

**Metabolic analysis and development of
efficient gene-targeting systems in oleaginous fungi
for useful lipid production**

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ABBREVIATIONS

14:0	Tetradecanoic acid
16:0	Hexadecanoic acid
16:1	Hexadecenoic acid
16:2	Hexadecadienoic acid
16:3	Hexadecatrienoic acid
18:0	Octadecanoic acid
18:1	Octadecenoic acid
18:2	Octadecadienoic acid
18:3	Octadecatrienoic acid
20:0	Eicosanoic acid
20:1	Eicosenoic acid
20:2	Eicosadienoic acid
20:3	Eicosatrienoic acid
20:4	Eicosatetraenoic acid
20:5	Eicosapentaenoic acid
22:0	Docosanoic acid
24:0	Tetracosanoic acid
ALA	α -Linolenic acid, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-octadecatrienoic acid (18:3 ω 3)
ARA	Arachidonic acid, <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-eicosatetraenoic acid (20:4 ω 6)
DGLA	Dihomo- γ -linolenic acid, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-eicosatrienoic acid (20:3 ω 6)
EA	<i>cis</i> -11-Eicosenoic acid (20:1 ω 9)
EPA	Eicosapentaenoic acid, <i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-eicosapentaenoic acid (20:5 ω 3)
ETA	ω 3-Eicosatetraenoic acid, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> -17-eicosatetraenoic acid (20:4 ω 3)
GLA	γ -Linolenic acid, <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-octadecatrienoic acid (18:3 ω 6)
LA	Linoleic acid, <i>cis</i> -9, <i>cis</i> -12-octadecadienoic acid (18:2 ω 6)
OA	Oleic acid, <i>cis</i> -9-octadecenoic acid (18:1 ω 9)
SDA	Stearidonic acid, <i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-octadecatetraenoic acid (18:4 ω 3)

ABBREVIATIONS

TAG	Triacylglycerol
FFA	Free fatty acid
DAG	Diacylglycerol
PL	Polar lipid
5-FOA	5-Fluoroorotic acid
ATMT	<i>Agrobacterium tumefaciens</i> -mediated transformation
bp	Base pair(s)
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DSB	DNA double strand break
$\Delta 5ds$	$\Delta 5$ -desaturase
GC	Gas-liquid chromatography
HR	homologous recombination
kb	Kilobase(s)
LB medium	Luria-Bertani medium
MMS	Methyl methanesulfonate
mRNA	Messenger RNA
NHEJ	non-homologous end joining
<i>maw3</i>	$\omega 3$ -desturase gene from <i>Mortierella alpina</i> 1S-4
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acid
RNA	Ribonucleic acid
SdhB	Succinate dehydrogenase subunit B

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) play important roles as structural component of membrane phospholipids, as well as precursors of signaling molecules eicosanoids including prostaglandins, thromboxanes and leukotrienes [1]. The representative PUFAs are γ -linolenic acid (18:3 ω 6, GLA), α -linolenic acid (18:3 ω 3, ALA), dihomogamma-linolenic acid (20:3 ω 6, DGLA), arachidonic acid (20:4 ω 6, ARA), and eicosapentaenoic acid (20:5 ω 3, EPA). Mammals including human synthesize eicosanoids which are involved in inflammatory responses, reproductive function, immune response, and repression of blood pressure [2].

PUFAs are produced from a natural source, such as fish oils, sea animals, and plant oils. However, these sources have some disadvantages, including unstable and limited supply, lower PUFAs content, and undesirable contaminations. Recent investigations have focused on PUFAs production by alternative source such as oleaginous bacteria, fungi, plants and microalgae [3, 4]. In particular, oleaginous microorganisms are more suitable as an alternative source for PUFAs production than conventional sources, because these microorganisms can be cultivated easily and rapidly on a large scale and produce considerable amounts of high-quality PUFAs.

Mortierella spp. are known to produce PUFAs like GLA and ARA. In particular, *M. alpina* possesses five kinds of fatty acid desaturases, i.e., Δ 9, Δ 12, Δ 6, Δ 5, and ω 3-desaturases [5-10], and three kinds of elongases, i.e., GLELO, MAELO, and MALCE1 [11-13], involved in PUFAs biosynthetic pathways. ARA comprises more than 30% of the total fatty acids in *M. alpina* but, in contrast, EA comprises less than 3% [14-16].

In this study, I focus on oleaginous fungi as functional fatty acid producers and carried out screening of eicosanoic acid-producing fungi, characterization of a fatty acid desaturase for ω 3-PUFA synthesis, construction of an efficient gene-targeting system, and molecular breeding of selective PUFA-producers by using the gene-targeting system.

Chapter I describes the screening of fungi producing *cis*-11-eicosanoic acid (20:1 ω 9, EA) which is beneficial as a raw material for medical supplies and a moisturizing component of cosmetic creams. In this chapter, I found some EA-producing fungi by screening of about 300 fungal strains.

Chapter II describes characterization of a ω 3-fatty acid desaturase gene (*maw3*) from the oil-producing zygomycete *Mortierella alpina* 1S-4. In this chapter, I investigated the products of

maw3 catalyzing reaction with endogenous and exogenous fatty acids in the yeast transformant.

Chapter III describes construction of an efficient gene-targeting system by *ku80* disruption in *M. alpina* 1S-4. I identified the *ku80* gene encoding the Ku80 protein and constructed *ku80* gene-disrupted strains via single-crossover homologous recombination. Dihomo- γ -linolenic acid-producing strains were constructed by disruption of the $\Delta 5$ -desaturase gene, encoding a key enzyme of bioconversion of DGLA to ARA, using the $\Delta ku80$ strain as a host strain.

Chapter IV describes the development of an efficient gene-targeting system by *lig4* disruption in *M. alpina* 1S-4. I identified the *lig4* gene encoding a DNA ligase 4 homolog related to the general pathway of non-homologous end joining and constructed *lig4* gene-disrupted strains for improvement of gene-targeting efficiency. The gene-targeting efficiency was estimated in the *lig4* disruptant.

Chapter V describes molecular breeding for DGLA-producer using an efficient gene-targeting system in *M. alpina* 1S-4. I constructed DGLA-producing strains with disruption of the $\Delta 5$ -desaturase gene by efficient gene-targeting system using the *lig4* disruptant as a host.

CHAPTER I

Production of *cis*-11-eicosenoic acid by *Mortierella* fungi

cis-11-Eicosenoic acid (20:1 ω 9, EA) is a monounsaturated fatty acid called gondoic acid that is contained in a variety of plant oils and nuts. In particular, EA represents 70% of the total fatty acids in jojoba oil, and eicosenol derived from EA represents more than 40% of the total fatty alcohols [17]. Jojoba oil is remarkable for its industrial usage, i.e., in engine lubricating oil, pharmaceutical compounds and cosmetics. EA is also expected to be a raw material for medical supplies and a moisturizing component of cosmetic creams. In higher plants, EA is a precursor of erucic acid (22:1 ω 9, *cis*-13-docosenoic acid). Erucic acid is beneficial for various materials such as creams, cosmetics, lubricating oil, biodiesel, and therapeutic medicine [18, 19]. It is supposed that EA is synthesized by a Δ 9-fatty acid elongase (Δ 9EL) from oleic acid (18:1 ω 9, OA) as a precursor. In the previous study, it was revealed that Δ 9EL derived from *Thraustochytrium aureum* converts fatty acid substrates with the first double bond at the 9 carbon atom position away from the carboxyl end (Δ 9-position) such as OA, linoleic acid (18:2 ω 6, LA), and α -linolenic acid (18:3 ω 3, ALA) through the sequential addition of C₂ units from malonyl-CoA [20]. As for higher plants, a fatty acid elongase 1 (*FAEI*) showing Δ 9EL-like activity catalyzes the condensation of malonyl-CoA with a long chain acyl-CoA to give a 3-ketoacyl-CoA, which is reduced to 3-hydroxyacyl-CoA, dehydrated to enoyl-CoA and finally reduced to an elongated acyl-CoA [21].

Mortierella spp. are known to produce polyunsaturated fatty acids (PUFAs) like γ -linolenic acid (18:3 ω 6, GLA) and arachidonic acid (20:4 ω 6, ARA). In particular, *Mortierella alpina* possesses five kinds of fatty acid desaturases [5-10] and three kinds of elongases [11-13], involved in PUFA biosynthetic pathways as described in INTRODUCTION. ARA comprises more than 30% of the total fatty acids in *M. alpina* but, in contrast, EA comprises less than 3% [14-16].

In this study, I screened for EA-producing fungi among about 300 fungal strains by fatty acid analysis with GC. *Mortierella chlamydospora* CBS 529.75 was selected as a candidate to produce EA. This fungus was an EA-producing fungus isolated from soil and belongs to the *Mortierellales* [22]. I investigated the EA productivity of *M. chlamydospora* CBS 529.75. This is the first report concerning EA-producing microorganisms.

MATERIALS AND METHODS

Microorganisms

The fungi tested in this study were purchased from the Centraalbureau voor Schimmelcultures (CBS) in the Netherlands, and the Biological Resource Center in the National Institute of Technology and Evaluation (NBRC), and the National Institute of Agrobiological Sciences (NIAS) in Japan.

Growth media, and culture conditions

For screening of EA-producing strains, they were cultured on GY agar medium (comprising 10 g/L glucose, 5 g/L yeast extract, 15 g/L agar, and 50 µl/L Triton X-100, pH 6.0). For evaluation of EA productivity by *M. chlamydospora* CBS 529.75, it was cultured in GY liquid medium (comprising 20 g/L glucose and 10 g/L yeast extract, pH 6.0). Individual culture conditions are given in each figure legend and table.

Fatty acid analysis

Fatty acid productions and compositions in transformants were analyzed as described previously [5] with a slight modification. The mycelia of the *lig4* gene-disrupted strain and its host strain were inoculated into 10 ml of GY liquid medium and cultivated at 28°C with reciprocal shaking at 300 rpm for 7 days. The fungal strains after cultivation were harvested by filtration and dried at 120°C for 3 h. The dried cells were directly transmethylated with 10% methanolic HCl at 55°C for 2 h. The resulting fatty acid methyl esters (FAMES) were extracted with *n*-hexane, concentrated and analyzed by GC-2010 Plus gas chromatography (GC; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (GL Science Inc., Tokyo, Japan). Fatty acid quantification was calculated with tricosanoic acid as an internal standard. All experiments were carried out in triplicate.

Identification of EA from *M. chlamydospora* CBS 529.75

The FAMES prepared from *M. chlamydospora* CBS 529.75 were separated on a Cosmosil 5C18-AR column (20 × 250 mm, Nacalai Tesque, Kyoto, Japan) by HPLC with a LC-10A system (Shimadzu, Kyoto, Japan) with a 90% (v/v) acetonitrile aqueous solution as the mobile phase at

a flow rate of 4.0 ml/min. The temperature in the column oven (CTO-20AC, Shimadzu) was set at 35°C, and the absorbance at 205 nm was monitored with a UV/VIS detector (SPD-20A, Shimadzu). The pyrrolidine derivative of the purified EA was prepared as described by Andersson and Holman [23], and analyzed with a GC-MS QP5050 system (Shimadzu). An HR-1 column (0.5 mm I.D. $\times$ 50 m; Shinwa Chemical Industries) was used for separation of the pyrrolidine derivative. The initial column temperature was set at 180°C and was raised to 300°C at a rate of 5°C/min. The apparatus provides the electron impact mode at 70 eV with a source temperature of 250°C. The injection temperature was set at 250°C.

Lipid analysis

Total lipids from *M. chlamydospora* CBS 529.75 were extracted by means of the Bligh-Dyer method [24]. The lipids were analyzed by thin layer chromatography (TLC) using a solvent system of *n*-hexane/diethyl ether/acetic acid (80/30/1, v/v/v). TLC glass plates (Silica gel 60, 20 $\times$ 20 cm) were purchased from Merck (Darmstadt, Germany). After separation of lipids, each plate was sprayed with 0.01% (w/v) primulin in an 80% (v/v) acetone aqueous solution for UV detection of each lipid at 254 nm. The lipid fractions were collected from the plate, directly transmethylated, and quantitatively analyzed for their fatty acids by GC as described above. All data are the means of triplicate experiments.

RESULTS

Screening of EA-producing fungi

About three-hundred fungal strains were tested for EA production, being cultivated on GY agar medium at 28°C for 2 days and then at 12°C for 2 days. The FAMES prepared from each fungal strain were analyzed by GC to determine the content of EA in the total fatty acids. The seven strains listed in Table 1-1 belonging to *Mortierella kuhlmanii*, *Mortierella lignicola*, and *Mortierella chlamydospora* were found to show high contents of EA in the total fatty acids. In some strains, the EA contents increased with the shift in cultivation temperature from 20°C to 4°C (Table 1-1). At the lower temperature, the EA contents of the five strains of *M. chlamydospora* reached more than 20% of the total fatty acids. To evaluate the EA productivities of the five strains of *M. chlamydospora*, the FAMES prepared from each strain cultivated in 5 ml of GY liquid medium were quantitatively analyzed by GC (Fig. 1-1). At the cultivation temperature of 28°C, *M. chlamydospora* CBS 529.75 showed the highest production of EA (0.14 mg/ml of broth). With shift of the cultivation temperature to 12°C, *M. chlamydospora* NBRC 32544 accumulated 0.25 mg/ml EA. The low temperature enhanced the accumulation of EA in the *M. chlamydospora* strains.

Table 1-1 EA contents in total fatty acids of selected strains.

Strain		EA content in total fatty acids (%)	
		20°C ^a	4°C ^b
<i>Mortierella kuhlmanii</i>	CBS 157.71	9.4	14.9
<i>Mortierella lignicola</i>	CBS 100594	6.8	10.5
<i>Mortierella chlamydospora</i>	CBS 529.75	17.4	23.6
<i>Mortierella chlamydospora</i>	NBRC 32542	25.0	24.5
<i>Mortierella chlamydospora</i>	NBRC 32544	27.2	23.5
<i>Mortierella chlamydospora</i>	CBS 751.83	24.0	26.1
<i>Mortierella chlamydospora</i>	MAFF425003	20.0	23.9

^a Strains were cultivated at 20°C for 4 days.

^b Strains were cultivated at 20°C for 4 days and then at 4°C for 3 days.

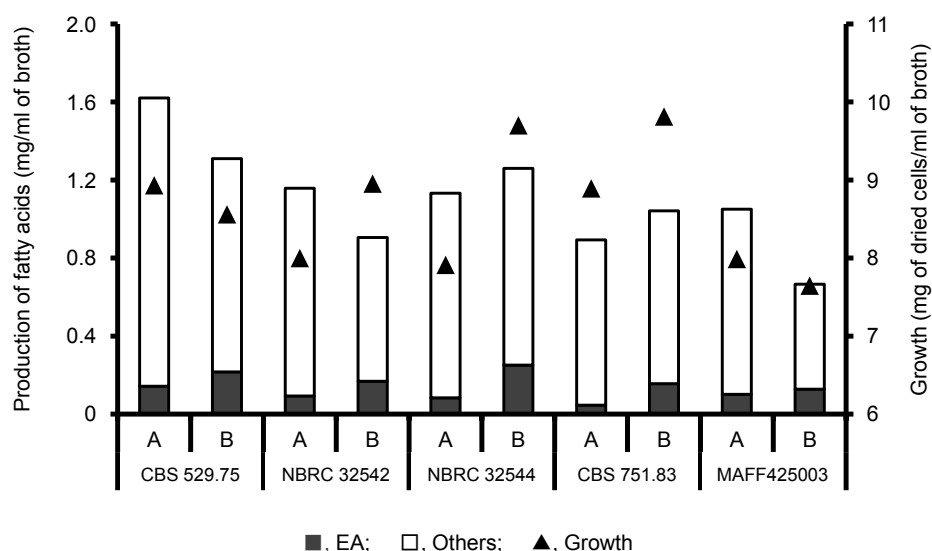


Fig. 1-1 EA production by *Mortierella* fungi. The fungi were cultured in 5 ml of GY liquid medium at 28°C for 7 days (A) or 28°C for 4 days and then at 12°C for 3 days (B) with shaking at 100 rpm. The bars indicate production of EA (■) or other fatty acids (□), and the diamonds indicate the growth (▲) of each strain.

The major FAME from *M. chlamydospora* CBS 529.75, assigned as EA based on comparison of its retention time on the GC chromatogram with that of authentic *cis*- and *trans*-EA, was purified by HPLC on a Cosmosil 5C18-AR column and then converted to pyrrolidine derivatives. GC-MS analysis of the pyrrolidine derivatives showed a molecular ion at m/z 363, and a gap of 26 atomic mass unit between m/z 224 and 250 (Fig. 1-2), indicating that the structure was that of a C20 monounsaturated fatty acid having one double bond at the $\Delta 11$ -position. Thus, the major fatty acid in *M. chlamydospora* CBS 529.75 was determined to be EA.

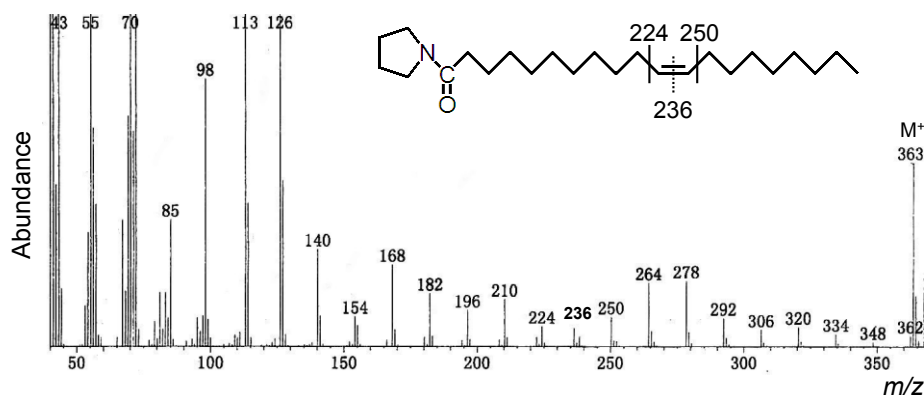


Fig. 1-2 GC-MS analysis of the pyrrolidide derivative of a major fatty acid produced by *M. chlamydospora* CBS 529.75.

EA content in each lipid fraction from *M. chlamydospora* CBS 529.75

M. chlamydospora CBS 529.75 was selected as a representative EA producer, and the total lipids extracted from the strain cultivated at 28°C for 7 days, or 28°C for 4 days and then at 12°C for 3 days were extracted. The individual lipid fractions, such as triacylglycerols (TAG), free fatty acids (FFA), diacylglycerols (1,3-DAG and 1,2-DAG), and polar lipids (PL), were separated by TLC as described under Materials and Methods. The lipid compositions and EA contents in total fatty acids are shown in Table 1-2. TAG was the major lipid in *M. chlamydospora* CBS 529.75 under both cultivation conditions. High contents of EA were detected in TAG fractions. At the cultivation temperature of 12°C, the EA contents in all lipid fractions were higher than those at 28°C.

Table 1-2 Lipid compositions and EA contents in individual lipid fractions from *M. chlamydospora* CBS 529.75^a.

Lipid ^b	Lipid composition (%)		EA content in total lipids (%)	
	28°C ^c	12°C ^d	28°C ^c	12°C ^d
TAG	66.92	60.09	5.57	12.83
FFA	12.69	12.71	1.01	2.60
1,3-DAG	7.10	7.89	0.27	0.96
1,2-DAG	1.56	2.26	0.05	0.25
PL	11.52	16.83	0.27	2.02

^a The strain was grown in 500 ml of GY liquid medium with shaking at 100 rpm.

^b TAG, triacylglycerols; FFA, free fatty acids; 1,3-DAG, 1,3-diacylglycerols; 1,2-DAG, 1,2-diacylglycerols; PL, polar lipids.

^c The strain was cultivated at 28°C for 7 days.

^d The strain was cultivated at 28°C for 4 days and then at 12°C for 3 days.

Effect of cultivation temperature on EA accumulation

To investigate the effects of cultivation temperature on EA production, the total fatty acid compositions in *M. chlamydospora* CBS 529.75 that was cultivated at various cultivation temperatures (4, 12, 15, 20 or 28°C) were analyzed (Table 1-3). At the cultivation temperature of 28°C, OA and ARA were the major unsaturated fatty acids in *M. chlamydospora* CBS 529.75, while the content of EA was only 6.8%. Below the cultivation temperature of 20°C, the amounts of EA and eicosapentaenoic acid (EPA) were increased, whereas that of ARA was significantly decreased. At the cultivation temperature of 4°C, the content of EA reached 25.3% of the total fatty acids.

Table 1-3 Effects of cultivation temperature on fatty acid compositions in *M. chlamydospora* CBS 529.75^a.

Fatty acid ^b	Fatty acid composition (%)				
	4°C	12°C	15°C	20°C	28°C
14:0	0.7	2.4	4.4	3.2	1.3
16:0	9.3	19.1	19.7	22.9	19.1
18:0	5.4	4.0	2.9	3.4	3.6
OA	19.9	21.2	23.0	32.3	26.5
LA	7.5	4.1	6.5	6.7	10.8
GLA	6.2	5.5	2.5	2.4	3.1
EA	25.3	23.6	19.6	12.8	6.8
DGLA	1.3	1.6	1.0	1.0	1.8
ARA	9.2	7.1	6.0	8.4	20.7
EPA	5.6	2.3	2.7	2.0	0.5
Others	9.6	9.1	11.7	5.0	5.9

^a*M. chlamydospora* CBS 529.75 was grown in 4 ml of GY liquid medium at 15, 20, or 28°C for 7 days, or at 28°C for 2 days and then at 4 or 12°C for 5 days with shaking.

^bOA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid; EA, *cis*-11-eicosenoic acid; DGLA, dihomogamma-linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid.

