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Review

Arachidonic acid production by the oleaginous fungus *Mortierella alpina* 1S-4: A review

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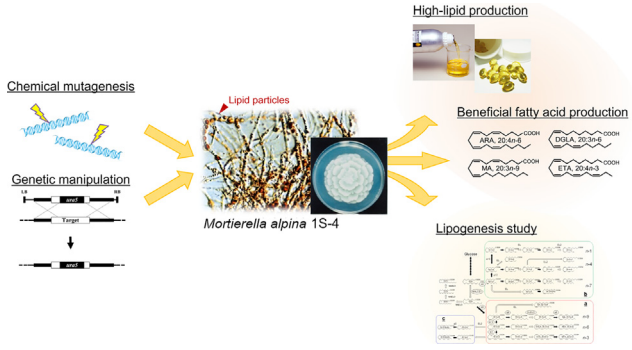
GRAPHICAL ABSTRACT



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ABSTRACT

The filamentous fungus *Mortierella alpina* 1S-4 is capable of accumulating a large amount of triacylglycerol containing C20 polyunsaturated fatty acids (PUFAs). Indeed, triacylglycerol production by *M. alpina* 1S-4 can reach 20 g/L of culture broth, and the critical cellular signaling and structural PUFA arachidonic acid (ARA) comprises 30%–70% of the total fatty acid. The demonstrated health benefits of functional PUFAs have in turn encouraged the search for rich sources of these compounds, including fungal strains showing enhanced production of specific PUFAs. Screening for mutants and targeted gene manipulation of *M. alpina* 1S-4 have elucidated the functions of various enzymes involved in PUFA biosynthesis and established lines with improved PUFA productivity. In some cases, these strains have been used for indistrial-scale production of PUFAs, including ARA. In this review, we described practical ARA production through mutant breeding, functional analyses of genes encoding enzymes involved in PUFA biosynthesis, and recent advances in the production of specific PUFAs through molecular breeding of *M. alpina* 1S-4.

Introduction

Fatty acids containing more than one carbon double bond, termed polyunsaturated fatty acids (PUFAs), are critical sources of metabolic energy, major structural components of membrane

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phospholipids, and precursors of the eicosanoid signaling molecules prostaglandins, thromboxanes, and leukotrienes. Fish oils, animal fats, and algal cells are among the most readily available lipid sources rich in 20-carbon (C20) PUFAs. Among PUFAs, ARA (ARA, C20:4*n*-6) is the most abundant C20 PUFA in humans, especially in the brain, muscles, and liver. ARA has multiple physiological functions and is an important nutrient for infants and the elderly [1,2]. ARA-derived lipid mediators can play various roles in establishing homeostasis for the humans [3]. However, most of the ARA in the humans is usually taken from dietary animal sources such as meat and eggs [4], and the PUFA contents of these conventional sources are insufficient for practical large-scale production. Alternatively, γ-linolenic acid (GLA, 18:3*n*-6)-containing oils have been produced using *Mucor* fungi as the first attempt at microbial PUFA production [5,6]. *Mortierella* fungi, such as *M. alpina* ATCC32222 [7], were found as ARA producer and have been used as commercial ARA producers. Recently, the various innovations on metabolic engineering using gene engineering and metabolomics for PUFA production by *Mortierella* fungi have reported, e.g. overexpression of malic enzyme increased the fatty acid content in *M. alpina* ATCC32222 [8–11].

The oil-producing filamentous fungus *M. alpina* 1S-4 is also a promising source of PUFAs such as ARA. *M. alpina* 1S-4 is the first strain found as the high ARA producer and can accumulate various PUFAs through the *n*-6 PUFA biosynthetic pathway as well as eicosapentaenoic acid (EPA, 20:5*n*-3) through the *n*-3 PUFA biosynthetic pathway [12–14]. In *M. alpina* 1S-4, most PUFAs are stored in lipid droplets as triacylglycerols, while some are present in the form of phospholipids as structural components of membranes. Given the high ARA content of *M. alpina* 1S-4, this fungus is one of the fungal models for both fundamental and applicative studies on fatty acid biosynthesis, including the development of strains suitable for large-scale production of specific PUFAs. In fact, ARA, dihomo-γ-linolenic acid (DGLA, 20:3*n*-6), and Mead acid (MA, 20:3*n*-9) have been commercially produced by *Mortierella* fungi [15–19].

