**Microbial production of dihomono- γ -linolenic acid by $\Delta 5$ -desaturase
gene-disruptants of *Mortierella alpina* 1S-4**

Hiroshi Kikukawa^a, Eiji Sakuradani^{a,b}, Akinori Ando^{a,c}, Tomoyo Okuda^a, Sakayu
Shimizu^{a,d}, and Jun Ogawa^{a,c*}

^aDivision of Applied Life Sciences, Graduate School of Agriculture, Kyoto University,
Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan;

^bInstitute of Technology and Science, Tokushima University, 2-1 Minami-Josanjima, Tokushima
770-8506, Japan;

^cResearch Unit for Physiological Chemistry, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku,
Kyoto 606-8502, Japan;

^dDepartment of Bioscience and Biotechnology, Faculty of Bioenvironmental Science, Kyoto Gakuen
University, 1-1 Nanjo, Sogabe, Kameoka 621-8555, Japan;

Running title: Construction of DGLA-producing strains from *M. alpina*

*Corresponding author: Jun Ogawa

Present address: Division of Applied Life Science, Graduate School of Agriculture, Kyoto
University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto 606-8502, Japan

Tel: +81 75 753 6115, Fax: +81 75 753 6113,

E-mail: ogawa@kais.kyoto-u.ac.jp

24 **ABSTRACT**

25 We constructed dihomog- γ -linolenic acid (DGLA)-producing strains with disruption
26 of the $\Delta 5$ -desaturase ($\Delta 5ds$) gene, which encodes a key enzyme catalyzing the
27 bioconversion of DGLA to arachidonic acid (ARA), by efficient gene-targeting, using
28 *Alig4* strain of *Mortierella alpina* 1S-4 as the host. In previous study, we had already
29 identified and disrupted the *lig4* gene encoding DNA ligase 4, which involves in
30 non-homologous end joining, in *M. alpina* 1S-4, and the *Alig4* strain had showed
31 efficient gene-targeting. In this study, the uracil auxotroph of *Alig4* strain was
32 constructed, and then transformed for disruption of $\Delta 5ds$. The isolation of nine $\Delta 5ds$
33 disruptants out of 18 isolates indicated that the disruption efficiency was 50%. The
34 ratio of DGLA among the total fatty acids of the $\Delta 5ds$ disruptants reached 40.1%;
35 however, no ARA was detected. To our knowledge, this is the first study to report the
36 construction of DGLA-producing transformants by using the efficient gene-targeting
37 system in *M. alpina* 1S-4.

38

39

40 **Key words**

41 *Mortierella alpina*, gene-targeting, $\Delta 5$ -desaturase, polyunsaturated fatty acid,
42 dihomog- γ -linolenic acid

43 INTRODUCTION

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

52 The oil-producing fungus *Mortierella alpina* 1S-4 is a producer of carbon 20
53 polyunsaturated fatty acids (PUFAs) such as arachidonic acid (20:4 ω 6, ARA) and
54 eicosapentaenoic acid (20:5 ω 3, EPA), which are rich in triacylglycerols (7). Moreover,
55 this fungus produces large amount of lipids. Therefore, it has been used as a model
56 oleaginous microorganism for studying the biosynthesis and accumulation of lipids,
57 including PUFAs (8-16). In previous studies, various PUFA-producing strains have
58 been constructed by chemical mutation and/or molecular breeding (17, 18). A
59 *Δ 5-desaturase (Δ 5ds, Fig. 1) gene-defective mutant (S14) was constructed by chemical*
60 *mutation, and showed high DGLA production. (19, 20).* However, some mutants and
61 recombinants showed unfavorable characteristics with respect to mycelia growth, spore
62 germination, and PUFA productivity, since the mutations and/or insertion of the plasmid
63 vector might occur at random positions, thereby introducing defects in essential genes.

64 Recently, it was reported that disruption of *lig4*, which encodes DNA ligase 4, a
65 key enzyme in non-homologous end joining, led to an improvement in gene-targeting
66 efficiency through homologous recombination in various filamentous fungi. *Lig4*

67 disruptants have been used as hosts for various applications (21-25). We previously
68 developed an efficient gene-targeting system through homologous recombination in *M.*
69 *alpina* 1S-4 by disrupting *lig4* to avoid unfavorable gene mutations and random
70 insertions (26).

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

76

77 MATERIALS AND METHODS

78 Enzymes and chemicals

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

82

83 Strains, media, and growth conditions

84 *M. alpina* 1S-4 has been deposited in the Graduate School of Agriculture of Kyoto
85 University (17). Czapek-Dox agar medium containing 0.05 mg/ml uracil, was used for
86 sporulation of the uracil auxotrophic (*ura5⁻*) strain, as described previously (27).

