Title: POLYNUCLEOTIDE CODING FOR AN HOMOLOGUE OF ACYL-COA SYNTHETASE AND ITS USE

The present invention relates to an acyl-CoA synthetase homolog protein from microorganisms of the genus Mortierella, a polynucleotide encoding the protein, and so on. The invention provides polynucleotides comprising an acyl-CoA synthetase...
(57) Abrégé(suite)/Abstract(continued):

homolog protein gene from, e.g., Mortierella alpina, expression vectors comprising these polynucleotides and transformants thereof a method for producing lipids or fatty acids using the transformants, food products containing the lipids or fatty acids produced by the method, etc.
ABSTRACT

The present invention relates to an acyl-CoA synthetase homolog protein from microorganisms of the genus Mortierella, a polynucleotide encoding the protein, and so on. The invention provides polynucleotides comprising an acyl-CoA synthetase homolog protein gene from, e.g., Mortierella alpina, expression vectors comprising these polynucleotides and transformants thereof, a method for producing lipids or fatty acids using the transformants, food products containing the lipids or fatty acids produced by the method, etc.
DESCRIPTION

POLYNUCLEOTIDE ENCODING ACYL-COA SYNTHETASE HOMOLOG AND USE THEREOF

This application is a division of Canadian Application Serial No. 2,787,832 filed February 1, 2011 (parent application).

It should be understood that the expression “the present invention” or the like used in this specification may encompass not only the subject matter of this divisional application, but that of the parent application also.

TECHNICAL FIELD

The present invention relates to a polynucleotide encoding an acyl-CoA synthetase homolog and use thereof.

BACKGROUND ART

Fatty acids containing two or more unsaturated bonds are collectively referred to as polyunsaturated fatty acids (PUFAs) and known to specifically include arachidonic acid (ARA), dihomo-γ-linolenic acid (DGLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), etc. Some of these polyunsaturated fatty acids cannot be synthesized in the animal body. It is therefore necessary to compensate these polyunsaturated fatty acids as essential amino acids from food.

Polyunsaturated fatty acids are widely distributed; for instance, arachidonic acid can be separated from lipids extracted from the adrenal glands and livers of animals. However, polyunsaturated fatty acids contained in animal organs are only in a small quantity and cannot be obtained sufficiently for large supplies when simply extracted or separated from animal organs. For this reason, microbial techniques have been developed for obtaining polyunsaturated fatty acids by cultivation of various microorganisms. Above all, microorganisms of the genus Mortierella are known to produce lipids containing polyunsaturated fatty acids such as arachidonic acid and the like.

Other attempts have also been made to produce polyunsaturated fatty acids in plants. Polyunsaturated fatty acids constitute storage lipids such as triacylglycerols and are known to be accumulated within microorganism mycelia or plant seeds.
Acyl-CoA synthetase (ACS) is an enzyme catalyzing the thioesterification of fatty acids and coenzyme A (CoA) and catalyzes the following reaction.

\[
\text{Fatty acid} + \text{CoASH} + \text{ATP} \rightarrow \text{Acyl-CoA} + \text{AMP} + \text{PPI}
\]

Acyl-CoA produced by ACS is involved in various life phenomena including the biosynthesis and remodeling of lipids, energy production by \(\beta\)-oxidation, acylation of proteins, expression regulation by fatty acids, etc. Furthermore, ACS is reportedly associated with extracellular uptake of fatty acids,
intracellular transport of fatty acids, etc. (Non-Patent Documents 1 and 2). In view of the foregoing, it is considered to control the activity of ACS when polyunsaturated fatty acids or the like are produced by utilizing microorganisms or plants.

In the yeast Saccharomyces cerevisiae used as a model eukaryote, six (6) acyl-CoA synthetase genes (ScFAA1, ScFAA2, ScFAA3, ScFAA4, ScFAT1 and ScFAT2) are known (Non-Patent Document 1). The proteins encoded by these genes are different in substrate specificity, timing of expression, intracellular localization and function.

Patent Document 1 discloses nine (9) genes as the acyl-CoA synthetase gene (ScACS) derived from Schizochytrium sp. Patent Document 1 also discloses an increased production of DPA (n-6) (docosapentanoic acid (n-6)) or DHA when the gene encoding the Schizochytrium sp. PUFA synthase system is co-expressed with ScACS, as compared to the case where the co-expression with ScACS is not involved.

In addition, acyl-CoA synthetase genes derived from animals and plants are also reported (Non-Patent Document 2 and Patent Document 2).


DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, it has been desired to isolate a novel gene that increases the amount of the fatty acids produced in a host cell or changes the composition of fatty acids produced, when the gene is expressed in the host cell.

As a result of extensive investigations, the present inventors have succeeded in cloning a gene encoding an ACS homolog of lipid-producing fungus Mortierella alpina (hereinafter "M. alpina") (MaACS), and accomplished the present invention. That is, the present invention provides the following polynucleotides, proteins, expression vectors, transformants, and a method for producing lipids or lipid compositions and foods, etc. using the transformants, as well as foods produced by the method, etc.
That is, the present invention relates to the following aspects:

[1] A polynucleotide according to any one selected from the group consisting of (a) to (c) below:

(a) a polynucleotide comprising the nucleotide sequence set forth as SEQ ID NO: 51 or 56;

(b) a polynucleotide encoding a protein consisting of the amino acid sequence set forth as SEQ ID NO: 52 or 57;

(c) a polynucleotide encoding a protein consisting of an amino acid sequence wherein 1 to 100 amino acids are deleted, substituted, inserted and/or added in the amino acid sequence SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell;

(d) a polynucleotide encoding a protein having an amino acid sequence having at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(e) a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence set forth as SEQ ID NO: 51 or 56 under stringent conditions, and which encodes a protein having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

[2] The polynucleotide according to aspect [1], which is either one defined in (f) or (g) below:

(f) a polynucleotide encoding a protein consisting of an amino acid sequence wherein 1 to 10 amino acids are deleted, substituted, inserted and/or added in the amino acid sequence set forth as SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(g) a polynucleotide encoding a protein having an amino acid sequence having at least 90% identity to the amino acid sequence set forth as SEQ ID NO: 52 or 57, and an acyl-CoA
synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

[3] The polynucleotide according to [1] above, comprising the nucleotide sequence set forth as SEQ ID NO: 51 or 56.

[4] The polynucleotide according to [1] above, encoding a protein consisting of the amino acid sequence set forth as SEQ ID NO: 52 or 57.

[5] The polynucleotide according to any one of [1] to [4] above, which is a DNA.


[9] A method for producing a lipid or fatty acid composition, which comprises collecting the lipid or fatty acid composition from the culture of the transformant according to [8] above.

[10] The method according to [9] above, wherein the lipid is a triacylglycerol.

[11] The method according to [9] above, wherein the fatty acid is a polyunsaturated fatty acid having at least 18 carbon atoms.

[12] A food product, pharmaceutical, cosmetic or soap comprising the lipid or fatty acid composition obtained by the production method according to [9] above.

The polynucleotide of the present invention can be used for transformation of an appropriate host cell. The transformant thus produced can be used to produce fatty acid compositions, food products, cosmetics, pharmaceuticals, soaps, etc.

More specifically, the transformant of the present invention provides an extremely high production efficiency of lipids and fatty acids. Accordingly, the present invention can be effectively used to manufacture pharmaceuticals or health foods which require a large quantity of lipids or fatty acids.

BRIEF DESCRIPTION OF DRAWINGS
FIG. 1 shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-1.

FIG. 2A shows the alignment between the genome sequence and CDS sequence of MaACS-1.

FIG. 2B is a continuation from FIG. 2A.

FIG. 3A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-2.

FIG. 3B is a continuation from FIG. 3A.

FIG. 4A shows the alignment between the genome sequence and CDS sequence of MaACS-2.

FIG. 4B is a continuation from FIG. 4A.

FIG. 4C is a continuation from FIG. 4B.

FIG. 5 shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-3.

FIG. 6A shows the alignment between the genome sequence and CDS sequence of MaACS-3.

FIG. 6B is a continuation from FIG. 6A.

FIG. 7A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-4.

FIG. 7B is a continuation from FIG. 7A.

FIG. 8A shows the alignment between the genome sequence and CDS sequence of MaACS-4.

FIG. 8B is a continuation from FIG. 8A.

FIG. 8C is a continuation from FIG. 8B.

FIG. 9A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-5.

FIG. 9B is a continuation from FIG. 9A.

FIG. 10A shows the alignment between the genome sequence and CDS sequence of MaACS-5.

FIG. 10B is a continuation from FIG. 10A.

FIG. 11A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-6.

FIG. 11B is a continuation from FIG. 11A.

FIG. 12A shows the alignment between the genome sequence and CDS sequence of MaACS-6.

FIG. 12B is a continuation from FIG. 12A.

FIG. 13 shows the correspondence between the cDNA sequence and putative
amino acid sequence of MaACS-7.

FIG. 14A shows the alignment between the genome sequence and CDS sequence of MaACS-7.

FIG. 14B is a continuation from FIG. 14A.

FIG. 15A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-8.

FIG. 15B is a continuation from FIG. 15A.

FIG. 16A shows the alignment between the genome sequence and CDS sequence of MaACS-8.

FIG. 16B is a continuation from FIG. 16A.

FIG. 16C is a continuation from FIG. 16B.

FIG. 17 shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-9.

FIG. 18A shows the alignment between the genome sequence and CDS sequence of MaACS-9.

FIG. 18B is a continuation from FIG. 18A.

FIG. 19A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-10.

FIG. 19B is a continuation from FIG. 19A.

FIG. 20A shows the alignment between the genome sequence and CDS sequence of MaACS-10.

FIG. 20B is a continuation from FIG. 20A.

FIG. 20C is a continuation from FIG. 20B.

FIG. 21A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-11.

FIG. 21B is a continuation from FIG. 21A

FIG. 22A shows the alignment between the genome sequence and CDS sequence of MaACS-11.

FIG. 22B is a continuation from FIG. 22A.

FIG. 23A shows the correspondence between the cDNA sequence and putative amino acid sequence of MaACS-12.

FIG. 23B is a continuation from FIG. 23A

FIG. 24A shows the alignment between the genome sequence and CDS sequence of MaACS-12.

FIG. 24B is a continuation from FIG. 24A

FIG. 25A shows the alignment between MaACS having relatively high amino acid sequence homology to S. cerevisiae-derived FAA protein (FAA: fatty
acid activation) and the FAA protein. The single underlined and double underlined sequences denote the ATP-AMP motif and the FACS/VLACS-FATP motif, respectively.

FIG. 25B is a continuation from FIG. 25A.

FIG. 25C is a continuation from FIG. 25B.

FIG. 26A shows the alignment between MaACS having relatively high amino acid sequence homology to S. cerevisiae-derived FAT protein (FAT: fatty acid transferase) and the FAT protein. The single underlined and double underlined sequences denote the ATP-AMP motif and the FACS/VLACS-FATP motif, respectively.

FIG. 26B is a continuation from FIG. 26A.

FIG. 27 shows changes with the passage of time in lipid production (FIG. 27A) and arachidonic acid production (FIG. 27B), per mycelia in MaACS-10-overexpressed M. alpina.

FIG. 28 shows changes with the passage of time in lipid production (FIG. 28A) and arachidonic acid production (FIG. 28B), per mycelia in MaACS-11-overexpressed M. alpina.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

This application claims priority to the Japanese Patent Application (No. 2010-19967) filed February 1, 2010.