Although such successes over the last 30 years have generated much interest in the development of microbial fermentation processes for the large-scale production of specific PUFAs, improved yields require more efficient biotechnological strategies for metabolic engineering of microorganism lipogenesis. This article reviews recent advances in the breeding of commercially viable PUFA-producing *M. alpina* strains by conventional chemical mutagenesis, the development of gene manipulation systems for *M. alpina* 1S-4, and the latest molecular breeding strategies for producing rare fatty acids using molecular genetics.

ARA-producing *Mortierella* sp.

Since the first reports of *Mortierella* strains producing ARA in 1987 [14,20], this genus has been studied extensively as a promising single-cell oil (SCO) source for various types of PUFAs [21,22].

Table 1
Arachidonic acid (ARA) production by various *Mortierella* strains.

Microorganism	ARA productivity	Scale	Ref.
Mortierella alpina 1S-4	3.6 g/L/7 days	5 L fermentor	[29]
	3.0 g/L/10 days	2 kL fermentor	[13]
	13 g/L/10 days	10 kL fermentor	[25]
M. alpina ATCC32221	11 g/L/16 days	500 L fermentor	[28]
M. alpina ATCC32222	11 g/L/11 days	250 mL flask	[7]
M. alpina DSA-12	18.8 g/L/12.5 days	12 L fermentor	[26]
M. alpina ME-1	19.8 g/L/7 days	5 L fermentor	[27]
Mortierella elongata 1S-5	1.0 g/L/4 days	500 mL flask	[14]
Mortierella schmuckeri S12	2.3 g/L/3 days	14 L fermentor	[24]
Mortierella alliacea YN-15	7.1 g/L/6 days	50 L fermentor	[23]

In particular, *M. alpina* 1S-4 has been studied for fundamental and applicative purposes, and has been used successfully for the commercial production of ARA-enriched SCO (Table 1) [7,13,14,23–29]. *Mortierella alpina* 1S-4 has the unique capacity to synthesize a wide range of PUFAs (Fig. 1), and has several additional advantages as both a model organism for studies on fungal lipid metabolism and an industrial lipid producer demonstrating particularly high yields of multiple PUFAs under energetically favorable culture conditions.

The total lipid fraction of *M. alpina* 1S-4 contains *n*-9, *n*-6, and *n*-3 PUFAs. The predominant PUFA, ARA, is synthesized from 16:0 by four desaturases and two elongases. Under culture conditions optimal for large-scale production, the total amount of lipid can reach 500–600 mg/g dry cell weight or 20 g/L of culture broth. Moreover, the ARA composition ranges from 30% to 70% of the total cellular fatty acid (70%–90% of which is present in triacylglycerols) [25,30,31]. This strain also produces EPA (approximately 10% of total fatty acids) with cultivation below 20 °C and exhibits higher EPA production upon the addition of α-linolenic acid (18:3*n*-3)-containing oils, such as linseed oil, to the medium [32].

Enzymes involved in ARA biosynthesis in *M. alpina* 1S-4

Arachidonic acid biosynthesis requires the activity of several fatty acid desaturases and elongases. The primary substrate hexadecanoic acid (16:0) is converted to ARA in sequential steps catalyzed by elongase 1 (MALCE1), Δ9 desaturase, Δ12 desaturase, Δ6 desaturase, elongase 2 (GLELO), and Δ5 desaturase, respectively (Fig. 1 and Table 2). Some of these enzymatic steps in *M. alpina* 1S-4 contain a NADH-cytochrome b_5 reductase and cytochrome b_5 as an electron transport system for fatty acid desaturation [33–35]. Cytochrome b_5 is a small hemoprotein which is an integral component of the microsomal membranes and functions as an electron carrier in a number of microsomal oxidation/reduction reactions, including fatty acid desaturation, cholesterol biosynthesis and reduction of cytochrome P450.