87 Strains used in this research were listed in Table 1. Synthetic complete (SC) medium
88 was used as a uracil-free synthetic medium to cultivate the transformants derived from
89 the uracil auxotrophs at 28°C (27). GY medium (2% [w/vol] glucose and 1% yeast
90 extract) was used for fatty acid analysis and extraction of genomic DNA. GY agar
91 plates containing 0.1% 5-fluoroorotic acid (5-FOA) were used to obtain uracil
92 auxotrophs from the *lig4* gene-deleted (*Δlig4::ura5*) strain (26). Uracil-free SC agar
93 plates containing 0.03% Nile blue A (Sigma-Aldrich, St. Louis, MO) and GY agar plates
94 containing 10 μg/ml carboxin and 0.03% Nile blue A were used to select transformants.

95 *Escherichia coli* strain DH5α was used for DNA manipulation and was grown on LB
96 agar plates containing 50 μg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was
97 used to transform the *M. alpina* 1S-4 *ura5⁻* strain. LB-Mg agar medium, minimal
98 medium, and induction medium were used for transformation, cultivation, and infection
99 of *A. tumefaciens*, respectively (28). All solid media were made with 2.0% agar.

100

101 **Isolation of uracil auxotrophs of the *Δlig4::ura5* strain from *M. alpina* 1S-4**

102 Uracil auxotrophs were isolated as described previously (29). The *Δlig4* strain
103 from *M. alpina* 1S-4 was incubated on Czapek-Dox agar medium at 28 °C for a month,
104 and allowed to sporulate at 12°C for a month. Spores of the *Δlig4::ura5* strain were
105 harvested from the surface of Czapek-Dox medium (2.6×10^8 spores/225 cm²); $2.6 \times$
106 10^7 spores were spread on GY agar medium containing 5-FOA (1.0 mg/ml) and uracil
107 (0.05 mg/ml).

108

109 **Identification of a mutation site on *ura5* integrated in the *lig4* genomic gene locus**

110 The *lig4* gene locus containing the *ura5* gene marker cassette was amplified with
111 Lig4 up F and Lig4 down R primers (Table 2), using genomic DNA isolated from the
112 uracil auxotroph of the *lig4* gene-deleted strain as a template. The PCR product was
113 sequenced with a Beckman-Coulter CEQ8000 system using Hispro stop F, *ura5* 300 F,
114 *ura5* 400 R, and SdhBt R primers (Table 2).

115

116 **Genomic DNA preparation**

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

120

121 **Construction of the plasmid vector for *Δ5ds* gene targeting**

122 A T-DNA binary vector for *Δ5ds* gene targeting was constructed by modifying the
123 *M. alpina* 1S-4 transformation vector pBIG35Zh (28). The *Δ5ds* front fragment
124 (1.2-kb) and *Δ5ds* rear fragment (2.4-kb) were amplified with primer sets, *Δ5* F1 *Apa*I

125 and $\Delta 5$ R1 *Apa*I, and $\Delta 5$ F2 *Xba*I and $\Delta 5$ R2 *Xba*I (Table 2), by using the genomic DNA
126 of *M. alpina* 1S-4 as a template. The *Δ5ds* rear fragment was digested with *Xba*I and
127 ligated into pBIG35Zh digested with same restriction enzyme. Then, the *Δ5ds* front
128 fragment was digested with *Apa*I and ligated into the resulting plasmid vectors digested
129 with same restriction enzyme and designated pBIG35Z Δ 5 (Fig. 2). The sequences
130 between the left and right borders were integrated into the genomic DNA of spores of *M.*
131 *alpina* 1S-4 through *A. tumefaciens*.

132

133 **Transformation of the *Alig4* strain of *M. alpina* 1S-4**

134 Spores of the uracil auxotroph of the *Alig4* strain was freshly prepared from
135 cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil,
136 and the suspension was filtered through Miracloth (Calbiochem) (27).

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

139

140 **Fatty acid analysis**

141 Fatty acid productivity and composition of the transformants were analyzed as
142 described previously (26). The mycelia of the *Alig4* uracil auxotroph strain used as a
143 host strain, the *Δ5ds* gene-defective mutant S14 (20), and the *Δ5ds* disruptants were
144 inoculated into 10 ml of GY liquid medium and cultivated at 28°C with reciprocal
145 shaking at 300 rpm for 7 days. After cultivation, the fungal strains were harvested by
146 filtration and used for fatty acid analysis. All experiments were performed in triplicate,
147 and the average of three separate experiments is presented as the mean $\pm$ SD in Table 3.

