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(71) Applicant *(for all designated States except US)*: **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, Delaware 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **XUE, Zhixiong** [CN/US]; 111 Harvey Lane, Chadds Ford, Pennsylvania 19317 (US). **YADAV, Narendra, S.** [US/US]; 3704 Knole Lane, Chadds Ford, Pennsylvania 19317 (US). **ZHU, Quinn, Qun** [US/US]; 544 Revere Road, West Chester, Pennsylvania 19382 (US).

(74) Agent: **FELTHAM, S., Neil**; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

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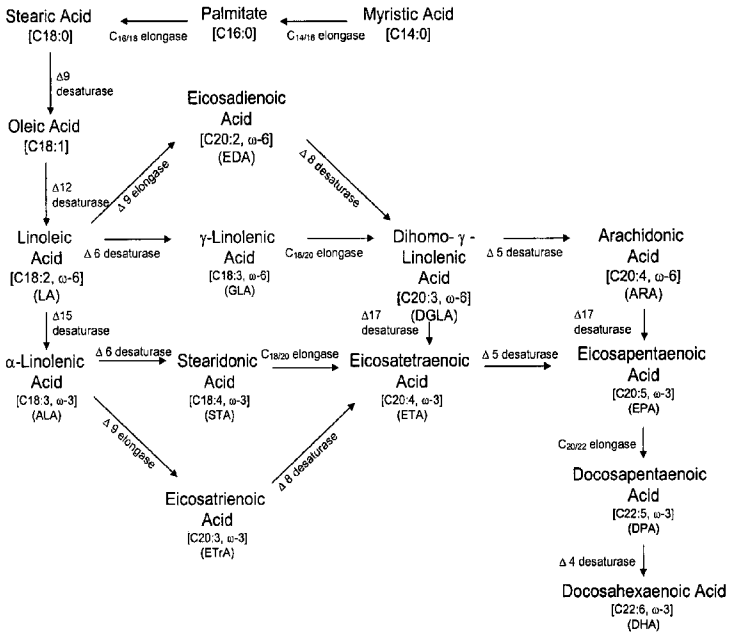
(54) **Title:** Δ17 DESATURASE AND ITS USE IN MAKING POLYUNSATURATED FATTY ACIDS



(57) **Abstract:** The present invention relates to Δ17 desaturases, which have the ability to convert ω-6 fatty acids into their ω-3 counterparts (i.e., conversion of arachidonic acid [20:4, ARA] to eicosapentaenoic acid [20:5, EPA]). Isolated nucleic acid fragments and recombinant constructs comprising such fragments encoding Δ17 desaturases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) using these Δ17 desaturases in oleaginous yeast are disclosed.

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TITLE

Δ17 DESATURASE AND ITS USE IN MAKING POLYUNSATURATED FATTY ACIDS

This application claims the benefit of United States Provisional Patent Application 60/855177, filed October 30, 2006.

FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this invention pertains to the identification of a nucleic acid fragment encoding a Δ17 fatty acid desaturase enzyme and the use of this desaturase in making long-chain polyunsaturated fatty acids (PUFAs).

BACKGROUND OF THE INVENTION

The importance of PUFAs is undisputed. For example, certain PUFAs are important biological components of healthy cells and are recognized as: "essential" fatty acids that cannot be synthesized *de novo* in mammals and instead must be obtained either in the diet or derived by further desaturation and elongation of linoleic acid (LA;18:2 ω-6) or α-linolenic acid (ALA; 18:3 ω-3); constituents of plasma membranes of cells, where they may be found in such forms as phospholipids or triacylglycerols; necessary for proper development (particularly in the developing infant brain) and for tissue formation and repair; and, precursors to several biologically active eicosanoids of importance in mammals (e.g., prostacyclins, eicosanoids, leukotrienes, prostaglandins). Additionally, a high intake of long-chain ω-3 PUFAs produces cardiovascular protective effects (Dyerberg, J. et al., *Amer. J. Clin. Nutr.*, 28:958-966 (1975); Dyerberg, J. et al., *Lancet,* 2(8081):117-119 (July 15, 1978); Shimokawa, H., *World Rev. Nutr. Diet*, 88:100-108 (2001); von Schacky, C. and Dyerberg, J., *World Rev. Nutr. Diet*, 88:90-99 (2001)). Numerous other studies document wide-ranging health benefits conferred by administration of ω-3 and/or ω-6 PUFAs against a variety of symptoms and diseases (e.g., asthma, psoriasis, eczema, diabetes, cancer).