The two Δ9 desaturase homologues (designated as Δ9-1 and Δ9-2) in *M. alpina* 1S-4 have a cytochrome b_5-like domain linked to the carboxyl terminus, similar to yeast Δ9 desaturase [36]. The *M. alpina* 1S-4 Δ9-1 exhibits 45% amino acid sequence similarity with the yeast *Saccharomyces cerevisiae* homologue and 34% with the rat homologue, suggesting that *M. alpina* Δ9-1 is a conserved membrane-bound protein using acyl-CoA as substrate. Both Δ9-1 and Δ9-2 desaturate 18:0 to oleic acid (18:1*n*-9). Although the *Δ9-2* gene is not transcribed in the wild-type, Δ9-2 protein was expressed and exhibited Δ9 desaturation activity in a *Δ9-1* gene-defective mutant [37]. The *M. alpina* Δ12 and ω3 desaturases, both of which lack a cytochrome b_5-like domain, have been characterized by heterologous gene expression systems. The *M. alpina* Δ12 desaturase was confirmed to catalyze the desaturation of 18:1*n*-9 to 18:2*n*-6 in both *S. cerevisiae* and *Aspergillus oryzae* [38]. The *M. alpina* ω3 desaturase shows 51% sequence identity with *M. alpina* Δ12 desaturase. It converts *n*-6 PUFAs to *n*-3 PUFAs with C18 and C20 chain lengths, and is particularly efficient at converting ARA to EPA [39]. Furthermore, the *M. alpina* ω3 desaturase exhibits two additional activities when expressed in *S. cerevisiae*, insertion of C=C double bonds at the Δ12-position and Δ15-position of hexadecenoic acid (16:1*n*-7) [40].

The *M. alpina* Δ5 and Δ6 desaturases have a cytochrome b_5-like domain linked to the N-terminus. A complementary DNA (cDNA) encoding Δ5 desaturase has been isolated from two *M. alpina* strains, CBS210.32 and ATCC32221 [41,42]. *Mortierella alpina* Δ5 desaturase inserts C=C double bond at the Δ5-position of PUFAs, thereby converting DGLA into ARA. Two Δ6 desaturase homologues (designated Δ6-1 and Δ6-2) are also present in *M. alpina*