As will be later described in detail in EXAMPLES below, the present inventors have succeeded for the first time in cloning the full-length cDNA of lipid-producing fungus M. alpina-derived ACS homolog genes (MaACS-1–12). The present inventors have also identified the nucleotide sequences of genomic DNAs of MaACS-1–12 from M. alpina and putative amino acid sequences thereof. The ORF sequences, putative amino acid sequences, CDS sequences, cDNA sequences and genome sequences of MaACS-1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12 are SEQ ID NOs: 1, 6, 11, 16, 21, 26, 31, 36, 41, 46, 51 and 56 (hereinafter these sequences are
collectively referred to as "ORF sequences of MaACS-1~12"), SEQ ID NOs: 2, 7, 12, 17, 22, 27, 32, 37, 42, 47, 52 and 57 (hereinafter these sequences are collectively referred to as "amino acid sequences of MaACS-1~12"), SEQ ID NOs: 3, 8, 13, 18, 23, 28, 33, 38, 43, 48, 53 and 58 (hereinafter these sequences are collectively referred to as "CDS sequences of MaACS-1~12"), SEQ ID NOs: 4, 9, 14, 19, 24, 29, 34, 39, 44, 49, 54 and 59 (hereinafter these sequences are collectively referred to as "cDNA sequences of MaACS-1~12") and SEQ ID NOs: 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 (hereinafter these sequences are collectively referred to as "genome sequences of MaACS-1~12"), respectively. These polynucleotides and proteins may be obtained by the methods described in EXAMPLES below, known genetic engineering techniques, known methods for synthesis, and so on.

1. Polynucleotide of the Invention

First, the present invention provides the polynucleotide described in any one selected from the group consisting of (a) to (g) below:

(a) a polynucleotide comprising any one nucleotide sequence selected from the group consisting of the ORF sequences of MaACS-1~12;

(b) a polynucleotide comprising any one nucleotide sequence selected from the group consisting of the cDNA sequences of MaACS-1~12;

(c) a polynucleotide encoding a protein consisting of any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12;

(d) a polynucleotide encoding a protein consisting of an amino acid sequence wherein 1 to 100 amino acids are deleted, substituted, inserted and/or added in any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell;

(e) a polynucleotide encoding a protein having an amino acid sequence having at least 60% identity to any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(f) a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to any one nucleotide sequence selected from the group consisting of the ORF sequences of MaACS-1~12 under stringent
conditions, and which encodes a protein having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(g) a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to any one nucleotide sequence selected from the group consisting of the cDNA sequences of MaACS-1--12 under stringent conditions, and which encodes a protein having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

As used herein, the term "polynucleotide" means a DNA or RNA.

As used herein, the term "polynucleotide which hybridizes under stringent conditions" refers to a polynucleotide obtained by the colony hybridization method, plaque hybridization method, Southern hybridization method or the like, using as a probe, for example, a polynucleotide consisting of a nucleotide sequence complementary to any one nucleotide sequence selected from the group consisting of the ORF sequences of MaACS-1--12 or any one nucleotide sequence selected from the group consisting of the cDNA sequences of MaACS-1--12, or the whole or part of a polynucleotide consisting of the nucleotide sequence encoding any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1--12. For the methods of hybridization, there are used the methods described in, e.g., "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001," "Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions and high stringent conditions. The term "low stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 32°C. The term "moderate stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 42°C, or 5x SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42°C. The term "high stringent conditions" are, for example, 5x SSC, 5x Denhardt's solution, 0.5% SDS, 50% formamide at 50°C or 0.2 x SSC, 0.1% SDS at 65°C. Under these conditions, a DNA with higher identity is expected to be obtained efficiently at higher temperatures, though multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and a person skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example,
an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55°C to detect the hybridized DNA. Alternatively, in producing a probe based on the nucleotide sequence complementary to any one nucleotide sequence selected from the group consisting of the ORF sequences of MaACS-1~12 or any one nucleotide sequence selected from the group consisting of the cDNA sequences of MaACS-1~12, or based on the entire or part of the nucleotide sequence encoding any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to those described above, other polynucleotides that can be hybridized include DNAs having 50% or higher, 51% or higher, 52% or higher, 53% or higher, 54% or higher, 55% or higher, 56% or higher, 57% or higher, 58% or higher, 59% or higher, 60% or higher, 61% or higher, 62% or higher, 63% or higher, 64% or higher, 65% or higher, 66% or higher, 67% or higher, 68% or higher, 69% or higher, 70% or higher, 71% or higher, 72% or higher, 73% or higher, 74% or higher, 75% or higher, 76% or higher, 77% or higher, 78% or higher, 79% or higher, 80% or higher, 81% or higher, 82% or higher, 83% or higher, 84% or higher, 85% or higher, 86% or higher, 87% or higher, 88% or higher, 89% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the DNA for any one nucleotide sequence selected from the group consisting of the ORF sequences of MaACS-1~12 or for any one nucleotide sequence selected from the group consisting of the cDNA sequences of MaACS-1~12, or with the DNA encoding any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, as calculated by a homology search software, such as FASTA, BLAST, etc. using default parameters.

Identity between amino acid sequences or nucleotide sequences may be determined using FASTA (Science 227 (4693): 1435-1441, (1985)), algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA, 87: 2264-2268, 1990; Proc. Natl. Acad. Sci. USA, 90: 5873, 1993). Programs called blastn, blastx, blastp, tblastn and tblastx based on the BLAST
algorithm have been developed (Altschul S. F. et al., J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using blastn, the parameters are, for example, score=100 and wordlength=12. When an amino acid sequence is sequenced using blastp, the parameters are, for example, score=50 and wordlength=3. When BLAST and Gapped BLAST programs are used, default parameters for each of the programs are employed.

The polynucleotides of the present invention described above can be obtained by known genetic engineering techniques or known methods for synthesis.

2. Protein of the Invention

The present invention provides the proteins shown below.

(i) A protein encoded by the polynucleotide of any one of (a) to (g) above.

(ii) A protein comprising any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12.

(iii) A protein consisting of an amino acid sequence wherein one or more amino acids are deleted, substituted, inserted and/or added in any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

(iv) A protein having an amino acid sequence having at least 90% identity to any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.


As used herein, the "protein consisting of an amino acid sequence wherein one or several acids are deleted, substituted, inserted and/or added in any one amino acid sequence selected from the group consisting of the amino acid sequences of
MaACS-1~12, and having an acyl-CoA synthetase activity or an activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell" includes proteins consisting of an amino acid sequence wherein, e.g., 1 to 100, 1 to 90, 1 to 80, 1 to 70, 1 to 60, 1 to 50, 1 to 40, 1 to 39, 1 to 38, 1 to 37, 1 to 36, 1 to 35, 1 to 34, 1 to 33, 1 to 32, 1 to 31, 1 to 30, 1 to 29, 1 to 28, 1 to 27, 1 to 26, 1 to 25, 1 to 24, 1 to 23, 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9 (1 to several), 1 to 8, 1 to 7, 1 to 6, 1 to 5, 1 to 4, 1 to 3, 1 to 2, or one amino acid is/are deleted, substituted, inserted and/or added in any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having the acyl-CoA synthetase activity or the activity of increasing the amount and/or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell. In general, the number of deletions, substitutions, insertions, and/or additions is preferably smaller.

Such proteins include a protein having an amino acid sequence having the identity of approximately 60% or higher, 61% or higher, 62% or higher, 63% or higher, 64% or higher, 65% or higher, 66% or higher, 67% or higher, 68% or higher, 69% or higher, 70% or higher, 71% or higher, 72% or higher, 73% or higher, 74% or higher, 75% or higher, 76% or higher, 77% or higher, 78% or higher, 79% or higher, 80% or higher, 81% or higher, 82% or higher, 83% or higher, 84% or higher, 85% or higher, 86% or higher, 87% or higher, 88% or higher, 89% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher, or 99.9% or higher, to any one amino acid sequence selected from the group consisting of the amino acid sequences of MaACS-1~12, and having the diacylglycerol acyltransferase activity. As the identity percentage described above is higher, the protein is preferable in general.

The term deletion, substitution, insertion and/or addition of one or more amino acid residues in the amino acid sequence of the protein of the invention is intended to mean that one or more amino acid residues are deleted, substituted, inserted and/or added at optional and one or more positions in the same sequence. Two or more types of deletions, substitutions, insertions and additions may occur at the same time.

Examples of the amino acid residues which are mutually substitutable are given below. Amino acid residues in the same group are mutually substitutable.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic
acid, methionine, o-methylserine, t-butylglycine, t-butyllalanine and
cyclohexylalanine; Group B: aspartic acid, glutamic acid, isoaspartic acid,
isoglutamic acid, 2-aminoacidic acid and 2-aminosuberic acid; Group C: asparagine
and glutamine; Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid and
2,3-diaminopropionic acid; Group E: proline, 3-hydroxyproline and
4-hydroxyproline; Group F: serine, threonine and homoserine; and Group G:
phenylalanine and tyrosine.

The protein of the present invention may also be produced by chemical
synthesis methods such as the Fmoc method (fluorenylmethyloxycarbonyl method),
the tBoc method (t-butyloxycarbonyl method), etc. In addition, peptide synthesizers
available from Advanced Automation Peptide Protein Technologies, Perkin Elmer,
Protein Technologies, PerSeptive, Applied Biosystems, SHIMADZU Corp., etc. may
also be used for the chemical synthesis.

The protein encoded by the polynucleotide of the invention and the protein
of the invention are both ACS homolog proteins and considered to have the acyl-CoA
synthetase activity since the ATP-AMP motif and FACS/VLACS-FATP motif, which
are important for the acyl-CoA synthetase activity, are conserved. As used herein,
ATP, AMP, FACS, VLACS and FATP are intended to mean adenosine triphosphate,
adenosine monophosphate, fatty acyl-CoA synthetase, very long chain acyl-CoA
synthetase and fatty acid transport protein, respectively. Specific amino acid
sequences of the ATP-AMP motif and FACS/VLACS-FATP motif contained in the
protein of the present invention are shown in FIGS. 25 and 26 at the single
underlined and double underlined sequences, respectively. With regard to
representative amino acid sequences of the ATP-AMP motif and
FACS/VLACS-FATP motif, reference may be made to databases including pfam, etc.

As used herein, the term "acyl-CoA synthetase activity (ACS activity)" is
intended to mean the activity of promoting the acyl-CoA-forming reaction through
formation of a thioester bond between a fatty acid and coenzyme A (chemical
reaction equation below).

Fatty acid + Coenzyme A → Acyl-CoA + H₂O

The acyl-CoA synthetase activity can be quantitatively confirmed, for
example, by cultivating for a certain period of time host cells, into which the
polypeptide of the present invention is introduced, preparing the lysate of the host
cells, mixing the cell lysate with a labeled fatty acid (e.g., polyunsaturated fatty acid

13
labeled with a radioactive isotope, etc.) and coenzyme A, reacting them for a certain period of time, then extracting free fatty acids with n-heptane, and quantifying the fatty acyl-CoA which is formed during the above reaction and remained in the aqueous fraction, using a scintillation counter. For details of the method for confirming the acyl-CoA synthetase activity, reference may be made to Black P. N., et al. (J. B. C., 272 (8), 4896-4903, 1997). Alternatively, the acyl-CoA synthetase activity may also be assayed by the method described in "Evaluation of ACS Activity" of EXAMPLE 2, which involves no radioactive label.

The "activity of increasing the amount of the fatty acids produced in a host cell when expressed in the host cell" is intended to mean the activity that, when the polynucleotide of the present invention or the polynucleotide encoding the protein of the present invention is introduced (transformed) into a host cell and expressed in the host cell, increases the total fatty acid production, as compared to a reference cell (control) derived from the same strain as the host cell in which the polynucleotide described above is not introduced.

The "activity of changing the composition of the fatty acids produced in a host cell when expressed in the host cell" is intended to mean the activity that, when the polynucleotide of the present invention or the polynucleotide encoding the protein of the present invention is introduced (transformed) into a host cell and expressed in the host cell, changes the amount or ratio of various fatty acids produced, as compared to a reference cell (control) derived from the same strain as the host cell in which the polynucleotide described above is not introduced.