Time course of EA production

M. chlamydospora CBS 529.75 was cultivated in GY liquid medium at 28°C or 12°C, and the amounts of EA were determined according to the standard protocol (Fig. 1-3). The EA production reached the maximum level at the 7th day at both temperatures. As shown in Fig. 1-3, *M. chlamydospora* CBS 529.75 on cultivation at 28°C had accumulated 17.6 mg/g by the 7th day. In contrast, after a shift the cultivation temperature to 12°C at the 4th day, the EA production by *M. chlamydospora* CBS 529.75 reached 36.3 mg/g at the 7th day. After the 7th day, the amounts of EA and the other fatty acids (data not shown) gradually decreased under the conditions at both different temperatures.

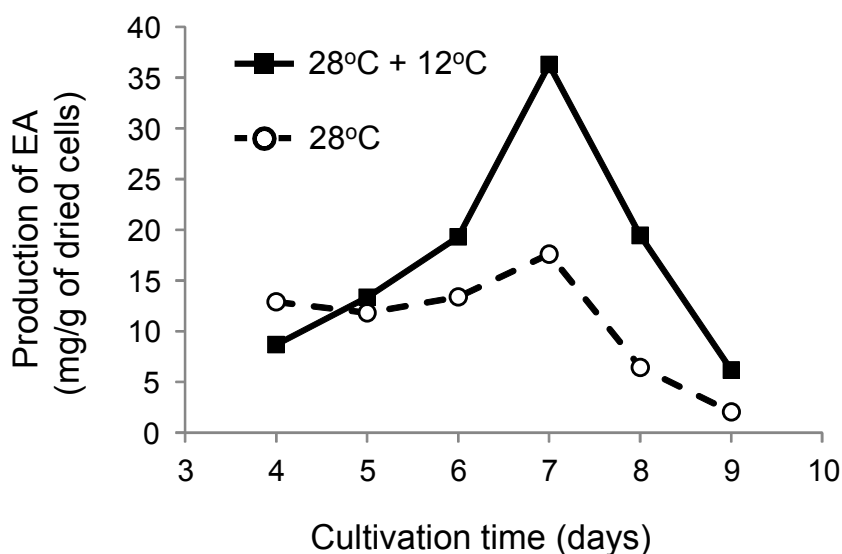


Fig. 1-3 Time course of EA production by *M. chlamydospora* CBS 529.75. *M. chlamydospora* CBS 529.75 was cultivated in 10 ml of GY liquid medium at 28°C (○), or 28°C for 4 days and then at 12°C for 5 days (■) with shaking at 100 rpm. Data are the means for triplicate experiments.

DISCUSSION

I found seven *Mortierella* strains producing high amounts of EA on screening of about three-hundred fungi. These strains biosynthesize EA as well as C20 PUFAs such as ARA. *M. chlamydospora* CBS 529.75 produced 36.3 mg of EA/g of dried cells on cultivation at 28°C for 4 days and then at 12°C for 3 days (Fig. 1-3). After the 7th day, the amount of EA and the other fatty acids decreased under both conditions, suggesting that the fatty acids might be consumed as carbon sources after *M. chlamydospora* CBS 529.75 had exhausted glucose as carbon source in the medium. Most fatty acids were accumulated as triacylglycerols in cells. When carbon sources are completely consumed in the medium, oleaginous microorganisms start to use their own storage lipids. A cultivation temperature below 20°C improved the EA production by *M. chlamydospora* CBS 529.75, suggesting that not PUFA but EA biosynthesis was enhanced at a low cultivation temperature. In contrast, the total contents of C20 PUFAs [dihomo- γ -linolenic acid (DGLA), ARA, and EPA] decreased at a lower cultivation temperature, as shown in Table 1-3. The contents of C20 PUFAs in *M. alpina*, an industrial strain for ARA production, reached more than 50% of total fatty acids when it was cultivated below the temperature of 20°C [25].

Two biosynthetic pathways for EA might be proposed, one is fatty acid elongation of OA, and the other is ω 9-fatty acid desaturation of eicosanoic acid (20:0), as shown in Fig. 1-4. *M. chlamydospora* CBS 529.75 accumulated a significant amount of OA and a slight amount of 20:0, indicating that the route of elongation of OA catalyzed by Δ 9EL might be the dominant pathway for EA biosynthesis in *M. chlamydospora* CBS 529.75. The ω 9-desaturase from *M. alpina* converts 24:0 and 26:0 to 24:1 ω 9 and 26:1 ω 9, respectively, on heterologous expression in fungus *Aspergillus oryzae* [26]. It remains unclear whether the ω 9-desaturase converts 20:0 to EA or not. The mechanism of activation of EA biosynthesis on a shift of the temperature to a low one has not yet been elucidated. I investigated the EA content in total fatty acids in *M. chlamydospora* CBS 529.75 under different culture conditions. The contents of EA in TAG (reserve lipids) were higher than those in PL (membrane lipids) on cultivation at both 28°C and 12°C. The C20 PUFAs in *M. alpina* cultivated at 12°C comprise more than 70% of the total fatty acids in PL, which are related with the maintenance of membrane fluidity against low temperature. It also remains unclear why *M. chlamydospora* CBS 529.75 accumulates a large amount of EA at a low cultivation temperature.

This is the first report of microbial production of EA. In higher plants, EA is a precursor of erucic acid (22:1 ω 9, *cis*-13-docosenoic acid), and most of EA producing plants excluding jojoba produce more erucic acid than EA. Therefore, microbial production of EA contributes to supply of EA as a pharmaceutical material for medicine and a moisturizing component of cosmetic creams. In future, sufficient supply of microbial EA lead to discovery of novel physiological functions of EA on further researches.

M. alpina possesses at least one Δ 9EL, named MALCE1, which converts LA and ALA to the corresponding C20 PUFAs at high efficiencies [12]. However, the activity of elongation of OA to EA is lower in *M. alpina*. The Δ 9EL from *M. chlamydospora* CBS 529.75 is assumed to exhibit high activity as to the conversion of OA to EA. Further research on the isolation and characterization of a Δ 9EL gene from *M. chlamydospora* CBS 529.75 is necessary. Breeding of and optimization of the culture conditions for the strain will lead to microbial production of EA.

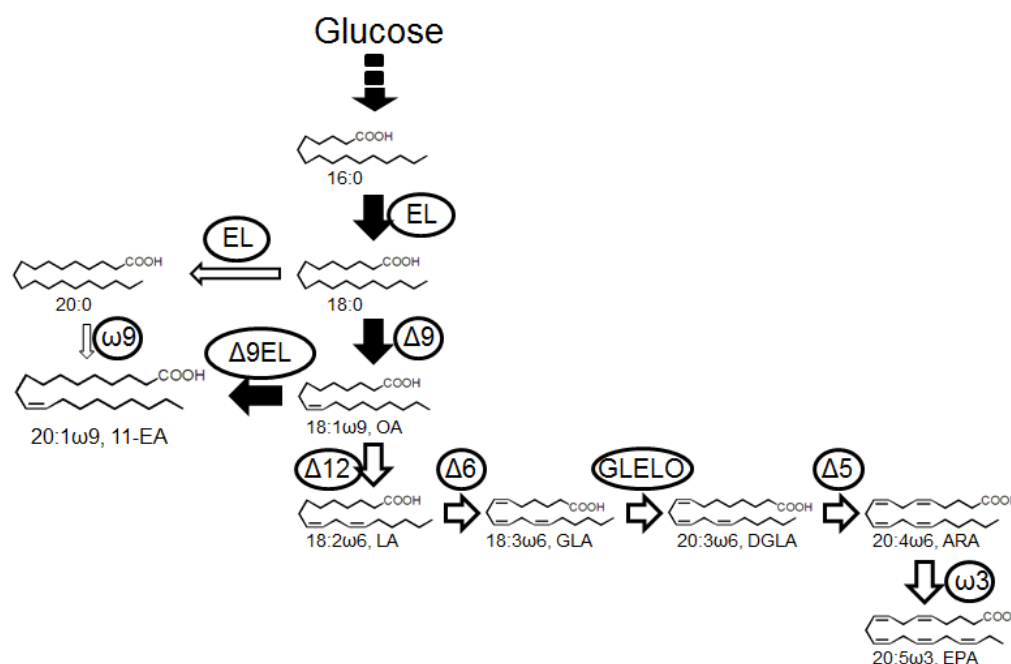


Fig. 1-4 Proposed biosynthetic pathways for EA and PUFAs in *M. chlamydospora* CBS 529.75. The solid arrows indicate hypothetical EA synthetic routes. Δ 9, Δ 12, Δ 6, Δ 5, ω 3, and ω 9-desaturase show the step of desaturation at each position of fatty acids, and EL and Δ 9EL show the step of elongation of fatty acids. Δ 9EL, Δ 9-fatty acid elongase; OA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid; ALA, α -linolenic acid; EA, *cis*-11-eicosenoic acid; DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid; EPA, eicosapentaenoic acid.

SUMMARY

cis-11-Eicosenoic acid (20:1 ω 9, EA) is a monounsaturated fatty acid that is contained in a variety of plant oils and nuts. EA is beneficial as a raw material for medical supplies and a moisturizing component of cosmetic creams. It is supposed that EA is synthesized by a Δ 9-fatty acid elongase from oleic acid as a precursor that is an intermediate in the biosynthetic pathways for polyunsaturated fatty acids. In this study, I found some EA-producing fungi by screening of about 300 fungal strains. In particular, *Mortierella chlamydospora* CBS 529.75 produced a high amount of EA (36.3 mg/g of dried cells) on cultivation at 28°C for 4 days and then at 12°C for 3 days. Most of the EA was a component of triacylglycerols, not phospholipids.

CHAPTER II

Characterization of a trifunctional fatty acid desaturase from oleaginous filamentous fungus *Mortierella alpina* 1S-4 using a yeast expression system

Biosynthetic pathways of fatty acids have been determined in many species by identification of their genes encoding fatty acid desaturases [12, 27-29]. An oleaginous filamentous fungus, *Mortierella alpina* 1S-4, has been shown to contain $\Delta 5$ -, $\Delta 6$ -, $\Delta 9$ -, $\Delta 12$ -, and $\omega 3$ -fatty acid desaturases and four kinds of elongases involved in polyunsaturated fatty acids (PUFAs) biosynthesis [30]. In *M. alpina* 1S-4, $\Delta 5$ -, $\Delta 6$ -, $\Delta 9$ -, and $\Delta 12$ -desaturases exhibit their desaturation activity at a cultural temperature of 28°C, whereas $\omega 3$ -fatty acid desaturase exhibits its activity only below a cultural temperature of 20°C [30].

M. alpina 1S-4 is capable of producing triacylglycerols rich in arachidonic acid [ARA (20:4 ω 6), *cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4)] with the amount ranging of 3-20 g/L of culture broth and 30-70% in the total fatty acid composition. On the other hand, this fungus accumulates a small amount of eicosapentaenoic acid [EPA (20:5 ω 3), *cis*-5, *cis*-8, *cis*-11, *cis*-14, *cis*-17-eicosapentaenoic acid (20:5)] with 0.3 g/L of culture broth and 10% in total fatty acids below a cultural temperature of 20°C [30]. My research group had previously reported isolation of the gene (*maw3*) encoding an $\omega 3$ -desaturase, involved in biosynthesis of $\omega 3$ -PUFAs, from *M. alpina* 1S-4, and have confirmed that the gene product (MAW3) converts $\omega 6$ -PUFAs into $\omega 3$ -PUFAs [7]. MAW3 possesses three histidine rich-cluster motifs containing eight histidine residues that are proposed to form an active-site domain and two hydrophobic membrane-spanning regions in its deduced amino acid sequence [7, 31]. Similar to $\Delta 12$ - and $\omega 3$ -desaturases found in other organisms, MAW3 has no cytochrome *b*₅ motif in its structure [7, 32, 33]. Overexpression of *maw3* gene in *M. alpina* 1S-4 through *Agrobacterium tumefaciens*-mediated transformation led to an increase in EPA production contributing to 40% of total fatty acid content [34].

Fatty acid desaturases are known to have five specificities: chemoselectivity (desaturation or hydroxylation), regioselectivity (double bond position), stereoselectivity (*cis* or *trans*), chain length specificity, and acyl carrier specificity (acyl-CoA, acyl-lipid, or acyl-ACP substrate) [35].

Three types of regioselectivities have been defined [35]. The Δx -desaturases such as $\Delta 9$ -desaturases insert a first double bond at x carbon atom position away from the carboxyl end. On the other hand, the ωy -desaturases such as $\omega 3$ -desaturases insert a double bond at position y from the methyl end. The $v + z$ -desaturases require a preexisting double bond as a mark (v) and catalyze the formation of a new double bond z carbon atoms further along the acyl chain, either toward the methyl end (*i.e.* $\Delta 12$ -desaturase) or toward the carboxyl end (*i.e.* $\Delta 6$ -desaturase). The aforementioned types of regioselectivity are explained for bifunctional fungal $\Delta 12/\omega 3$ -desaturases from *Fusarium moniliforme* [36] and *Aspergillus nidulans* [37]. These bifunctional enzymes desaturate oleic acid [OA (18:1 ω 9), *cis*-9-octadecenoic acid (18:1)] at $\Delta 12$ -position to form linoleic acid [LA (18:2 ω 6), *cis*-9,*cis*-12-octadecadienoic acid (18:2)] and further at the $\Delta 15$ -position to form α -linolenic acid [ALA (18:3 ω 3), *cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3)]. Additionally, these desaturases also convert $\omega 6$ -PUFAs: LA, γ -linolenic acid [GLA (18:3 ω 6), *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid (18:3)], dihomogamma-linolenic acid [DGLA (20:3 ω 6), *cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid (20:3)], and ARA to their corresponding $\omega 3$ -PUFAs with preference of the 18-carbon PUFAs. Other bifunctional $\Delta 12/\Delta 15$ -desaturases that catalyze the synthesis of unusual $\omega 1$ hexadecatrienoic acid [*cis*-9,*cis*-12,*cis*-15-hexadecatrienoic acid (16:3)] have been reported in the free-living soil protozoa *Acanthamoeba castellanii* [38], ascomycota *Claviceps purpurea* [39], basidiomycota *Coprinus cinereus* [40], and nematoda *Caenorhabditis elegans* [41].

In this study, I attempted to characterize the MAW3 using the yeast *Saccharomyces cerevisiae* expression system. Two unusual fatty acids synthesized *de novo* in *maw3*-expressing yeast transformant were identified by GC-MS and NMR. The MAW3 in yeast transformant inserted a double bond at $\Delta 12$ -position and further at $\Delta 15$ -position for endogenous 16-carbon fatty acids as well as at $\omega 3$ -position of exogenous 18- and 20-carbon PUFAs. The *M. alpina* 1S-4 $\omega 3$ -desaturase was found to be a trifunctional $\Delta 12/\Delta 15/\omega 3$ -desaturase that mainly acts as $\Delta 15$ - and $\omega 3$ -desaturase, and as a $\Delta 12$ -desaturase for 18- and 20-carbon PUFAs in addition to 16-carbon fatty acids. The finding is different from other bifunctional desaturases so far reported [36, 38-41].

MATERIALS AND METHODS

Strains, plasmid, growth media, and culture conditions

The *maw3* gene from *M. alpina* 1S-4 [42] was registered in the DDBJ database under the accession number AB182163 [7]. *S. cerevisiae* EH1315 (α *trp1*) was used as the recipient strain in transformation experiments [43]. Plasmid pYE22m, used as a shuttle vector, carried the ampicillin resistance gene (for selection in *Escherichia coli*) and *TRP1* (for tryptophan prototrophy selection in *S. cerevisiae* EH1315), with an expression of a target gene controlled under the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and the GAP terminator from *S. cerevisiae*.

M. alpina 1S-4 strain was cultured in GY medium (containing 20 g/L of glucose, 10 g/L of yeast extract, pH 6.0) for 5 days at 28°C. Yeast strains were cultured in a yeast synthetic complete medium (SC medium; containing 20 g/L of glucose, 5 g/L of ammonium sulfate, 1.7 g/L of yeast nitrogen base without amino acids and ammonium sulfate, 60 mg/L of isoleucine, 60 mg/L of leucine, 60 mg/L of phenylalanine, 50 mg/L of threonine, 40 mg/L of lysine, 30 mg/L of tyrosine, 20 mg/L of adenine, 20 mg/L of arginine, 20 mg/L of uracil, 20 mg/L of histidine, and 10 mg/L of methionine) for 2 days at 15°C (or at 28°C).

maw3 expression in yeast

The full-length *maw3* cDNA was amplified by PCR in a total reaction volume of 50 μ l sample containing < 0.2 μ g of plasmid pYMAW3 [7] as template DNA, 0.5 μ l of *PrimeSTAR HS* DNA polymerase (Takara Bio, Shiga, Japan), 10 μ l of 5 $\times$ *PrimeSTAR* buffer (Mg²⁺ plus), 200 μ M of each dNTP and 200 pM of each primer: ω 3-1F (5'-ATGGCCCCCCTCACGTTGTCGACGAACA-3') containing an ATG start site (underline) and ω 3-3R (5'-TAAATGCTTGTAAAACACTACATCTCC-3') containing a TAA stop site (underline). PCR amplification was performed as follows: initial denaturation at 94°C for 2 min, followed by 25 cycles of 98°C for 10 sec, 55°C for 15 sec, and 72°C for 2 min. The resultant 1,212-bp PCR fragment was digested with *EcoRI* and treated with DNA Blunting Kit (Takara Bio) for blunt-end ligation into the yeast expression vector pYE22m to result in the construction of a plasmid, designated as pYE22MAW3, which was further transformed into the *S. cerevisiae* EH1315 strain by means of the lithium acetate-mediated transformation protocol [44].

Transformants were selected for tryptophan (Trp) auxotrophy on SC (-Trp) medium.

The transformants were grown at 28°C or 15°C with shaking for 48 h in SC medium. To test for the substrate specificities of MAW3 in yeast, various free fatty acids (FFAs) were added to the culture broth at 0.1 mM concentration after 24 h of cultivation. The yeast transformant with an empty vector was used as the control strain.

Fatty acid analysis

Total fatty acid compositions of the yeast transformants were analyzed as described in chapter I. The conversion rate of substrate into the product was calculated as the enzymatic activity of the desaturase [conversion rate (%) = $100 \times \text{product} / (\text{product} + \text{substrate})$].

Total lipids were extracted from wet yeast cells using water/chloroform/methanol (2/2.5/2.5, v/v/v) according to the Bligh-Dyer method [24]. The extracted lipids were saponified to obtain their FFAs using 0.5 N KOH in 90% MeOH. The unidentified FFAs were separated by HPLC with a LC-20A system (Shimadzu). A Develosil C30-UG-5 column (4.6 × 250 mm, Nomura chemical, Aichi, Japan) was used for FFA separation by HPLC with 80% (v/v) acetonitrile aqueous solution as a mobile phase at a flow rate of 1.0 ml/min. The column oven temperature was maintained at 35°C, and the absorbance was measured by UV/VIS detector at 205 nm. The mixture of FFAs separated by HPLC were transmethylated with methanol/benzene/1% diazomethane (4/6/1, v/v/v) for 30 min at room temperature for preparation of FAMES. Through the Discovery Ag-Ion SPE column (SUPELCO, Bellefonte, PA, USA), most of FAMES in the UK2 fraction except for the UK2 were removed with acetone and the UK2 was eluted with acetonitrile. The 4,4-Dimethyloxazoline (DMOX) derivatives of the UK1 and UK2 obtained through the HPLC purification was prepared and analyzed by GC-MS as described by Yu et al. [45]. A GC-MS QP2010 (Shimadzu) with GC-2010 gas chromatograph was used for MS analysis. The SPB^{TM-1} column (0.25 mm I.D. × 30 m, SUPELCO) was used for separation of DMOX derivatives. Initial column temperature was set at 180°C and was raised to 300°C later at a rate of 5°C/min. The apparatus provides the electron impact mode at 70 eV with a source temperature of 250°C. The injection temperature was 250°C. The ¹H-NMR and DQF-COSY experiments were performed with a Bruker Advance 500 (500 MHz). Samples were dissolved in chloroform-*d* (Sigma, St. Louis, MO, USA). Chemical shifts were assigned relative to the solvent signal.

RESULTS

Functional analysis of the *maw3* gene product in the yeast transformant

To characterize the *maw3* gene product from *M. alpina* 1S-4 by using a yeast expression system, the yeast transformant with pYE22MAW3 containing the *maw3* cDNA was obtained. The yeast transformants were grown in SC medium, and their total fatty acid compositions were analyzed by GC (Fig. 2-1A and 2-1B). The *maw3*-expressing yeast transformant accumulated two unusual fatty acids, named UK1 and UK2 respectively, which were not detected in the control strain. The UK1 was expected to be *cis*-9,*cis*-12-hexadecadienoic acid (16:2), because the retention time of the UK1 was consistent with that of an authentic standard (Fig. 2-1C).

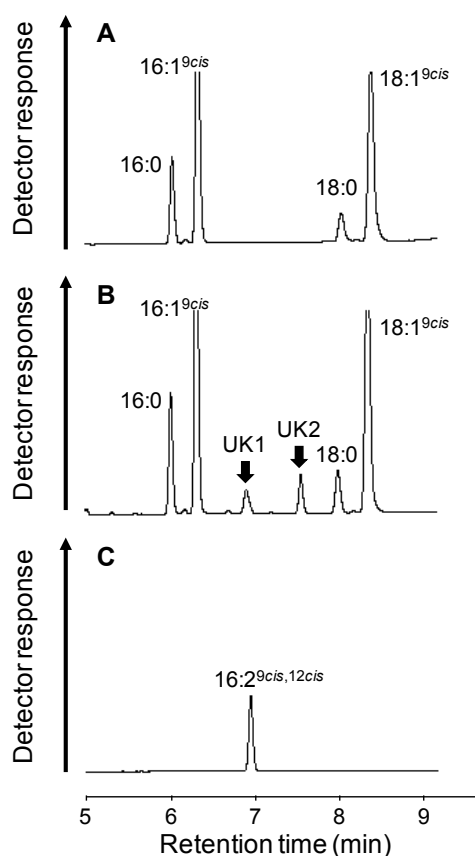


Fig. 2-1 GC chromatograms of FAMES prepared from total lipids of the control strain (pYE22m) (A), the *maw3*-expressing yeast transformant (pYE22MAW3) (B), and the FAMES of *cis*-9,*cis*-12-16:2 (C).