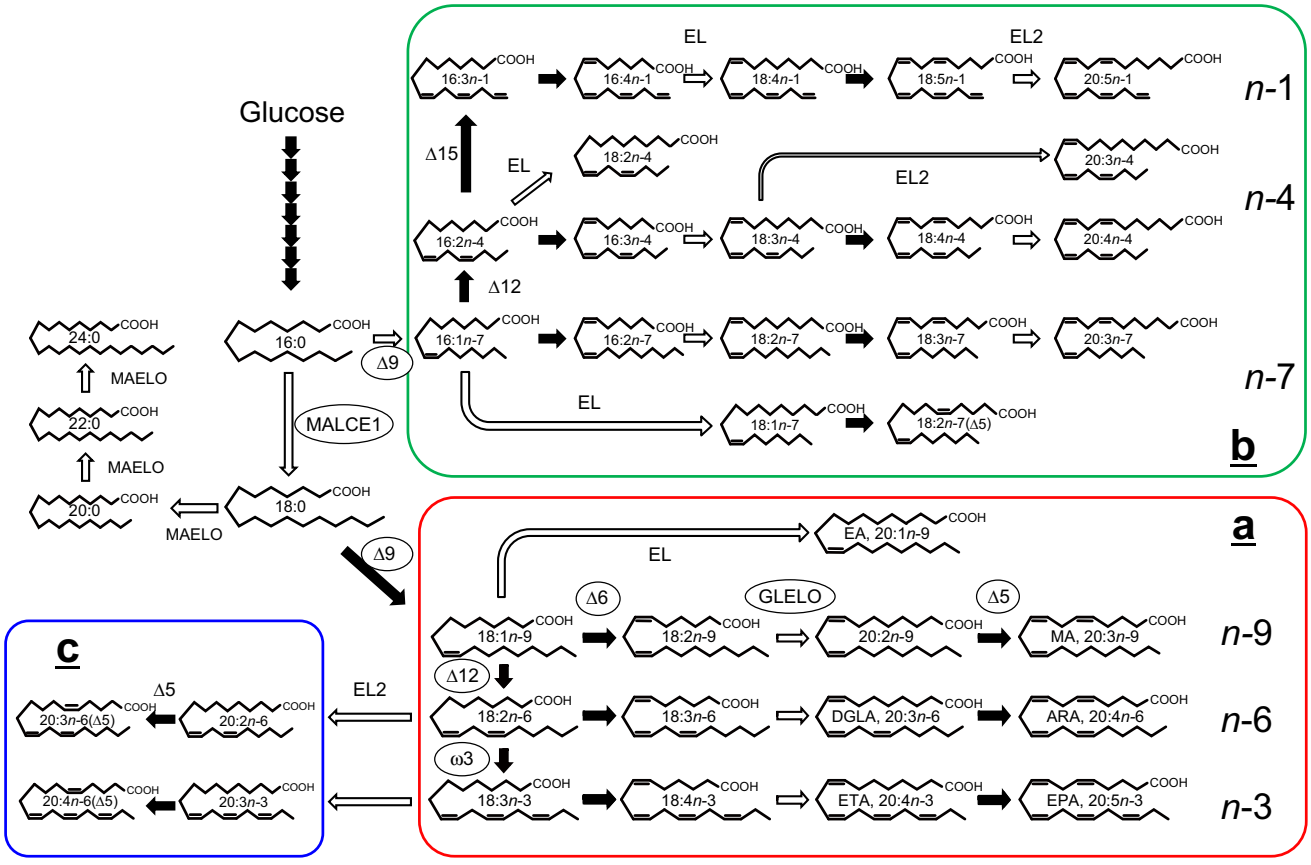

Fig. 1. Biosynthetic pathway of PUFAs in *Mortierella alpina* 1S-4. ARA is biosynthesized through desaturation by Δ9, Δ12, Δ6, and Δ5 desaturases and elongation by MALCE1 and GLELO. The *n*-3, *n*-6, and *n*-9 PUFAs derived from 18:1*n*-9 (a), the *n*-1, *n*-4, and *n*-7 PUFAs derived from 16:1*n*-7 (b), and the non-methylene-interrupted PUFAs detected in Δ6 desaturase-defective mutants (c). ΔX, ΔX desaturase; ω3, ω3 desaturase; EL, fatty acid elongase; ARA, arachidonic acid; DGLA, dihomo-γ-linolenic acid; EPA, eicosapentaenoic acid; ETA, ω3 eicosatetraenoic acid; MA, Mead acid.

Table 2
Substrates and products of enzymes involved in arachidonic acid (ARA) biosynthesis in *M. alpina* 1S-4.

Type	Isozyme	Substrate	Product
Δ9 desaturase	Δ9-1	18:0	18:1*n*-9
	Δ9-2	18:0	18:1*n*-9
Δ12 desaturase	–	18:1*n*-9	18:2*n*-6
Δ6 desaturase	Δ6-1	18:2*n*-6	GLA
	Δ6-2	18:2*n*-6	GLA
Δ5 desaturase	–	DGLA	ARA
ω3 desaturase	–	*n*-6 PUFA	*n*-3 PUFA
		16:1*n*-7	16:2*n*-4, 16:3*n*-1
MALCE1	–	16:0	18:0
GLELO	–	GLA	DGLA
Cyt.b_5 reductase	Cyt.b_5 reductase-1	–	–
	Cyt.b_5 reductase-2	–	–
Cyt.b_5	–	–	–

1S-4 [43,44]. Expression of the full-length cDNA clone in *A. oryzae* resulted in greater accumulation of GLA, reaching 25.2% of the total fatty acid content. The amino acid sequence homology between Δ6-1 and Δ6-2 is very high (92%). Usually, *Δ6-1* gene transcription is 2-fold to 17-fold higher than *Δ6-2* gene transcription in *M. alpina* 1S-4. However, transcription of the *Δ6-2* gene was enhanced up to 8-fold in *Δ6-1* gene-silenced *M. alpina* 1S-4 compared to the wild-type, suggesting that Δ6-2 may compensate when Δ6-1 activity is deficient [45]. Two fatty acid elongases, MALCE1 and GLELO, are also involved in the ARA biosynthetic pathway. GLELO is a Δ6 elongase that catalyzes the elongation of both C18 *n*-3 and C18 *n*-6 PUFAs to the corresponding C20 PUFAs [46]. The *M. alpina malce1* gene was confirmed to encode a fatty acid elongase that efficiently catalyzed the elongation of 16:1*n*-7, 18:2*n*-6, and 18:3*n*-3 when expressed in *S. cerevisiae*. Furthermore, MALCE1 also catalyzes the elongation of 16:0 to 18:0 in *M. alpina* 1S-4. Indeed, this is its primary activity in *M. alpina* 1S-4 [47].