As used herein, the term "fatty acid" is intended to mean an aliphatic monocarboxylic acid (a carboxylic acid having one carboxylic residue and carbon atoms connected to each other in a chain) represented by general formula RCOOH (wherein R is an alkyl). The fatty acid includes a saturated fatty acid having no double bond and an unsaturated fatty acid containing a double bond(s) in the hydrocarbon chain. The fatty acid is preferably an unsaturated fatty acid, and more preferably, a polyunsaturated fatty acid containing a plurality of double bonds in the hydrocarbon chain. The polyunsaturated fatty acid includes preferably an unsaturated fatty acid having carbon atoms of 18 or more, e.g., an unsaturated fatty acid having carbon atoms of 18 or 20, and examples include, but not limited to, oleic acid, linoleic acid, linolenic acid (γ-linolenic acid, dihomo-γ-linolenic acid, etc.), arachidonic acid, and the like. The polyunsaturated fatty acids are particularly preferably linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid and arachidonic acid, more preferably, linoleic acid, dihomo-γ-linolenic acid and arachidonic acid,
and most preferably, dihomo-γ-linolenic acid and arachidonic acid.

In the present invention, the "host cell" is not particularly limited so long as the cell is capable of expressing the polynucleotide of the invention when the polynucleotide is introduced. The cells include cells derived from mammals (excluding human), insects, plants, fungi, bacteria, etc., preferably cells from plants and fungi, more preferably, cells from fungi, and most preferably, lipid-producing fungi or yeast.

The lipid-producing fungi which can be used are the lipid-producing fungi described in, e.g., MYCOTAXON, Vol. XLIV, No. 2, pp. 257-265 (1992). Specific examples include, but not limited to, microorganisms belonging to the genus Mortierella including microorganisms belonging to the subgenus Mortierella, e.g., Mortierella elongata IFO8570, Mortierella exigua IFO8571, Mortierella hygrophila IFO5941, Mortierella alpina IFO8568, ATCC16266, ATCC32221, ATCC42430, CBS 219.35, CBS224.37, CBS250.53, CBS343.66, CBS527.72, CBS528.72, CBS529.72, CBS608.70 and CBS754.68, etc., or microorganisms belonging to the subgenus Micromucor, e.g., Mortierella isabellina CBS194.28, IFO6336, IFO7824, IFO7873, IFO7874, IFO8286, IFO8308 and IFO7884, Mortierella nana IFO8190, Mortierella ramanniana IFO5426, IFO8186, CBS112.08, CBS212.72, IFO7825, IFO8184, IFO8185 and IFO8287, Mortierella vinacea CBS236.82, etc. Among others, Mortierella alpina is preferable.

Specific examples of the yeast include the genus Saccharomyces, the genus Candida, the genus Zygosaccharomyces, the genus Pichia and the genus Hansenula, and preferably, Saccharomyces cerevisiae in the genus Saccharomyces. In wild strains of yeast such as Saccharomyces cerevisiae, etc., saturated fatty acids or monovalent fatty acids having mainly 18 or less carbon atoms can be synthesized within the cells, but polyunsaturated fatty acids cannot be synthesized therein. For this reason, when yeast such as Saccharomyces cerevisiae, etc. is used as a host cell, it is preferred to impart the ability to synthesize polyunsaturated fatty acids to the yeast cells by genetic engineering, etc. The ability to synthesize polyunsaturated fatty acids can be imparted by introducing a gene encoding a protein derived from an organism that already possesses the ability to synthesize polyunsaturated fatty acids and takes part in fatty acid synthesis.

The "organism that already possesses the ability to synthesize polyunsaturated fatty acids" includes, for example, lipid-producing fungi. Specific examples of the lipid-producing fungi are the same as those given hereinabove.

Examples of the gene encoding a protein derived from an organism that already possesses the ability to synthesize polyunsaturated fatty acids and "gene
encoding the protein that takes part in fatty acid synthesis" include, but not limited to, 
\( \Delta 12 \) fatty acid desaturase gene, \( \Delta 6 \) fatty acid desaturase gene, GLELO fatty acid 
elongase gene and \( \Delta 5 \) fatty acid desaturase gene, etc. The nucleotide sequences of 
\( \Delta 12 \) fatty acid desaturase gene, \( \Delta 6 \) fatty acid desaturase gene, GLELO fatty acid 
elongase gene and \( \Delta 5 \) fatty acid desaturase gene are available by having access to 
databases including GenBank, etc. For example, in GenBank, Accession No. 
AB020033, No. AB020032, No. AB193123 and No. AB188307 are entered to access 
the respective sequences.

The genes for fatty acid synthesis-related proteins described above are 
inserted into appropriate vectors (e.g., pESC (Stratagene), pYES (Invitrogen), etc.), 
which are then introduced into yeast by the electroporation method, the spheroplast 
method (Proc. Natl. Acad. Sci. USA, 75 p1929 (1978)), the lithium acetate method (J. 
Harbor Laboratory Course Manual, etc.

Fatty acids can be extracted from the host cells transformed by the 
polynucleotides of the present invention or the polynucleotide encoding the protein of 
the present invention in the following manner. A host cell is cultured and then treated 
in a conventional manner, e.g., by centrifugation, filtration, etc. to obtain cultured 
cells. The cells are thoroughly washed with water and preferably dried. Drying may 
be accomplished by lyophilization, air-drying, etc. Depending upon necessity, the 
dried cells are disrupted using a Dynomil or by ultrasonication, and then extracted 
with an organic solvent preferably in a nitrogen flow. Examples of the organic 
 solvent include ether, hexane, methanol, ethanol, chloroform, dichloromethane, 
petroleum ether and so on. Alternatively, good results can also be obtained by 
alternating extraction with methanol and petroleum ether or by extraction with a 
single-phase solvent system of chloroform-methanol-water. Removal of the organic 
solvent from the extract by distillation under reduced pressure may give fatty 
acid-containing lipids. The fatty acids extracted may be converted into the methyl 
esters by the hydrochloric acid methanol method, etc.

The quantity or ratio of various fatty acids may be determined by analyzing 
the fatty acids extracted as described above using various chromatography 
techniques. Examples of the chromatography techniques include, but not limited to, 
high performance liquid chromatography and gas chromatography, and particularly 
preferably, gas chromatography.

3. Vector of the Invention and Vector-Introduced Transformants
In another embodiment, the present invention further provides the expression vector comprising the polynucleotide of the invention.

The vector of the invention is generally constructed to contain an expression cassette comprising:

(i) a promoter that can be transcribed in a host cell;
(ii) any of the polynucleotides defined in (a) to (g) above that is linked to the promoter; and,
(iii) an expression cassette comprising as a component a signal that functions in the host cell with respect to the transcription termination and polyadenylation of RNA molecule.

The vector thus constructed is introduced into a host cell. Examples of host cells which may be appropriately used in the present invention are the same as described above.

In these host cells transformed by the vector of the present invention, the ACS activity is more increased, fatty acids are more produced or the quantity or ratio of various fatty acids contained in the cells are changed, when compared to the host cells which are not transformed by the vector of the present invention.


Any vector is available as the vector used to introduce into the yeast and not particularly limited so long as it is a vector capable of expressing the insert in the yeast cells. The vector includes, e.g., pYE22m (Biosci. Biotech. Biochem., 59, 1221-1228, 1995).

Promoters/terminators for regulating gene expression in host cells may be used in an optional combination as far as they function in the host cells. For example, a promoter of the histone H4.1 gene, a promoter of the glyceraldehyde-3-phosphate dehydrogenase, etc. may be used.

As selection markers used for the transformation, there may be utilized auxotrophic markers (ura5, niaD), hygromycin-resistant gene, zeocin-resistant gene, genecitin-resistant gene (G418r), copper-resistant gene (CUP1) (Marin et al., Proc. Natl. Acad. Sci. USA, 81, 337 1984), cerulenin-resistant gene (fas2m, PDR4) (Junji Inokoshi, et al., Biochemistry, 64, 660, 1992; and Hussain et al., Gene, 101: 149, 1991, respectively), and the like.

For the transformation of host cells, generally known methods may be used. In lipid-producing fungi, the transformation may be performed, e.g., by the electroporation method (Mackenzie, D. A. et al., Appl. Environ. Microbiol., 66,
4655-4661, 2000) and the particle delivery method (the method described in JPA 2005-287403 "Method of Breeding Lipid-Producing Fungus"). On the other hand, the electroporation method, the spheroplast method (Proc. Natl. Acad. Sci. USA, 75 p1929 (1978)) and the lithium acetate method (J. Bacteriology, 153 p163 (1983)) as well as the methods described in Proc. Natl. Acad. Sci. USA, 75 p1929 (1978), Methods in yeast genetics, 2000 Edition: A Cold Spring Harbor Laboratory Course Manual, etc) may be used for the transformation of yeast. However, the method for transformation is not limited to those described above.


4. Method for Producing the Lipid or Fatty Acid Composition of the Invention

In another embodiment, the present invention further provides a method for preparing a lipid or fatty acid composition which comprises using the transformant described above.

As used herein, the term "lipid" is intended to mean a simple lipid including a compound (e.g., a glyceride) which is composed of a fatty acid and an alcohol attached via an ester linkage, or its analog (e.g., a cholesterol ester), etc.; a complex lipid in which phosphoric acid, amino acid(s), saccharide(s) or the like are bound to a part of the simple lipid; or a derived lipid which is a hydrolysate of the lipid and is insoluble in water.

As used herein, the term "oil and fat" is intended to mean an ester of glycerol and a fatty acid (glyceride).

The term "fatty acid" is the same as defined above.

The method for extracting the lipid or fatty acid composition of the present invention is the same as the method for extracting fatty acids described above.

Fatty acids can be separated from the above fatty acid-containing lipids in a state of mixed fatty acids or mixed fatty acid esters by concentration and separation in a conventional manner (e.g., urea addition, separation under cooling, column chromatography, etc.).

The lipids produced by the method of the present invention include preferably unsaturated fatty acids, and more preferably, polyunsaturated fatty acids.

Preferred examples of the polyunsaturated fatty acids are unsaturated fatty acids having 18 or more carbon atoms, e.g., unsaturated fatty acids having 18 to 20 carbon atoms, and include, but not limited to, oleic acid, linoleic acid, linolenic acid
(γ-linolenic acid and dihomo-γ-linolenic acid, etc.), arachidonic acid, etc. Particularly preferred polyunsaturated fatty acids are linoleic acid, γ-linoleic acid, dihomo-γ-linoleic acid and arachidonic acid, more preferably, linoleic acid, dihomo-γ-linoleic acid and arachidonic acid, and most preferably, dihomo-γ-linolenic acid and arachidonic acid.

The lipids produced by the method of the present invention and the composition of the fatty acids contained in the lipids may be confirmed by the lipid extraction method or fatty acid separation method described above, or a combination thereof.

The lipid or fatty acid composition obtained by the production method of the present invention can be provided for use in producing, e.g., food products, pharmaceuticals, industrial materials (raw materials for cosmetics, soaps, etc.), which contain oils and fats, in a conventional manner.

In a still other embodiment, the present invention provides a method for preparing food products, cosmetics, pharmaceuticals, soaps, etc. using the transformant of the present invention. The method involves the step of forming lipids or fatty acids using the transformant of the present invention.

Food products, cosmetics, pharmaceuticals, soaps, etc. containing the lipids or fatty acids produced are prepared in a conventional manner. As such, the food products, cosmetics, pharmaceuticals, soaps, etc. produced by the method of the present invention contain the lipids or fatty acids produced using the transformant of the present invention. The present invention further provides the food products, cosmetics, pharmaceuticals, soaps, etc. produced by such a method.

The form of the cosmetic (composition) or pharmaceutical (composition) of the present invention is not particularly limited and may be any form including the state of a solution, paste, gel, solid or powder. The cosmetic composition or pharmaceutical composition of the present invention may also be used as cosmetics or topical agents for the skin, including an oil, lotion, cream, emulsion, gel, shampoo, hair rinse, hair conditioner, enamel, foundation, lipstick, face powder, facial pack, ointment, perfume, powder, eau de cologne, tooth paste, soap, aerosol, cleansing foam, etc., an anti-aging skin care agent, anti-inflammatory agent for the skin, bath agent, medicated tonic, skin beauty essence, sun protectant, or protective and improving agent for skin troubles caused by injury, chapped or cracked skin, etc.

