separate experiments is presented as the mean ± SD in Table 3.

Identification of gene disruption by PCR and Southern blot analysis Recombinants transformed with the pBIG35ZΔD5 vector were repeatedly cultured on fresh SC agar medium containing no uracil at least three times to prevent contamination of abortive recombinants. *Δ5ds*-disruptants were selected for PCR analysis by using the *Δ5ds* genomic gene locus amplified with the Δ5 up F and Δ5 down R primers (Table 2) as the template and primer sets, Δ5 F1 *Apa*I and Δ5

TABLE 2. Primers used in this study.

Primer	Sequence (5′ to 3′)
Lig4 up F	GTGTGCCATCAACACGTTGTCTGC
Lig4 down R	CCAAGAACGACATGTCAATGTACG
Hispro stop F	CTCACCCAACACTCTCTCAAC
ura5 300 F	CTGCACCACCGTCATTGCCTTG
ura5 400 R	CATGGTCCTTCTTTTCCTTGC
SdhBt R	GAGGTAGTTGCGTGGGTCGTCGTAC
Δ5 F1 *Apa*I	GCGGGCCCTTTTGTTTCCTTGATCCTCACATTC[a]
Δ5 R1 *Apa*I	GCGGGCCCAAGAACTTTGTGACATCGTATAC
Δ5 F2 *Xba*I	GCTCTAGATCTGTTCCCGAACGTTTCCCAGC
Δ5 R2 *Xba*I	GGTCTAGAGCGATAATTATGAAAACCTTACC
Δ5 up F	CGTCCTCTACGATCCTCTGACCGAC
Δ5 down R	CCACATGGTACCAAAGCGTGGGCTG
SdhBt stop F	CGAGATGTCGCAGCTGAGCTAAGC
Δ5 down R2	GCTCTCTCATCCTGTCACATGC
ura5 start F	GGATTACCAGCGCGAGTTC
ura5 stop R	TTAAACACCGTACTTCTCGCGG

[a] The underlined sequences represent the synthesized restriction enzyme sites.

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