Gene manipulation in *M. alpina* 1S-4

A transformation system for *M. alpina* 1S-4 has been developed using *M. alpina* uracil auxotrophs as the host strain and a complementary gene as a selection marker [48]. Transformation with *M. alpina* 1S-4 spores and a vector containing the *M. alpina* 1S-4 *ura5* gene as a marker was achieved with high efficiency (transformant frequency of 0.4/mg of vector DNA) using microprojectile bombardment [49,50]. Southern blot analysis revealed that most of the integrated plasmids in stable transformants were present as multiple copies at ribosomal DNA (rDNA) positions and/or at random positions in the chromosomal DNA. An *Agrobacterium tumefaciens*-mediated transformation system for *M. alpina* 1S-4 has also been developed [51] in which the *ura5* gene is used as a selectable marker under control of the homologous histone H4.1 promoter in the transfer-DNA region. The frequency of transformation reached more than 400/10^8 spores using this system, and Southern blot analysis revealed that most of the integrated transfer-DNAs appeared as a single copy at random position in the chromosomal DNA.

Mortierella alpina 1S-4 exhibits resistance to various antibiotics used to destroy other filamentous fungi. However, Zeocin- and Carboxin-resistance markers have been developed for selection of *M. alpina* 1S-4 [52,53]. A high concentration of Zeocin (20 mg/mL)

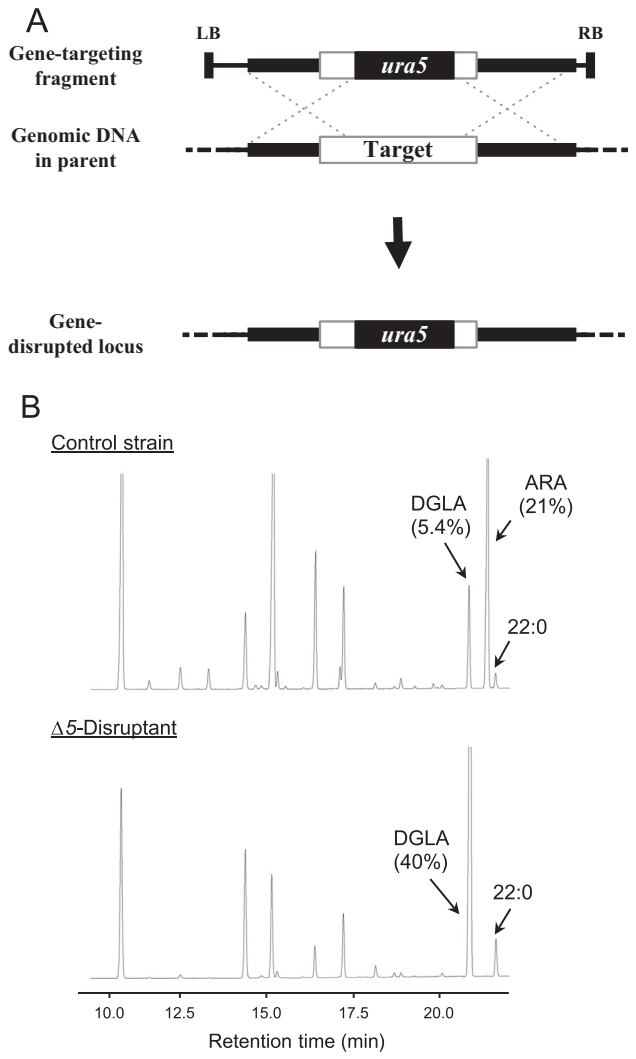

Fig. 2. Gene-disruption through double crossing-over HR (A) and chromatograms of fatty acid methyl esters prepared from a control strain (*lig4* disruptant) and *Δ5 desaturase* gene-disrupted strain (B).

completely inhibited the germination of *M. alpina* 1S-4 spores, and decreased the growth rate of fungal filaments. On the other hand, the fungicide Carboxin (100 mg/mL) completely inhibited *M. alpina* 1S-4 hyphal growth and spore germination. These genes for Zeocin and Carboxin resistance have proven useful as selective markers for the transformation of both the parental strain and mutants.