A variety of different hosts including plants, algae, fungi and yeast are being investigated as means for commercial PUFA production.

Genetic engineering has demonstrated that the natural abilities of some hosts (even those natively limited to LA and ALA fatty acid production) can be substantially enhanced to produce high levels of e.g., γ-linolenic acid (GLA; 18:3 ω-6), dihomo-γ-linolenic acid (DGLA; 20:3 ω-6), arachidonic acid (ARA; 20:4 ω-6), eicosapentaenoic acid (EPA; 20:5 ω-3), docosapentaenoic acid (DPA; 22:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3).

Whether ω-3/ω-6 PUFA production is the result of natural abilities or recombinant technology, both strategies may require conversion of ω-6 PUFAs into their ω-3 counterparts. Specifically, a Δ15 desaturase is responsible for the conversion of LA to ALA, while a Δ17 desaturase is responsible for the conversion of ARA to EPA (although some Δ17 desaturases can also use DGLA) as a substrate to produce eicosatetraenoic acid (ETA; 20:4 ω-3)). Both of these enzymes have a role in the Δ6 desaturase/Δ6 elongase pathway (which is predominantly found in algae, mosses, fungi, nematodes and humans and which is characterized by the production of GLA and/or stearidonic acid (STA; 18:4 ω-3)) and the Δ9 elongase/Δ8 desaturase pathway (which operates in some organisms, such as euglenoid species and which is characterized by the production of eicosadienoic acid (EDA; 20:2 ω-6) and/or eicosatrienoic acid (ETrA; 20:3 ω-3)) (Figure 1).

Because of the role Δ17 desaturase enzymes play in enabling the synthesis of ω-3 fatty acids, there has been considerable effort to identify and characterize these enzymes from various sources. However, only a few Δ17 desaturases are presently known and these have been isolated from only two different taxonomic genera. Specifically, Patent Publication No. US 2003/0190733 describes a Δ17 desaturase from *Saprolegnia diclina* (see also GenBank Accession No. AY373823). PCT Publication No. WO 2005/083053 describes a *Phytophthora infestans* "ω3 desaturase" (see also GenBank Accession No. CAJ30870), while PCT Publication No. WO 2006/100241 describes a *Phytophthora sojae* "ω3 desaturase", both of which appear to function as Δ17 desaturases. Also, commonly owned,

co-pending application having U.S. Patent Application No. 11/787772 (filed April 18, 2007) discloses nucleic acid and amino acid sequences for Δ17 desaturases from *Phytophthora sojae* and *Phytophthora ramorum*. Thus, there is need for the identification and isolation of additional genes encoding Δ17 desaturases that will be suitable for heterologous expression in a variety of host organisms for use in the production of ω-3 fatty acids.

Applicants have solved the stated problem by isolating the gene encoding Δ17 desaturase from the oomycete, *Pythium aphanidermatum*.

SUMMARY OF THE INVENTION

The present invention relates to new genetic constructs encoding polypeptides having Δ17 desaturase activity, and their use in plants, bacteria, algae, fungi and yeast for the production of PUFAs and particularly ω-3 fatty acids.

Accordingly, the invention provides an isolated nucleic acid molecule selected from the group consisting of:

a.) an isolated nucleotide molecule encoding a Δ17 desaturase enzyme, selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3;

b.) an isolated nucleotide molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS; or,

an isolated nucleotide molecule that is completely complementary to (a) or (b).