148

149 **Identification of gene disruption by PCR and Southern blot analysis**

150 Recombinants transformed with the pBIG35Z Δ D5 vector were repeatedly cultured
151 on fresh SC agar medium containing no uracil at least three times to prevent
152 contamination of abortive recombinants. *Δ5ds* disruptants were selected for PCR
153 analysis by using the *Δ5ds* genomic gene locus amplified with the Δ 5 up F and Δ 5 down
154 R primers (Table 2) as the template and primer sets, Δ 5 F1 *Apa*I and Δ 5 R1 *Apa*I, and
155 SdhBt stop F and Δ 5 down R2 (Table 2). When the deletion fragment from
156 pBIG35Z Δ D5 was inserted into the targeted *Δ5ds* gene locus, a 2.5-kb fragment was
157 amplified with SdhBt stop F and Δ 5 down R2 primers, indicating that the fragment was
158 integrated into the targeted *Δ5ds* gene locus (Fig. S1).

159 A single correct homologous integration resulting in partial replacement of the
160 *Δ5ds* gene with the introduced sequence from pBIG35Z Δ D5 was confirmed by
161 Southern blot analysis. The 0.6-kb partial *ura5* gene amplified with primers *ura5* start
162 F and *ura5* stop R (Table 2) using *ura5* from *M. alpina* 1S-4 as a template was used as a
163 probe for hybridization. Southern blot hybridization was performed as described
164 previously (28). Genomic DNA (10 μ g) digested with *Apa*I was size-fractionated by
165 electrophoresis in 1% agarose gel and transferred to Amersham Hybond-N⁺ membrane
166 (GE Healthcare Ltd., Buckinghamshire, UK). Southern hybridization was performed
167 with Amersham AlkPhos Direct™ Labeling Reagents (GE Healthcare) and
168 Amersham™ CDP-Star Detection Reagent (GE Healthcare), and chemical
169 luminescence was detected with the ImageQuant™ LAS 4000 mini system (GE
170 Healthcare).

171

172 RESULTS

173 Selection of a uracil auxotroph (*ura5*) from the *Δlig4::ura5* strain

174 To recycle the uracil marker system, a uracil auxotroph of the *Δlig4::ura5* strain
175 was selected on GY medium plate containing 0.1% 5-FOA. Spores of the *Δlig4::ura5*
176 strain were spread on GY plates containing 0.1% 5-FOA, and single colonies that
177 appeared after 10 days of cultivation were transferred onto GY plates containing 0.05%
178 5-FOA. To confirm the mutations on *ura5*, the sequence of the *ura5* marker fragment
179 amplified with the *Δlig4* gene locus was determined with a Beckman-Coulter CEQ8000
180 system. A base replacement was recognized in a selected uracil auxotroph; the
181 replacement of guanine with adenine occurred at the +212 nucleotide position, leading
182 to G71D.

183

184 Construction and confirmation of *Δ5ds* gene-disruption in the uracil auxotroph of 185 the *Δlig4::ura5* strain

186 To construct *Δ5ds* disruptants which accumulate high concentrations of DGLA,
187 spores of the uracil auxotroph of the *Δlig4::ura5* strain were transformed with
188 pBIG35ZΔD5 (Fig. 2) by the ATMT method (Fig. 3A). Integration of the disruption
189 fragment from pBIG35ZΔD5 was detected by PCR in 18 transformants (Fig. S1).
190 Total FAMES prepared from the transformants were analyzed by GC; nine transformants
191 accumulated high concentrations of DGLA, along with decreased ARA concentration.

192 To confirm *Δ5ds* disruption in the transformants, Southern blot analyses were
193 performed with their genomic DNAs (Fig. 3A and 3B). When the genomic DNAs
194 were digested with *ApaI*, the 5.0-kb positive signal corresponding to the replaced *Δ5ds*
195 locus was detected only in the transformants (Fig. 3B). The 10 kb signal illustrated

196 endogenous *ura5* gene locus, and the 7.9 kb signal illustrated the *ura5* gene that had
197 been used for disruption of *lig4* gene-disruption. We had confirmed that *lig4*
198 gene-disruption resulted a new 7.9 kb signal in previous study (26). This result
199 indicated that $\Delta 5ds$ had been successfully disrupted with the *ura5* marker from the
200 pBIG35Z Δ D5 plasmid vector in the transformants. In addition, nine transformants
201 exhibited genotypes of $\Delta 5ds$ gene-deletion, suggesting that the targeting efficiency of
202 the deletion in this study was 50% in the uracil auxotroph of the $\Delta lig4::ura5$ strain
203 (Table 4).

204

205 **Effect of $\Delta 5ds$ gene-disruption on DGLA production**

206 Transformants showed sufficient mycelial growth, sporulation, and spore
207 germination compared to wild-type strain (data not shown). Furthermore, we
208 compared the fatty acid productivities and compositions of the transformants with those
209 of the uracil auxotroph of $\Delta lig4::ura5$ strain (host) and the $\Delta 5ds$ gene-defective mutant
210 S14 (Table 3). The transformants showed improved productivities of total fatty acid in
211 GY liquid medium compared with the host strain and the $\Delta 5ds$ gene-defective mutant
212 S14 derived from wild-type strain by using chemical mutagen. The ratio of DGLA in
213 the total fatty acid content increased in the transformants. In particular, the DGLA
214 production in transformant #10 and #12 reached 884 mg/L broth (38.7%) and 778 mg/L
215 broth (40.1%), respectively, with no accumulation of ARA. By activation of the
216 endogenous $\omega 3$ -desaturase which converts DGLA to $\omega 3$ -eicosatetraenoic acid (20:4 $\omega 3$,
217 ETA) only at low temperature in *M. alpina* 1S-4, the $\Delta 5ds$ disruptants and mutant S14
218 accumulated ETA, calculated as 4% of the total fatty acid content, indicating that the
219 ratios were improved by 10-fold compared with that of the host strain (Table 3).