To develop a more effective gene expression system for *M. alpina* 1S-4, the transcriptional activity of each promoter was evaluated using the β-glucuronidase (GUS) reporter assay system [54]. The GUS gene was synthesized with optimized codon usage for *M. alpina* and inserted into a basic vector under control of the histone H4.1 promoter and *SdhB* gene terminator for reporter assays. Approximately 30 promoter regions were replaced with the histone H4.1 promoter and evaluated for expression activity. Seven promoters with high-level constitutive or time-dependent expression were selected, and deletion analysis determined the promoter regions required to retain the expression activities. Furthermore, using an inducible *GAL10* promoter, an approximately 50-fold increase in GUS activity was achieved by addition of galactose to the culture media at any cultivation phase [55].

The integration of exogenous DNA into chromosomes occurs through two DNA double-strand break repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ) [56]. In HR, exogenous DNA is integrated into the chromosome using homologous regions as templates for precise gene insertion. The HR method is used frequently for insertion of exogenous expression constructs to disrupt target genes (gene targeting) (Fig. 2A). However, these two pathways are independent of one another and often function competitively [57]. Gene targeting systems have also been developed by disruption of key proteins involved in NHEJ [58,59], such as Ku80 or DNA ligase IV (lig4). We identified and disrupted the *ku80* and *lig4* genes in *M. alpina* 1S-4 to improve gene-targeting efficiency. These gene-disrupted strains showed no defect in vegetative growth, spore formation, or fatty acid production. Importantly, the efficiency of gene-targeting through HR was improved only in the *lig4*-disrupted strain, where it was 21-fold (67%) greater than that of the host strain. Metabolic engineering using *lig4* gene-disrupted strains as hosts is expected to produce higher levels of rare and beneficial PUFAs and contribute to basic research on fungal lipogenesis.

PUFA production by *M. alpina* 1S-4 mutants and transformants

Numerous desaturase-deficient and (or) elongase-deficient mutants have been isolated by treating *M. alpina* 1S-4 spores with the chemical mutagen *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Table 3) [60–65]. The *M. alpina* 1S-4 wild-type can accumulate *n*-3 PUFAs only when cultivated at low temperature (below 20 °C), while the ω3 desaturase-defective mutants are unable to synthesize *n*-3 PUFAs even when grown at low temperature [60,66]. The wild-type usually shows the highest ARA yield at 20 °C, although a portion of the accumulated ARA is further converted to EPA, so the resultant oil includes a small amount of EPA (ca. 3%). Therefore, these mutants (e.g., Y11 and Y61 strain) are superior to the wild-type for production of SCO with a relatively higher ARA content [64,66]. Additionally, rare fatty acids accumulated in *M. alpina* 1S-4 by suppression of MALCE1-mediated 16:0 elongation to 18:0 or by supplementation of exogenous fatty acids such as 16:1*n*-7 into the culture medium (Fig. 1b).

This practical transformation system for *M. alpina* 1S-4 allows overexpression, RNA interference (RNAi), and disruption of genes involved in PUFA biosynthesis for improved production of desired PUFAs. Several valuable *M. alpina* mutants were directly transformed with drug resistance markers, or their uracil auxotrophs were transformed with the *ura5* marker. Molecular breeding of *M. alpina* 1S-4 and its mutants yielded unique fatty acid profiles and high productivities of valuable PUFAs (Table 3 and 4). Mutant JT-180 exhibits no Δ12 desaturase activity and enhanced Δ5 and

Table 3
Mutants described in the present review.

Mutant	Deficient enzyme	Product	Productivity and characteristics	Ref.
Y11	ω3 desaturase	ARA	1.5 g/L, 45% of total fatty acid with no *n*-3 PUFAs	[64,66]
Y61	ω3 desaturase	ARA	1.8 g/L	[66]
JT-180	Δ12 desaturase	MA	2.6 g/L, 49%	[65]
S14	Δ5 desaturase	DGLA	Enhanced activities of Δ5 and Δ6 desaturases 4.1 g/L and low ARA content (<1%)	[61]

Table 4
Polyunsaturated fatty acid (PUFA) production by mutants and transformants derived from *M. alpina* 1S-4.