In another embodiment the invention provides isolated nucleic acid molecules encoding Δ17 desaturase enzyme, selected from the group consisting of SEQ ID NO:1 and 4 or isolated nucleic acid molecules which encoding Δ17 desaturase enzyme as set forth in SEQ ID NO:2, wherein at least 175 codons are codon-optimized for expression in *Yarrowia*. Additionally the invention provides an isolated nucleic acid molecule comprising a first nucleotide sequence encoding a Δ17 desaturase

enzyme of at least 359 amino acids that has at least 75.3% identity based on Clustal W algorithms when compared to a polypeptide having the sequence as set forth in SEQ ID NO:2;

or a second nucleotide sequence comprising the complement of the first nucleotide sequence.

In other embodiments the invention provides chimeric genes comprising the isolated nucleic acid molecules of the invention and transformed hosts comprising the same.

In another embodiment the invention provides a method for the production of eicosapentaenoic acid comprising:

a.) providing a host cell comprising:

(i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase

polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

(ii) a source of arachidonic acid;

b.) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the a bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the arachidonic acid is converted to eicosapentaenoic acid; and,

c.) optionally recovering the eicosapentaenoic acid of step (b).

Similarly the invention provides A method for the production of eicosatetraenoic acid comprising:

a.) providing a host cell comprising:

(i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

(ii) a source of dihomo-γ–linolenic acid;

b.) growing the host cell of step (a) under conditions wherein the

nucleic acid molecule encoding the a bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the dihomo-γ–linolenic acid is converted to eicosatetraenoic acid; and,

c.) optionally recovering the eicosatetraenoic acid of step (c).

Alternatively the invention provides A method for the production of polyunsaturated fatty acids comprising:

a) providing a host cell comprising:

i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

ii) a source of fatty acid selected from the group consisting of: linoleic acid and eicosadienoic acid;

b) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the linoleic acid is converted to α-linolenic acid and the eicosadienoic acid is converted to eicosatrienoic acid; and,

c) optionally recovering the fatty acid of step (b).

In another embodiment the invention provides an isolated nucleic acid fragment comprising a nucleic acid sequence encoding a Δ17 desaturase polypeptide comprising at least one amino acid sequence motifs selected from the group consisting of:

a) F T X G H D X G H (SEQ ID NO:96);

b) H R H H H K N T G (SEQ ID NO:97); and,

c) I G T H Q X H H L F P (SEQ ID NO:98);

wherein X can be any amino acid, and

wherein the Δ17 desaturase polypeptide does not have the amino acid sequence as set forth in SEQ ID NOs:43 and 95.

Alternatively the invention provides a Δ17 desaturase polypeptide comprising at least one amino acid motif selected from the group consisting of SEQ ID NO:96-98.

In other embodiments the invention provides methods for the identification and isolation of a Δ17 desaturase polypeptide comprising:

a) probing a genomic library with:

i) an isolated nucleic acid fragment encoding an amino acid sequence selected from the group consisting of SEQ ID NO:96-98; or,

ii) an isolated nucleic acid fragment that is complementary to (i);

b) identifying a DNA clone that hybridizes with the nucleic acid fragment of step (a); and,

c) sequencing the genomic fragment that comprises the clone identified in step (b);

wherein the sequenced genomic fragment encodes a Δ17 desaturase polypeptide, or alternatively,

a) synthesizing at least one oligonucleotide primer corresponding to a portion of an isolated nucleic acid sequence encoding an amino acid motift selected from the group consisting of SEQ ID NOs 96-98; and,

b) amplifying an insert present in a cloning vector using the oligonucleotide primer of step (a);

wherein the amplified insert encodes a portion of an amino acid sequence encoding a Δ17 desaturase enzyme.

BIOLOGICAL DEPOSITS

The following biological material has been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, and bears the following designation, accession number and date of deposit.

Biological Material	Accession No.	Date of Deposit
Yarrowia lipolytica Y2047	ATCC PTA-7186	October 26, 2005

The biological material listed above was deposited under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The listed deposit will be maintained in the indicated international depository for at least 30 years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

Figure 1 illustrates the ω-3/ω-6 fatty acid biosynthetic pathway.