220 DISCUSSION

221 To use the *Δlig4* strain as a host strain for molecular breeding, we constructed a
222 uracil auxotroph from the *Δlig4::ura5* strain of *M. alpina* 1S-4 *Δlig4::ura5* strain. An
223 exchange of glycine to aspartic acid at the 71st amino acid position, which is highly
224 conserved in orotate phosphoribosyl transferases, may lead to inactivation of the
225 enzyme. The uracil auxotroph of the *Δlig4::ura5* strain showed no defect in mycelial
226 growth rate, formation of spores, and exhibited slightly improved fatty acid productivity
227 (Table 3), which indicate that this fungus may function as a suitable host for PUFA
228 production.

229 We constructed DGLA-producing recombinants derived from *M. alpina* 1S-4 by
230 *Δ5ds* gene-disruption using gene targeting. Similar to the *ku80* gene-disruption
231 reported previously, we used biolistic particle bombardment for transformation in an
232 earlier study; however, this method was limited by the fact that numerous vectors were
233 integrated in the genomic DNA of *M. alpina* 1S-4 (30). In the present research, *Δ5ds*
234 gene-disruption was achieved by the ATMT method (28) through double-crossover
235 homologous recombination using the *Δlig4* strain, which is a suitable host for efficient
236 gene-targeting (26). By this method, *Δ5ds* was successfully replaced with a single
237 vector fragment in *M. alpina* 1S-4 with a gene-targeting efficiency of 50%. The
238 targeting efficiency was as high as that of *lig4*-replacement in the *Δlig4::ura5* strain
239 from *M. alpina* 1S-4. As a result of *Δ5ds* disruption, DGLA composition of total fatty
240 acid was drastically increased with no conversion to ARA because of no *Δ5*-desaturase
241 activity. The ratio of DGLA in the total fatty acid content reached 40.1%, with no
242 accumulation of ARA (Table 3), suggesting that a single integration of the vector in
243 *Δ5ds* did not affect the growth rate and PUFA productivities. In the earlier study on

244 *Δ5ds*-defective mutants including the S14 strain, the mutation sites on their *Δ5ds* genes
245 were found to be point mutations of amino acids or frameshifts in translation (31).
246 Although the mutants exhibited increased DGLA accumulation, low concentrations of
247 ARA were also detected. Separation of DGLA from ARA is difficult during PUFA
248 purification, because of their similar properties. Therefore, the *Δ5ds*-disruptant
249 reported here may have applications in the industrial production of DGLA.

250 In summary, we successfully constructed a high DGLA-producer from *M. alpina*
251 1S-4 by *Δ5ds* gene-disruption. To the best of our knowledge, this is the first study to
252 report the construction of DGLA-producing transformants using the efficient
253 gene-targeting system in *M. alpina* 1S-4. The DGLA producer may have applications
254 in industrial DGLA production. Moreover, the uracil auxotroph from the *Δlig4::ura5*
255 strain may be used as a host for the construction of various PUFA-producing
256 recombinants.

257

258 **ACKNOWLEDGMENTS**

259 This work was partially supported by Grants-in Aid for Scientific Research of
260 Japan (no. 22380051 to E. Sakuradani and no. 23248014 to J. Ogawa), the Program for
261 Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry
262 (BRAIN) of Japan, the Science & Technology Research Promotion Program for
263 Agriculture, Forestry, Fisheries, and Food Industry from the Ministry of Agriculture,
264 Forestry & Fisheries of Japan, the Advanced Low Carbon Technology Research and
265 Development Program (ALCA) of Japan, and the Japan Society for the Promotion of
266 Science (JSPS).