Fatty acid	Target gene[a]	Parent[b]	Method[c]	Productivity
ARA	*Δ12*	JT-180	OE	Higher production (2.0 g/L/7 days, 39% of total fatty acids) than the *M. alpina* 1S-4 wild-type (1.2 g/L/7 days, 21%)
	malce1	1S-4	OE	Higher ARA production (0.76 g/L/6 days, 34%) than the wild-type (0.68 g/L/6 days, 28%)
	glelo	1S-4	OE	Higher ARA production (3.6 g/L/10 days, 28%) than the wild-type (1.9 g/L/10 days, 19%)
	PavΔ5	1S-4	OE	Higher ARA composition (39%) and lower DGLA composition in the transformant than the wild-type (19% and 4%, respectively)
	OstΔ6	1S-4	OE	Higher ARA composition (37%) in the transformant than the wild-type (19%)
	TriΔ12	1S-4	OE	Higher ARA composition (36%) in the transformant than the wild-type (19%)
18:1*n*-9	*Δ6-1*	JT-180	Ri	2.76 g/L/6 days, 68% of total fatty acid
EPA	*ω3*	1S-4	OE	0.68 g/L, 38.2% of total fatty acid
	sdd17m	ST1358	OE	1.8 g/L, 26.4% of total fatty acid
ETA	*sdd17m*	S14	OE	2.76 g/L/6 days, 68% of total fatty acid
DGLA	*Δ5*	1S-4	GT	Higher DGLA composition (40%) than the mutant S14 strain (27%), with no ARA accumulation versus 0.2% in the mutant S14
MA	*Δ12*	1S-4	GT	Higher MA composition (8.4%) than the mutant JT-180 (4.5%), with no *n*-6 and *n*-3 PUFAs

[a] The genes, except for *PavΔ5*, *OstΔ6*, *TriΔ12*, and *sdd17m*, were derived from *M. alpina* 1S-4. *ΔX*, *ΔX desaturase* gene; *PavΔ5*, *Pavlova salina Δ5 desaturase*; *OstΔ6*, *Ostreococcus lucimarinus Δ6 desaturase*; *TriΔ12*, *Tribolium castaneum Δ12 desaturase*; *sdd17m*, *Saprolegnia diclina Δ17 desaturase*.
[b] JT-180, Δ12 desaturase-defective mutant; ST1358, ω3 desaturase-defective mutant; S14, Δ5 desaturase-defective mutant.
[c] OE, overexpression; Ri, RNAi; GT, targeted gene-disruption (gene-targeting).

Δ6 desaturase activities, resulting in the efficient production of Mead acid (MA, 20:3*n*-9) [65]. With overexpression of the endogenous *Δ12 desaturase* gene, JT-180 accumulated a larger amount of ARA (2.0 g/L/7 days, 39% of total fatty acids) but little MA compared to the wild-type (1.2 g/L/7 days, 21%) due to enhanced Δ5 and Δ6 desaturation. Overexpression of the endogenous *malce1* gene in *M. alpina* 1S-4 also led to faster and greater ARA accumulation (0.76 g/L/6 days, 34%) than in the wild-type (0.68 g/L/6 days, 28%). In addition, overexpression of the gene encoding GLELO, which has been suggested to catalyze the rate limiting step in ARA biosynthesis [67], was successfully performed in *M. alpina* 1S-4 [68]. The resulting transformants yielded more ARA (3.6 g/L/10 days, 28%) than the wild-type (1.9 g/L/10 days, 19%). Overexpression of both *malce1* and *glelo* genes had substantial effects on ARA production by *M. alpina* 1S-4. The exogenous Δ5 and Δ6 desaturases (PavΔ5, OstΔ6) from the microalgae *Pavlova salina* and *Ostreococcus lucimarinus* and the Δ12 desaturase (TriΔ12) from the beetle *Tribolium castaneum* have desaturation activities for fatty acyl-CoA substrates. On the other hand, the homologous desaturases from *M. alpina* use phospholipids as substrates. By expressing these exogenous desaturases, higher ARA yields were obtained (unpublished data) [69]. For instance, overexpression of the *PavΔ5* gene in the wild-type led to a markedly high ARA/DGLA ratio, while overexpression of the *OstΔ6* gene in the wild-type led to higher 18:3*n*-6, DGLA, and ARA contents as proportions of total fatty acid compared to the wild-type. Similarly, overexpression of the *TriΔ12* gene in the wild-type led to greater proportions of 18:2*n*-6, 18:3*n*-6, DGLA, and ARA compared to the wild-type.

The RNAi method using double-strand RNA has been applied to silence gene expression in *M. alpina* 1S-4 [70]. By suppressing endogenous *Δ6-1* gene expression by RNAi in the mutant JT-180, 18:1*n*-9 accumulation reached 68.0% of total fatty acid content, and 18:1*n*-9 production in broth reached 2.76 g/L [45].