Each FFA of UK1 and UK2 was purified by HPLC equipped with a Develosil C30-UG-5 column, followed by the further purification through Discovery Ag-Ion SPE column for UK2. The positions of the double bonds in UK1 and UK2 were determined by GC-MS analysis of their DMOX derivatives (Fig. 2-2). MS analysis of UK1 and UK2 DMOX derivatives in Fig. 2-2 revealed molecular ions of m/z 305 and 303, and the gaps of 26 atomic mass unit (amu) between m/z 196 and 222, and m/z 236 and 262, indicating double bond at the $\Delta 9$ - and $\Delta 12$ -positions in their structures, respectively. The gap of 41 amu between m/z 262 and 303 in the mass spectrum of UK2 (Fig. 2-2B) suggested a double bond at the $\Delta 14$ - or $\Delta 15$ -position. To determine a position of a double bond at methyl terminal of UK2 completely, the FAME of UK2 showing 95% purity on GC chromatogram was applied for ^1H -NMR analysis (Fig. 2-3) and DQF-COSY analysis (Fig. 2-4). ^1H -NMR δ_{H} (CDCl_3) spectral data of UK2: 5.82 (1H, *tt*, $-\text{CH}_2-\text{CH}=\text{CH}_2$, H-15), 5.38 (4H, *m*, $-\text{CH}_2-\text{CH}=\text{CH}-$, H-9, H-10, H-12, H-13), 5.02 (2H, *d*, $J = 10.1, 17.0$ Hz, $-\text{CH}_2-\text{CH}=\text{CH}_2$, H-16), 3.67 (3H, *s*, $-\text{C}(=\text{O})-\text{O}-\text{CH}_3$), 2.83 (2H, *dd*, $J = 6.07, 6.07$ Hz, $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$, H-14), 2.79 (2H, *dd*, $J = 6.07, 6.07$ Hz, $=\text{CH}-\text{CH}_2-\text{CH}=\text{CH}-$, H-11), 2.30 (2H, *t*, $J = 7.28$ Hz, $-\text{C}(=\text{O})-\text{CH}_2-\text{CH}_2-$, H-2), 2.05 (2H, *dt*, $J = 6.78, 6.78$ Hz, $-\text{CH}_2-\text{CH}_2-\text{CH}=\text{CH}-$, H-8), 1.679 (2H, *tt*, $J = 7.33, 7.41$ Hz, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, H-3), and 1.31 (8H, *m*, $-\text{CH}_2-\text{CH}_2-\text{CH}_2-$, H-4, H-5, H-6, H-7). The absorption peaks at δ 5.82 ($-\text{CH}_2-\text{CH}=\text{CH}_2$, H-15) and 5.02 ($-\text{CH}_2-\text{CH}=\text{CH}_2$, H-16) in Fig. 2-3 revealed a double bond at the $\Delta 15$ -position in the structure of UK2. From these analyses, UK1 and UK2 were identified as *cis*-9,*cis*-12-16:2 and *cis*-9,*cis*-12,*cis*-15-16:3, respectively.

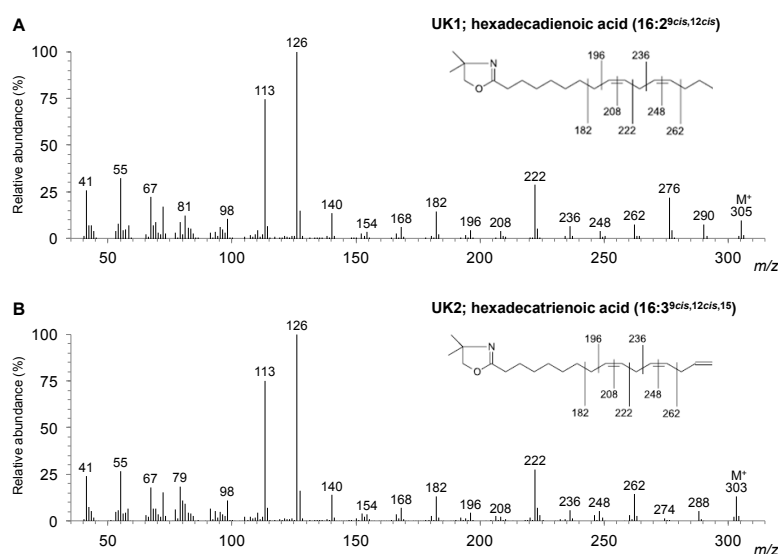


Fig. 2-2 Mass spectra of DMOX derivatives of (A) UK1 and (B) UK2 obtained from the yeast transformant (pYE22MAW3). The specific mass fragments of authentic *cis*-9,*cis*-12-16:2 were m/z 113, 126, 140, 154, 168, 182, 196, 208, 222, 236, 248, 262, 276, 276, 290, and 305.

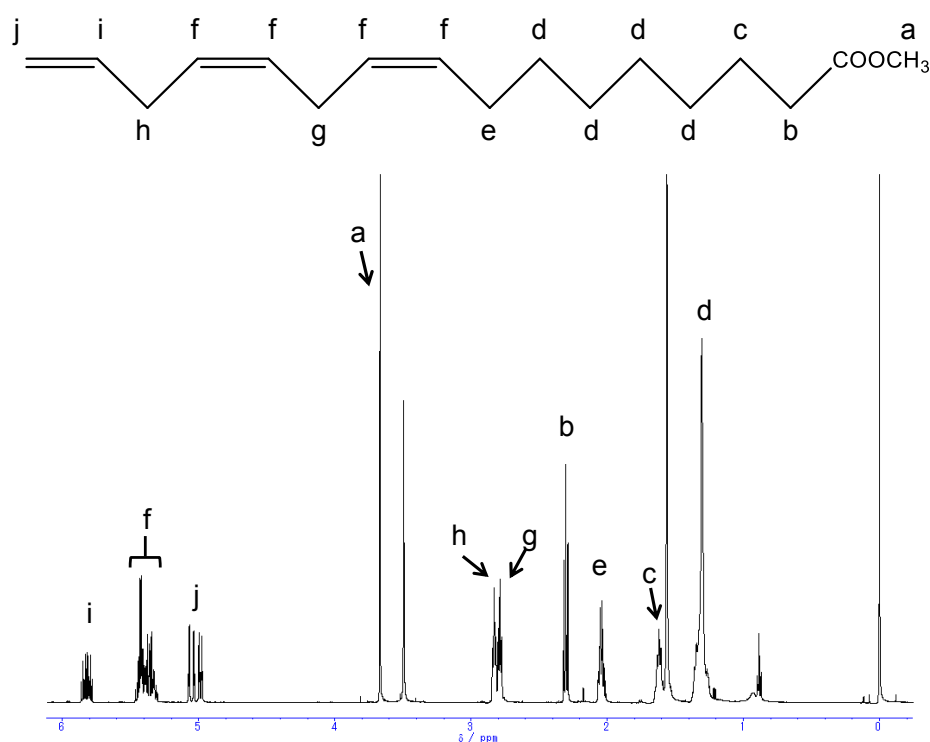


Fig. 2-3 ^1H -NMR analysis of the UK2 methyl ester.

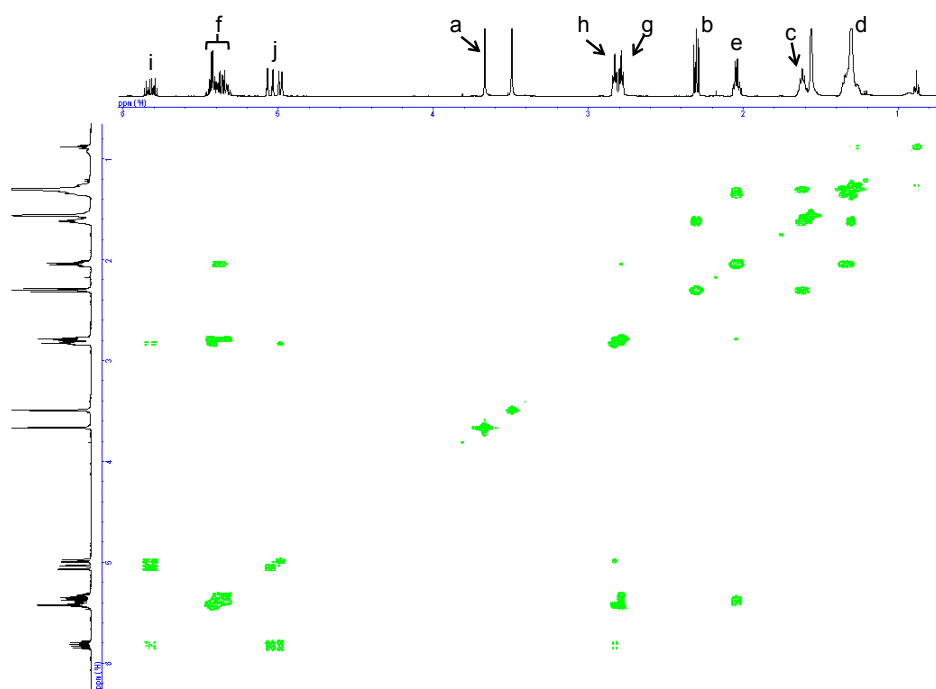


Fig. 2-4 DQF-COSY analysis of the UK2 methyl ester.

As shown in Table 2-1, the fatty acid composition of the *maw3*-expressing yeast transformant was different from that of the control strain. The *cis*-9,*cis*-12-16:2 and *cis*-9,*cis*-12,*cis*-15-16:3 comprised 0.8% and 4.0% of the total fatty acid content, respectively, which were not detected in the control strain. The percentage of *cis*-9-hexadecenoic acid (16:1) was decreased slightly in the yeast transformant, compared to that in the control strain. Neither LA nor ALA was detected in both yeast strains, suggesting that MAW3 in the transformant prefers a 16-carbon fatty acid like *cis*-9-16:1 to an 18-carbon fatty acid like OA.

Table 2-1 Fatty acid compositions of the yeast transformants.

Fatty acid	Fatty acid composition (%)	
	Control strain (pYE22m)	<i>maw3</i> Transformant (pYE22MAW3)
16:0	12.4	11.9
<i>cis</i> -9-16:1	45.4	40.2
<i>cis</i> -9, <i>cis</i> -12-16:2	N.D. ^a	0.8
<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-16:3	N.D.	4.0
18:0	5.7	5.2
<i>cis</i> -9-18:1 (OA)	34.6	32.8

^a N.D., not detected

Catalytic properties of MAW3 gene product analyzed in the transformant

To investigate the substrate preference of MAW3, the yeast transformants were grown in SC medium containing 0.1 mM of exogenous FFA substrates such as *cis*-9,*cis*-12-16:2, *cis*-9-18:1 (OA), crepenynic acid (*cis*-9-octadecen-12-ynoic acid, *cis*-9,*yn*-12-18:1), octadecadienoic acid (18:2) isomers [*cis*-6,*cis*-9-18:2, *cis*-9,*cis*-12-18:2 (LA), *cis*-9,*trans*-11-18:2, *trans*-9,*cis*-12-18:2, *trans*-9,*trans*-12-18:2, and *trans*-10,*cis*-12-18:2], *cis*-6,*cis*-9,*cis*-12-18:3 (GLA), *cis*-9,*cis*-12-eicosadienoic acid (20:2), *cis*-8,*cis*-11,*cis*-14-20:3 (DGLA), and *cis*-5,*cis*-8,*cis*-11,*cis*-14-20:4 (ARA). Conversions of the substrates by the *maw3*-expressing yeast transformant were carried out during cultivation at 15°C or 28°C (Table 2-2). *M. alpina* 1S-4 has no ω 3-desaturation activity at 28°C cultivation, whereas the yeast transformant exhibited the activity for the substrates even at 28°C. The MAW3 in the yeast transformant desaturated the following PUFAs: *cis*-9,*cis*-12-16:2, *cis*-9,*cis*-12-18:2 (LA), *cis*-6,*cis*-9,*cis*-12-18:3 (GLA), *cis*-8,*cis*-11,*cis*-14-20:3 (DGLA), and *cis*-5,*cis*-8,*cis*-11,*cis*-14-20:4 (ARA) to *cis*-9,*cis*-12,*cis*-15-16:3, *cis*-9,*cis*-12,*cis*-15-18:3 (ALA), stearidonic acid [SDA (18:4 ω 3), *cis*-6,*cis*-9,*cis*-12,*cis*-15-octadecatetraenoic acid (18:4)], ω 3-eicosatetraenoic acid [ETA (20:4 ω 3), *cis*-8,*cis*-11,*cis*-14,*cis*-17-eicosatetraenoic acid (20:4)], and *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-20:5 (EPA)]. Putative Δ 15-desaturation products of *cis*-9,*cis*-15-octadecadien-12-ynoic acid (*cis*-9,*yn*-12,*cis*-15-18:2), *trans*-9,*cis*-12,*cis*-15-18:3, and *trans*-10,*cis*-12,*cis*-15-18:3 from *cis*-9,*yn*-12-18:1, *trans*-9,*cis*-12-18:2, *trans*-10,*cis*-12-18:2, respectively, were also observed on GC chromatograms. The ω 6-PUFAs (LA, GLA, DGLA, and ARA) were more effectively converted into ω 3-PUFAs (ALA, SDA, ETA, and EPA) at a low temperature (15°C) cultivation of *M. alpina* 1S-4 than at 28°C temperature. In addition, conversion rates for *cis*-9,*cis*-12-16:2 and *cis*-9,*yn*-12-18:1 (crepenynic acid) were observed at similar or higher levels at 28°C as compared to the ones at 15°C. Neither Δ 15-desaturation nor ω 3-desaturation products were detected for *cis*-9,*cis*-12-20:2 in the yeast transformant.

The Δ 12-desaturation catalyzed by MAW3 was observed only for *cis*-9-16:1, and not for *cis*-9-18:1 and *cis*-6,*cis*-9-18:2. The high conversion rate with more than 60% was shown for *cis*-9,*cis*-12-16:2 to *cis*-9,*cis*-12,*cis*-15-16:3 by Δ 15-desaturation of MAW3 in the yeast transformant. Furthermore, the ω 3-desaturation of MAW3 was observed for 18- and 20-carbon PUFAs having a preexisting 12*cis*-double bond or 12-triple bond and a preexisting 14*cis*-double bond, respectively.

Table 2-2 Conversion of various fatty acids by the *maw3*-expressing yeast transformant^a.

Substrate	Product	Conversion rate (%) ^b	
		15°C	28°C
Endogenous fatty acid			
<i>cis</i> -9-16:1	<i>cis</i> -9, <i>cis</i> -12-16:2	10.2 ± 0.2	N.D. ^c
<i>cis</i> -9, <i>cis</i> -12-16:2	<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-16:3	83.0 ± 0.5	N.D.
Exogenous fatty acid			
<i>cis</i> -9, <i>cis</i> -12-16:2	<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-16:3	68.8 ± 0.6	60.3 ± 0.6
<i>cis</i> -9-18:1 (OA)	N.D.	-	-
<i>cis</i> -9, <i>yn</i> -12-18:1	<i>cis</i> -9, <i>yn</i> -12, <i>cis</i> -15-18:2 ^d	11.0 ± 0.4	19.5 ± 1.5
<i>cis</i> -6, <i>cis</i> -9-18:2	N.D.	-	-
<i>cis</i> -9, <i>cis</i> -12-18:2 (LA)	<i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:3 (ALA)	65.2 ± 0.6	24.4 ± 0.4
<i>cis</i> -9, <i>trans</i> -11-18:2	N.D.	-	-
<i>trans</i> -9, <i>cis</i> -12-18:2	<i>trans</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:3 ^d	63.8 ± 0.2	40.0 ± 3.7
<i>trans</i> -9, <i>trans</i> -12-18:2	N.D.	-	-
<i>trans</i> -10, <i>cis</i> -12-18:2	<i>trans</i> -10, <i>cis</i> -12, <i>cis</i> -15-18:3 ^d	41.3 ± 0.2	18.1 ± 0.5
<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12-18:3 (GLA)	<i>cis</i> -6, <i>cis</i> -9, <i>cis</i> -12, <i>cis</i> -15-18:4 (SDA)	74.8 ± 0.1	32.6 ± 0.7
<i>cis</i> -9, <i>cis</i> -12-20:2	N.D.	-	-
<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14-20:3 (DGLA)	<i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> 17-20:4 (ETA)	57.7 ± 1.1	6.7 ± 0.3
<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> 14-20:4 (ARA)	<i>cis</i> -5, <i>cis</i> -8, <i>cis</i> -11, <i>cis</i> -14, <i>cis</i> 17-20:5 (EPA)	52.5 ± 2.6	8.7 ± 0.7

^a All exogenous free fatty acid substrates were added at 0.1 mM. All the values are for three independent samples (mean ± SD).

^b Conversion rate (%) = 100 × [product]/[product + substrate].

^c N.D., not detected.

^d Putative product.

DISCUSSION

The fungus *M. alpina* 1S-4 not only produces a great number of ω 6-PUFAs rich in ARA, but also accumulates ω 3-PUFAs such as EPA below a cultural temperature of 20°C [46]. The ω 3-PUFAs in *M. alpina* 1S-4 *in vivo* are expected to be synthesized from ω 6-PUFAs through ω 3-desaturation. The *maw3* gene was previously isolated and characterized as to its function of ω 6-PUFAs conversion to ω 3-PUFAs in *M. alpina* 1S-4 [7].

In this study, the *maw3*-expressing yeast transformant was found to accumulate two newly synthesized fatty acids, *cis*-9,*cis*-12-16:2 and *cis*-9,*cis*-12,*cis*-15-16:3. Thus far, I had no idea that the MAW3 had Δ 12-desaturase activity as an alternative function. The accumulation of *cis*-9,*cis*-12-16:2 and *cis*-9,*cis*-12,*cis*-15-16:3 suggested that the yeast transformant had both Δ 12-desaturation activity with a conversion rate of 10.2% (calculated as $100 \times [16:2 + 16:3]/[16:1 + 16:2 + 16:3]$) for endogenous *cis*-9-16:1, and Δ 15-desaturation one at ω 1-position, not ω 3, with a conversion rate of 83.0% (calculated as $100 \times [16:3]/[16:2 + 16:3]$) for the resultant *cis*-9,*cis*-12-16:2. Once the *cis*-9,*cis*-12-16:2 was formed from *cis*-9-16:1 by Δ 12-desaturation, *cis*-9,*cis*-12-16:2 was demonstrated to be rapidly converted to *cis*-9,*cis*-12,*cis*-15-16:3.

The analysis of substrate specificities (Table 2-2) showed that the MAW3 not only had ω 3-desaturation activity for 18- and 20-carbon PUFAs, but also demonstrated bifunctional Δ 12/ Δ 15-desaturation activities for 16-carbon fatty acids. The Δ 12-, Δ 15-, or ω 3-desaturation activity of MAW3 requires a preexisting double bond at Δ 9-, Δ 12-, or ω 6-position, respectively. Hence, MAW3 is classified as $v + 3$ desaturase (v indicating the position of the preexisting double bond) which inserted a new double bond 3 carbon atoms further along the acyl chain toward the methyl end.

Compared to other bifunctional Δ 12/ Δ 15-desaturases (amoeba *A. castellanii* [38]; basidiomycete *C. cinereus* [40]; nematode *C. elegans* [41]), MAW3 in the yeast transformant exhibited Δ 12-desaturation for *cis*-9-16:1, not *cis*-9-18:1, and in addition to that, also exhibited Δ 15-desaturation for *cis*-9,*cis*-12-16:2 with a high conversion rate (83.0%). The previously reported bifunctional Δ 12/ Δ 15-desaturases mainly act as a Δ 12-desaturase, except for the fungal ω 3-desaturases described by Damude et al. [36] and Hoffmann et al. [37], however do not possess Δ 12-desaturation activity for the 16-carbon fatty acid. The ratios of ω 3/ ω 6 fatty acids (calculated as $[16:3 + 18:3]/[16:2 + 18:2]$) in yeast transformant were calculated as follows: 5.0

(MAW3, described here), 21 (ω 3-desaturase from *F. moniliforme* [36]), 0.0080 (Δ 12-desaturase from *F. moniliforme* [36]), 2.1 (ω 3-desaturase from *A. nidulans* [37]), 0.48 (Δ 12-desaturase from *A. castellanii* [38]), 0.042 (Δ 12-desaturase from *C. cinereus* [40]), and 0.090 (Δ 12-desaturase from *C. elegans* [41]) respectively based on the information derived from each report. The bifunctional Δ 12/ Δ 15-desaturases from *F. moniliforme* and that has a high ratio of 21, mainly work as a ω 3-desaturase, which prefers the 18-carbon PUFAs as substrates and not the 16-carbon fatty acids. On the other hand, the bifunctional Δ 12/ Δ 15-desaturase from *C. purpurea* [39] introduces a double bond for both 16- and 18-carbon fatty acids, demonstrating increased activity of Δ 12-desaturation as compared to Δ 15-desaturation.

On investigation of substrate specificities, the characteristic feature of MAW3 was shown to have both the Δ 12/ Δ 15-desaturation activity for the 16-carbon fatty acids and ω 3-desaturation activity for 18- and 20-carbon PUFAs, indicating its substrate specificities that differed from the other bifunctional Δ 12/ Δ 15-desaturases [36-41]. It is of interest to note that, the *maw3*-expressing yeast transformant exhibited the ω 3-desaturation activity on cultivation at 28°C, whereas *M. alpina* 1S-4 had no such ω 3-desaturation activity under similar culture conditions. This may imply the possibility of some regulation in the translation process and/or localization of MAW3 in *M. alpina* 1S-4 *in vivo*. The localization of MAW3 might be detected in cells of *M. alpina* 1S-4 by means of labeling techniques. I expect that the elucidation of the mechanism for the difference in MAW3 activity (depending on the culture temperature) may contribute to the accumulation of basic information on membrane-bound desaturases and to efficient production of ω 3-PUFAs by *M. alpina* breeding.