267

268

269 REFERENCES

270

- 271 1. **Horrobin, D. F.:** Pharmaceutical compositions containing one or more of
 272 gamma-linolenic acid dihomogamma-linolenic and an arachidonic acid, European patent,
 273 **EP 0678299 A2**, (1995).
- 274 2. **Melnik, B. and Plewig, G.:** Agent for the atopy prophylaxis, European patent, **EP**
 275 **0391218 A2**, (1990).
- 276 3. **Shimizu, S., Shinmen, Y., Kawashima, H., Akimoto, K. and Yamada, H.:** Fungal
 277 mycelia as a novel source of eicosapentaenoic acid: Activation of enzyme(s) involved in
 278 eicosapentaenoic acid production at low temperature. *Biochem. Biophys. Res. Commun.*, **150**,
 279 335-341 (1988).
- 280 4. **Fu-Lin, E. and Dupuy, J. L.:** The fatty acid composition of three unicellular algal
 281 species used as food sources for larvae of the American oyster. *Lipids*, **15**, 356-364 (1980).
- 282 5. **Hulanicka, D., Erwin, J. and Bloch, K.:** Lipid metabolism of *Euglena gracilis*. *J.*
 283 *Biol. Chem.*, **239**, 2778-87 (1964).
- 284 6. **Mead, J. F.:** The metabolism of the polyunsaturated fatty acids, p. 159-192. In
 285 Holman, R. T. (ed.), *Progress in the Chemistry of Fats and other Lipids*, vol. 9. Oxford,
 286 Pergamon Press, Inc. (1971).
- 287 7. **Sakuradani, E., Ando, A., Ogawa, J. and Shimizu, S.:** Improved production of
 288 various polyunsaturated fatty acids through filamentous fungus *Mortierella alpina* breeding.
 289 *Appl. Microbiol. Biotechnol.*, **84**, 1-10 (2009).
- 290 8. **Kawashima, H., Akimoto, K., Fujita, T., Naoki, H., Konishi, K. and Shimizu, S.:**
 291 Preparation of ¹³C-labeled polyunsaturated fatty acids by an arachidonic acid-producing
 292 fungus *Mortierella alpina* 1S-4. *Anal. Biochem.*, **229**, 317-22 (1995).
- 293 9. **Kikukawa, H., Sakuradani, E., Kishino, S., Park, S.-B., Ando, A., Shima, J., Ochiai,**
 294 **M., Shimizu, S. and Ogawa, J.:** Characterization of a trifunctional fatty acid desaturase from
 295 oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system. *J.*

296 Biosci. Bioeng., **116**, 672-676 (2013).

297 10. **Sakuradani, E., Abe, T., Iguchi, K. and Shimizu, S.:** A novel fungal
 298 omega3-desaturase with wide substrate specificity from arachidonic acid-producing
 299 *Mortierella alpina* 1S-4. Appl. Microbiol. Biotechnol., **66**, 648-54 (2005).

300 11. **Sakuradani, E., Kobayashi, M., Ashikari, T. and Shimizu, S.:** Identification of
 301 Delta12-fatty acid desaturase from arachidonic acid-producing *Mortierella* fungus by
 302 heterologous expression in the yeast *Saccharomyces cerevisiae* and the fungus *Aspergillus*
 303 *oryzae*. Eur. J. Biochem., **261**, 812-20 (1999).

304 12. **Sakuradani, E., Kobayashi, M. and Shimizu, S.:** Delta 9-fatty acid desaturase from
 305 arachidonic acid-producing fungus. Unique gene sequence and its heterologous expression in
 306 a fungus, *Aspergillus*. Eur. J. Biochem., **260**, 208-16 (1999).

307 13. **Sakuradani, E., Kobayashi, M. and Shimizu, S.:** Delta6-fatty acid desaturase from
 308 an arachidonic acid-producing *Mortierella* fungus. Gene cloning and its heterologous
 309 expression in a fungus, *Aspergillus*. Gene, **238**, 445-53 (1999).

310 14. **Sakuradani, E., Murata, S., Kanamaru, H. and Shimizu, S.:** Functional analysis of
 311 a fatty acid elongase from arachidonic acid-producing *Mortierella alpina* 1S-4. Appl.
 312 Microbiol. Biotechnol., **81**, 497-503 (2008).

313 15. **Sakuradani, E., Nojiri, M., Suzuki, H. and Shimizu, S.:** Identification of a novel
 314 fatty acid elongase with a wide substrate specificity from arachidonic acid-producing fungus
 315 *Mortierella alpina* 1S-4. Appl. Microbiol. Biotechnol., **84**, 709-16 (2009).

316 16. **Sakuradani, E. and Shimizu, S.:** Gene cloning and functional analysis of a second
 317 delta 6-fatty acid desaturase from an arachidonic acid-producing *Mortierella* fungus. Biosci.
 318 Biotechnol. Biochem., **67**, 704-11 (2003).

319 17. **Sakuradani, E.:** Advances in the production of various polyunsaturated fatty acids
 320 through Oleaginous Fungus *Mortierella alpina* breeding. Biosci. Biotechnol. Biochem., **74**,
 321 908-17 (2010).

322 18. **Sakuradani, E., Ando, A., Shimizu, S. and Ogawa, J.:** Metabolic engineering for the

323 production of polyunsaturated fatty acids by oleaginous fungus *Mortierella alpina* 1S-4. J.
324 Biosci. Bioeng., **116**, 417-22 (2013).