The cosmetic composition of the present invention may further be formulated appropriately with other oils and fats and/or dyes, fragrances, preservatives, surfactants, pigments, antioxidants, etc., if necessary. The formulation ratio of these materials may be appropriately determined by those skilled in the art,
depending upon purpose (for example, oils and fats may be contained in the
composition in 1 to 99.99 wt %, preferably, 5 to 99.99 wt %, and more preferably, 10
to 99.95 wt%). If necessary, the pharmaceutical composition of the present invention
may also contain other pharmaceutically active components (e.g., anti-inflammatory
components) or aid components (e.g., lubricants or vehicle components). Examples
of the other components commonly used in a cosmetic or a skin preparation for
external use include an agent for acne, an agent for preventing dandruff or itching, an
antiperspirant and deodorant agent, an agent for burn injury, an anti-mite and lice
agent, an agent for softening keratin, an agent for xeroderma, an antiviral agent, a
percutaneous absorption promoting agent, and the like.

The food product of the present invention includes a dietary supplement,
health food, functional food, food product for young children, baby food, infant
modified milk, premature infant modified milk, geriatric food, etc. As used herein,
the food or food product is intended to mean a solid, fluid and liquid food as well as
a mixture thereof, and collectively means an edible stuff.

The term dietary supplement refers to food products enriched with specific
nutritional ingredients. The term health food refers to food products which are
healthful or beneficial to health, and encompasses dietary supplements, natural foods,
diet foods, etc. The term functional food refers to a food product for replenishing
nutritional ingredients which assist body control functions and is synonymous with a
food for specified health use. The term food for young children refers to a food
product given to children up to about 6 years old. The term geriatric food refers to a
food product treated to facilitate digestion and absorption when compared to
untreated foods. The term infant modified milk refers to modified milk given to
children up to about one year old. The term premature infant modified milk refers to
modified milk given to premature infants until about 6 months after birth.

The form of these food products includes natural foods (treated with fats and
oils) such as meat, fish and nuts; foods supplemented with fats and oils during
cooking, e.g., Chinese foods, Chinese noodles, soups, etc.; foods prepared using fats
and oils as heating media, e.g., tempura or deep-fried fish and vegetables, deep-fried
foods, fried bean curd, Chinese fried rice, doughnuts, Japanese fried dough cookies
or karinto; fat- and oil-based foods or processed foods supplemented with fats and
oils during processing, e.g., butter, margarine, mayonnaise, dressing, chocolate,
instant noodles, caramel, biscuits, cookies, cakes, ice cream; and foods sprayed or
coated with fats and oils upon finishing, e.g., rice crackers, hard biscuits, sweet bean
paste bread, etc. However, the food product is not limited to foods containing fats
and oils, and other examples include agricultural foods such as bakery products,
noodles, cooked rice, sweets (e.g., candies, chewing gums, gummies, sweet tablets, Japanese sweets), bean curd or tofu and processed products thereof; fermented foods such as Japanese rice wine or sake, medicinal liquor, sweet cooking sherry or mirin, vinegar, soy sauce and bean paste or miso, etc.; livestock food products such as yoghurt, ham, bacon, sausage, etc.; seafood products such as minced and steamed fish cake or kamaboko, deep-fried fish cake or ageten and puffy fish cake or hanpen, etc.; as well as fruit drinks, soft drinks, sports drinks, alcoholic beverages, tea, etc.

The food product of the present invention may also be in the form of pharmaceutical preparations such as capsules, etc., or in the form of a processed food such as natural liquid diets, defined formula diets and elemental diets formulated with the oil and fat of the present invention together with proteins, sugars, trace elements, vitamins, emulsifiers, aroma chemicals, etc., health drinks, enteral nutrients, and the like.

As described above, fatty acids can be efficiently produced by expressing the ACS homolog gene of the present invention in host cells.

Furthermore, the expression level of the gene can be used as an indicator to study conditions for cultivation, cultivation control, etc. for efficient fatty acid production.

EXAMPLES

Hereinafter, the present invention is described in more detail with reference to EXAMPLES but it should be understood that the invention is not deemed to limit the scope of the invention to these EXAMPLES.

[EXAMPLE 1]

Genome Analysis of M. alpina

The M. alpina 1S-4 strain was plated on 100 ml of GY2:1 medium (2% glucose and 1% yeast extract, pH 6.0) followed by shake culture at 28°C for 2 days. The mycelial cells were collected by filtration, and genomic DNA was prepared using DNeasy (QIAGEN). The nucleotide sequence of the genomic DNA described above was determined using a Roche 454 GS FLX Standard. On this occasion, nucleotide sequencing of a fragment library was performed in two runs and nucleotide sequencing of a mate paired library in three runs. The resulting nucleotide sequences were assembled to give 300 supercontigs.

Synthesis of cDNA and Construction of cDNA Library

The M. alpina strain 1S-4 was plated on 100 ml of medium (1.8% glucose,
1% yeast extract, pH 6.0) and precultured for 3 days at 28°C. A 10 L culture vessel (Able Co., Tokyo) was charged with 5 L of medium (1.8% glucose, 1% soybean powder, 0.1% olive oil, 0.01% Adekanol, 0.3% KH₂PO₄, 0.1% Na₂SO₄, 0.05% CaCl₂·2H₂O and 0.05% MgCl₂·6H₂O, pH 6.0), and the whole amount of the pre-cultured product was plated thereon, followed by aerobic spinner culture under conditions of 300 rpm, 1vvm and 26°C for 8 days. On Days 1, 2 and 3 of the cultivation, glucose was added in an amount corresponding to 2%, 2% and 1.5%, respectively. The mycelial cells were collected at each stage on Days 1, 2, 3, 6 and 8 of the cultivation to prepare total RNA by the guanidine hydrochloride/CsCl method.

Using an Oligotex-dT30<sup>®</sup>mRNA Purification Kit (Takara Bio Inc.), poly(A)+RNA was purified from the total RNA. A cDNA library was constructed for each stage using a ZAP-cDNA Gigapack III Gold Cloning Kit (STRATAGENE).

**Search for ACS Homolog**

Using as a query the amino acid sequences of ScFAA1 (YOR317W), ScFAA2 (YER015W), ScFAA3 (YIL009W), ScFAA4 (YMR246W), ScFAT1 (YBR041W) and ScFAT2 (YBR222C), which are ACS from yeast, a tblastn search was performed against the genome nucleotide sequence of the M. alpina strain 1S-4.

As a result, hits were found in twelve (12) sequences. That is, hit was found on supercontigs containing the sequence shown by SEQ ID NO: 5, SEQ ID NO: 10, SEQ ID NO: 15, SEQ ID NO: 20, SEQ ID NO: 25, SEQ ID NO: 30, SEQ ID NO: 35, SEQ ID NO: 40, SEQ ID NO: 45, SEQ ID NO: 50, SEQ ID NO: 55 or SEQ ID NO: 60. The genes bearing SEQ ID NO: 5, SEQ ID NO: 10, SEQ ID NO: 15, SEQ ID NO: 20, SEQ ID NO: 25, SEQ ID NO: 30, SEQ ID NO: 35, SEQ ID NO: 40, SEQ ID NO: 45, SEQ ID NO: 50, SEQ ID NO: 55 and SEQ ID NO: 60 were designated respectively as MaACS-1, MaACS-2, MaACS-3, MaACS-4, MaACS-5, MaACS-6, MaACS-7, MaACS-8, MaACS-9, MaACS-10, MaACS-11 and MaACS-12.

**Cloning of ACS Homolog**

For cloning of the cDNAs corresponding to the MaACS-1~12 genes, screening of the cDNA library described above was performed. Probe labeling was performed by PCR using an ExTaq™ (Takara Bio Inc.). That is, digoxigenin (DIG)-labeled amplified DNA probes were prepared using a PCR Labeling Mix (Roche Diagnostics) instead of dNTP mix attached to ExTaq.

Conditions for hybridization were set as follows.

- **Buffer**: 5x SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formaldehyde;
Temperature: 42°C (overnight);
Wash conditions: 0.2x SSC, in 0.1% SDS solution (65°C) for 20 mins. x 3

Detection was performed using a DIG Nucleic Acid Detection Kit (Roche Diagnostics). Phage clones were obtained by screening and plasmids were excised from the phage clones by in vivo excision to give the respective plasmid DNAs.

Primers for preparing the probes used for screening of the respective genes, the number of nucleotides in CDS of the respective genes, the number of amino acids in the amino acid sequences deduced from the nucleotide sequences of CDS, and the number of exons and introns by comparison of genomic DNA sequences with CDS sequences are given below.

(1) MaACS-1
Primer ACS-1-1F: 5'-GTCGGCTCCAAGCTTGAATCC-3' (SEQ ID NO: 61)
Primer ACS-1-2R: 5'-GGACAGCTGCCAGCAGCTGGA-3' (SEQ ID NO: 62)
cDNA (SEQ ID NO: 4)
CDS (SEQ ID NO: 3): 1857 bp
ORF (SEQ ID NO: 1): 1854 bp
Amino acid sequence (SEQ ID NO: 2): 618 amino acids (see FIG. 1)
Number of exons: 5, number of introns: 4 (see FIG. 2)

(2) MaACS-2
Primer ACS-2-1F: 5'-GACCACGGAATTCCCCAAAGGCTGC-3' (SEQ ID NO: 63)
Primer ACS-2-2R: 5'-CTTGGTGCGCCCTTGGTCCTGGCCAC-3' (SEQ ID NO: 64)
cDNA (SEQ ID NO: 9)
CDS (SEQ ID NO: 8): 1929 bp
ORF (SEQ ID NO: 6): 1926 bp
Amino acid sequence (SEQ ID NO: 7): 642 amino acids (see FIG. 3)
Number of exons: 8, number of introns: 7 (see FIG. 4)

(3) MaACS-3
Primer ACS-3-1F: 5'-TACAGCTTTTGGCTGTCCCCATC-3' (SEQ ID NO: 65)
Primer ACS-3-2R: 5'-GATGATGGGTGTGCTTGCAAGATC-3' (SEQ ID NO: 66)
  cDNA (SEQ ID NO: 14)
  CDS (SEQ ID NO: 13): 1653 bp
  ORF (SEQ ID NO: 11): 1650 bp
  Amino acid sequence (SEQ ID NO: 12): 550 amino acids (see FIG. 5)
  Number of exons: 9, number of introns: 8 (see FIG. 6)

(4) MaACS-4
  Primer ACS-4-1F: 5'-AACCCAAAGCTGCAGCCAGGCTTGCC-3' (SEQ ID NO: 67)
  Primer ACS-4-2R: 5'-TTACGGCTGATTCTTTTGATGG-3' (SEQ ID NO: 68)
  cDNA (SEQ ID NO: 19)
  CDS (SEQ ID NO: 18): 2067 bp
  ORF (SEQ ID NO: 16): 2064 bp
  Amino acid sequence (SEQ ID NO: 17): 688 amino acids (see FIG. 7)
  Number of exons: 7, number of introns: 6 (see FIG. 8)

(5) MaACS-5
  Primer ACS-5-1F: 5'-GTCGTGCGCCGATGAGACGC-3' (SEQ ID NO: 69)
  Primer ACS-5-2R: 5'-TCAGTGGATCCGTTACCTACAG-3' (SEQ ID NO: 70)
  cDNA (SEQ ID NO: 24)
  CDS (SEQ ID NO: 23): 1980 bp
  ORF (SEQ ID NO: 21): 1977 bp
  Amino acid sequence (SEQ ID NO: 22): 659 amino acids (see FIG. 9)
  Number of exons: 6, number of introns: 5 (see FIG. 10)