SUMMARY

A ω 3-fatty acid desaturase gene (*maw3*) which is involved in biosynthesis of ω 3-polyunsaturated fatty acids (PUFAs) was previously isolated from *Mortierella alpina* 1S-4. In this report, I investigated the products of MAW3 catalyzing reaction with endogenous and exogenous fatty acids in the yeast transformant. Two unusual fatty acids *de novo* synthesized in the yeast transformant expressing *maw3* gene were identified as ω 4 hexadecadienoic acid (*cis*-9,*cis*-12-16:2) and ω 1 hexadecatrienoic acid (*cis*-9,*cis*-12,*cis*-15-16:3) by GC-MS and $^1\text{H-NMR}$ analyses. In addition to the desaturation activity at the ω 3-position for 18- and 20-carbon PUFAs, MAW3 in the yeast transformant inserted a double bond at Δ 12-position of endogenous *cis*-9-16:1 and further at Δ 15-position of the resulting *cis*-9,*cis*-12-16:2 to result in the formation of *cis*-9,*cis*-12,*cis*-15-16:3 leading to a bifunctional Δ 12/ Δ 15-desaturase for 16-carbon fatty acids. Moreover, I evaluated the activity of MAW3 in the yeast transformant under different temperatures. The MAW3 did not have desaturation activities in *M. alpina* 1S-4 at 28°C but it had in the yeast transformant for various fatty acids. The MAW3 was demonstrated to be a trifunctional Δ 12/ Δ 15/ ω 3-desaturase, exhibiting Δ 12-desaturation for *cis*-9-16:1, Δ 15-desaturation for 16- and 18-carbon fatty acids that had a preexisting *cis*-double bond at Δ 12 position, and ω 3-desaturation for 20-carbon fatty acids having that at Δ 14-position. It is the first report that the fatty acid desaturase (MAW3) is shown to have Δ 12- and Δ 15-desaturation activities for a 16-carbon fatty acid, in addition to its major function, ω 3-desaturation activity.

CHAPTER III

**Improvement of gene-targeting in the oil-producing fungus
Mortierella alpina 1S-4 and construction of a strain producing
a valuable polyunsaturated fatty acid by gene targeting**

Integration of exogenous DNA into the chromosome in all organisms follows two pathways of DNA double-strand break (DSB) repair: homologous recombination (HR) and non-homologous end joining (NHEJ) pathways [47]. The repair of DSBs is induced by both exogenous and endogenous triggers and causes detrimental DNA lesions [48]. In the mechanism of the HR pathway, the homologous region is used as a template and the exogenous DNA is integrated into the chromosome. In contrast, in the mechanism of the NHEJ pathway, the strand ends of the exogenous DNA are directly ligated into DSBs without a requirement of sequence identity. These two mechanisms for DSB repair are independent of each other and are considered to function competitively [49]. The repair of DSBs requires many associated proteins, such as the Rad protein group including Rad54, Rad51, Rad52, Mre11, and Xrs2 in the HR pathway [50, 51], and Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), DNA ligase 4 (Lig4), and X-ray repair cross-complementing protein 4 (Xrcc4) in the NHEJ pathway [52, 53]. A pathway similar to HR has been confirmed ubiquitously in various organisms (bacteria, yeast, and human) [54, 55]. In addition, important discoveries such as the Rad51-independent HR and Ku80-independent NHEJ pathways and the occurrence of all non-homologous chromosomal integration under the control of Lig4 have been reported [56].

The yeast *Saccharomyces cerevisiae* mainly utilizes the HR system for DSB repair. Accordingly, gene targeting through the HR pathway in *S. cerevisiae* exhibits quite high efficiency [57]. In contrast, many other organisms, including mammals, plants, insects, and filamentous fungi, predominantly use the NHEJ pathway for DSB repair, and exogenous DNA, even if it consists of a long homologous sequence, can be integrated into nonspecific regions in chromosomes. Disruption of the *ku70*, *ku80*, or *lig4* gene leads to an increase in the frequency of HR in filamentous fungi [56, 58-64]. In particular, disruption of the *ku80* and/or *lig4* gene in *Neurospora crassa* and the *lig4* gene in *Aspergillus oryzae* have led to 100% targeting efficiency [56, 61, 62].

The oil-producing filamentous fungus *Mortierella alpina* 1S-4 is a producer of carbon 20 (C20) polyunsaturated fatty acids (PUFAs), such as arachidonic acid (20:4 ω 6, ARA) and eicosapentaenoic acid (20:5 ω 3, EPA), which are rich in triacylglycerols [65]. In addition, the lipid productivity of this fungus reaches 600 mg/g of dried mycelia. For these reasons, the fungus has been used as a model oleaginous microorganism for biosynthesis and accumulation of lipids, including PUFAs [5, 7, 9, 13, 15, 30, 66]. In previous studies, several techniques for gene manipulation in this fungus, such as a host–vector system [67-69], RNA interference [70], and transformation systems [34, 71], have been established. By use of such transformation systems, plasmid vectors are integrated randomly into the fungal genome.

To construct a high-producing strain of beneficial PUFAs from this fungus by metabolic engineering, an efficient gene-targeting system using HR is necessary. However, gene targeting by HR in this fungus is rarely attempted, given that NHEJ is predominant and the efficiency of HR is low. In this study, to develop an efficient gene-targeting system in *M. alpina* 1S-4, I identified the *ku80* gene and constructed *ku80* gene-disrupted strains via single-crossover HR. Moreover, to evaluate the improvement of gene-targeting efficiency by *ku80* gene-disruption, I investigated the construction of a dihomo- γ -linolenic acid (20:3 ω 6, DGLA)-producing strain by $\Delta 5$ -desaturase ($\Delta 5ds$) gene-disruption using the $\Delta ku80$ strain as a host strain.

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

M. alpina 1S-4 is deposited in the Graduate School of Agriculture of Kyoto University, Japan [30] and the uracil auxotrophic strain (*ura5⁻* strain) [71] was used as a host strain. Czapek–Dox agar medium containing 0.05 mg/ml uracil was used for sporulation of the *ura5⁻* strain, as described previously [71]. Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of transformants derived from the *M. alpina* 1S-4 *ura5⁻* strain at 28°C [71]. GY medium (20 mg/ml glucose and 10 mg/ml yeast extract) was used for fatty acid composition analysis and extraction of genomic DNA. GY agar medium containing 0.75 mg/ml 5-fluoroorotic acid (5-FOA) and 0.05 mg/ml uracil were used to confirm the growth of *ku80*-disrupted transformants [72-74]. GY agar medium containing 100 µg/ml carboxin was used for selection of *ku80*-disrupted transformants. *Escherichia coli* strain DH5α was used for DNA manipulation and grown on LB agar plates containing 50 µg/ml kanamycin. All solid media contained 2% agar.

Genomic DNA preparation

M. alpina 1S-4 host strain and transformants were cultivated in 100 ml of GY liquid medium at 28°C for 5 days with shaking at 100 rpm. Fungal mycelia were harvested by suction filtration and washed with sterile water. Preparation of genomic DNA was performed by a previously described method [75].

Cloning and identification of the *ku80* gene from *M. alpina* 1S-4

Two highly degenerate primers, *ku80* F and *ku80* R (Table 3-1), were synthesized for cloning of the *ku80* cDNA, based on the amino acid sequences of Ku80 homologs from two filamentous fungi, *Rhizopus delemar* (accession EIE88285) and *Aspergillus clavatus* (accession XP_001272945). The sequences of the primers correspond to regions that encode IAIQMIVT

and PFAGDVNTY peptides. PCR amplification was performed in a total volume of 50 μ l containing 1 μ g of genomic DNA, 0.25 μ l of *Takara EX taq* polymerase (Takara Bio), 5 μ l of 10 $\times$ *EX Taq* buffer, 200 μ M of each dNTP, and 5 pM of primers, and performed as 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by one cycle of extension at 72°C for 5 min. The resulting 0.7-kb fragment was cloned into the pT7Blue T-Vector (Novagen, Merck KGaA, Darmstadt, Germany), and the nucleotide sequence was determined with a Beckman Coulter CEQ8000 system (Beckman Coulter, Fullerton, CA, USA). For cDNA synthesis and construction of a cDNA library, RNA extraction reagent Isogen (Nippon Gene, Tokyo, Japan) and a PrimeScript High Fidelity RT-PCR Kit (Takara Bio) were used, following the supplier's instructions.

Table 3-1 Primers used in this study.

Primer	Sequence (5' to 3')
ku80 F	ATYGCNATYCARATGATYGTIAC
ku80 R	GTRTTVACRTCDCCIGCRAAIGG
ku80 IPCR F	GAGTGCACTCCGTGAGGTCGGT
ku80 IPCR R	CATCATATCCAACGACACGATCAGTCC
ku80 start	ATGGCATCGAAAGAAGCGACAATTTACATC
ku80 <i>Xho</i> I R	<u>GCTCGAG</u> GGGCAAAACCGTCAAGCTTC
$\Delta 5$ <i>Eco</i> RI F	CCGAATT <u>CG</u> TATGGCCCCGATTGC
$\Delta 5$ <i>Xho</i> I R	GCCTCGAGGGCACCTTGTAAGTCTGCTGCAGGTG
ura5 stop R	TTAAACACCGTACTTCTCGCGG
ku80 F2	CGGATAGCGAAGACGTGGAAG
ku80 R2	GTCAGCCGCTGGTTGTGCGTTGATC
$\Delta 5$ F	CTCGCGGCGCATAACACCGAGGACAGCC
$\Delta 5$ R	GGTCCAGAGGTGCGAATCGTGGGCG

^aThe underlined sequences indicate synthesized restriction enzyme sites.

To isolate whole *ku80* genomic DNA from *M. alpina* 1S-4, inverse PCR was performed with primers, ku80 IPCR F and ku80 IPCR R (Table 3-1). The *Sal*I/*Xho*I-digested genomic fragment was self-ligated and then used as a template. PCR amplification was performed in a total volume of 50 μ l containing 500 ng of the template, 0.25 μ l of *Takara EX taq* polymerase (Takara Bio), 5 μ l of 10 $\times$ *EX Taq* buffer, 200 μ M of each dNTP, and 5 pM of each primer, and

performed as follows: initial denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 3 min and one cycle of extension period at 72°C for 10 min. The amplified 3.3-kb fragment was cloned into the pT7Blue T-Vector and identified completely with the Beckman Coulter CEQ8000 system.

Construction of the plasmid vector for *ku80* gene-targeting

A binary vector pKSUku80 was constructed for *ku80* gene-targeting on the backbone of pKSU, which is pBluescript II KS (+) (Stratagene, La Jolla, CA, USA) ligated with a *ura5* gene marker cassette derived from the *M. alpina* 1S-4 transformation vector. A 4.3-kb fragment containing a partial *ku80* gene, amplified with *ku80* start and *ku80 XhoI* R primers (Table 3-1) using *M. alpina* 1S-4 genomic DNA as a template, was cloned into the pUC118 using a Mighty Cloning Kit (Blunt End) (Takara bio). The resulting plasmid (pUC118ku80) was digested with *HindIII* and the digested partial *ku80* fragment was ligated into pKSU vector digested with the same restriction enzyme. The resulting plasmid was named pKSUku80 (Fig. 3-3A).

A pKSCD5 vector for the *Δ5ds* gene-targeting was constructed on the backbone of the *M. alpina* 1S-4 transformation vector pKSC. The *CBXB* gene expression cassette from pSBZNCBXB [67], digested with *EcoRI* and *XbaI*, was ligated into pBluescript II KS (+) (Stratagene) digested with the same restriction enzymes, and the resulting plasmid was named pKSC. The partial *Δ5ds* gene (2024-bp) was amplified with two primers, *Δ5 EcoRI* F and *Δ5 XhoI* R primers (Table 3-1) and *M. alpina* 1S-4 genomic DNA as a template. The resulting fragment was digested with *EcoRI* and *XhoI* and ligated into the pKSC plasmid digested with the same restriction enzymes. The resulting plasmid was named pKSCD5 (Fig. 3-6A).

Transformation of *M. alpina* 1S-4

The gene-targeting vectors were introduced into spores of *M. alpina* 1S-4 by biolistic particle bombardment with PDS-1000/He Particle Delivery System (Bio-Rad, Hercules, CA, USA) [71]. Given that linear plasmids are completely integrated into chromosomes by HR [76], the vectors were digested with restriction enzymes and introduced into the spores: the pKSUku80 vector was digested with *NcoI* and the pKSCD5 vector was digested with *NruI*. Spores (1.5×10^8) were spread on an agar plate using SC uracil-free medium for transformation with pKSUku80 or GY containing 5-FOA for transformation with pKSCD5. After bombardment, the plates were

incubated at 28°C for 5 days. Transformants were transferred to a new GY plate containing 5-FOA.

Mutagen sensitivity

Sensitivity to chemical mutagen toxicity in gene-manipulated transformants was evaluated by spot test [61, 77]. Methyl methanesulfonate (MMS) was added to GY agar medium at final concentrations of 0, 0.01, 0.02, 0.025, 0.05, and 0.1%.

Identification of gene-disruption by PCR and Southern blot analysis

The *ku80* gene-disrupted candidates were evaluated by means of colony-PCR using the extracted genomic DNA as a template and the primers *ku80* start and *ura5* stop R (Table 3-1). When integration into the genomic *ku80* gene locus was successful, a 4.3-kb fragment was amplified using the primers and genomic DNA from the transformants.

Correct homologous integration in the genomic *ku80* gene was confirmed by Southern blot analysis. The 1.0-kb partial *ku80* gene amplified with primers *ku80* F2 and *ku80* R2 (Table 3-1) using *M. alpina* 1S-4 genomic DNA as a template was used as a probe for hybridization. Southern blot hybridization was performed as described previously [10]. Genomic DNA (10 µg) digested with *Xho*I was size-fractionated by electrophoresis in 1% agarose gel and transferred to Amersham Hybond-N⁺ membrane (GE Healthcare Ltd., Buckinghamshire, UK) using a VacuGene XL Vacuum Blotting System (GE Healthcare). Southern hybridization was performed using the Gene Images AlkPhos Direct Labeling and Detection System (GE Healthcare).

For Southern blotting analysis of *Δ5ds* gene-disruption, 1.7 kb of the *Δ5ds* gene fragment amplified with primers *Δ5* F and *Δ5* R (Table 3-1) using *M. alpina* 1S-4 genomic DNA as a template was used as a probe for hybridization. Genomic DNA (10 µg) was digested with the pair *Nar*I and *Xba*I or *Cla*I and *Xho*I. Southern blot hybridization was then performed as described above.

Fatty acid analysis

Fatty acid production and composition of transformants were analyzed as described in chapter I. In this study, mycelia of the transformants and the host strain were inoculated into 3

CHAPTER III

ml of GY liquid medium in a 20-ml Erlenmeyer flask and cultivated at 28°C with reciprocal shaking at 120 rpm for 7 days.

Nucleotide sequence accessions

The *ku80* gene from *M. alpina* 1S-4 has been registered in the DNA Data Bank of Japan (DDBJ) database as accession LC009413. The *Δ5ds* genomic gene of *M. alpina* 1S-4 has been deposited in GenBank/EMBL/DDBJ as accession AB188307.

RESULTS

Identification and phylogenetic analysis of the *ku80* gene from *M. alpina* 1S-4

To isolate the *ku80* partial gene fragment, a 0.7-kb gene fragment was amplified by PCR using highly degenerate primers and *M. alpina* 1S-4 genomic DNA as a template. The predicted amino acid sequence encoded by the partial gene fragment showed similarity to those of Ku80 proteins from other organisms. To identify the whole *ku80* gene from *M. alpina* 1S-4, inverse PCR was performed with *M. alpina* 1S-4 genomic DNA. The open reading frame of *ku80* gene from *M. alpina* 1S-4 was found to consist of 3366 bp. Based on the whole *ku80* gene information from genomic DNA, the full-length cDNA of the *ku80* gene was obtained by PCR. The *ku80* cDNA with 2511-bp length was predicted to encode a protein consisting of 836 amino acids. These results suggested that the *M. alpina* 1S-4 *ku80* genomic gene has nine exons (1–129, 257–311, 400–543, 653–821, 906–1467, 1580–1795, 1877–2043, 2141–3116, 3274–3366) and eight introns. The predicted amino acid sequence of *M. alpina* 1S-4 Ku80 shares low identities with those of metazoa (*Mus musculus*, 21%; *Rattus norvegicus*, 21%; *Homo sapiens*, 25%; *Tigriopus japonicas*, 21%), higher plants (*Hordeum vulgare*, 24%; *Triticum aestivum*, 25%; *Oryza sativa*, 25%; *Arabidopsis thaliana*, 22%), oleaginous yeast (*Rhodospiridium toruloides*, 24%), fungi (*Neurospora crassa*, 29%; *N. tetrasperma*, 29%; *Lecanicillium* sp., 29%; *Aspergillus oryzae*, 28%; *A. sojae*, 28%; *A. fumigatus*, 29%; *Penicillium digitatum*, 28%). Compared with various Ku80 proteins from these organisms, the Ku80 from *M. alpina* 1S-4 is located in the expected position in the phylogenetic tree (Fig. 3-1).

Disruption of the *ku80* gene of *M. alpina* 1S-4 with pKSUku80 vector

A vector for *ku80* gene disruption, pKSUku80, digested with *Nco*I was delivered into spores of *M. alpina* 1S-4 *ura5*[−] strain by biolistic particle bombardment with a PDS-1000/He Particle Delivery System. To confirm integration of a *ura5* gene marker, all transformants grown on an SC uracil-free plate were inoculated onto GY medium containing 5-FOA. Finally, 77 transformants were obtained under these conditions. The transformants were selected by colony PCR with primers *ku80* start and *ura5* stop R (Table 3-1), and each genomic DNA as a template. Fragments of approximately 4.3-kb, which were formed presumably by integration via HR, were observed in only two transformants (3 and 6), but not in the host strain (Fig. 3-2).

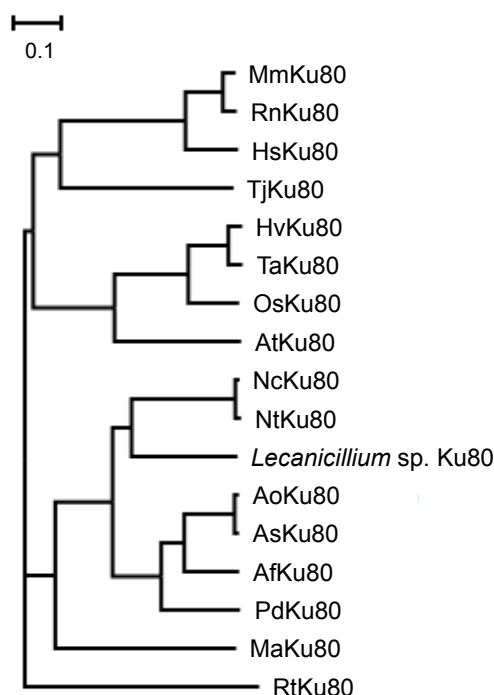


Fig. 3-1 Phylogenetic tree of Ku80 proteins. The tree was created by the neighbor-joining (NJ) method with 10,000 bootstrap replicates using the sequence analysis software GENETYX 11.0 (Genetyx corp., Tokyo, Japan). MmKu80 (*Mus musculus*), AAH51660; RnKu80 (*Rattus norvegicus*), NP_803154; HsKu80 (*Homo sapiens*), P13010; TjKu80 (*Tigriopus japonicus*), AIL94178; HvKu80 (*Hordeum vulgare* subsp. *vulgare*), AEO86624; TaKu80 (*Triticum aestivum*), ADO00729; OsKu80 (*Oryza sativa* Japonica Group), Q75IP6; AtKu80 (*Arabidopsis thaliana*), AEE32242; MaKu80 (*Mortierella alpina* 1S-4), LC009413; RtKu80 (*Rhodospiridium toruloides*), AIA21644; NcKu80 (*Neurospora crassa*), AFM68948; NtKu80 (*N. tetrasperma* FGSC 2508), EGO57771; *Lecanicillium* sp. Ku80 (*Lecanicillium* sp. HF627), AHY22503; AoKu80 (*Aspergillus oryzae*), BAE78503; AsKu80 (*A. sojae*), BAE78504; AfKu80 (*A. fumigatus* Af293), Q4WI96; PdKu80 (*Penicillium digitatum*), AGT79985.

The genome integration patterns of transformants 3 and 6 were confirmed by Southern blot analysis. Their genomic DNAs were digested with *Xho*I and a 1.0-kb fragment consisting of partial *ku80* gene was used as a probe (Fig. 3-3A). The 5.2-kb hybridization signal on the host strain was not detected in the two transformants (Fig. 3-3B). However, the expected 4.0- and 8.3-kb signals resulting from single-crossover HR were detected only in transformant 3. These results suggest that a single pKSUku80 vector was successfully integrated into *ku80* genomic DNA of transformant 3. In contrast, some of the introduced pKSUku80 vectors appear to have been integrated ectopically into the *ku80* gene locus in transformant 6. Thus, transformant 3 was used as a host strain for *Δ5ds* gene disruption in the present research. The gene-targeting efficiency of pKSUku80 vector integration in *M. alpina* 1S-4 was calculated as 1.3% (Table 3-2).