Overexpression systems using promoters that exhibit high transcriptional activities may facilitate further improvements in PUFA production. Usually, *M. alpina* can express ω3 desaturation activity and accumulate *n*-3 PUFAs when cultured at low temperatures (below 20 °C), with an EPA ratio of approximately 10%, while no accumulation of *n*-3 PUFAs was observed at 28 °C. However, overexpression of the endogenous ω3 desaturase gene in *M. alpina* 1S-4 at 20 °C increased EPA accumulation to 40% of total fatty acid [51]. Expression of the heterologous *Saprolegnia diclina* Δ17 desaturase (sdd17m) gene in the ω3 desaturase-defective mutant ST1358 [71] resulted in EPA content as high as 26.4% of total fatty acid or 1.8 g/L at 28 °C [72]. While wild *M. alpina* accumulates only a small amount of the *n*-3 eicosatetraenoic acid (ETA, 20:4*n*-3) at low temperature (below 20 °C), this ETA was successfully produced by molecular breeding [73]. Further, by overexpression of the heterologous *sdd17m* gene controlled by an SSA2 promoter showing high transcriptional activity, ETA productivity in a Δ5 desaturase-defective mutant S14 reached 24.9% of total fatty acid at 28 °C [61].

Gene targeting may also be a valuable strategy for development of *M. alpina* strains producing SCO containing rare PUFAs. DGLA-producing transformants were constructed by disruption of the *Δ5 desaturase* gene, which encodes a key enzyme catalyzing the bioconversion of DGLA to ARA, in the *lig4* gene-disrupted strain of *M. alpina* 1S-4 [74]. The uracil auxotroph of the *lig4* gene-disrupted strain was transformed for disruption of the *Δ5 desaturase* gene through double crossing-over HR, and the targeting efficiency was calculated as 50%. The ratio of DGLA to total fatty acid in this disruptant reached 40.1%; however, no ARA was detected (Fig. 2). Thus, DGLA oil can be produced without ARA contamination. Such disruptants are superior to defective mutants (e.g., *M. alpina* 1S-4 mutant S14 constructed by chemical mutagenesis) for practical production of DGLA. Using the same methodology, MA-producing disruptants were constructed by disruption of the *Δ12 desaturase* gene (unpublished data) [75]. These disruptants showed no defects in growth, spore germination, and fatty acid production, but exhibited higher MA composition (8.4% of the total fatty acid) than the MA-producing Δ12 desaturase-defective mutant JT-180 (4.5%), with no accumulation of *n*-6 and *n*-3 PUFAs. Further application of gene targeting in *M. alpina* strains should facilitate improved PUFA productivity and help elucidate the enzyme pathways of PUFA biosynthesis.

Conclusions and future perspectives

The present review summarizes studies on lipogenesis in *M. alpina* 1S-4, the development of efficient gene manipulation systems for this strain, and the utilization of various *M. alpina* 1S-4 mutants for the production of beneficial PUFAs, especially ARA. The *M. alpina* 1S-4 wild-type, derivative mutants, and transformants are potential sources of triacylglycerols containing various

PUFAs, including *n*-1, *n*-3, *n*-4, *n*-6, *n*-7, and *n*-9 PUFAs. By selective breeding of *M. alpina* and its mutants, it is possible to regulate the flow of both endogenous and exogenous fatty acids, thereby modifying the fatty acid profile and enhancing the production of desired (i.e., beneficial) PUFAs. Recent studies on *M. alpina* and its mutants have focused on molecular engineering of genes involved in PUFA biosynthesis and yielded strains with improved PUFA productivity. The molecular breeding of mutants and transgenic strains may make it possible to produce desired PUFAs efficiently. However, more efficient expression systems for enzymes involved in lipid synthesis, PUFA synthesis, and lipid conversion, as well as improved gene-silencing and targeted gene-disruption systems are needed to facilitate the breeding of *M. alpina* strains for large-scale production of functional lipids with industrial applications.

Conflict of interest

The authors declare no conflict of interest.

Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

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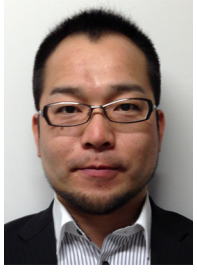

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