Figure 2 shows a pairwise alignment of the amino acid sequences of the *Phytophthora sojae* Δ17 desaturase (SEQ ID NO:45) and the *Phytophthora ramorum* Δ17 desaturase (SEQ ID NO:47), created using default parameters of Vector NTI®'s AlignX program (Invitrogen Corporation, Carlsbad, CA).

Figure 3 provides plasmid maps for the following: (A) pKUNFmkF2; (B) pDMW287F; (C) pDMW214; and, (D) pFmD8S.

Figure 4A diagrams the development of *Yarrowia lipolytica* strain Y2047, producing 11% ARA in the total lipid fraction. Figure 4B provides a plasmid map for pKUNF12T6E, while Figure 4C provides a plasmid map for pDMW271.

Figures 5A and 5B show a comparison of the DNA sequence of the *Phytophthora aphanidermatum* Δ17 desaturase gene (designated as "PaD17"; SEQ ID NO:1) and the synthetic gene (designated as "PaD17S"; SEQ ID NO:4) codon-optimized for expression in *Yarrowia lipolytica*.

Figure 6A diagrams the development of *Yarrowia lipolytica* strain Y4070, producing 12% ARA in the total lipid fraction. Figure 6B provides a plasmid map for pZKLeuN-29E3, while Figure 6C provides a plasmid map for pY116.

Figure 7 provides plasmid maps for the following: (A) pKO2UF8289; and, (B) pZKSL-555R.

Figure 8 provides plasmid maps for the following: (A) pFBAIN-MOD-1; and, (B) pY6.GPD.Leu2.

Figure 9 shows a comparison of the DNA sequence of the *Phytophthora sojae* Δ17 desaturase gene (designated as "PsD17"; SEQ ID NO:44) and the synthetic gene (designated as "PsD17S"; SEQ ID NO:81) codon-optimized for expression in *Y. lipolytica*.

Figure 10 shows a comparison of the DNA sequence of the *Phytophthora ramorum* Δ17 desaturase gene (designated as "PrD17"; SEQ ID NO:46) and the synthetic gene (designated as "PrD17S"; SEQ ID NO:84) codon-optimized for expression in *Y. lipolytica*.

Figure 11 provides plasmid maps for the following: (A) pY130; (B) pY138; (C) pY139; and, (D) pY140.

Figure 12 provides plasmid maps for the following: (A) pY137; and, (B) pY117.

Figure 13 is a graph showing the ω-6 fatty acid substrate specificity of the following ω-3 desaturases: *Fusarium moniliforme* Δ15 desaturase (FmD15; SEQ ID NOs:86 and 87); a synthetic Δ17 desaturase derived from *Phytopthora ramorum*, codon-optimized for expression in *Yarrowia lipolytica* (PrD17S; SEQ ID NOs:84 and 47); a synthetic Δ17 desaturase derived from *Phytopthora sojae*, codon-optimized for expression in *Yarrowia lipolytica* (PsD17S; SEQ ID NOs:81 and 82); and the synthetic Δ17 desaturase derived from *Pythium aphanidermatum*, codon-optimized for expression in *Yarrowia lipolytica* (PaD17S; SEQ ID NOs:4 and 2).

Figure 14 shows a Clustal V alignment (with default parameters) of the of the following ω-3 desaturases: *Phytophthora infestans* Δ17 desaturase (PiD17; SEQ ID NO:43); *Phytopthora ramorum* Δ17 desaturase (PrD17; SEQ ID NO:47); synthetic Δ17 desaturase derived from *Phytopthora sojae*, codon-optimized for expression in *Yarrowia lipolytica* (PsD17S; SEQ ID NO:82); *Saprolegnia diclina* Δ17 desaturase, (SdD17; SEQ ID NO:95); and the *Pythium aphanidermatum* Δ17 desaturase of the instant invention (PaD17S; SEQ ID NO:2). Sequence regions shown in boxes correspond to delta-17 motifs #1, #2 and #3,

respectively. The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions, which form a part of this application.

The following sequences comply with 37 C.F.R. §1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NOs:1-8, 42-53, 56-95 and 102 are ORFs encoding genes or proteins or plasmids, as identified in Table 1.