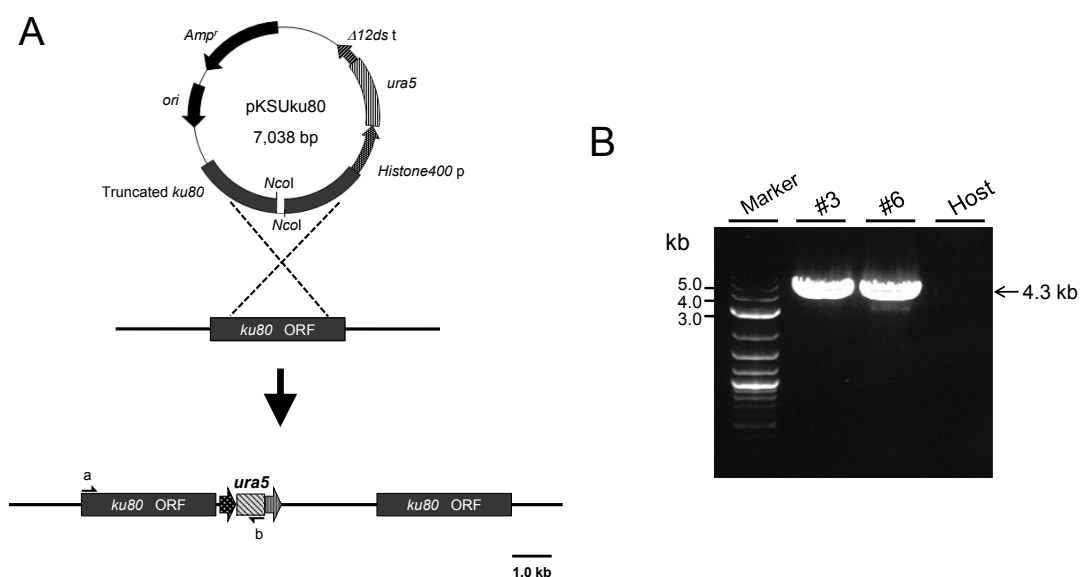


Fig. 3-2 Construction scheme of *ku80* gene disruptant and selection by Southern blot analysis. (A) The figure illustrates the homologous integration of pKSUku80 vector into the *ku80* genomic gene locus in *M. alpina* 1S-4. Two arrows, a and b, indicate the position of oligonucleotide primers used for PCR. (B) Selection of *ku80* gene disruptant by PCR. PCR amplification was performed with primers *ku80* start and *ura5* stopR resulting 4.3-kb product. Lanes #3 and #6 indicates transformants as $\Delta ku80$ candidates.

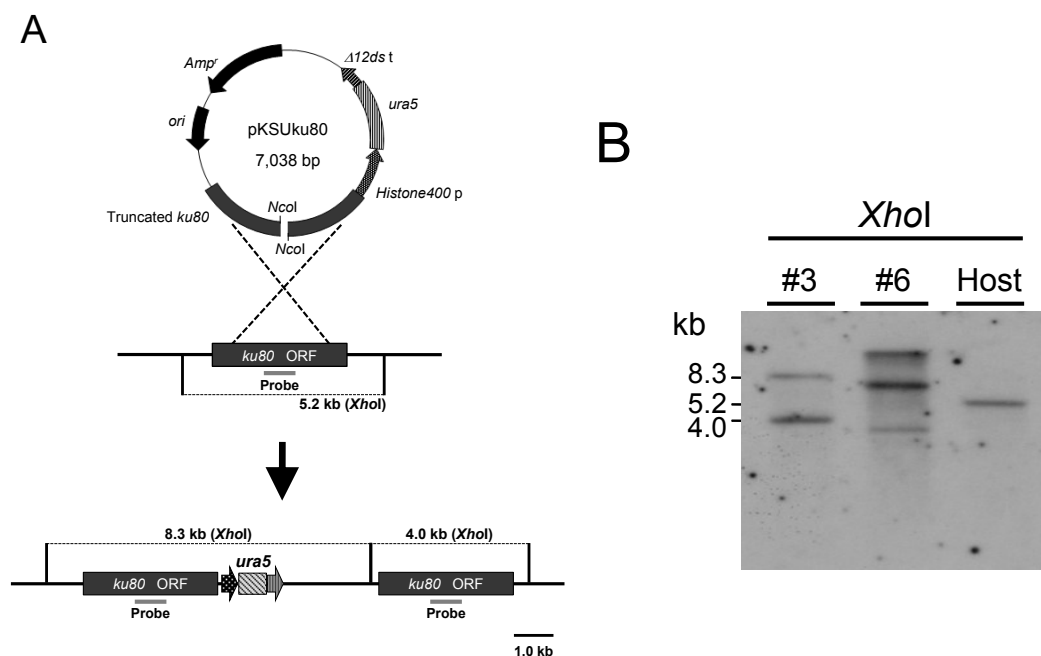


Fig. 3-3 Construction scheme of *ku80* gene disruptant and confirmation by Southern blot analysis. (A) The figure illustrates the homologous integration of pKSUku80 vector into the *ku80* genomic gene locus in *M. alpina* 1S-4. Gray short bar indicates the position hybridized by the probe. Dotted lines indicate the position and base lengths of hybridization signals. (B) Southern hybridization analysis of *ku80* gene-disrupted candidates. *XhoI*-digested genomic DNAs from transformants 3 and 6 and host strain were hybridized with the probe.

Table 3-2 Efficiency of gene targeting in *M. alpina* 1S-4 host strain and the $\Delta ku80$ strain.

Host	The number of transformant	Homologous replaced strain	Gene targeting efficiency (%) ^a
Wild	77	1	1.3
$\Delta ku80$	32	1	3.1

^aThe efficiency of gene targeting was calculated by the ratio of the number of homologously replaced strains to the number of transformants.

Growth characteristics and mutagen sensitivity

Given that Ku70, Ku80, and Lig4 proteins are involved in DSB repair through NHEJ in diverse organisms [78, 79] and telomere maintenance in some organisms [80], the growth characteristics and mutagen sensitivity of *M. alpina* 1S-4 $\Delta ku80$ strain were investigated. The growth rate of the $\Delta ku80$ strain did not decrease, compared with that of the wild strain both on plate medium and in liquid medium (data not shown). In addition, the germination rate of its spores was similar to that of the wild strain (data not shown). Furthermore, given that the fatty acid productivity and composition of the $\Delta ku80$ strain were similar to those of the wild strain, I infer that *ku80* gene disruption did not affect fatty acid productivity (Table 3-3). In *N. crassa*, deletion of the *ku70* homolog (*mus-51*), the *ku80* homolog (*mus-52*), and the *lig4* homolog (*mus-53*) gene resulted in an increase in sensitivity to chemical mutagens causing DSBs, such as MMS [56, 62]. The *M. alpina* 1S-4 $\Delta ku80$ strain showed no sensitivity to low ($\leq 0.02\%$) concentrations of MMS, but showed high sensitivity to 0.05% MMS (Fig. 3-4). These phenotypes of the *M. alpina* 1S-4 $\Delta ku80$ strain were consistent with that of the *lig4* gene disruptant of *A. oryzae* [61].

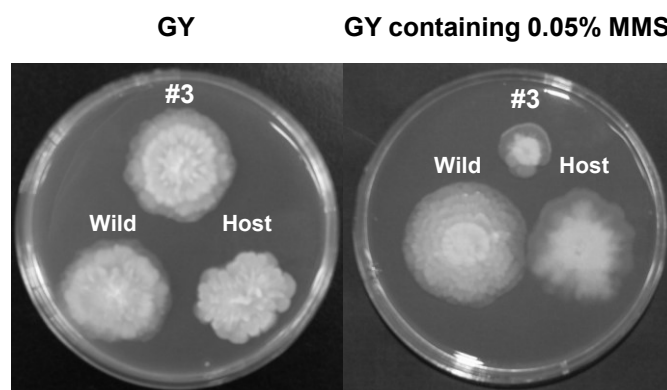


Fig. 3-4 Sensitivity of *M. alpina* 1S-4 wild strain, host strain (uracil auxotrophic mutant), and $\Delta ku80$ strain to methyl methanesulfonate (MMS). Their spores were spotted and grown on GY agar plate without MMS or containing 0.05% MMS for 4 days.

Table 3-3 Fatty acid productivity and composition in *M. alpina* 1S-4 host, $\Delta ku80$, $\Delta 5ds$ -defective mutant, and $\Delta 5ds$ -disrupted strain^a.

Strain	Total fatty acid		Fatty acid composition ^b (%)							
	mg/g	g/L	16:0	18:0	OA	LA	GLA	DGLA	ARA	24:0
Host	249.4 ± 21.1	5.0 ± 1.7	17.7 ± 1.3	6.8 ± 0.2	19.6 ± 0.9	7.2 ± 0.5	3.6 ± 0.2	3.3 ± 0.2	35.4 ± 2.1	2.2 ± 0.3
$\Delta ku80$	258.3 ± 77.0	5.1 ± 1.1	16.5 ± 0.6	6.9 ± 0.4	24.3 ± 0.5	7.2 ± 0.5	4.5 ± 0.5	3.1 ± 0.1	31.5 ± 1.5	2.2 ± 0.1
$\Delta 5ds$ -defective mutant	282.1 ± 35.6	3.7 ± 0.2	18.2 ± 0.5	6.8 ± 0.3	17.7 ± 1.3	2.7 ± 0.2	8.7 ± 0.3	38.0 ± 1.1	0.3 ± 0.0	2.6 ± 0.2
$\Delta 5ds$ -disruptant #15	263.4 ± 4.0	4.7 ± 1.0	19.8 ± 2.5	7.4 ± 0.7	17.0 ± 2.4	2.6 ± 0.8	4.3 ± 0.6	36.8 ± 6.2	3.4 ± 0.4	2.5 ± 0.4

^a The strains were grown in 3 ml of GY liquid medium for 7 days at 28°C with shaking at 120 rpm. All the values are for three independent samples (mean ± SD).^b OA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid; DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid.

Construction and characterization of $\Delta 5ds$ gene disruptant with pKSCD5 vector

To evaluate the improvement of gene-targeting efficiency in the $\Delta ku80$ strain and construct a strain producing valuable PUFAs by use of gene targeting, $\Delta 5ds$ gene disruption causing an increase in DGLA production and a decrease in ARA production (Fig. 3-5) was performed in the $\Delta ku80$ strain as a host strain (Fig. 3-6A). A vector for $\Delta 5ds$ gene disruption, pKSCD5, which contains the *CBXB* marker, was digested with *NruI* to enhance gene targeting efficiency and was introduced into spores of the $\Delta ku80$ strain on GY medium containing 100 $\mu\text{g/ml}$ carboxin by biolistic particle bombardment. After bombardment, the spores were cultivated at 28°C for 5 days. Finally, 32 stable transformants were obtained.

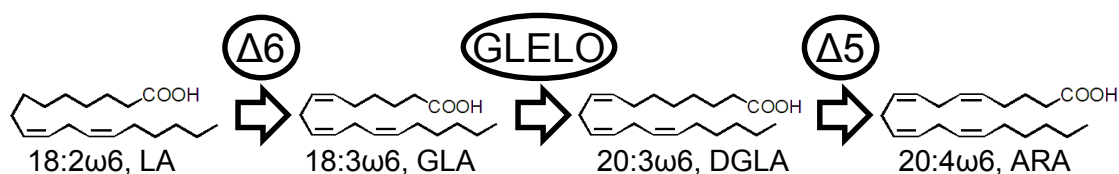


Fig. 3-5 Biosynthetic flow of ARA. ARA is biosynthesized by desaturation at the $\Delta 5$ -position of DGLA by $\Delta 5$ -desaturase. LA, linoleic acid; GLA, γ -linolenic acid; DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid; $\Delta 6$, $\Delta 6$ -desaturase; GLELO, $\Delta 6$ -elongase; $\Delta 5$, $\Delta 5$ -desaturase.

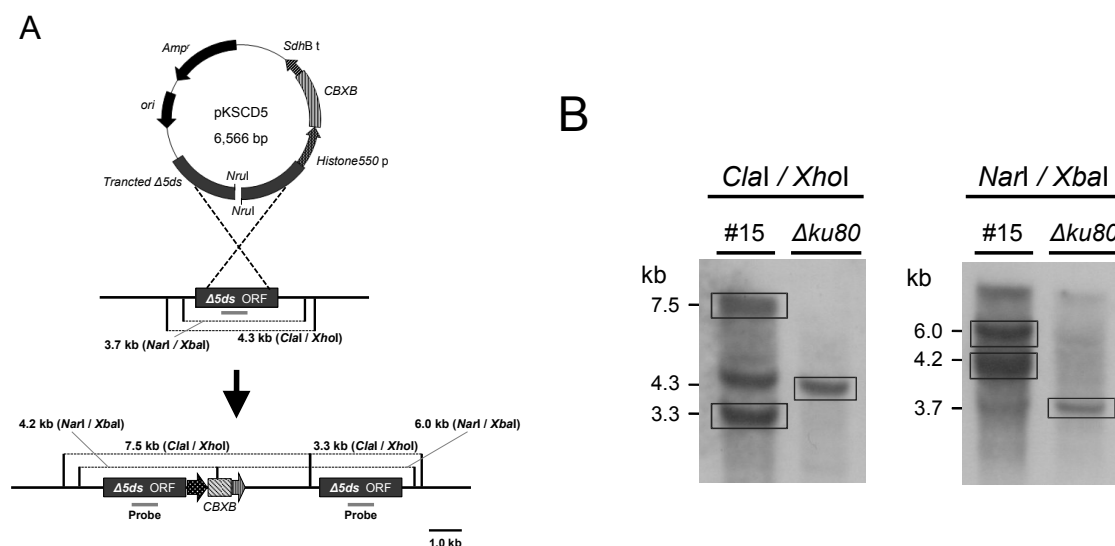


Fig. 3-6 Construction scheme of $\Delta 5ds$ gene disruptant and confirmation by Southern blot analysis. (A) The figure illustrates the homologous integration of the pKSCD5 vector into the $\Delta 5ds$ genomic gene locus in *M. alpina* 1S-4. Gray short bar indicates the position hybridized by the probe. Dotted lines indicate the position and base lengths of hybridization signals. (B) Southern hybridization analysis of $\Delta 5ds$ gene-disrupted candidates. *NarI*- and *XbaI*- or *Clal*- and *XhoI*-digested genomic DNAs were hybridized with the probe. Lane #15 and $\Delta ku80$ indicate a $\Delta 5ds$ gene-disrupted candidate and the host strain ($ku80$ -disruptant), respectively.

All stable transformants, the wild type, and the $\Delta ku80$ strain were cultivated in 3 ml of GY medium at 28°C for 7 days with reciprocal shaking, and their fatty acid productivities were determined by GC analysis. The ratio of DGLA to total fatty acids reached 36.8% in transformant 15, whereas that of ARA was only 3.4% (Fig. 3-7 and Table 3-3). Transformant 15 exhibited the same fatty acid composition as that of the $\Delta 5ds$ gene-defective mutant S14 isolated previously [81] (Table 3-3). To confirm the disruption of the $\Delta 5ds$ gene in transformant 15, Southern blot analyses were performed with genomic DNAs prepared from the $\Delta ku80$ strain and transformant 15 (Fig. 3-6B). When the genomic DNAs were digested with *Cla*I and *Xho*I, the 4.3-kb hybridization signal corresponding to the original $\Delta 5ds$ open reading frame was not detected in transformant 15, but the 3.3- and 7.5-kb signals were detected. When the genomic DNAs were digested with *Nar*I and *Xba*I, the 3.7-kb signal was not detected in transformant 15, but the expected 4.2- and 6.0-kb signals were detected. These results showed that the $\Delta 5ds$ gene in transformant 15 was successfully disrupted by integration of the pKSCD5 vector. Unexpected signals in transformant 15, however, were observed on the Southern blot. This finding may mean that several pKSCD5 vectors had been introduced into random sites in the genomic DNA of the $\Delta ku80$ strain by biolistic particle bombardment. The $ku80$ gene disruption improved the efficiency of gene targeting by 2.4-fold compared to the disruption of the $ku80$ gene in the *M. alpina* 1S-4 host strain (Table 3-2).

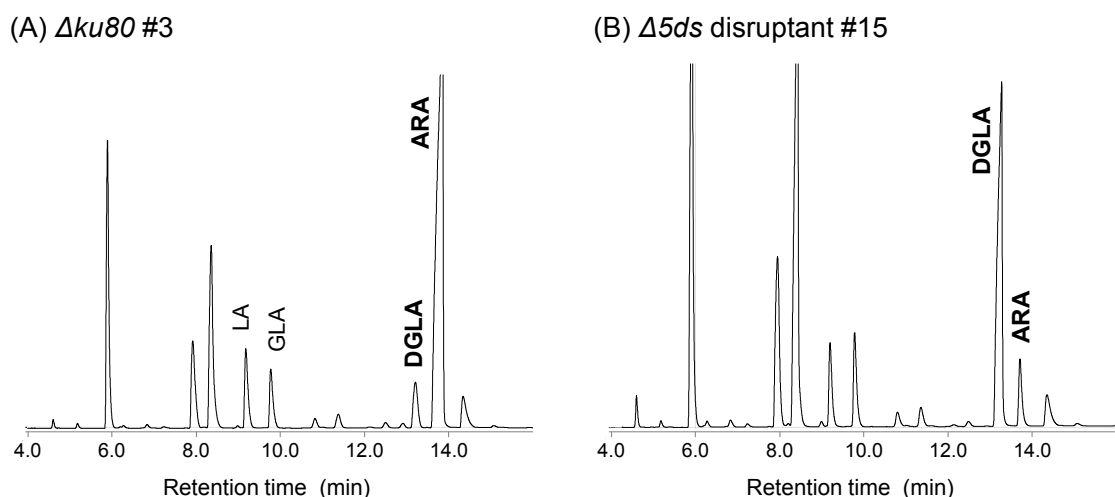


Fig. 3-7 GC chromatograms of fatty acid methyl esters prepared from total lipids of the $\Delta ku80$ #3 (A) used as a host strain and the $\Delta 5ds$ disruptant #15 (B).

DISCUSSION

To improve gene-targeting efficiency in *M. alpina* 1S-4, I cloned and identified the *ku80* gene encoding the Ku80 protein, which forms a Ku-protein complex with Ku70 protein, and is related to the NHEJ pathway. Ku80 homolog proteins of other organisms were classified by kingdom (Fig. 3-1). However, the predicted translation product of the *ku80* gene of this fungus shares low (<30%) identities with those of other organisms. I constructed a *ku80* gene disruptant (transformant 3 in Fig. 3-3B) via HR using the pKSUku80 vector. In transformant 6, I speculated that some of the introduced pKSUku80 vectors were integrated ectopically into the *ku80* gene locus (Fig. 3-3B). In general, one of the problems of biolistic particle bombardment is the delivery of many plasmids into cells. However, in view of the result for transformant 3, the biolistic particle bombardment method is applicable to the integration of a single vector into the genome via HR. The $\Delta ku80$ strain showed no marked differences in vegetative growth, formation of spores, or fatty acid productivity compared with the host strain (Table 3-3). Thus, I expect the $\Delta ku80$ strain to be a superior host strain for metabolic engineering for PUFA production. Furthermore, the $\Delta ku80$ strain exhibited a sensitivity to 0.05% MMS (Fig. 3-4) similar to those of *N. crassa*, *A. fumigatus*, and *A. aculeatus* [62, 64, 82]. Such sensitivity indicates that the NHEJ pathway in this strain is repressed. To evaluate the improvement of gene-targeting efficiency and construct a beneficial PUFA-producing strain, the disruption of $\Delta 5ds$ gene was performed using the $\Delta ku80$ strain as a host strain. The $\Delta 5ds$ gene disruptant, transformant 15, produced a large amount of DGLA. The DGLA productivity of transformant 15 was at the same level (Table 3-3) as that of a $\Delta 5ds$ gene-defective mutant obtained by chemical mutagenesis [81]. However, chemical mutagens cause mutation in multiple locations in the genome and often suppress growth, spore germination, and PUFA production. The ARA production of the $\Delta 5ds$ gene disruptant was drastically decreased. Given that the pKSCD5 vector was integrated via single-crossover HR, the incomplete $\Delta 5$ -desaturase may act in catalyzing the conversion of DGLA to ARA. For this reason, future gene targeting should be performed via double-crossover HR. Southern blot analysis using the genomic DNA from the $\Delta 5ds$ gene disruptant suggested that several vectors were integrated into ectopic sites on the chromosome. To obtain a complete disruptant with a single plasmid, more transformants should be isolated and checked, or gene targeting should be performed by an alternative transformation method such as an *A. tumefaciens*-

mediated method, introducing a single vector into a spore of the host strain.

The efficiency of *ku80* gene targeting was calculated as 1.3% (Table 3-2). Use of the *ku80* gene-disrupted strain as a host led to an efficiency of *Δ5ds* gene targeting calculated as 3.1%, representing a 2.4-fold improvement. In the same way, the efficiency of gene targeting in *Δku80* strains from *A. sojae*, *A. oryzae*, *A. fumigatus*, *A. niger*, and *Lecanicillium* sp. was not improved to 100% [58, 63, 82, 83]. In a previous study in *N. crassa*, chromosomal integration of exogenous DNA was achieved via two types of HR and two types of NHEJ, the Ku80-dependent major pathway and the Ku80-independent minor pathway [56]. In the *Δku80* strain from *M. alpina* 1S-4, the minor pathway may operate and reduce gene-targeting efficiency. When the *Δku80* strain was used as a host, the incomplete Ku80 protein formed a Ku-protein complex with the Ku70 protein, and the pKSCD5 vector was integrated ectopically via the NHEJ pathway. The loss of Lig4 activity involved in both the major and minor NHEJ pathways raised the targeting efficiency to 100% in *A. oryzae* and *A. luchuensis* [61, 84]. Further improvement in targeting efficiency in the *Δku80* strain, such as by simultaneous disruption of the *lig4* gene, might facilitate metabolic engineering and reverse-genetic studies in *M. alpina* 1S-4.

In summary, I succeeded in improving gene targeting efficiency in *M. alpina* 1S-4 by construction of a *ku80* gene disruptant showing normal growth, germination, and lipid production. This report is the first to date to describe the identification and disruption of the *ku80* gene in Mucoromycotina fungi. I demonstrated the utility of the NHEJ-defective strain by constructing a *Δ5ds* gene disruptant using the *Δku80* strain as a host strain. This efficient gene-targeting system may contribute to the construction of various PUFA-producing strains via metabolic engineering.