325 19. **Jareonkitmongkol, S., Kawashima, H., Shirasaka, N., Shimizu, S. and Yamada, H.:**
326 Production of Dihomo-gamma-Linolenic Acid by a Delta5-Desaturase-Defective Mutant of
327 *Mortierella alpina* 1S-4. Appl. Environ. Microbiol., **58**, 2196-200 (1992).

328 20. **Jareonkitmongkol, S., Sakuradani, E. and Shimizu, S.:** A Novel
329 Delta5-Desaturase-Defective Mutant of *Mortierella alpina* 1S-4 and Its
330 Dihomo-gamma-Linolenic Acid Productivity. Appl. Environ. Microbiol., **59**, 4300-4 (1993).

331 21. **Kawauchi, M., Nishiura, M. and Iwashita, K.:** Fungus-specific sirtuin HstD
332 coordinates secondary metabolism and development through control of LaeA. Eukaryot. Cell,
333 **12**, 1087-96 (2013).

334 22. **Maruyama, J. and Kitamoto, K.:** Multiple gene disruptions by marker recycling
335 with highly efficient gene-targeting background (DeltaligD) in *Aspergillus oryzae*.
336 Biotechnol. Lett., **30**, 1811-7 (2008).

337 23. **Mizutani, O., Kudo, Y., Saito, A., Matsuura, T., Inoue, H., Abe, K. and Gomi, K.:** A
338 defect of LigD (human Lig4 homolog) for nonhomologous end joining significantly improves
339 efficiency of gene-targeting in *Aspergillus oryzae*. Fungal Genet. Biol., **45**, 878-89 (2008).

340 24. **Yoon, J., Aishan, T., Maruyama, J. and Kitamoto, K.:** Enhanced production and
341 secretion of heterologous proteins by the filamentous fungus *Aspergillus oryzae* via
342 disruption of vacuolar protein sorting receptor gene *Aovps10*. Appl. Environ. Microbiol., **76**,
343 5718-27 (2010).

344 25. **Ishibashi, K., Suzuki, K., Ando, Y., Takakura, C. and Inoue, H.:** Nonhomologous
345 chromosomal integration of foreign DNA is completely dependent on MUS-53 (human Lig4
346 homolog) in *Neurospora*. Proc. Natl. Acad. Sci. USA, **103**, 14871-6 (2006).

347 26. **Kikukawa, H., Sakuradani, E., Ando, A., Okuda, T., Ochiai, M., Shimizu, S. and**
348 **Ogawa, J.:** Disruption of *lig4* improves gene targeting efficiency in the oleaginous fungus
349 *Mortierella alpina* 1S-4. J. Biotechnol., **208**, 63-69 (2015).

350 27. **Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H.,**
351 **Ashikari, T. and Shimizu, S.:** Establishment of an overall transformation system for an
352 oil-producing filamentous fungus, *Mortierella alpina* 1S-4. Appl. Microbiol. Biotechnol., **65**,
353 419-25 (2004).

354 28. **Ando, A., Sumida, Y., Negoro, H., Suroto, D. A., Ogawa, J., Sakuradani, E. and**
355 **Shimizu, S.:** Establishment of Agrobacterium tumefaciens-mediated transformation of an
356 oleaginous fungus, *Mortierella alpina* 1S-4, and its application for eicosapentaenoic acid
357 producer breeding. Appl. Environ. Microbiol., **75**, 5529-35 (2009).

358 29. **Takeno, S., Sakuradani, E., Murata, S., Inohara-Ochiai, M., Kawashima, H.,**
359 **Ashikari, T. and Shimizu, S.:** Cloning and sequencing of the *ura3* and *ura5* genes, and
360 isolation and characterization of uracil auxotrophs of the fungus *Mortierella alpina* 1S-4.
361 Biosci. Biotechnol. Biochem., **68**, 277-85 (2004).

362 30. **Kikukawa, H., Sakuradani, E., Ando, Nakatani, M., A., Okuda, T., Sakamoto, T.,**
363 **Ochiai, M., Shimizu, S. and Ogawa, J.:** Gene targeting in the oil-producing fungus
364 *Mortierella alpina* 1S-4 and construction of a strain producing a valuable polyunsaturated
365 fatty acid. Curr. Genet., <http://dx.doi.org/10.1007/s00294-015-0481-2> (Published online 18
366 March 2015).

367 31. **Abe, T., Sakuradani, E., Ueda, T. and Shimizu, S.:** Identification of mutation sites
368 on $\Delta 5$ desaturase genes from *Mortierella alpina* 1S-4 mutants. J. Biosci. Bioeng., **99**, 296-299
369 (2005).