Table 1

Summary Of Gene And Protein SEQ ID Numbers

Description and Abbreviation	Nucleic acid SEQ ID NO.	Protein SEQ ID NO.
Pythium aphanidermatum Δ17 desaturase ("PaD17")	1 (1080 bp)	2 (359 AA)
Pythium aphanidermatum Δ17 desaturase ("PaD17*")	--	3 (359 AA)
Synthetic Δ17 desaturase derived from *Pythium aphanidermatum*, codon-optimized for expression in *Yarrowia lipolytica* ("PaD17S")	4 (1080 bp)	2 (359 AA)
Pythium aphanidermatum PaD17—internal cDNA fragment	5 (614 bp)	--
Pythium aphanidermatum PaD17 —5' genomic fragment	6 (739 bp)	--
Pythium aphanidermatum PaD17 —3' cDNA fragment	7 (512 bp)	--
Pythium aphanidermatum PaD17 contig—coding sequence corresponds to nucleotides 388-1467	8 (1533 bp)	--
Phytophthora infestans Δ17 desaturase ("PiD17") (GenBank Accession No. CAJ30870)	42 (1085 bp)	43 (361 AA)
Phytophthora sojae Δ17 desaturase	44	45

("PsD17") (U.S. Patent Application No. 11/787772)	(1092 bp)	(363 AA)
Phytophthora ramorum Δ17 desaturase ("PrD17") (U.S. Patent Application No. 11/787772)	46 (1086 bp)	47 (361 AA)
Plasmid pKUNFmkF2	48 (7145 bp)	--
Plasmid pDMW287F	49 (5473 bp)	--
Plasmid pDMW214	50 (9513 bp)	--
Plasmid pFmD8S	51 (8910 bp)	--
Synthetic Δ8 desaturase, derived from *Euglena gracilis*, codon-optimized for expression in *Yarrowia lipolytica* ("EgD8S") (equivalent to SEQ ID NOs:112 and 113 in PCT Publication No. WO 2006/012326)	52 (1272 bp)	53 (422 AA)
Plasmid pKUNF12T6E	56 (12,649 bp)	--
Synthetic $C_{18/20}$ elongase derived from *Thraustochytrium aureum* (U.S. Patent 6,677,145), codon-optimized for expression in *Yarrowia lipolytica* ("EL2S")	57 (819 bp)	58 (272 AA)
Plasmid pDMW271	59 (13,034 bp)	--
Synthetic Δ5 desaturase derived from *Homo sapiens* (GenBank Accession No. NP_037534), codon-optimized for expression in *Yarrowia lipolytica*	60 (1335 bp)	61 (444 AA)
Plasmid pPaD17S	62 (3800 bp)	--
Plasmid pZKLeuN-29E3	63 (14,655 bp)	--
Synthetic Δ9 elongase derived from *Euglena gracilis* (U.S. Patent Applications No. 11/601563 and No. 11/601564), codon-optimized for expression in *Yarrowia lipolytica* ("EgD9eS")	64 (777 bp)	65 (258 AA)
Escherichia coli LoxP recombination site, recognized by a Cre recombinase enzyme	66 (34 bp)	--
Synthetic $C_{16/18}$ elongase derived from *Mortierella alpina* ELO3 (U.S. Patent Application No. 11/253882), codon-optimized for expression in *Yarrowia lipolytica* ("ME3S")	67 (828 bp)	68 (275 AA)
Plasmid pY116	69 (8739 bp)	--