(6) MaACS-6
  Primer ACS-6-1F: 5'-CGTCCCCCTCTATGATACATTG-3' (SEQ ID NO: 71)
  Primer ACS-6-2R: 5'-GGGGATGCCAGGACGCAACATCG-3' (SEQ ID NO: 72)
  cDNA (SEQ ID NO: 29)
  CDS (SEQ ID NO: 28): 1980 bp
ORF (SEQ ID NO: 26): 1977 bp
Amino acid sequence (SEQ ID NO: 27): 659 amino acids (see FIG. 11)
Number of introns: at least 5 (see FIG. 12)

(7) MaACS-7
Primer ACS-7-1F: 5'-GGATGCGAACAACAGCGTG-3' (SEQ ID NO: 73)
Primer ACS-7-2R: 5'-GCACCCTCCTCAGAAACAGCCCTC-3' (SEQ ID NO: 74)
cDNA (SEQ ID NO: 34)
CDS (SEQ ID NO: 33): 1827 bp
ORF (SEQ ID NO: 31): 1824 bp
Amino acid sequence (SEQ ID NO: 32): 608 amino acids (see FIG. 13)
Number of exons: 5, number of introns: 4 (see FIG. 14)

(8) MaACS-8
Primer ACS-8-1F: 5'-CAGTCGAGTACATTGTCAACCACG-3' (SEQ ID NO: 75)
Primer ACS-8-2R: 5'-GCGGGAGAGGCGGAGGCACAGC-3' (SEQ ID NO: 76)
cDNA (SEQ ID NO: 39)
CDS (SEQ ID NO: 38): 2079 bp
ORF (SEQ ID NO: 36): 2076 bp
Amino acid sequence (SEQ ID NO: 37): 692 amino acids (see FIG. 15)
Number of exons: 8, number of introns: 7 (see FIG. 16)

(9) MaACS-9
Primer ACS-9-1F: 5'-GTTCATCTTCTGCTGGCTGGTCTC-3' (SEQ ID NO: 77)
Primer ACS-9-2R: 5'-GTTGCGTGTGCACCGGGAATCC-3' (SEQ ID NO: 78)
cDNA (SEQ ID NO: 44)
CDS (SEQ ID NO: 43): 1851 bp
ORF (SEQ ID NO: 41): 1848 bp
Amino acid sequence (SEQ ID NO: 42): 616 amino acids (see FIG. 17)
Number of exons: 5, number of introns: 4 (see FIG. 18)
(10) MaACS-10
Primer ACS-10-1F: 5'-ATGGAAACCTTGGTTAACGGGAAAG-3' (SEQ ID NO: 79)
Primer ACS-10-2R: 5'-TCAGCAAAAGATGGCCCTTGGGCTTG-3' (SEQ ID NO: 80)
cDNA (SEQ ID NO: 49)
CDS (SEQ ID NO: 48): 2076 bp
ORF (SEQ ID NO: 46): 2073 bp
Amino acid sequence (SEQ ID NO: 47): 691 amino acids (see FIG. 19)
Number of exons: 8, number of introns: 7 (see FIG. 20)

(11) MaACS-11
Primer ACS-11-1F: 5'-GTCAAGGGCGAGACTCGCATCC-3' (SEQ ID NO: 81)
Primer ACS-11-2R: 5'-CGGTGACGATGGTCATGGACTGC-3' (SEQ ID NO: 82)
cDNA (SEQ ID NO: 54)
CDS (SEQ ID NO: 53): 2043 bp
ORF (SEQ ID NO: 51): 2040 bp
Amino acid sequence (SEQ ID NO: 52): 680 amino acids (see FIG. 21)
Number of exons: 3, number of introns: 2 (see FIG. 22)

(12) MaACS-12
Primer ACS-12-1F: 5'-GCAGGACCCGCATCCGCGCCGCTCC-3' (SEQ ID NO: 83)
Primer ACS-12-2R: 5'-GACCGTCCTCGCCAGGGTGTCG-3' (SEQ ID NO: 84)
cDNA (SEQ ID NO: 59)
CDS (SEQ ID NO: 58): 2043 bp
ORF (SEQ ID NO: 56): 2040 bp
Amino acid sequence (SEQ ID NO: 57): 680 amino acids (see FIG. 23)
Number of exons: 3, number of introns: 2 (see FIG. 24)

Sequencing Analysis
The identity between the CDS nucleotide sequences of 12 ACS homologs from M. alpina is shown in TABLE 1 and the identity between the amino acid sequences is shown in TABLE 2. MaACS-11 and MaACS-12 showed high identity
of 80.2% in the nucleotide sequence and 84.3% in the amino acid sequence.

TABLE 1  Sequence identity among CDS nucleotide sequences of ACS homologs from M. alpina

<table>
<thead>
<tr>
<th>MaACS-1</th>
<th>MaACS-2</th>
<th>MaACS-3</th>
<th>MaACS-4</th>
<th>MaACS-5</th>
<th>MaACS-6</th>
<th>MaACS-7</th>
<th>MaACS-8</th>
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<td>44.6</td>
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TABLE 2  Sequence identity among amino acid sequences of ACS homologs from M. alpina

<table>
<thead>
<tr>
<th>MaACS-1</th>
<th>MaACS-2</th>
<th>MaACS-3</th>
<th>MaACS-4</th>
<th>MaACS-5</th>
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<th>MaACS-7</th>
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<th>MaACS-9</th>
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</tr>
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</table>

Using as query sequences the putative amino acid sequences for the CDS sequences of MaACS-1~12, BLASTp search was performed against the amino acid sequences registered in GenBank. The proteins having the amino acid sequence which matched the putative amino acid sequences of MaACS-1~12 with highest score and the identity between these proteins and the putative amino acid sequences of MaACS-1~12 are shown in TABLE 3. The identity of the putative amino acid sequences of MaACS-1~12 with the amino acid sequences of S. cerevisiae-derived acyl-CoA synthetases are also shown in TABLE 4.

TABLE 3  Sequence identity between the amino acid sequences of M. alpina-derived ACS homologs and known amino acid sequences

27
| MaACS-1  | 41.8  | 71014575 | Putative protein from Ustilago maydis |
| MaACS-2  | 35.4  | 71014575 | Putative protein from Ustilago maydis |
| MaACS-3  | 23.5  | 71895089 | Chick ACS long-chain family member 5 |
| MaACS-4  | 36.9  | 115487304 | Putative protein from Oryza sativa |
| MaACS-5  | 42.5  | 168085128 | Putative protein from Physcomitrella patens |
| MaACS-6  | 40.9  | 13516481 | Long-chain acyl-CoA synthetase from Arabidopsis thaliana |
| MaACS-7  | 45.7  | 120612991 | Putative protein from Acidovorax avenae subsp. citrulli |
| MaACS-8  | 40.0  | 13516481 | Long-chain acyl-CoA synthetase from Arabidopsis thaliana |
| MaACS-9  | 37.8  | 67599044 | Putative protein from Aspergillus nidulans |
| MaACS-10 | 33.2  | 171682488 | Putative protein from Podospora anserina |
| MaACS-11 | 48.8  | 169854433 | Putative protein from Coprinopsis atramentarius |
| MaACS-12 | 45.1  | 156045509 | Putative protein from Sclerotinia sclerotiorum |

**TABLE 4** Comparison of amino acid sequences of *M. alpina*-derived ACS homologs and amino acid sequences of *S. cerevisiae*-derived ACS

<table>
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<tr>
<th></th>
<th>ScFAA1</th>
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<th>ScFAA3</th>
<th>ScFAA4</th>
<th>ScFAT1</th>
<th>ScFAT2</th>
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<td>25.8</td>
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<td>14.4</td>
<td>16.2</td>
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</tbody>
</table>

FIG. 25 shows the alignment between MaACS from MaACS-1~12, which have relatively high amino acid sequence homology to the *S. cerevisiae*-derived FAA proteins, and the FAA proteins. FIG. 26 shows the alignment of the ACS homologs having relatively high amino acid sequence homology to *S. cerevisiae*-derived FAT proteins. The regions of the ATP-AMP motif and FACS/VLACS-FATP motif, which are important motifs for the ACS activity, are highly conserved in both groups shown in FIGS. 25 and 26.

**Construction of Expression Vector**

Vectors for expressing MaACS-1, MaACS-10, MaACS-11, MaACS-6, MaACS-8 and MaACS-9, respectively, in yeast were constructed as follows, using the expression vector pYE22m (Biosci. Biotech. Biochem., 59, 1221-1228, 1995).

The plasmid containing SEQ ID NO: 29, which was obtained by screening MaACS-6, was digested with restriction enzymes BamHI and XhoI. The resulting DNA fragment of approximately 2.1 kbp was ligated to the DNA fragment obtained
by digestion of vector pYE22m with restriction enzymes BamHI and Sall using a Ligation High (TOYOBO) to give plasmid pYE-ACS-6.

Using the plasmid containing cDNA of MaACS-8 as a template, PCR was performed with the primers below using ExTaq (Takara Bio Inc.). The thus amplified DNA fragment was cloned by a TOPO-TA Cloning Kit (Invitrogen).

Primer EcoRI-ACS-8-F: 5'-GGATCCATGCTTCCCTCAAACGTAAACC-3' (SEQ ID NO: 85)
Primer SmaI-ACS-8-R: 5'-CCCAGGCAAAGAGTTTCTATCTACAGCT-3' (SEQ ID NO: 86)

The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes EcoRI and SmaI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 2.1 kbp was ligated to the DNA fragment obtained by digesting vector pYE22m with restriction enzymes EcoRII and SmaI to give plasmid pYE-ACS-8.

Using the plasmid containing cDNA of MaACS-9 as a template, PCR was performed with the primers below using ExTaq (Takara Bio Inc.). The thus amplified DNA fragment was cloned by a TOPO-TA Cloning Kit (Invitrogen).

Primer EcoRI-ACS-9-F: 5'-GAATTCATGGTGTCTTCCCACTCG-3' (SEQ ID NO: 87)
Primer BamHI-ACS-9-R: 5'-GGATCCCTACTATAGCCTTGCCCTTGCC-3' (SEQ ID NO: 88)

The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes EcoRI and BamHI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 2.0 kbp was ligated to the DNA fragment obtained by digesting vector pYE22m with restriction enzymes EcoRII and BamHI to give plasmid pYE-ACS-9.

Using the plasmid containing cDNA of MaACS-1 as a template, PCR was performed with the primers below using ExTaq (Takara Bio Inc.). The thus amplified DNA fragment was cloned by a TOPO-TA Cloning Kit (Invitrogen).

Primer EcoRI-ACS-1-F: 5'-GGATCCATGATGTCAAGCTTGC-3' (SEQ ID NO: 89)
Primer SalI-ACS-1-R: 5’-GTCGACTCAAGCCTGGCTTTGCCGCTGACG-3’ (SEQ ID NO: 90)

The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes EcoRI and SalI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 1.9 kbp was ligated to the DNA fragment obtained by digesting vector pYE22m with restriction enzymes EcoRI and SalI to give plasmid pYE-ACS-1.

Using the plasmid containing cDNA of MaACS-10 as a template, PCR was performed with the primers below using ExTaq (Takara Bio Inc.). The thus amplified DNA fragment was cloned by a TOPO-TA Cloning Kit (Invitrogen).

Primer ACS-10-1F: 5’-GGATCCATGGAAACCTTTGGTTAACGGAAG-3’ (SEQ ID NO: 91)
Primer KpnI-ACS-10-R: 5’-GGTACCTAGAAGTCTTCCACATCTCCTC-3’ (SEQ ID NO: 92)

The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes EcoRI and KpnI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 2.1 kbp was ligated to the DNA fragment obtained by digesting vector pYE22m with restriction enzymes EcoRI and KpnI. Plasmid pYE-ACS-10 was obtained by screening for the orientation that the GAPDH promoter of vector pYE22m was located at its 5’ end of CDS of MaACS-10.

Using the plasmid containing cDNA of MaACS-11 as a template, PCR was performed with the primers below using ExTaq (Takara Bio Inc.). The thus amplified DNA fragment was cloned by a TOPO-TA Cloning Kit (Invitrogen).