SUMMARY

To develop an efficient gene-targeting system in *Mortierella alpina* 1S-4, I identified the *ku80* gene encoding the Ku80 protein, which is involved in the non-homologous end joining pathway in genomic double-strand break (DSB) repair, and constructed *ku80* gene-disrupted strains via single-crossover homologous recombination. The $\Delta ku80$ strain from *M. alpina* 1S-4 showed no negative effects on vegetative growth, formation of spores, or fatty acid productivity and exhibited high sensitivity to methyl methanesulfonate, which causes DSBs. Dihomo- γ -linolenic acid (DGLA)-producing strains were constructed by disruption of the $\Delta 5$ -desaturase gene, encoding a key enzyme of bioconversion of DGLA to ARA, using the $\Delta ku80$ strain as a host strain. The disruption efficiency of 3.1% was improved by 2.4-fold compared with the disruption of the *ku80* gene in the *M. alpina* 1S-4 host strain. This report describes the first study on the identification and disruption of the *ku80* gene in zygomycetes and construction of a DGLA-producing transformant using a gene-targeting system in *M. alpina* 1S-4.

CHAPTER IV

An efficient gene-targeting system in a *lig4* disruptant derived from oleaginous fungus *Mortierella alpina* 1S-4

In chapter III, I constructed *ku80* disruptant from *Mortierella alpina* 1S-4 and evaluated the improvement of gene-targeting efficiency in the *ku80* disruptant. However, the efficiency in the *ku80* disruptant was not sufficient for application in *M. alpina* 1S-4. On the gene-targeting, multi copy of vector were detected on the genome of transformant by means of biolistic particle bombardment, which might lead to defects in essential genes.

Here, I remarked *lig4* disruption through homologous recombination. Recently, it was reported that disruption of *lig4* gene encoding DNA ligase 4 which is a key enzyme in the non-homologous end joining (NHEJ) led to an improvement of the gene-targeting efficiency through homologous recombination in various filamentous fungi, and their *lig4* disruptants contributed to basic researches and applications as the hosts [56, 60, 61, 85, 86]. On *lig4* disruption, *Agrobacterium tumefaciens*-mediated transformation (ATMT) method is useful for insertion of a single copy of vector in this fungus instead of means of biolistic particle bombardment [34].

In this study, I tried to develop more efficient gene-targeting system by *lig4* disruption through double-crossover HR by ATMT method in this fungus. Then, I evaluated gene-targeting efficiency in *M. alpina* 1S-4 without Lig4 activity.

MATERIALS AND METHODS

Enzymes and chemicals

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

Strains, media, and growth conditions

M. alpina 1S-4 is deposited in the Graduate School of Agriculture of Kyoto University [30] and was used as a host strain in this study. Czapek-Dox agar medium containing 0.05 mg/ml uracil, was used for sporulation of the uracil auxotrophic (*ura5⁻*) strain, as described previously [71]. Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of the transformants derived from *M. alpina* 1S-4 *ura5⁻* strain at 28°C [71]. GY medium (2% glucose and 1% yeast extract) was used for fatty acid composition analysis and extraction of genomic DNA. Uracil-free SC agar plates containing 0.03% Nile blue A (Sigma-Aldrich, St. Louis, MO) and GY agar plates containing 10 µg/ml carboxin and 0.03% Nile blue A were used for selection of transformants. *Escherichia coli* DH5α was used for DNA manipulation and grown on LB agar plates containing 50 µg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was used for the transformation of the *M. alpina* 1S-4 *ura5⁻* strain. LB-Mg agar medium, minimal medium (MM), and induction medium (IM) were used for transformation, cultivation and infection of *A. tumefaciens*, respectively [34]. All solid media were made by solidification with 2.0% agar.

Genomic DNA preparation

M. alpina 1S-4 was cultivated in 100 ml of GY liquid medium at 28°C for 5 days with shaking at 100 rpm. Preparation of genomic DNA was performed as described in chapter III.

Cloning and identification of the *lig4* gene from *M. alpina* 1S-4

Two highly degenerate primers were synthesized for cloning of the *lig4* gene using Lig4 F1 and Lig4 R1 primer (Table 4-1). The primers were designed based on the conserved amino acid sequences of Lig4 homologs from *Rhizopus delemar* (accession no. EIE79010), *Canis lupus* (accession no. XP_542663), *Laccaria bicolor* (accession no. XP_001874645), *Pan troglodytes*

(accession no. XP_509726), *Schizosaccharomyces pombe* (accession no. NP_587888). The sequences of the sense and antisense primers correspond to the regions that encode LIVGGY and TLRFPK peptides, respectively. PCR amplification was carried out in a total volume of 50 μ l containing 1 μ g of genomic DNA, 0.25 μ l of *Takara EX taq* polymerase (Takara Bio Inc.), 5 μ l of 10 $\times$ *EX Taq* buffer, 200 μ M of each dNTP, and 5 pM of primers, and performed as 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min, followed by 1 cycle of extension period at 72°C for 5 min. The resulting 571-bp fragment was cloned into the pT7Blue T-Vector (Novagen, Madison, WI, USA) and sequenced the nucleotide sequence was determined with a Beckman-Coulter CEQ8000 system (Beckman-Coulter, Fullerton, CA, USA). For RNA isolation and cDNA synthesis, RNA extraction reagent Isogen (Nippon Gene, Tokyo, Japan) and a PrimeScript™ High Fidelity RT-PCR Kit (Takara Bio Inc.) were used according to the standard protocols.

To isolate the whole *lig4* genomic DNA from *M. alpina* 1S-4, inverse PCR was performed with Lig4 IPCR F and Lig4 IPCR R primers (Table 4-1), corresponding to 117-162 and 373-412 positions of the *lig4* gene fragment, respectively. The *Bam*HI-digested genomic fragment was self-ligated and then used as a template. PCR amplification was carried out in a total volume of 50 μ l containing 500 ng of the template, 0.25 μ l of *Takara EX taq* polymerase (Takara Bio Inc.), 5 μ l of 10 $\times$ *EX Taq* buffer, 200 μ M of each dNTP, and 5 pM of each primer, and performed as followed: initial denaturation at 94°C for 1min, followed by 35 cycles of 94°C for 40 sec, 57°C for 40 sec, and 72°C 3 min and 1 cycle of extension period at 72°C for 10 min. The amplified 3.0-kb fragment was cloned into the pT7Blue T-Vector and confirmed by determination of its nucleotide sequences with a Beckman-Coulter CEQ8000 system.

Construction of the plasmid vector for *lig4* gene-targeting

A T-DNA binary vector for *lig4* gene-targeting was constructed on the backbone of *M. alpina* 1S-4 transformation vector pBIG35Zh [34, 87] or pBIG3CBZ (This study). Firstly, the carboxin resistance *CBXB* gene cassette [67] on the *M. alpina* 1S-4 transformation vector pSDZNCBXB was digested with *Eco*RI and *Xba*I, ligated into pBIG35Zh vector digested with same restriction enzymes, and the resulting plasmid was designated pBIG3CBZ. The 5'-*lig4* fragment (1.6-kb) and 3'-*lig4* fragment (1.8-kb) were amplified with primer sets, Lig4 F1 *Apa*I and Lig4 R1 *Apa*I, and Lig4 F2 *Xba*I and Lig4 R2 *Nhe*I (Table 4-1), by using genomic DNA of

M. alpina 1S-4 as a template, respectively. The 3'-*lig4* fragment was digested with *Xba*I and *Nhe*I, and ligated into pBIG35Zh or pBIG3CBZ digested with same restriction enzymes. Then, the 5'-*lig4* fragment was digested with *Apa*I, and ligated into the resulting plasmid vectors digested with the same restriction enzymes and designated pBIG35ZΔL4 and pBIG3CBZ, respectively (Fig. 4-1). The pBIG3CBZ vector was constructed in order to estimate targeting efficiencies in the *lig4* gene-disruptant. The sequences between left border and right border were integrated into genomic DNA of *M. alpina* 1S-4.

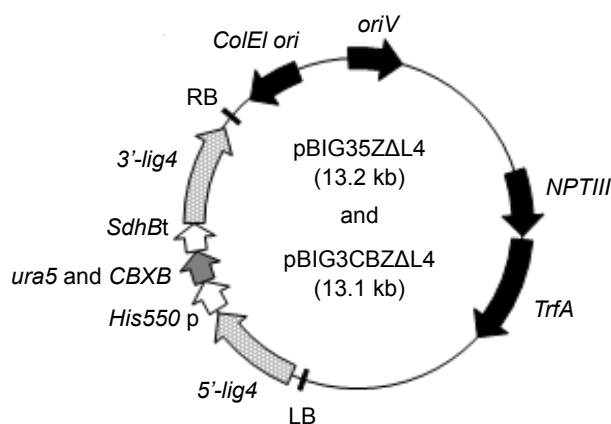


Fig. 4-1 The pBIG35ZΔL4 and pBIG3CBZΔL4 vector used for *lig4* gene-disruption. LB, left border; RB, right border; *His550p*, *M. alpina* 1S-4 histone H4.1 promoter short fragment; *ura5*, orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *CBXB*, the mutated *sdhB* gene for carboxin resistance; *SdhBt*, *M. alpina* 1S-4 *sdhB* translation terminator; *NPTIII*, neomycin phosphotransferase III gene; *TrfA*, *trfA* locus, which produces two proteins that promote replication of the plasmid; *ColEI ori*, *ColEI* origin of replication; *oriV*, pRK2 origin of replication.

Transformation of *M. alpina* 1S-4

Spores of the *M. alpina* 1S-4 uracil auxotrophic strain was freshly prepared from cultures growing on Czapek-Dox agar medium supplemented with 0.05 mg/ml uracil, and the spore suspension was filtered through Miracloth (Calbiochem) [71].

Transformation of the *M. alpina* 1S-4 was carried out by means of the ATMT method as described previously [34] with slight modification. After co-cultivation of *A. tumefaciens* cells and the spores of *M. alpina* 1S-4, the membranes were transferred onto uracil-free SC agar plates containing 0.03% Nile blue A or GY agar plates containing 10 µg/ml carboxin. After 4 days of incubation at 28°C, aerial hyphae of fungal colonies were transferred to fresh uracil-free SC agar

plates or GY agar plates containing 100 µg/ml carboxin.

Selection of the gene-disrupted strain by PCR

The recombinants transformed with pBIG35ZΔL4 were repeatedly cultured on fresh SC agar medium containing none of uracil at least three times. The *lig4* gene-disrupted candidates were selected by PCR. Firstly, the *lig4* genomic gene locus was amplified with Lig4 up F and Lig4 down R primers (Table 4-1) and genomic DNA prepared from transformants as a template. Selection of *Δlig4* candidates was performed by PCR using the resulting fragment as a template and three primer sets, Lig4 F1 *Apa*I and Lig4 R1 *Apa*I, Lig4 in F and Lig4 in R, and Lig4 F1 *Apa*I and *ura5* 400 R (Table 4-1 and Fig. 4-3).

The replacement of *ura5* marker to *CBXB* marker with pBIG3CBZΔL4 were confirmed by colony-PCR using the extracted genomic DNA as a template and Lig4 up F and CBXB stop R primers (Table 4-1).

Table 4-1 Primers used in this study.

Primer	Sequence (5' to 3')
Lig4 F1	YTIATYGTBGTBTA
Lig4 R1	AAYYTIMGITTYCCIMG
Lig4 IPCR F	CCAGAGAGGTATGCTGCACCTATTTACACCAACCAATCC
Lig4 IPCR R	GCCAAACATCTCGAACGTAATTCCTGATCCCACTTTCGAGAAGGTC
Lig4 F1 <i>Apa</i> I	GCGGGCCCTGACCTAACAGCCCCGACAATAC
Lig4 R1 <i>Apa</i> I	GCGGGCCCAATATCATGTATCACACAATGTCAG
Lig4 F2 <i>Xba</i> I	GCTCTAGAAGACAGCCGTCACCTCATACG
Lig4 R2 <i>Nhe</i> I	GCGCTAGCAACTACTTCGACACCGCAAC
Lig4 up F	GTGTGCCATCAACACGTTGTCTGC
Lig4 down R	CCAAGAACGACATGTCAATGTACG
Lig4 in F	TCCACAGATCTTCCCAAAGG
Lig4 in R	CGATCAGCACGTCCAAACTA
<i>ura5</i> 400 R	CATGGTCCTTCTTTTCCTTGC
Lig4 F2	GCTGTTACGGCTTACCATC
<i>ura5</i> start F	GGATTACCAGCGCGAGTTC
<i>ura5</i> stop R	TTAAACACCGTACTTCTCGCGG
CBXB stop R	GATTACTCCAAAGCCATGGTC

^aThe underlined sequences show synthesized restriction enzyme site.

Southern blot analysis

Probes used for hybridization were the 0.8-kb part of 5'-fragment of *lig4* gene and the 0.6-kb part of *ura5* gene, amplified with primer sets, Lig4 F2 and Lig4 R1 *Apa*I, and *ura5* start F and *ura5* stop R (Table 4-1), respectively. Genomic DNA (10 µg) were digested with *Sac*I and *Xho*I, size-fractionated by electrophoresis in 1% agarose gel, and transferred to Amersham Hybond-N⁺ membrane (GE Healthcare Ltd., Buckinghamshire, UK). Southern blot hybridization was carried out as described in chapter III.

Mutagen sensitivity of *Δlig4::ura5* strain

Sensitivity to chemical mutagen toxicity was evaluated by spot test as described in chapter III. Methyl methanesulfonate (MMS) was added in concentration of 0, 0.01, 0.02, 0.03, 0.04, and 0.005% [w/v] to SC agar medium supplemented with 0.05 mg/ml uracil. Spores were spotted and grown on the agar plates for 8 days.

Fatty acid analysis

The mycelia of the *lig4* gene-disrupted strain and its host strain were inoculated into 10 ml of GY liquid medium and cultivated at 28°C with reciprocal shaking at 300 rpm for 7 days. Fatty acid productions and compositions in the strains were analyzed as described in chapter I.

Nucleotide sequence accession number

The *lig4* gene from *M. alpina* 1S-4 was registered in the DNA Data Bank of Japan (DDBJ) database under the accession number LC009448.

RESULTS

Identification and phylogenetic analysis of the *lig4* homolog gene from *M. alpina* 1S-4

To clone the *lig4* gene from *M. alpina* 1S-4, a 571-bp fragment amplified using the degenerate primers designed on the basis of highly-conserved amino acid sequences of other Lig4 homologs and total cDNAs prepared from *M. alpina* 1S-4 was isolated and its nucleotide sequences were determined. The whole nucleotide sequence of *lig4* gene was identified by inverse PCR. The open reading frame of the *M. alpina lig4* gene consists of a 3,309-bp has ten exons (1-103, 217-691, 791-901, 1,009-1,212, 1,312-1,354, 1,449-1,659, 1,776-1,881, 1,974-2,079, 2,178-2,872, and 2,964-3,309) which generate a mRNA in a length of 2,400-bp. The deduced protein consisting 799 amino acids shares low identities with other Lig4 from ascomycetes (*Aspergillus nidulans*, 32%; *Aspergillus oryzae*, 31%; *Aspergillus fumigatus*, 29%), basidiomycetes (*Cryptococcus neoformans*, 24%; *Coprinopsis cinerea*, 29%), mammals (*Mus musculus*, 31%; *Rattus norvegicus*, 30%; *Homo sapiens*, 31%), and higher plants (*Arabidopsis thaliana*, 26%; *Oryza sativa*, 33%). The phylogenetic tree of various Lig4 homolog proteins from these organisms indicated that the Lig4 homologs were clustered in each organism group, such as fungi, mammal, and plant, whereas the Lig4 protein (MaLig4) from *M. alpina* 1S-4 was remotely related to those from other organisms (Fig. 4-2).

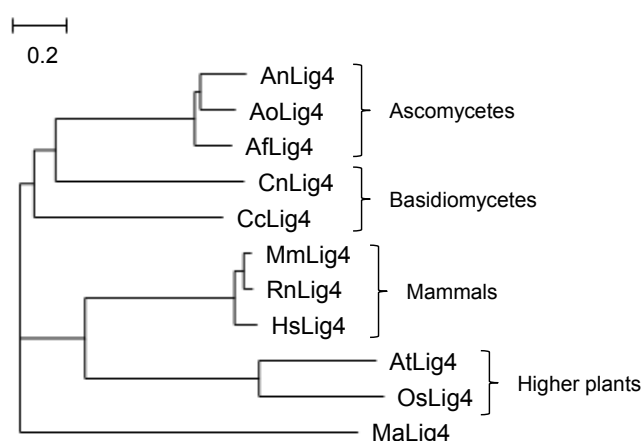


Fig. 4-2 The phylogenetic tree of Lig4 proteins. The tree was created by using neighbor-joining (NJ) method with 10000 bootstrap replicates by a sequence analysis software GENETYX ver. 11.0 (Genetyx corp., Tokyo, Japan). *Aspergillus nidulans* (AnLig4), Q5BH83; *Aspergillus oryzae* (AoLig4), BAE62914; *Aspergillus fumigatus* (AfLig4), EAL91408; *Cryptococcus neoformans* (CnLig4), AAW46139; *Coprinopsis cinerea* (CcLig4), Q7Z7W5; *Mus musculus* (MmLig4), NP_795927; *Rattus norvegicus* (RnLig4), NP_001099565; *Homo sapiens* (HsLig4), NP_001091738; *Arabidopsis thaliana* (AtLig4), NP_568851; *Oryza sativa* (OsLig4), Q7X7E9; *Mortierella alpina* 1S-4 (MaLig4), LC009448.

Construction and characterization of the *lig4* gene-disruptants from the host strain

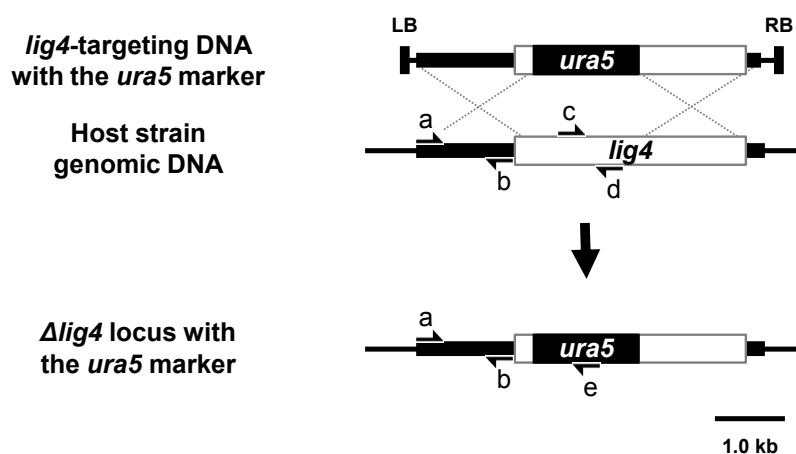
To construct a $\Delta lig4$ strain ($\Delta lig4::ura5$) from *M. alpina* 1S-4 uracil auxotrophic strain, the spores of this fungal strain were transformed with pBIG35Z Δ L4 (Fig. 4-1) by means of the ATMT method (Fig. 4-4A). The *lig4*-gene disruption in 93 transformants obtained were checked by PCR (Fig. 4-3). When the disruption fragment from pBIG35Z Δ L4 is inserted into the *lig4* gene locus, a 0.2-kb fragment from intact *lig4* gene is not amplified by using a pair of primers, Lig4 in F and Lig4 in R, but a 2.5-kb fragment is amplified using a pair of primers, Lig4 F1 *Apa*I and *ura5* 400 R, indicating that the fragment is successfully integrated into the targeted *lig4* gene locus (Fig. 4-4A). As a result, 3 transformants showed the genotypes from *lig4* gene-disruptants: defect of a part of *lig4* gene and integration of the *ura5* marker of the plasmid in *lig4* gene locus (Figure 4-2A and 4-2B). By Southern blot analysis, I investigated whether a single homologous integration into the *lig4* gene was correctly occurred (Fig. 4-4A and 4-4B). The Southern analysis illustrated the hybridization signal at 1.0-kb not 2.9-kb by using probe A in the lane of the genomic DNAs digested with *Xho*I, 1.9-kb by using probe B in the lane of the genomic DNAs digested with *Xho*I, and 10.7-kb by using probe B in the lane of the genomic DNAs digested with *Sac*I (Fig. 4-4B). These results suggested that gene replacement with *ura5* marker had successfully occurred at the *lig4* gene locus in the host strain of *M. alpina* 1S-4. The targeting efficiency of the *lig4* gene in the uracil auxotrophic strain was calculated as 3.2% (Table 4-2).

Table 4-2 The efficiency of gene targeting through homologous recombination at the *lig4* locus with pBIG3CBZ Δ L4 in *M. alpina* 1S-4 wild strain and $\Delta lig4$ strain.

Host	The number of transformant	Homologous replaced strain	Gene targeting efficiency (%) ^a
Wild	93	3	3.2
$\Delta lig4::ura5$	3	2	66.7

^aThe efficiency of gene targeting was calculated by the ratio of the number of homologous replaced strain to the number of the transformant.