370

371

372

373 **Figure legends**

374

375 **FIG. 1.** Polyunsaturated fatty acid (PUFA) biosynthetic pathways in *Mortierella*
376 *alpina* 1S-4. ARA is synthesized by the desaturation of DGLA at the $\Delta 5$ -position by
377 $\Delta 5$ -desaturase. LA, linoleic acid; GLA, γ -linolenic acid; DGLA, dihomo- γ -linolenic
378 acid; ARA, arachidonic acid; ETA, $\omega 3$ -eicosatetraenoic acid; EPA, eicosapentaenoic
379 acid; $\Delta 6$, $\Delta 6$ -desaturase; GLELO, $\Delta 6$ -elongase; $\Delta 5$, $\Delta 5$ -desaturase; $\omega 3$, $\omega 3$ -desaturase.

380

381 **FIG. 2.** The pBIG35Z Δ D5 vector used for *$\Delta 5ds$* gene-disruption. LB, left border;
382 RB, right border; *His550p*, *M. alpina* 1S-4 histone H4.1 promoter short fragment; *ura5*,
383 orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *SdhBt*, *M. alpina* 1S-4 *sdhB*
384 translation terminator; *NPTIII*, neomycin phosphotransferase III gene; *TrfA*, *trfA* locus,
385 which produces two proteins that promote replication of the plasmid; *ColEI ori*, ColEI
386 origin of replication; *oriV*, pRK2 origin of replication.

387

388 **FIG. 3.** *$\Delta 5ds$* gene disruption and confirmation of the disruption. (A) The figure
389 illustrates the homologous integration of the pBIG35Z Δ D5 vector into the *$\Delta 5ds$* gene
390 locus in *M. alpina* 1S-4. The *gray short bar* indicates the position hybridized by the
391 probe. *Dotted lines* indicate the positions and base lengths of the hybridization signals.
392 (B) Southern hybridization analysis of the *$\Delta 5ds$* gene disruptant. *ApaI*- and
393 *XbaI*-digested genomic DNAs were hybridized with the probes. Lane 1, wild-type;
394 Lane 2, *$\Delta 5ds$* gene disruptant.

395

396 **Figure S1.** Construction scheme and confirmation of *$\Delta 5ds$* disrupted strains. (A)

397 The figure illustrates the integration of the pBIG35Z Δ D5 vector into the *Δ5ds* gene
398 locus in *M. alpina* 1S-4. *Black arrows* (a–d) indicate the position of the
399 oligonucleotide primers (Table 2) used for the PCR analysis: a, SdhBt stop F; b, Δ 5
400 down R2; c, Δ 5 F1 *Apa*I; d, Δ 5 R1 *Apa*I. (B) Selection of the *Δ5ds* disruptant by PCR
401 analysis.

402

403

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

2	Strain	Parental strain	Genotype	Reference
3	<i>M. alpina</i> 1S-4	-	Wild-type	(7)
4	Uracil auxotroph of wild -type	Wild-type	<i>ura5⁻</i>	(7)
5	<i>Δ5ds</i> -defective mutant S14	Wild-type	<i>Δ5ds⁻</i>	(20)
6	<i>Δlig4</i>	Uracil auxotroph of wild-type	<i>ura5⁻ Δlig4::ura5</i>	(26)
7	Uracil auxotroph of <i>Δlig4</i>	<i>Δlig4</i>	<i>ura5⁻ Δlig4::ura5⁻</i>	This study
8	<i>Δ5ds</i> -disputants	Uracil auxotroph of <i>Δlig4</i>	<i>ura5⁻ Δlig4::ura5⁻ ΔΔ5ds::ura5</i>	This study

9

10

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
$\Delta 5$ F1 <i>Apa</i> I	GCGGGCCCTTTTGTTCCTTGATCCTCACATTC
$\Delta 5$ R1 <i>Apa</i> I	GCGGGCCCAAGAAGTTTGTGACATCGTATAC
$\Delta 5$ F2 <i>Xba</i> I	GCTCTAGATCTGTTCCCGAACGTTTCCCAGC
$\Delta 5$ R2 <i>Xba</i> I	GGTCTAGAGCGATAATTATGAAAACCTTACC
$\Delta 5$ up F	CGTCCTCTACGATCCTCTGACCGAC
$\Delta 5$ down R	CCACATGGTACCAAAGCGTGGGCTG
SdhBt stop F	CGAGATGTCGCAGCTGAGCTAAGC
$\Delta 5$ down R2	GCTCTCTCATCCTGTCACATGC
ura5 start F	GGATTACCAGCGCGAGTTC
ura5 stop R	TTAAACACCGTACTTCTCGCGG

^aThe underlined sequences represent the synthesized restriction enzyme sites.