Primer SacI-ACS-11-F: 5’-GAGCTCATGCAAAATGCTTTACCAGTCAACG-3’ (SEQ ID NO: 93)
Primer BamHI-ACS-11-R: 5’-GGATCCTACTTGGACGCATAGATCTGCTTG-3’ (SEQ ID NO: 94)

The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes SacI and BamHI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 2.0 kbp was ligated to the DNA fragment obtained by digesting vector.
pYE22m with restriction enzymes SacI and BamHI to give plasmid pYE-ACS-11.

**Expression in Yeast**

**Acquisition of Transformants**

The yeast *S. cerevisiae* EH13-15 strain (trp1, MATα) (Appl. Microbiol. Biotechnol., 30, 515-520, 1989) was transformed with plasmids pYE22m, pYE-MaACS-6, pYE-MaACS-8 and pYE-MaACS-9, respectively, by the lithium acetate method. The transformants were screened for the ability to grow on SC-Trp agar medium (2% agar) (per liter, 6.7 g Yeast Nitrogen Base w/o Amino Acids (DIFCO), 20 g glucose, 1.3 g amino acid powders (a mixture of 1.25 g adenine sulfate, 0.6 g arginine, 3 g aspartic acid, 3 g glutamic acid, 0.6 g histidine, 1.8 g leucine, 0.9 g lysine, 0.6 g methionine, 1.5 g phenylalanine, 11.25 g serine, 0.9 g tyrosine, 4.5 g valine, 6 g threonine and 0.6 g uracil).

**Cultivation of Yeast**

One each from the transformants obtained using the respective plasmids was provided for the following cultivation experiment.

One platinum loop of the yeast was plated on 10 ml of SC-Trp and cultured with shaking for preincubation at 30°C for a day. After 1 ml of the preincubation was added to the SC-Trp medium, main cultivation was performed by shake culturing at 30°C for a day.

**Analysis of Fatty Acids in Mycelia**

The yeast culture broth was centrifuged to recover the mycellal cells. After washing with 10 ml of sterile water, the mycellal cells were again centrifuged, recovered and lyophilized. The fatty acids in the mycellal cells were converted into the methyl esters by the hydrochloric acid-methanol method followed by extraction with hexane. After hexane was removed by distillation, the fatty acids were analyzed by gas chromatography.

The fatty acid production per medium is shown in TABLE 5. In the strains transformed by ppYE-MaACS-6, pYE-MaACS-8 or pYE-MaACS-9, the fatty acid production per medium was increased as compared to the control which was transformed by pYE22m.

**TABLE 5** Fatty Acid Production by Transformant per Medium

<table>
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<tr>
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<th>MaACS-6</th>
<th>MaACS-8</th>
<th>MaACS-9</th>
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<tbody>
<tr>
<td>Fatty acid production (mg/L)</td>
<td>135</td>
<td>159</td>
<td>196</td>
<td>187</td>
</tr>
</tbody>
</table>

31
Expression in Arachidonic Acid-Producing Yeast

(1) Breeding of Arachidonic Acid-Producing Yeast Strains

To breed arachidonic acid-producing yeast strain (S. cerevisiae), the following plasmids were constructed.

First, using the cDNA prepared from M. alpina strain 1S-4 as a template, PCR was performed with ExTaq using the primer pair of Δ12-f and Δ12-r, Δ6-f and Δ6-r, GLELO-f and GLELO-r, or Δ5-f and Δ5-r to amplify the Δ12 fatty acid desaturase gene (GenBank Accession No. AB020033) (hereinafter "Δ12 gene"), the Δ6 fatty acid desaturase gene (GenBank Accession No. AB020032) (hereinafter "Δ6 gene"), the GLELO fatty acid elongase gene (GenBank Accession No. AB193123) (hereinafter "GLELO gene") and the Δ5 fatty acid desaturase gene (GenBank Accession No. AB188307) (hereinafter "Δ5 gene") in the M. alpina strain 1S-4.

Δ12-f: 5'-TCTAGAATGGCACCCTCTCCCCACACTATTG-3' (SEQ ID NO: 95)
Δ12-r: 5'-AAGCTTTTACTCTTGAAAAAGACCCAGTC-3' (SEQ ID NO: 96)
Δ6-f: 5'-TCTAGAATGGCTGCTGCTCCCCAGTGAGG-3' (SEQ ID NO: 97)
Δ6-r: 5'-AAGCTTTTACTGTGCTGCTGCTCCCATCTTGG-3' (SEQ ID NO: 98)
GLELO-f: 5'-TCTAGAATGGAGTCTGATTGGAATCC-3' (SEQ ID NO: 99)
GLELO-r: 5'-GAGCTTTTACTGCAACCTTCTTGGGCAATTCG-3' (SEQ ID NO: 100)
Δ5-f: 5'-TCTAGAATGGGTGGCCAGACAGGAAGAAAAAC-3' (SEQ ID NO: 101)
Δ5-r: 5'-AAGCTTTTACTCTTTCTGGAAGACC-3' (SEQ ID NO: 102)

These genes were cloned with the TOPO-TA-Cloning Kit. The clones were confirmed by their nucleotide sequences. The clones containing the nucleotide sequences of the Δ12 gene, Δ6 gene, GLELO gene and Δ5 gene were designated as plasmids pCR-MAΔ12DS (containing the nucleotide sequence of the Δ12 gene), pCR-MAΔ6DS (containing the nucleotide sequence of the Δ6 gene), pCR-MAGLELO (containing the nucleotide sequence of the GLELO gene) and pCR-MAΔ5DS (containing the nucleotide sequence of the Δ5 gene), respectively.

On the other hand, the plasmid pURA34 (JPA 2001-120276) was digested with restriction enzyme HindIII. The resulting DNA fragment of approximately 1.2 kb was inserted into the HindIII site of the vector, which was obtained by digesting pUC18 vector (Takara Bio Inc.) with restriction enzymes EcoRI and SphI, then blunt ending and self ligating said vector. The clone in which the EcoRI site of the vector was located at its 5' end of URA3 was designated as pUC-URA3. Also, the DNA fragment of approximately 2.2 kb, which was obtained by digesting YEp13 with
restriction enzymes Sall and XhoI, was inserted into the Sall site of vector pUC18. The clone in which the EcoRI site of the vector was located at its 5' end of LUE2 was designated as pUC-LEU2.

Next, the plasmid pCR-MAAΔ12DS was digested with restriction enzyme HindIII, followed by blunt ending and further digestion with restriction enzyme XbaI. The resulting DNA fragment of approximately 1.2 kbp was ligated to the DNA fragment of approximately 6.6 kbp, which was obtained by digesting vector pESC-URA (STRATAGENE) with restriction enzyme SacI, blunt ending and further digesting with restriction enzyme SpeI. Thus, the plasmid pESC-U-Δ12 was obtained.

The plasmid pCR-MAAΔ6DS was digested with restriction enzyme XbaI, followed by blunt ending and further digestion with restriction enzyme HindIII. The resulting DNA fragment of approximately 1.6 kbp was ligated to the DNA fragment of approximately 8 kbp, which was obtained by digesting the plasmid pESC-U-Δ12 with restriction enzyme Sall, blunt ending and further digesting with restriction enzyme HindIII, thereby to give the plasmid pESC-U-Δ12:Δ6. This plasmid was partially digested with restriction enzyme PvuII. The resulting fragment of approximately 4.2 kb was inserted into the Smal site of pUC-URA3 to give the plasmid pUC-URA-Δ12:Δ6.

Also, the plasmid pCR-MAGLELO was digested with restriction enzymes XbaI and SacI. The resulting DNA fragment of approximately 0.95 kbp was ligated to the DNA fragment of approximately 7.7 kbp, which was obtained by digesting vector pESC-LEU (STRATAGENE) with restriction enzymes XbaI and SacI. Thus, the plasmid pESC-L-GLELO was obtained. The plasmid pCR-MAAΔ5DS was digested with restriction enzyme XbaI, followed by blunt ending and further digestion with restriction enzyme HindIII. The resulting DNA fragment of approximately 1.3 kbp was ligated to the DNA fragment of approximately 8.7 kbp, which was obtained by digesting the plasmid pESC-L-GLELO with restriction enzyme Apal, blunt ending and further digesting with restriction enzyme HindIII, thereby to give the plasmid pESC-L-GLELO:Δ5. This plasmid was digested with restriction enzyme PvuII and the resulting fragment of approximately 3.2 kbp was inserted into the Smal site of pUC-LEU2 to give plasmid pUC-LEU-GLELO:Δ5. The Saccharomyces cerevisiae strain YPH499 (STRATAGENE) was co-transformed by the plasmid pUC-URA-Δ12:Δ6 and plasmid pUC-LEU-GLELO:Δ5. The transformants were screened for the ability to grow on SC-Leu,Ura agar medium.

Among the transformants thus obtained, random one strain was designated as the strain ARA3-1. By cultivating the strain in a galactose-supplemented medium, the strain became capable of expressing from the GAL1/10 promoter the Δ12 fatty acid.
desaturase gene, the Δ6 fatty acid desaturase gene, the GLELO gene and the Δ5 fatty acid desaturase gene.

(2) Transformation into Arachidonic Acid-Producing Yeast and Analysis

The ARA3-1 strain was transformed by plasmids pYE22m, pYE-ACS-1, pYE-ACS-10 and pYE-ACS-11, respectively. Transformants were screened for the ability to grow on SC-Trp,Leu,Ura agar medium (2 % agar) (per liter, 6.7 g Yeast Nitrogen Base w/o Amino Acids (DIFCO), 20 g glucose and 1.3 g amino acid powders (a mixture of 1.25 g adenine sulfate, 0.6 g arginine, 3 g aspartic acid, 3 g glutamic acid, 0.6 g histidine, 0.9 g lysine, 0.6 g methionine, 1.5 g phenylalanine, 11.25 g serine, 0.9 g tyrosine, 4.5 g valine and 6 g of threonine). Random four strains from the respective plasmid-transfected strains were used for the subsequent cultivation.

These strains were cultivated at 30°C for a day in 10 ml of the

SC-Trp,Leu,Ura liquid medium described above. One milliliter of the culture was plated on 10 ml of SG-Trp,Leu,Ura liquid medium (per liter, 6.7 g Yeast Nitrogen Base w/o Amino Acids (DIFCO), 20 g galactose and 1.3 g amino acid powders (a mixture of 1.25 g adenine sulfate, 0.6 g arginine, 3 g aspartic acid, 3 g glutamic acid, 0.6 g histidine, 0.9 g lysine, 0.6 g methionine, 1.5 g phenylalanine, 11.25 g serine, 0.9 g tyrosine, 4.5 g valine and 6 g of threonine) and then cultivated at 15°C for 6 days.

The mycelial cells were collected, washed with water and then lyophilized. After the fatty acids in the dried mycelial cells were converted to the methyl esters by the hydrochloric acid-methanol method, the analysis of fatty acids was performed by gas chromatography. The ratio of each PUFA to the total fatty acids in the control strain transformed by plasmid pYE22m, and in the strains transformed by each ACS homolog from Mortierella is shown in TABLE 6.

<table>
<thead>
<tr>
<th>TABLE 6</th>
<th>% Ratio of PUFA in ACS homolog expression strains from Mortierella</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
</tr>
<tr>
<td>18:2</td>
<td>7.23 ± 0.11</td>
</tr>
<tr>
<td>18:3(n-6)</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>DGLA</td>
<td>0.41 ± 0.01</td>
</tr>
<tr>
<td>ARA</td>
<td>0.42 ± 0.01</td>
</tr>
</tbody>
</table>

As shown in TABLE 6, the ratio of fatty acids could be modified by expressing the ACS homolog from Mortierella. Particularly in the MaACS-11 expression strain, the ratios of arachidonic acid, linoleic acid and γ-linolenic acid were increased by about 1.8 times, about 1.5 times and about 2.4 times, respectively,
as compared to the control strain. In the MaACS-1 expression strain, the ratio of arachidonic acid was increased by about 1.5 times, as compared to the control strain. Further in the MaACS-10 expression strain, the ratios of linoleic acid and γ-linolenic acid were increased by about 2 times and about 4 times, respectively, as compared to the control strain.