A



B

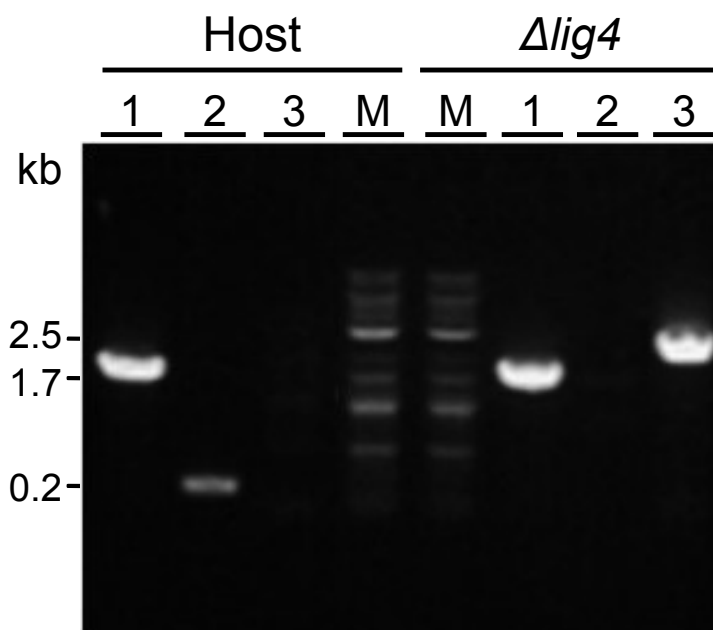
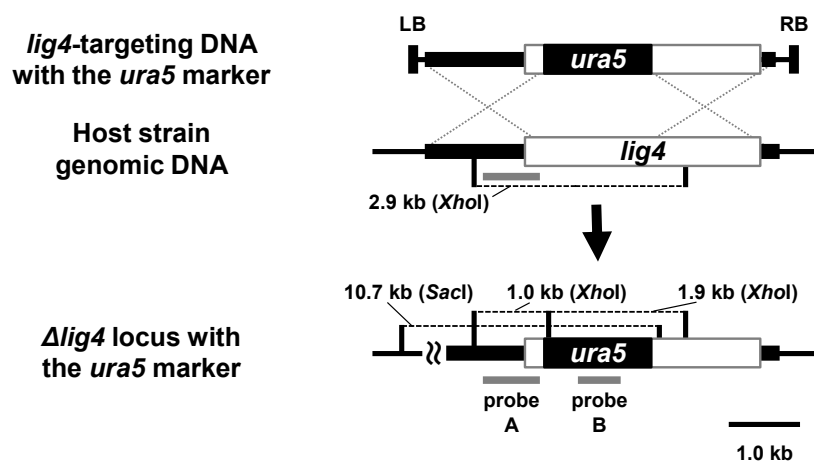


Fig. 4-3 Construction scheme and confirmation of $\Delta lig4$ strains. (A) The figure illustrates integration of pBIG35 Δ L4 vector into the *lig4* genomic gene locus in *M. alpina* 1S-4. Black arrows, a ~ e, indicate the position of oligonucleotide primers used for PCR: a, Lig4 F1 Apal; b, Lig4 R1 Apal; c, Lig4 in F; d, Lig4 in R; e, *ura5* 400 R. (B) Selection of *lig4* disruptant by PCR. Lane 1, primer a and b; Lane 2, primer c and d; Lane 3, primer a and e.

A



B

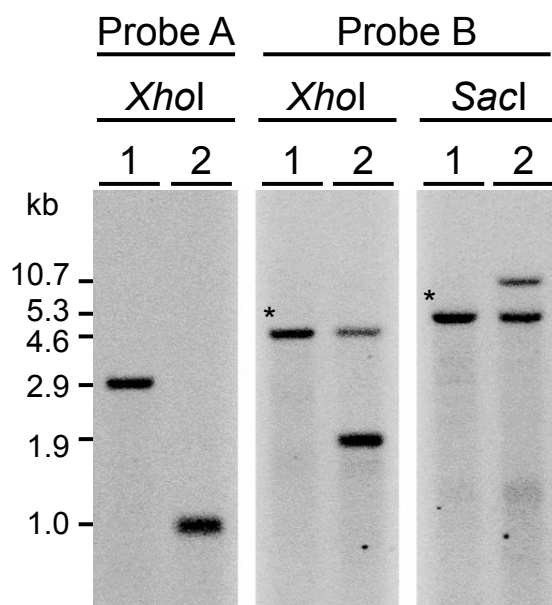


Fig. 4-4 The *lig4* gene-disruption and confirmation of the disruption. (A) The figure illustrates homologous integration of pBIG35ZΔL4 vector into the *lig4* genomic gene locus in *M. alpina* 1S-4. Gray short bar indicates the position hybridized by probe. Dotted lines indicate the position and base length of hybridization signals. (B) Southern hybridization analysis of the *lig4* gene-disrupted strain. *Xho*I- or *Sac*I-digested genomic DNAs were hybridized with the probes. The asterisks demonstrate signals of an endogenous *ura5* gene. Lane 1, host (*ura5*⁺) strain; Lane 2, Δ *lig4* strain.

Evaluation of the gene-targeting efficiency

To evaluate the improvement of the gene-targeting efficiency in the $\Delta lig4::ura5$ strain, the replacement of *ura5* marker to *CBXB* marker on the $\Delta lig4$ locus was carried out in the $\Delta lig4::ura5$ strain (Fig. 4-5A). The pBIG3CBZ Δ L4 vector was introduced into spores of the $\Delta lig4::ura5$ strain on GY medium containing 100 μ g/ml carboxin by ATMT method. The integration patterns of the vector in genomes prepared from three transformants obtained were investigated by colony-PCR, and the successful replacement resulted in the amplification of a 3.5-kb fragment (Fig. 4-5A and 4-5B). As a result, two transformants showed the expected replacement on the $\Delta lig4$ locus. Thus, the targeting efficiency was calculated as 66.7%, and improved to 20.7-fold as high as that of isolation of $\Delta lig4$ strain from *M. alpina* 1S-4 host strain (Table 4-2).

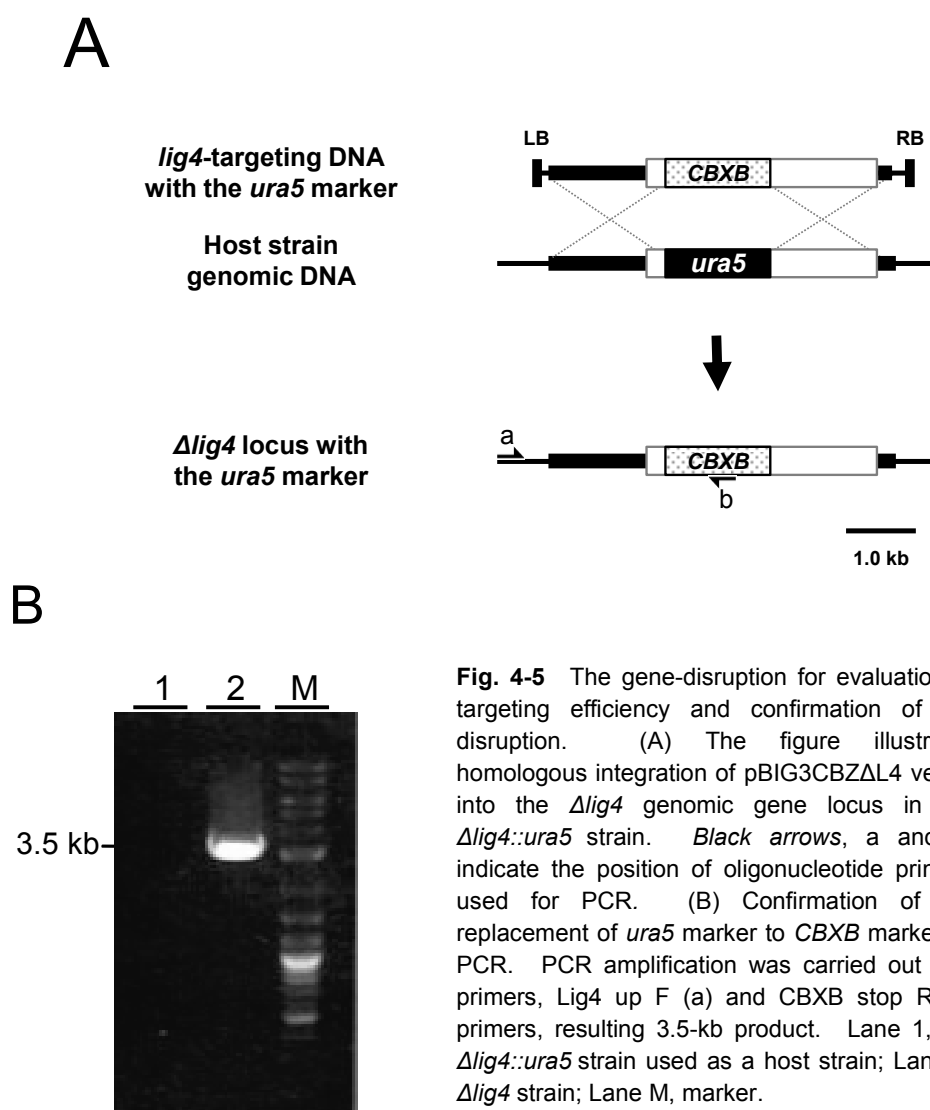


Fig. 4-5 The gene-disruption for evaluation of targeting efficiency and confirmation of the disruption. (A) The figure illustrates homologous integration of pBIG3CBZ Δ L4 vector into the $\Delta lig4$ genomic gene locus in the $\Delta lig4::ura5$ strain. Black arrows, a and b, indicate the position of oligonucleotide primers used for PCR. (B) Confirmation of the replacement of *ura5* marker to *CBXB* marker by PCR. PCR amplification was carried out with primers, Lig4 up F (a) and CBXB stop R (b) primers, resulting 3.5-kb product. Lane 1, the $\Delta lig4::ura5$ strain used as a host strain; Lane 2, $\Delta lig4$ strain; Lane M, marker.

Mutagen sensitivity, growth, and fatty acid productivity of the *Δlig4::ura5* strain

The *Δlig4::ura5* strain showed sufficient sporulation, and the spores germinated on MMS-free SC agar medium (Fig. 4-6). However, growth rate of the *Δlig4::ura5* strain significantly decreased on SC agar plates containing MMS, compared with that of host strain on the same medium (Fig. 4-6). The *Δlig4::ura5* strain was not capable of growing on the plates containing the higher concentration than 0.03% of MMS, whereas the host strain grew on the plates containing 0.03% MMS. Furthermore, I compared fatty acid productivities and growth rates of the *Δlig4::ura5* strain with those of wild strain. The *Δlig4::ura5* strains showed the same fatty acid-productivities and growth rates in GY liquid medium, as those of host strain (Table 4-3). The *Δlig4::ura5* strain formed the same white aerial hyphae as the host strain (Table 4-3 and under condition of 0% MMS in Fig. 4-6). The *Δlig4::ura5* strain showing general amounts of PUFAs, such as linoleic acid (LA), γ -linoleic acid (GLA), dihomo- γ -linolenic acid (DGLA), and ARA, is expected as a recipient for the molecular breeding (Table 4-3).

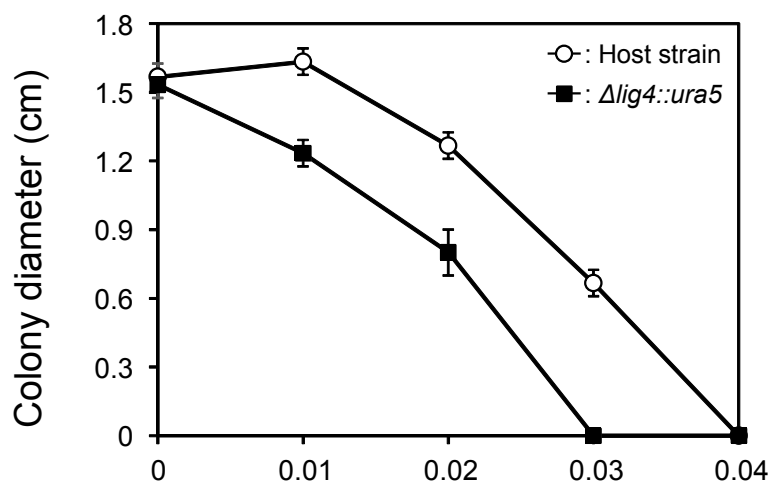


Fig. 4-6 Sensitivity of *M. alpina* 1S-4 host strain and *Δlig4* strain to methyl methanesulfonate (MMS). Spores were placed and cultivated on SC agar plates with or without MMS for 8 days.

Table 4-2 The fatty acid productivity and composition in *M. alpina* 1S-4 host strain, uracil auxotroph of *lig4* disruptant, $\Delta 5ds$ -defective mutant, and $\Delta 5ds$ -disruptant^a.

Strain	DCW ^b		Total fatty acid		Fatty acid composition ^c (%)							
	mg		mg/g	mg/L	16:0	18:0	OA	LA	GLA	DGLA	ARA	Others
Host	92.7 ± 0.6		30.6 ± 0.9	284.0 ± 7.0	21.4 ± 1.0	7.9 ± 0.7	16.7 ± 2.2	7.8 ± 0.6	3.0 ± 0.0	4.2 ± 0.2	25.0 ± 3.1	13.9 ± 0.3
$\Delta lig4::ura5$	90.1 ± 0.8		37.7 ± 1.3	339.1 ± 12.3	22.4 ± 1.2	7.3 ± 0.2	17.8 ± 0.5	8.4 ± 0.6	3.3 ± 0.3	4.2 ± 0.4	22.1 ± 0.8	14.6 ± 0.2

^a The strains were grown in 10 ml of GY liquid medium for 7 days at 28°C with shaking at 300 rpm. All the values are for three independent samples (mean ± SD).

^b DCW means weight of dried cells.

^c OA, oleic acid; LA, linoleic acid; GLA, γ-linolenic acid; DGLA, dihomο-γ-linolenic acid; ARA, arachidonic acid.

DISCUSSION

I cloned and identified the *lig4* gene encoding DNA ligase 4 (Lig4) involved in all NHEJ pathways from *M. alpina* 1S-4. Lig4 homolog proteins of other organisms were classified with each kingdom (Fig. 4-2). In contrast, the Lig4 homolog (MaLig4) from *M. alpina* 1S-4 shares low identities (about 30%) with those of other organisms.

Then, I constructed a *lig4* gene-disruptant (the $\Delta lig4::ura5$ strain) from *M. alpina* 1S-4 through HR with pBIG35Z Δ L4 vector. In the previous study on *ku80* gene-disruption, a lot of vectors were introduced in genomes of *M. alpina* 1S-4 by means of biolistic particle bombardment [67]. In the present research, the *lig4* gene-disruption was constructed by ATMT method that can introduce single-copy of fragment between left border and right border of pBIG35Z Δ L4 into genome. On ATMT method, multiple integration occurs at quite low frequencies in genomes of *M. alpina* 1S-4 [34]. It was previously reported that the ratio of integration through HR was increased by ATMT method [88-91]. By using ATMT method, the $\Delta lig4::ura5$ strains were obtained with 3.2% of efficiency which was a slightly higher compared with *ku80* gene-disruption by using biolistic particle bombardment (Chapter III). Also, the targeting efficiency in the $\Delta lig4::ura5$ strain was calculated as 66.7%, which corresponded to 20.7-fold as higher as that in the disruption of the *lig4* gene in *M. alpina* 1S-4 host strain (Table 4-2). Compared with that of the $\Delta ku80$ strain, the gene-targeting efficiency was drastically improved in $\Delta lig4$ strain. It is expected that disruption of *lig4* gene through double-crossover HR led to complete inactivation of Lig4 and occurred the improvement of gene-targeting efficiency. However, the targeting efficiency did not reach 100% in the $\Delta lig4::ura5$ strain, whereas it was 100% in *N. crassa*, *A. oryzae*, and *Aspergillus luchuensis* [56, 61, 84]. It is unclear why the gene-targeting efficiency in several organisms including this fungus did not improve up to 100% [60, 89, 92]. Depending on the target gene locus, the targeting efficiency is quite different because eukaryotic chromosome is a mosaic of active and inactive domains [93]. In the future, I should evaluate more statistical data of the gene-targeting efficiency by disruption of various genes related to fatty acid synthesis and accumulation.

The $\Delta lig4::ura5$ strain showed no defect in vegetative growth, formation of spores, and fatty acid productivity (Table 4-3). Since this fungus is expected as a PUFA producer, such characteristic is suitable for the following molecular breeding. Lig4 protein is involved in NHEJ

repair of DSBs in phylogenetically diverse organisms [78, 79] and in telomere maintenance in some organisms [80]. The *lig4* disruption in some organisms led to sensitivity to MMS exhibiting mutagen toxicity [56, 62]. The $\Delta lig4::ura5$ strain exhibited high sensitivity to MMS (Fig. 4-6), and the sensitivity was similar to that of *N. crassa*, *A. oryzae*, and *A. kawachii* [56, 61, 92]. Since the $\Delta lig4::ura5$ strain showed high sensitivity for MMS, the repression of NHEJ pathway in this strain was indicated.

This study is the first report described on identification and characterization of a *lig4* gene from Mucoromycotina, and the improvement of the gene-targeting efficiency in the $\Delta lig4$ strain is expected to facilitate metabolic engineering for production of beneficial PUFAs and reverse genetic studies for basic research in *M. alpina* 1S-4.

SUMMARY

The oil-producing zygomycete *Mortierella alpina* 1S-4 is known to accumulate beneficial polyunsaturated fatty acids. I identified the *lig4* gene encoding a DNA ligase 4 homolog related to the general pathway of non-homologous end joining on genomic double strand breaks repair, and constructed *lig4* gene-disrupted strains for improvement of gene-targeting efficiency. The *M. alpina* 1S-4 *lig4* gene-disruptant showed no defect in vegetative growth, formation of spores, and fatty acid productivity, and exhibited high sensitivity to methyl methansulfonate causing DNA double-strand breaks. The replacement occurred in 67%, and the gene-targeting efficiency was improved to 21-fold compared to the disruption of *lig4* gene in *M. alpina* 1S-4 host strain. It is expected that the gene-targeting in the *lig4* gene-disruptant contributes to construction of a beneficial and rare PUFA-producing strains by metabolic engineering, and to basic research on zygomycete.

CHAPTER V

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

Dihomo- γ -linolenic (20:3 ω 6, DGLA) is a precursor of physiologically active eicosanoids, including group 1 prostaglandins and thromboxanes. DGLA attracted a great deal of interest because of its unique biological activities. For example, DGLA is used in combination with interferons for enhancement of the anti-virus, anti-cancer or anti-inflammatory effects [94] and in combination with prostaglandin E1 for treatment of atopy of the skin and mucosa [95]. A small amount of DGLA was detected as a component of cellular lipids in fungi [25], algae [96], protozoa [97], and animals [98].

Previously, my research group have isolated DGLA-producing strains that is $\Delta 5$ -desaturase ($\Delta 5ds$) gene-defective mutant [81, 99] from oil-producing fungus, *Mortierella alpina* 1S-4 (Fig. 5-1). In the previous studies, various PUFA-producing strains were constructed by mutation and/or molecular breeding [15, 30]. However, some mutants and recombinants showed unfavorable characterization in mycelia growth, spore germination, and PUFA productivity, since the mutations and/or insertion of plasmid vector might occur at random positions with defects in essential genes.

Chapter III describes to develop efficient gene-targeting system through homologous recombination in *M. alpina* 1S-4 by disruption of the *lig4* gene to avoid unfavorable gene mutation and random insertion (Chapter IV).

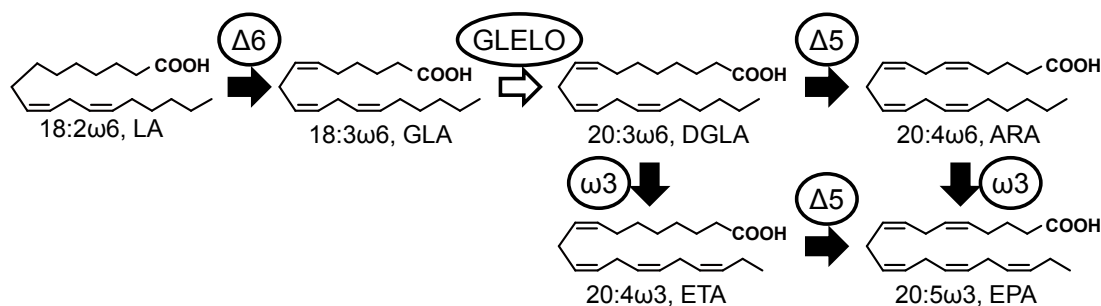


Fig. 5-1 PUFA biosynthetic pathways in *M. alpina* 1S-4. ARA is biosynthesized through desaturation at $\Delta 5$ -position of DGLA by $\Delta 5$ -desaturase. LA, linoleic acid; GLA, γ -linolenic acid; DGLA, dihomo- γ -linolenic acid; ARA, arachidonic acid; ETA, $\omega 3$ -eicosatetraenoic acid; EPA, eicosapentaenoic acid; $\Delta 6$, $\Delta 6$ -desaturase; GLELO, $\Delta 6$ -elongase; $\Delta 5$, $\Delta 5$ -desaturase; $\omega 3$, $\omega 3$ -desaturase.

CHAPTER V

In this study, I aimed to develop high DGLA-producing strain not having unwished mutations and gene-defections from *M. alpina* 1S-4 by gene targeting. I obtained uracil auxotrophic (*ura5*⁻) strain as a host strain in this study from the efficient gene-targeting strain (*Δlig4::ura5*) (Chapter IV), and tried to construct DGLA-producing strain by disruption of *Δ5ds* gene.

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

M. alpina 1S-4 is deposited in the Graduate School of Agriculture of Kyoto University [30]. Czapek-Dox agar medium containing 0.05 mg/ml uracil, was used for sporulation of uracil auxotrophic (*ura5*⁻) strain, as described previously [71]. Synthetic complete (SC) medium was used as a uracil-free synthetic medium for cultivation of the transformants derived from uracil auxotrophs at 28°C [71]. GY medium (2% [w/vol] glucose and 1% yeast 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-deleted (*Δlig4::ura5*) strain (Chapter IV). Uracil-free SC agar plates containing 0.03% Nile blue A (Sigma-Aldrich, St. Louis, MO) and GY agar plates containing 10 μg/ml carboxin and 0.03% Nile blue A were used for selection of transformants. *Escherichia coli* strain DH5α was used for DNA manipulation and grown on LB agar plates containing 50 μg/ml kanamycin. *Agrobacterium tumefaciens* C58C1 was used for the transformation of the *M. alpina* 1S-4 *ura5*⁻ strain. LB-Mg agar medium, minimal medium (MM), and induction medium (IM) were used for transformation, cultivation and infection of *A. tumefaciens*, respectively [34]. All solid media were made with 2.0% agar.