1 **Table 3** Fatty acid productivity and composition of the *M. alpina* 1S-4 host (*Alig4::ura5⁺*), *Δ5ds*-defective mutant, and *Δ5ds*-disruptants^a

2	Strain	Cultivation	Total fatty acid (g/L)	Fatty acid composition ^b (%)									
		temperature (°C)		16:0	18:0	OA	LA	GLA	DGLA	ARA	ETA	EPA	Others
4	<i>M. alpina</i> 1S-4 <i>Alig4</i>	28	1.83 ± 0.13	16.5 ± 0.8	7.1 ± 0.5	21.5 ± 1.1	6.6 ± 0.3	3.6 ± 0.1	5.4 ± 0.1	20.8 ± 1.1	N. D. ^c	N. D.	18.6 ± 0.7
5		12	0.20 ± 0.02	12.1 ± 1.0	2.8 ± 0.4	22.8 ± 0.6	8.5 ± 0.4	7.2 ± 0.7	3.8 ± 0.5	15.9 ± 1.1	0.4 ± 0.1	3.0 ± 0.3	26.3 ± 2.3
6	<i>Δ5ds</i> -defective mutant S14	28	1.88 ± 0.00	26.5 ± 3.9	6.8 ± 0.5	13.9 ± 1.8	3.7 ± 0.1	3.6 ± 0.9	26.7 ± 3.9	0.2 ± 0.0	N. D.	N. D.	18.7 ± 5.8
7		12	0.30 ± 0.00	17.5 ± 0.2	3.1 ± 0.4	21.4 ± 0.8	4.5 ± 0.1	9.3 ± 0.1	21.3 ± 0.5	0.2 ± 0.0	4.1 ± 0.2	N. D.	18.6 ± 1.2
8	<i>Δ5ds</i> -disruptant #6	28	2.10 ± 0.26	20.4 ± 0.4	7.0 ± 1.0	20.3 ± 1.2	3.5 ± 0.1	5.1 ± 0.5	30.4 ± 1.2	N. D.	N. D.	N. D.	13.3 ± 0.5
9		12	0.40 ± 0.16	17.1 ± 1.2	7.7 ± 1.1	18.0 ± 1.7	5.2 ± 0.6	8.6 ± 1.3	25.5 ± 3.3	N. D.	4.1 ± 0.9	N. D.	13.7 ± 2.0
10	#8	28	2.14 ± 0.24	20.6 ± 1.8	8.1 ± 0.9	20.4 ± 3.3	3.9 ± 0.3	4.6 ± 0.1	27.3 ± 5.0	1.2 ± 1.3	N. D.	N. D.	13.9 ± 0.8
11		12	0.37 ± 0.07	17.6 ± 0.6	6.5 ± 0.1	16.6 ± 0.5	5.5 ± 0.3	9.3 ± 0.7	27.3 ± 0.7	N. D.	4.5 ± 0.4	N. D.	12.7 ± 0.5
12	#10	28	2.29 ± 0.13	16.6 ± 0.4	8.8 ± 0.1	11.9 ± 0.3	3.2 ± 0.3	4.6 ± 0.1	38.7 ± 0.5	N. D.	N. D.	N. D.	16.2 ± 0.4
13		12	0.44 ± 0.08	16.3 ± 1.1	8.4 ± 0.3	16.2 ± 0.6	5.2 ± 0.2	8.5 ± 0.6	28.7 ± 0.3	N. D.	4.4 ± 0.7	N. D.	12.2 ± 0.6

#12	28	1.94 ± 0.28	16.1 ± 0.5	7.7 ± 0.1	11.5 ± 0.5	2.9 ± 0.1	4.7 ± 0.0	40.1 ± 1.2	N. D.	N. D.	N. D.	16.9 ± 0.4
	12	0.55 ± 0.14	17.5 ± 0.6	7.5 ± 0.4	17.2 ± 0.6	5.0 ± 0.1	8.3 ± 0.2	28.1 ± 1.2	0.0 ± 0.1	4.1 ± 0.1	N. D.	12.2 ± 1.4

^aThe strains were grown in 10 ml of GY liquid medium for 7 days at 12°C or 28°C with shaking at 120 rpm. All the values are for three independent samples (mean ± SD).

^bOA, 18:1, oleic acid; LA, 18:2, linoleic acid; GLA, 18:3, γ -linolenic acid; DGLA, 20:3, dihomog- γ -linolenic acid; ARA, ω 6-20:4, arachidonic acid; ETA, ω 3-20:4, eicosatetraenoic acid.

^cNot detected.

Table 4 Efficiency of gene targeting through homologous recombination at the *lig4* locus with pBIG3CBZΔL4 in the *M. alpina* 1S-4 wild type and Δ*lig4* strains.

Host strain	Number of transformants	Number of homologous recombinants	Gene targeting efficiency (%) ^a	Reference
<i>lig4</i> -disruption				
Wild-type	93	3	3.2	(26)
Δ <i>lig4::ura5</i>	3	2	66.7	(26)
Δ <i>5ds</i> -disruption	18	9	50.0	This study

^aThe efficiency of gene targeting was calculated by the ratio of the number of homologous recombinant strains to the total number of transformants obtained.