[EXAMPLE 2]  
Construction of Expression Vector

Expression Vector for Yeast

The vector pYE-ACS-12 for expressing MaACS-12 in yeast was constructed as follows. Using a plasmid containing the cDNA of MaACS-12 as a template, PCR was performed with the following primers using KOD-Plus-(TOYOBO).

Primer Eco-ACS-G-F: 5'-GAATTCATGACAAAGTGCCTCACCCTCG-3' (SEQ ID NO: 103)
Primer Sma-ACS-G-R: 5'-CCCGGACTTAGGCGGTTCAGTCAAGCTG-3' (SEQ ID NO: 104)

The amplified DNA fragment was cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The nucleotide sequence of the insert was verified and the plasmid containing the correct nucleotide sequence was digested with restriction enzymes EcoRI and SmaI. Using a Ligation High (TOYOBO), the resulting DNA fragment of approximately 2 kbp was ligated to the DNA fragment obtained by digesting vector pYE22m with restriction enzyme BamHI and then blunt ending with a Blunting Kit (TAKARA Bio) and further digesting with EcoRI, to give plasmid pYE-ACS-12.

Expression Vector for M. alpina

The vector for expressing MaACS-10 and MaACS-11 in M. alpina was constructed as follows.

First, pUC18 was digested with restriction enzymes EcoRI and HindIII and an adapter obtained by annealing oligo DNA MCS-for-pUC18-F2 with MCS-for-pUC18-R2 was inserted therein to construct plasmid pUC18-RF2.

MCS-for-pUC18-F2:
5'-AATTCATAAGATGGCGCGCTAAACTATTCTAGACTAGGTCGACGGCGCGCCA-3' (SEQ ID NO: 105)
MCS-for-pUC18-R2:
5'-AGCTTGCGCAGGGCTGACCTAGTCTAGAATAGTTTTAGCGGCCGACAT
TTATG-3' (SEQ ID NO: 106)

Using the genome DNA of M. alpina as a template, PCR was performed with the primers Not1-GAPDHt-F and EcoR1-Asc1-GAPDHt-R using KOD-Plus- (Toyobo). The amplified DNA fragment of about 0.5 kbp was cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). After the nucleotide sequence of the insert was verified, the DNA fragment of about 0.9 kbp obtained by digesting with restriction enzymes NotI and EcoRI was inserted into the NotI and EcoRI site of plasmid pUC18-RF2 to construct plasmid pDG-1.

Not1-GAPDHt-F: 5'-AGCGGCGCCGATAGGGGAGATCGAACC-3' (SEQ ID NO: 107)

EcoR1-Asc1-GAPDHt-R:
5'-AGATCGCGGCGCCATGCACGCGGTCCTTCTCA-3' (SEQ ID NO: 108)

Using the genome of M. alpina as a template, PCR was performed with the primers URA5g-F1 and URA5g-R1 using KOD-Plus- (Toyobo). The amplified DNA fragment was cloned using a Zero Blunt TOPO PCR Cloning Kit (Invitrogen). After the nucleotide sequence of the insert was verified, the DNA fragment of about 2 kbp obtained by digestion with Sall was inserted into the Sall site of plasmid pDG-1. The plasmid that the 5' end of URAS gene inserted was oriented toward the EcoRI side of the vector was designated as the plasmid pDuraG.

URA5g-F1: 5'-GTCGACCAGTGACTGTGTTTGCG-3' (SEQ ID NO: 109)
URA5g-R1: 5'-GTCGACTGGAAGACGACGACG-3' (SEQ ID NO: 110)

Subsequently, PCR was performed with KOD-Plus- (TOYOBO) using the genome of M. alpina as a template and the primers hisHp+URA5-F and
hisHp+Mgt-F. Using an In-Fusion (registered trade name) Advantage PCR Cloning Kit (TAKARA Bio), the amplified DNA fragment of about 1.0 kbp was ligated to the DNA fragment of about 5.3 kbp amplified by PCR with KOD-Plus- (TOYOBO) using pDuraG as a template and the primers pDuraSC-GAPt-F and URA5gDNA-F, to give plasmid pDura-RhG.

hisHp+URA5-F:
5'-GGCAAACTTGTACATGAAGCGAAAGAGAGATTATGAAAACAAGC-3' (SEQ ID NO: 111)
hisHp+MGt-F:
5'-CACTCCCTTTTCTTAATTGGTAGAGAGTTGGGTAGAGT-3' (SEQ ID NO: 112)
pDuracSC-GAPt-F: 5'-TAAGAAAAAGGGAGTGAATCGCATAAGGG-3' (SEQ ID NO: 113)
URA5gDNA-F: 5'-CATGACAAGTTTGGCAGAGATGCG-3' (SEQ ID NO: 114)

Using the plasmid pDUra-RhG as a template, the DNA fragment of about 6.3 kbp was amplified by PCR with KOD-Plus- (TOYOBO) using the primers pDuracSC-GAPt-F and pDurahG-hisp-R.

pDurahG-hisp-R: 5'-ATTGTTGAGAGAGTTGGGTAGAGTG-3' (SEQ ID NO: 115)

Using the plasmid containing cDNA of MaACS-10, the DNA fragment of about 2.1 kbp was amplified by PCR with KOD-Plus- (TOYOBO), using the primers below.

Primer ACS-10+hisp-F:
5'-CACTCTCTCAAACAATATGGAAACCTTGGTTAAGCAGAAGT-3' (SEQ ID NO: 116)
Primer ACS-10+MGt-R:
5'-CACTCCCTTTTCTTACTAGAAGACTTCTCCATCTCCTCAATATC-3' (SEQ ID NO: 117)

The resulting DNA fragment was ligated to the 6.3 kbp DNA fragment described above using an In-Fusion (registered trade name) Advantage PCR Cloning Kit (TAKARA BIO) to give plasmid pDurahRg-ACS-10.

Using the plasmid containing cDNA of MaACS-11 as a template, the 2.1 kbp DNA fragment was amplified by PCR with KOD-Plus- (TOYOBO) using the primers below.

Primer ACS-11+MGt-R:
5'-CACTCCCTTTTCTTATTACCTGGAGCCATAGATCTGCTTG-3' (SEQ ID
NO: 118)
Primer ACS-11+hisp-F:
5'-CACTCTCTCAACAATATGCCAAAGTGCTTTACCGTCAAC-3' (SEQ ID NO: 119)

The resulting DNA fragment was ligated to the 6.3 kbp DNA fragment described above using an In-Fusion (registered trade name) Advantage PCR Cloning Kit (TAKARA BIO) to give the plasmid pDUraRhG-ACS-11.

Evaluation of ACS Activity

The yeast EH13-15 was transformed by plasmids pYE22m, pYE-ACS-5, pYE-ACS-8, pYE-ACS-10, pYE-ACS-11 and pYE-ACS-12, respectively, and random two transformants obtained were cultivated as follows. One platinum loop of the mycelial cells were plated on 10 ml of SC-Trp medium and cultivated with shaking for preincubation at 30°C for a day. After 1% of the preincubation was added to 100 ml of the SD-Trp medium, main cultivation was performed by shake culturing at 28°C for a day.

The crude enzyme solution was prepared as follows. The mycelial cells were collected by centrifugation, washed with water and temporarily stored at -80°C. The mycelial cells were suspended in 5 ml of Buffer B (50 mM sodium sulfate buffer (pH 6.0), 10% glycerol and 0.5 mM PMSF). The mycelial cells were then disrupted with a French press (16 kPa, 3 times). Centrifugation was carried out at 1,500 xg at 4°C for 10 minutes and centrifuged. The supernatant obtained was used as the crude enzyme solution.

The ACS activity was determined by the following procedures based on the description of a reference literature (J.B.C., 272 (8), 1896-4903, 1997). The reaction solution contained 200 mM Tris-HCl (pH7.5), 2.5 mM ATP, 8 mM MgCl₂, 2 mM EDTA, 20 mM NaF, 0.1% TritonX-100, 50 µg/ml fatty acids, 50 µM CoA and 100 µl of the crude enzyme solution (suitably diluted in Buffer B), and was made 500 µl in total. The reaction was carried out at 28°C for 30 minutes. After completion of the reaction, 2.5 ml of stop solution (isopropanol : n-heptane : 1 M sulfuric acid (40: 20: 1)) was added and the mixture was thoroughly agitated. Furthermore, 2 ml of n-heptane was added thereto. After thoroughly mixing them, the mixture was centrifuged to recover the upper layer. Further 2 ml of n-heptane was added to the lower layer and treated in the same manner to recover the upper layer. The upper layers recovered were combined and evaporated to dryness using a centrifugal
concentrator. Then, 50 µl of 0.2 mg/ml tricosanoic acid (23:0) was added thereto as an internal standard. The fatty acids were converted into the methyl esters by the hydrochloric acid-methanol method, followed by fatty acid analysis using gas chromatography. The amount of the fatty acids, which were changed to acyl-CoA and thus distributed into the lower layer by the procedures above, was calculated from the amount of fatty acids detected. The results are shown in the table below. The ACS activity is expressed as the amount of fatty acids distributed into the lower layer by the procedures above, per weight of the protein in the crude enzyme solution. The control is the strain transformed by pYE22m and the others are the transformants in which the expression vectors of the respective genes were introduced.

### TABLE 7  ACS Activity on Palmitic Acid

<table>
<thead>
<tr>
<th></th>
<th>MaACS-5</th>
<th>MaACS-10</th>
<th>MaACS-11</th>
<th>MaACS-12</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
<td>#1</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>0.26</td>
<td>0.20</td>
<td>0.41</td>
<td>0.34</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>0.31</td>
<td>0.40</td>
<td>0.11</td>
<td>0.12</td>
<td></td>
</tr>
</tbody>
</table>

When palmitic acid was used as substrate, MaACS-5, MaACS-10, MaACS-11 and MaACS-12 showed the ACS activity of approximately 2 to 4 times the control.

### TABLE 8  ACS Activity on Oleic Acid

<table>
<thead>
<tr>
<th></th>
<th>MaACS-10</th>
<th>MaACS-11</th>
<th>MaACS-12</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>0.25</td>
<td>0.20</td>
<td>0.25</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>0.09</td>
<td>0.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

When oleic acid was used as substrate, MaACS-10, MaACS-11 and MaACS-12 showed the ACS activity of approximately twice the control.

### TABLE 9  ACS Activity on Linoleic Acid

<table>
<thead>
<tr>
<th></th>
<th>MaACS-5</th>
<th>MaACS-8</th>
<th>MaACS-10</th>
<th>MaACS-11</th>
<th>MaACS-12</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>0.47</td>
<td>0.42</td>
<td>0.42</td>
<td>0.38</td>
<td>0.54</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>0.34</td>
<td>0.20</td>
<td>0.59</td>
<td>0.78</td>
<td>0.14</td>
<td>0.14</td>
</tr>
</tbody>
</table>

When linoleic acid was used as substrate, MaACS-5, MaACS-8 and MaACS-12 showed the ACS activity of several times (approximately 3, 3 and 6 times, respectively) the control, whereas MaACS-10 and MaACS-11 showed the ACS activity of several tens times (approximately 40 and 20 times, respectively) the
control.

<table>
<thead>
<tr>
<th>TABLE 10</th>
<th>ACS Activity on γ-Linoleic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaACS-5</td>
<td>MaACS-8</td>
</tr>
<tr>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>0.16</td>
</tr>
</tbody>
</table>

When γ-linoleic acid was used as substrate, all of MaACS-5, MaACS-8, MaACS-10, MaACS-11 and MaACS-12 showed the ACS activity of approximately 2 to 10 times the control.