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

Isolation of uracil auxotrophs was performed as described previously [68]. 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 (2.6×10^8 spores/225 cm²): 2.6×10^7 spores were spread on a GY agar medium containing 5-FOA (1.0 mg/ml) and uracil (0.05 mg/ml).

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

The *lig4* gene locus containing *ura5* gene marker cassette was amplified with Lig4 up F and Lig4 down R primers (Table 5-1), using genomic DNA from uracil auxotroph of *lig4* gene-deleted strain genomic DNA 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 5-1).

Table 5-1 Primers used in this study.

Primer	Sequence (5' to 3')
Lig4 up F	GTGTGCCATCAACACGTTGTCTGC
Lig4 down R	CCAAGAACGACATGTCAATGTACG
Hispro stop F	CTCACCCAACACTCTCTCAAC
<i>ura5</i> 300 F	CTGCACCACCGTCATTGCCTTG
<i>ura5</i> 400 R	CATGGTCCTTCTTTTCCTTGC
SdhBt R	GAGGTAGTTGCGTGGGTCGTCGTAC
$\Delta 5$ F1 <i>Apal</i>	GCGGGCCCTTTTGTTCCTTGATCCTCACATTC
$\Delta 5$ R1 <i>Apal</i>	GCGGGCCCAAGAACTTTGTGACATCGTATAC
$\Delta 5$ F2 <i>XbaI</i>	GCTCTAGATCTGTTCCCGAACGTTTCCCAGC
$\Delta 5$ R2 <i>XbaI</i>	GGTCTAGAGCGATAATTATGAAAACCTTACC
$\Delta 5$ up F	CGTCCTCTACGATCCTCTGACCGAC
$\Delta 5$ down R	CCACATGGTACCAAAGCGTGGGCTG
SdhBt stop F	CGAGATGTCGCAGCTGAGCTAAGC
$\Delta 5$ down R2	GCTCTCTATCCTGTACATGC

^aThe underlined sequences show synthesized restriction enzyme site.

Genomic DNA preparation

M. 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 were prepared as described in chapter III.

Construction of the plasmid vector for $\Delta 5ds$ gene-targeting

A T-DNA binary vector for $\Delta 5ds$ gene targeting was constructed by modification of *M. alpina* 1S-4 transformation vector pBIG35Zh [34]. The $\Delta 5ds$ front fragment (1.2-kb) and $\Delta 5ds$ rear fragment (2.4-kb) were amplified with primer sets, $\Delta 5$ F1 *Apa*I and $\Delta 5$ R1 *Apa*I, and $\Delta 5$ F2 *Xba*I and $\Delta 5$ R2 *Xba*I (Table 5-1), by using genomic DNA of *M. alpina* 1S-4 as a template. The $\Delta 5ds$ rear fragment was digested with *Xba*I, and ligated into pBIG35Zh digested with same restriction enzyme. Then, the $\Delta 5ds$ front fragment was digested with *Apa*I, and ligated into the resulting plasmid vectors digested with same restriction enzyme and designated pBIG35Z Δ D5 (Fig. 5-2). The sequences between left border and right border were integrated into genomic DNA in spores of *M. alpina* 1S-4 through *A. tumefaciens*.

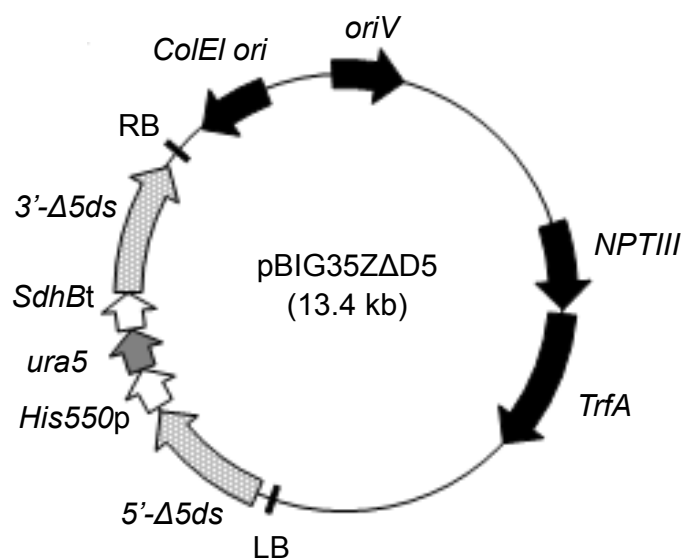


Fig. 5-2 The pBIG35Z Δ D5 vector used for $\Delta 5ds$ gene-disruption. LB, left border; RB, right border; *His550p*, *M. alpina* 1S-4 histone H4.1 promoter short fragment; *ura5*, orotate phosphoribosyl transferase gene of *M. alpina* 1S-4; *SdhBt*, *M. alpina* 1S-4 *sdhB* translation terminator; *NPTIII*, neomycin phosphotransferase III gene; *TrfA*, *trfA* locus, which produces two proteins that promote replication of the plasmid; *ColEI ori*, ColE1 origin of replication; *oriV*, pRK2 origin of replication.

Transformation of the *Δlig4* strain from *M. alpina* 1S-4

Transformation of the *M. alpina* strains were performed using the *Agrobacterium tumefaciens*-mediated transformation (ATMT) method as described in chapter IV.

Fatty acid analysis

Fatty acid productivity and composition of transformants were analyzed as described in chapter I. The mycelia of the *Δlig4* uracil auxotroph strain used as a host strain, the *Δ5ds* gene-defective mutant S14 [81], 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. The fungal strains after cultivation were harvested by filtration and applied for fatty acid analysis. All experiments were performed in triplicate, and the average of three separate experiments are presented in the table 5-2.

Identification of gene-disruption by PCR

The recombinants transformed with the pBIG35Z Δ D5 vector were repeatedly cultured on fresh SC agar medium containing no uracil at least three times to prevent contamination of abortive recombinants. The *Δ5ds* disruptants were selected for PCR using the *Δ5ds* genomic gene locus amplified with Δ 5 up F and Δ 5 down R primers (Table 5-1) as a template and primer sets, Δ 5 F1 *Apa*I and Δ 5 R1 *Apa*I, and SdhBt stop F and Δ 5 down R2 (Table 5-1). When the deletion fragment from pBIG35Z Δ D5 was inserted into the targeted *Δ5ds* gene locus, a 2.5-kb fragment was amplified with SdhBt stop F and Δ 5 down R2 primers, indicating that the fragment was integrated into the targeted *Δ5ds* gene locus.

RESULTS

Selection of uracil auxotroph (*ura5*) from the *Alig4::ura5* strain

To recycle the uracil marker system, a uracil auxotroph was selected on GY medium plate containing 0.1% 5-FOA from the *Alig4::ura5* strain. The spores of the *Alig4::ura5* strain were spread on GY plate containing 0.1% 5-FOA, and single colony was transferred onto fresh agar plates. To confirm mutations on the *ura5* gene used as a selectable marker, the sequence of *ura5* marker fragment amplified with the *Alig4* gene locus was determined with a Beckman-Coulter CEQ8000 system. A base replacement was recognized in a selected uracil auxotroph: the replacement of guanine to adenine occurred at the +212 nucleotide position, leading to G71D.

Construction and characterization of the *Δ5ds* gene-disrupted candidates

To construct *Δ5ds* disruptants in which DGLA is expected to be accumulated in high amounts, the spores of the uracil auxotroph from *Alig4::ura5* strain were transformed with pBIG35ZΔD5 (Fig. 5-2) by ATMT method (Fig. 5-3A). The integration of the disruption fragment from pBIG35ZΔD5 were detected by PCR in 18 transformants. Total FAMES prepared from the transformants were analyzed by GC, resulting that 9 transformants showed high accumulation of DGLA with a decrease of arachidonic acid (20:4ω6, ARA). On the other hand, the transformants showed good points in mycelial growth, sporulation, and spore germination (data not shown). Furthermore, I compared fatty acid productivities and compositions of the transformants with those of the uracil auxotroph from *Alig4::ura5* strain (host) and *Δ5ds* gene-defective mutant S14 (Table 5-2). The transformants showed same productivities of total fatty acid in GY liquid medium as those of host strain and *Δ5ds* gene-defective mutant S14. The ratio of DGLA in the total fatty acids increased and especially the ratio of DGLA in transformant #10 and #12 reached 38.7% and 40.1%, respectively, with no accumulation of ARA. On cultivation at low temperature, the *Δ5ds*-disrupted candidates and mutant S14 accumulated ω3-eicosatetraenoic acid (20:4ω3, ETA) calculated as 4% in total fatty acid, indicating that the ratios were improved to 10-fold compared with that of host strain (Table 5-2).

Table 5-2 Fatty acid productivity and composition in *M. alpina* 1S-4 host ($\Delta lig4::ura5^+$), $\Delta 5ds$ -defective mutant, and $\Delta 5ds$ -disruptants^a.

Strain	Cultivation temperature (°C)	Total fatty acid (g/L)	Fatty acid composition ^b (%)									
			16:0	18:0	OA	LA	GLA	DGLA	ARA	ETA	EPA	Others
Host	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
$\Delta 5ds$ -defective mutant	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
	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
$\Delta 5ds$ -disruptant #6	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
	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
	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
	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
#8	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
	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
#10	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
	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	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

^a The 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).^b OA, oleic acid; LA, linoleic acid; GLA, γ -linolenic acid; DGLA, dihomogamma-linolenic acid; ARA, arachidonic acid; ETA, eicosatetraenoic acid.^c Not detected.

Confirmation of the *Δ5ds* gene-disruption in the uracil auxotroph from *Δlig4::ura5* strain

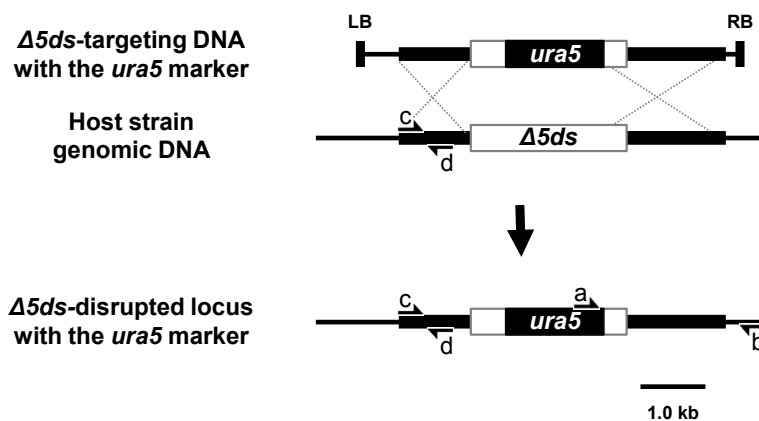
To confirm *Δ5ds* disruption in the transformants, PCR analyses were performed with their genomic DNAs (Fig. 5-3A and 5-3B). The integration patterns of the vector in genomes prepared from 18 transformants obtained were investigated by PCR, and the successful replacement resulted in the amplification of a 2.5-kb fragment. As a result, nine transformants showed the expected replacement on the *Δ5ds* locus. In addition, 9 transformants showed genotypes of *Δ5ds* gene-deletion, suggesting that the targeting efficiency of the deletion in this study was 50% in the uracil auxotroph from *Δlig4::ura5* strain (Table 5-3).

Table 5-3 Efficiency of gene targeting through homologous recombination at the *lig4* locus with pBIG3CBZΔL4 in *M. alpina* 1S-4 wild strain and *Δlig4* strain.

Host	The number of transformant	Homologous replaced strain	Gene targeting efficiency (%) ^a	Chapter
<i>lig4</i> -disruption				
Wild	93	3	3.2	Chapter IV
<i>Δlig4::ura5</i>	3	2	66.7	Chapter IV
<i>Δ5ds</i> -disruption	18	9	50.0	This chapter

^aThe efficiency of gene targeting was calculated by the ratio of the number of homologous replaced strain to the number of the Transformant.

A



B

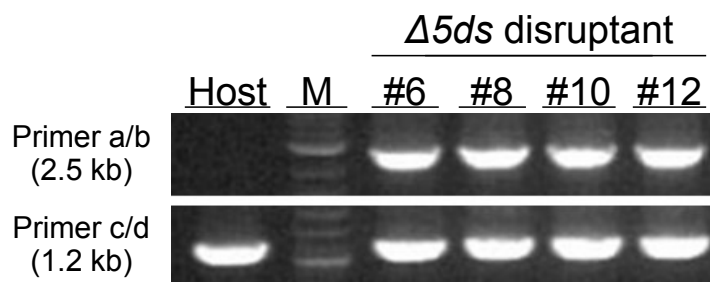


Fig. 5-3 Construction scheme and confirmation of $\Delta 5ds$ disrupted strains. (A) The figure illustrates integration of pBIG35Z $\Delta 5$ vector into the $\Delta 5ds$ genomic gene locus in *M. alpina* 1S-4. Black arrows, a ~ d, indicate the position of oligonucleotide primers used for PCR: a, SdhBt stop F; b, $\Delta 5$ down R2; c, $\Delta 5$ F1 *Apal*; d, $\Delta 5$ R1 *Apal*. (B) Selection of $\Delta 5ds$ disruptant by PCR.

DISCUSSION

To use *Δlig4* strain as a host strain for the following molecular breeding, I constructed the uracil auxotroph from *Δlig4::ura5* strain from the *M. alpina* 1S-4 *Δlig4::ura5* strain. An exchange of glycine to aspartic acid at 71 amino acid position which is highly conserved in orotate phosphoribosyl transferases may lead to incomplete conformation and inactivation of the enzyme. The uracil auxotroph from *Δlig4::ura5* strain showed no defect in mycelial growth rate, formation of spores, and fatty acid productivity (Table 5-2), which indicate that this fungus is expected to be PUFA producer, it was acceptable as a host for PUFA production.

I constructed DGLA-producing recombinants derived from *M. alpina* 1S-4 by *Δ5ds* gene-disruption using gene targeting. On *ku80* gene-disruption reported previously, one of the problems on biolistic particle bombardment was that a lot of vectors were integrated in genomic DNA of *M. alpina* 1S-4 (Chapter III). In the present research, the *Δ5ds* gene-disruption was achieved by ATMT method [34] through double-crossover homologous recombination using *Δlig4* strain showing efficient gene-targeting (Chapter IV). In the result, the *Δ5ds* gene was successfully replaced with a single vector fragment in *M. alpina* 1S-4 with 50% of the gene-targeting efficiency. The targeting efficiency was as high as that of *lig4*-replacement in the *Δlig4::ura5* strain from *M. alpina* 1S-4. The ratio of DGLA in the total fatty acids reached 40.1%, with no accumulation of ARA (Table 5-2). It suggests that a single integration of the vector in *Δ5ds* gene affected no defect on growth rate and PUFA productivities. In the previous research on *Δ5ds*-defective mutants including S14 strain, the mutation sites on their *Δ5ds* gene were found to occur point mutation of amino acids or frameshift in translation [100]. The mutants showed an increase in DGLA composition, but ARA was slightly detected in their mutants. On the process of PUFA purification, the separation of DGLA with ARA is quite difficult because of their similar properties. The DGLA-containing oils without ARA from the *Δ5ds*-disruptant in this chapter is useful for industrial production of DGLA. These results suggest that the selective PUFA-producers constructed by gene-targeting system were useful for industrial production of the PUFAs.

In summary, I succeeded to construct high DGLA-producer from *M. alpina* 1S-4 by *Δ5ds* gene-disruption. This is the first study on construction of DGLA-producing transformants using the efficient gene-targeting system in *M. alpina* 1S-4. The DGLA producer showed an

CHAPTER V

advantage for DGLA production. The uracil auxotroph from *Δlig4::ura5* strain is expected as a host for construction of various PUFA-producing recombinants.

SUMMARY

For microbial production of dihomo- γ -linolenic acid (DGLA), I obtained a uracil auxotroph from the efficient gene-targeting strain, *Δlig4* strain, and constructed DGLA-producing strains with disruption of the *Δ5-desaturase* (*Δ5ds*) gene encoding a key enzyme of bioconversion of DGLA to arachidonic acid (ARA) by efficient gene-targeting system using the uracil auxotroph isolated as a host. The isolation of nine *Δ5ds* disruptants out of eighteen isolates indicated that the disruption efficiency was 50%. The ratio of DGLA in the total fatty acids reached 40.1%, whereas no ARA was detected. This is the first study on construction of DGLA-producing transformants using the efficient gene-targeting system in *M. alpina* 1S-4.

CONCLUSIONS

In this thesis, I described screening of eicosenoic acid-producing fungi, characterization of the ω 3-desaturase from *Mortierella alpina* 1S-4, construction of an efficient gene-targeting system, and molecular breeding of selective polyunsaturated fatty acids (PUFAs)-producer by using the gene-targeting system in *M. alpina* 1S-4. The results described in each chapter are summarized as follows:

CHAPTER I

This chapter described the screening of fungi producing *cis*-11-eicosenoic acid (20:1 ω 9, EA) which is beneficial as a raw material for medical supplies and a moisturizing component of cosmetic creams. It is supposed that EA is synthesized by a Δ 9-fatty acid elongase from oleic acid as a precursor that is an intermediate in the biosynthetic pathways for polyunsaturated fatty acids. I found some EA-producing fungi by screening of about 300 fungal strains. In particular, *Mortierella chlamydospora* CBS 529.75 produced a high amount of EA (36.3 mg/g of dried cells) on cultivation at 28°C for 4 days and then at 12°C for 3 days. Most of the EA was a component of triacylglycerols, not phospholipids.

CHAPTER II

This chapter described characterization of a ω 3-fatty acid desaturase gene (*maw3*) from *Mortierella alpina* 1S-4. I investigated the products of *maw3* catalyzing reaction with endogenous and exogenous fatty acids in the yeast transformant. Two unusual fatty acids *de novo* synthesized in the yeast transformant expressing *maw3* gene were identified as ω 4 hexadecadienoic acid (*cis*-9,*cis*-12-16:2) and ω 1 hexadecatrienoic acid (*cis*-9,*cis*-12,*cis*-15-16:3) by GC-MS and ¹H-NMR analyses. In addition to the desaturation activity at the ω 3-position for 18- and 20-carbon PUFAs, MAW3 in the yeast transformant inserted a double bond at Δ 12-position of endogenous *cis*-9-16:1 and further at Δ 15-position of the resulting *cis*-9,*cis*-12-16:2 to result in the formation of *cis*-9,*cis*-12,*cis*-15-16:3 leading to a bifunctional Δ 12/ Δ 15-desaturase for 16-carbon fatty acids. It is the first report that the fatty acid desaturase (MAW3) is shown to have Δ 12- and Δ 15-desaturation activities for a 16-carbon fatty acid, in addition to its major function, ω 3-desaturation activity.

CHAPTER III

This chapter described construction of an efficient gene-targeting system by *ku80* disruption in *Mortierella alpina* 1S-4. I identified the *ku80* gene encoding the Ku80 protein, which is related to the non-homologous end joining pathway in genomic double-strand break (DSB) repair, and constructed *ku80* gene-disrupted strains via single-crossover homologous recombination. The $\Delta ku80$ strain from *M. alpina* 1S-4 showed no negative effects on vegetative growth, formation of spores, or fatty acid productivity and exhibited high sensitivity to methyl methanesulfonate, which causes DSBs. Dihomo- γ -linolenic acid (DGLA)-producing strains were constructed by disruption of the $\Delta 5$ -desaturase gene, encoding a key enzyme of bioconversion of DGLA to ARA, using the $\Delta ku80$ strain as a host strain. The disruption efficiency of 3.1% was improved by 2.4-fold compared with the disruption of the *ku80* gene in the *M. alpina* 1S-4 host strain. This report describes the first study on the identification and disruption of the *ku80* gene in zygomycetes and construction of a DGLA-producing transformant using a gene-targeting system in *M. alpina* 1S-4.

CHAPTER IV

This chapter described construction of an efficient gene-targeting system by *lig4* disruption in *M. alpina* 1S-4. I identified the *lig4* gene encoding a DNA ligase 4 homolog related to the general pathway of non-homologous end joining on genomic double strand breaks repair, and constructed *lig4* gene-disrupted strains for improvement of gene-targeting efficiency. The *M. alpina* 1S-4 *lig4* gene-disruptant showed no defect in vegetative growth, formation of spores, and fatty acid productivity, and exhibited high sensitivity to methyl methanesulfonate causing DNA double-strand breaks. The replacement occurred in 67%, and the gene-targeting efficiency was improved to 21-fold compared to the disruption of *lig4* gene in *M. alpina* 1S-4 host strain. It is expected that the gene-targeting in the *lig4* gene-disruptant contributes to construction of a beneficial and rare PUFA-producing strains by metabolic engineering, and to basic research on zygomycete.

CONCLUSIONS

CHAPTER V

This chapter described molecular breeding for DGLA-producer using an efficient gene-targeting system in *M. alpina* 1S-4. For microbial production of dihomo- γ -linolenic acid (DGLA), I obtained a uracil auxotroph from the efficient gene-targeting strain, *Δlig4* strain, and constructed DGLA-producing strains with disruption of the *Δ5-desaturase* (*Δ5ds*) gene encoding a key enzyme of bioconversion of DGLA to arachidonic acid (ARA) by efficient gene-targeting system using the uracil auxotroph isolated as a host. The isolation of nine *Δ5ds* disruptants out of eighteen isolates indicated that the disruption efficiency was 50%. The ratio of DGLA in the total fatty acids reached 40.1%, whereas no ARA was detected. This is the first study on construction of DGLA-producing transformants using the efficient gene-targeting system in *M. alpina* 1S-4.

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PUBLICATIONS

Kikukawa, H., Sakuradani, E., Nishibaba, Y., Okuda, T., Ando, A., Shima, J., Shimizu, S., and Ogawa, J.: Production of *cis*-11-eicosenoic acid by *Mortierella* fungi. J. Appl. Microbiol., in press.

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