<table>
<thead>
<tr>
<th>TABLE 11</th>
<th>ACS Activity on Dihomo-γ-Linoleic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaACS-10</td>
<td>MaACS-11</td>
</tr>
<tr>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>4.98</td>
</tr>
</tbody>
</table>

When dihomo-γ-linoleic acid was used as substrate, all of MaACS-10, MaACS-11 and MaACS-12 showed the ACS activity of several tens times (approximately 60 times, 40 times and 30 times, respectively) the control.

<table>
<thead>
<tr>
<th>TABLE 12</th>
<th>ACS Activity on Arachidonic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>MaACS-10</td>
<td>MaACS-11</td>
</tr>
<tr>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/mg protein</td>
<td>8.12</td>
</tr>
</tbody>
</table>

When arachidonic acid was used as substrate, MaACS-10, MaACS-11 and MaACS-12 showed the ACS activity of several tens times (approximately 90 times, 30 times and 10 times, respectively) the control.

As above, MaACS-10, MaACS-11 and MaACS-12 in particular showed a higher activity on polyunsaturated fatty acids of 20 carbon atoms such as dihomo-γ-linoleic acid or arachidonic acid.

**Arachidonic Acid Uptake Activity of ACS-Expressed Yeast**

The yeast EH13-15 was transformed by plasmids pYE22m, pYE -ACS-10, pYE -ACS-11 and pYE -ACS-12, respectively, and random two transformants obtained were cultivated as follows. One platinum loop of the cells were plated on 10 ml of SC-Trp medium and cultivated with shaking for preincubation at 30°C for a
day. After 100 μl of the preincubation was added to 10 ml of the SC-Trp medium in which 50 μg/ml of arachidonic acid was supplemented, main cultivation was performed by shake culturing at 25°C for a day. The mycelial cells were collected, lyophilized and subjected to fatty acid analysis. The ratio of arachidonic acid taken up into the mycelial cells to the added arachidonic acid was determined. The results are shown in TABLE 14. The control is the strain transformed by pYE22m and the others are the transformants in which the expression vectors of the respective genes were introduced.

<table>
<thead>
<tr>
<th>TABLE 13</th>
<th>Dry Mycelial Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MaACS-10</td>
</tr>
<tr>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>%</td>
<td>36.63</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TABLE 14</th>
<th>Ratio of Arachidonic Acid Taken Up into Mycelia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>MaACS-10</td>
</tr>
<tr>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>mg/10ml</td>
<td>15.30</td>
</tr>
</tbody>
</table>

Acquisition of M. Alpina Transformants

Using as a host the uracil-auxotrophic strain Δura-3 derived from M. alpina strain 1S-4 as described in PCT International Publication Pamphlet WO 2005/019437 entitled "Method of Breeding Lipid-Producing Fungus"), transformation was performed by the particle delivery method using the plasmids pDUraRhG-ACS-10 and pDUraRg-ACS-11, respectively. For screening of the transformants, SC agar medium was used (0.5% Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco), 0.17% ammonium sulfate, 2% glucose, 0.002% adenine, 0.003% tyrosine, 0.0001% methionine, 0.0002% arginine, 0.0002% histidine, 0.0004% lysine, 0.0004% tryptophan, 0.0005% threonine, 0.0006% isoleucine, 0.0006% leucine, 0.0006% phenylalanine, and 2% agar).

Evaluation of M. Alpina Transformants

The transformants obtained were plated on 4 ml of GY medium and cultured with shaking at 28°C for 2 days. The mycelial cells were collected by filtration, and RNA was extracted with an RNeasy Plant Kit (QIAGEN). A SuperScript First Strand System for RT-PCR (Invitrogen) was used to synthesize cDNA. To confirm expression of the respective genes from the introduced constructs, RT-PCR was
performed with the following primer pairs.

ACS10-RT1: 5'-GTCCCGAATGGTTCCCT-3' (SEQ ID NO: 120)
ACS10-RT2: 5'-AGCGGTTTCTACTTGC-3' (SEQ ID NO: 121)
ACS11-RT1: 5'-AACTACAACCGCGTCG-3' (SEQ ID NO: 122)
ACS11-RT2: 5'-CGGCATAAACGCAGAT-3' (SEQ ID NO: 123)

In the transformants that overexpression was confirmed, one transformant each was plated on 10 ml of GY medium (2% glucose and 1% yeast extract) and cultured with shaking at 28°C at 300 rpm for 3 days. The whole volume of the culture was transferred to 500 ml of GY medium (2 L Sakaguchi flask) and shake cultured at 28°C and 120 rpm. Three, seven, ten and twelve days after this day, 5 ml each and 10 ml each were taken and filtered. After the mycelial cells were dried at 120°C, fatty acids were converted into the methyl esters by the hydrochloric acid-methanol method and analyzed by gas chromatography. The fatty acid production and the amount of arachidonic acid produced, per dried mycelial cells were monitored with the passage of time. The transformant host strain Aura-3 was used as control. The results are shown in FIG. 27 (MaACS-10) and FIG. 28 (MaACS-11).

As shown in FIGS. 27 and 28, when MaACS-10 and MaACS-11 were overexpressed in M. alpina, both the amount of fatty acids and the amount of arachidonic acid per mycelia were increased as compared to the control.

INDUSTRIAL APPLICABILITY

The polynucleotide of the present invention is expressed in an appropriate host cell to efficiently produce fatty acids, in particular, polyunsaturated fatty acids. The fatty acids produced in host cells according to the present invention can be used to produce fatty acid compositions, food products, cosmetics, pharmaceuticals, soaps, etc.

[Sequence Listing]
SEQUENCE LISTING IN ELECTRONIC FORM

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 30179-221D1 Seq 17-APR-14 v1.txt).

A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

The sequences in the sequence listing in electronic form are reproduced in the following table.

SEQUENCE TABLE

110> SUNTORY HOLDINGS LIMITED
120> POLYNUCLEOTIDE ENCODYING ACYL-COA SYNTHETASE HOMOLOG AND USE THEREOF
130> 30179-221D1
140> Division of CA 2,707,832
141> 2011-02-01
150> JP 2010-19967
151> 2010-02-01
160> 123
170> PatentIn version 3.4

210> 1
211> 1854
212> DNA
213> Mortierella alpina

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<td><strong>Ala</strong></td>
<td><strong>Ile</strong></td>
<td><strong>Pro</strong></td>
<td><strong>Arg</strong></td>
<td><strong>Asp</strong></td>
<td><strong>Val</strong></td>
<td><strong>Lys</strong></td>
<td><strong>Leu</strong></td>
<td><strong>Ala</strong></td>
<td><strong>Lys</strong></td>
<td>20</td>
<td>25</td>
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<td></td>
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**Secondary Structure**

- **α-helix**: From position 1 to position 10
- **β-sheet**: From position 11 to position 15

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**Tertiary Structure**

- **_α_**: From position 1 to position 10
- **_β_**: From position 11 to position 15

**Overall Structure**

- **Coiled-Coil**: From position 1 to position 15
Gly Leu Leu His Ser Met Asn Glu Ala Glu Val Gly Thr Ala Tyr Thr
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Thr Leu Lys Arg Ile Val Tyr Asp Gly Glu Ala Asn Ala Ala Asp Val
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210  215  220
Glu Leu Lys Glu Leu Gly Val Asp His Pro Val Glu Pro Thr Pro Pro
225  230  235  240
Thr Ala Glu Asp Cys Ser Cys Ile Met Tyr Thr Ser Gly Ser Thr Gly
245  250  255
Asn Pro Lys Gly Val Ile Leu Thr His Gly Asn Leu Ala Ala Ala Ile
260  265  270
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275  280  285
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290  295  300
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305  310  315  320
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325  330  335
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355  360  365
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Mortierella alpina

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2569

DNA

Mortierella alpina

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(557),..(767)

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24

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Artificial sequence

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CLAIMS:

1. A polynucleotide according to any one selected from the group consisting of (a) to (e) below:

(a) a polynucleotide comprising the nucleotide sequence set forth as SEQ ID NO: 51 or 56;

(b) a polynucleotide encoding a protein consisting of the amino acid sequence set forth as SEQ ID NO: 52 or 57;

(c) a polynucleotide encoding a protein consisting of an amino acid sequence wherein 1 to 100 amino acids are deleted, substituted, inserted and/or added in the amino acid sequence SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell;

(d) a polynucleotide encoding a protein having an amino acid sequence having at least 60% identity to the amino acid sequence set forth as SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(e) a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence set forth as SEQ ID NO: 51 or 56 under stringent conditions, and which encodes a protein having an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

2. The polynucleotide according to claim 1, which is either one defined in (f) or (g) below:

(f) a polynucleotide encoding a protein consisting of an amino acid sequence wherein 1 to 10 amino acids are deleted, substituted, inserted and/or added in the amino acid sequence set forth as SEQ ID NO: 52 or 57, and having an acyl-CoA synthetase activity or an
activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell; and,

(g) a polynucleotide encoding a protein having an amino acid sequence having at least 90% identity to the amino acid sequence set forth as SEQ ID NO: 52 or 57, and an acyl-CoA synthetase activity or an activity of increasing the amount or changing the composition, of the fatty acids produced in a host cell when expressed in the host cell.

3. The polynucleotide according to claim 1, comprising the nucleotide sequence set forth as SEQ ID NO: 51 or 56.

4. The polynucleotide according to claim 1, encoding a protein consisting of the amino acid sequence set forth as SEQ ID NO: 52 or 57.

5. The polynucleotide according to any one of claims 1 to 4, which is a DNA.

6. A protein encoded by the polynucleotide according to any one of claims 1 to 5.

7. A vector comprising the polynucleotide according to any one of claims 1 to 5.

8. A non-human transformant, into which the polynucleotide according to any one of claims 1 to 5, or the vector according to claim 7 is introduced.

9. A method for producing a lipid or fatty acid composition, which comprises collecting the lipid or fatty acid composition from the culture of the transformant according to claim 8.

10. The method according to claim 9, wherein the lipid is a triacylglycerol.

11. The method according to claim 9, wherein the fatty acid is a polyunsaturated fatty acid having at least 18 carbon atoms.

12. A food product, pharmaceutical, cosmetic or soap comprising the lipid or fatty acid composition obtained by the production method according to claim 9.
Figure 8C

2801

genome  A

cds    A
Figure 9B

1901 CTATGAGAAGCCTGCTGGCTCATCCGGCTGCAAGAAAACATTCTGAAAAAGCTCAGAAGGTTTCTCAGCTGAAATGTGTGGAGGTTTTGAAGATATG

1901 YEELCAHPAVKETIIKLKELKEFRENDLKLKFEIL

2001 AAGAAATCTCATTGAAAGGGAACATCCTGCTGAGTAATGATATGTTCTTGGACACCCACATTCAACGCTGAAAGGAAACACGACAGGACAGAAATACATG

2001 KNINHTAEQFSIENDLLTTPTFKKLKRHTAKEKYIA

2101 CCGAGATTGAGCTGATGTATAAACGATGACGTAAGAAGCACAACCTTAATATCTTGTTTTTTAATACCTGTAACCATCAAAA

2101 EIENMYNG1H*

2201 AAAAAA AAAAAA
Figure 11B

2081  TTTACGCGCCACCTTCGATGCTGACAAATTCCCTAAGTAAAGTTTACGCTGTTTCAATTTATCTCTGACACATCTGACCTAGG
         LTPFKLKHAACKKYNAEIDMRWYAIA

2101  TTTACGCGCTATATAAAAAAA
Figure 18A
Figure 19B

1901
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-KSOAKAAAGLSSKSETVQGGVIIDDDSDOWNNTNGMTS-

2001
CAGCGCAAGSCTCAAGGAGACGCGGGGCAAGGAACACACACAGGAGATGGGAGATGGGAGATTTTGATGAT
-SSKVKKRREVRKAKNKKDIEEMWKKFK-
Figure 21B

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Figure 24A
Figure 25B
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Figure 28

A

Lipid production (mg/g dry mycelia)

- MaACS-11
- Control

Cultivation time (days)

B

AA production (mg/g dry mycelia)

- MaACS-11
- Control

Cultivation time (days)