Plasmid pKO2UF8289	70 (15,304 bp)	--
Synthetic mutant Δ8 desaturase ("EgD8S-23"; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326)	71 (1272 bp)	72 (422 AA)
Euglena gracilis Δ9 elongase (U.S. Patent Applications No. 11/601563 and No. 11/601564) ("EgD9e")	73 (777 bp)	65 (258 AA)
Plasmid pZKSL-555R	74 (13,707 bp)	--
Synthetic Δ5 desaturase derived from *Euglena gracilis* (U.S. Patent Application No. 11/748629), codon-optimized for expression in *Yarrowia lipolytica* ("EgD5S")	75 (1350 bp)	76 (449 AA)
Synthetic Δ5 desaturase derived from *Peridinium sp.* CCMP626 (U.S. Patent Application No. 11/748637), codon-optimized for expression in *Yarrowia lipolytica* ("RD5S")	77 (1392 bp)	78 (463 AA)
Euglena gracilis Δ5 desaturase (U.S. Patent Application No. 11/748629) ("EgD5")	79 (1350 bp)	76 (449 AA)
Plasmid pFBAIN-MOD-1	80 (7222 bp)	--
Synthetic Δ17 desaturase derived from *Phytophthora sojae*, codon-optimized for expression in *Yarrowia lipolytica* (U.S. Patent Application No. 11/787772) ("PsD17S")	81 (1086 bp)	82 (361 AA)
Plasmid pPsD17S	83 (3806 bp)	--
Synthetic Δ17 desaturase derived from *Phytophthora ramorum*, codon-optimized for expression in *Yarrowia lipolytica* (U.S. Patent Application No. 11/787772) ("PrD17S")	84 (1086 bp)	47 (361 AA)
Plasmid pPrD17S	85 (3806 bp)	--
Fusarium moniliforme (*Gibberella fujikuroi*) Δ15 desaturase (PCT Publication No. WO 2005/047480; GenBank Accession No. DQ272516.1)	86 (1209 bp)	87 (402 AA)
Plasmid pY6.GPD.Leu2	88 (7668 bp)	--
Plasmid pY130	89 (9048 bp)	--

Plasmid pY138	90 (8925 bp)	--
Plasmid pY139	91 (8925 bp)	--
Plasmid pY140	92 (8919 bp)	--
Plasmid pY137	93 (6267 bp)	--
Plasmid pY117	94 (9570 bp)	--
Saprolegnia diclina Δ17 desaturase (GenBank Accession No. AAR20444)	--	95 (358 AA)
Plasmid pFBAINPaD17S	102 (8067 bp)	--

SEQ ID NOs:9-11 correspond to SMART™ IV oligonucleotide primer, CDSIII/3' PCR primer and 5'-PCR primer, respectively, used for *Pythium aphanidermatum* cDNA synthesis.

SEQ ID NO:12 corresponds to degenerate oligonucleotide primer PD17-F1, which encodes the peptide set forth in SEQ ID NO:13.

SEQ ID NOs:14 and 15 correspond to degenerate oligonucleotide primers PD17-F2 and PD17-F3, respectively, both of which encode the peptide set forth in SEQ ID NO:16.

SEQ ID NOs:17 and 18 correspond to degenerate oligonucleotide primers PD17-F4 and PD17-F5, respectively, both of which encode the peptide set forth in SEQ ID NO:19.

SEQ ID NOs:20 and 21 correspond to degenerate oligonucleotide primers PD17-F6 and PD17-F7, respectively, both of which encode the peptide set forth in SEQ ID NO:22.

SEQ ID NOs:23 and 24 correspond to degenerate oligonucleotide primers PD17-R1 and PD17-R2, respectively, both of which encode the peptide set forth in SEQ ID NO:25.

SEQ ID NOs:26 and 27 correspond to degenerate oligonucleotide primers PD17-R3 and PD17-R4, respectively, both of which encode the peptide set forth in SEQ ID NO:28.

SEQ ID NOs:29 and 30 correspond to degenerate oligonucleotide primers PD17-R5 and PD17-R6, respectively, both of which encode the peptide set forth in SEQ ID NO:31.

SEQ ID NO:32 corresponds to degenerate oligonucleotide primer PD17-R7, which encodes the peptide set forth in SEQ ID NO:33.

SEQ ID NOs:34 and 35 correspond to the Universal GenomeWalker™ adaptor.

SEQ ID NOs:36, 37, 38 and 39 correspond to primers PUD17-5-1, Universal GenomeWalker™ primer AP1, PUD17-5-3 and Universal GenomeWalker™ primer AP2, respectively, used for PCR amplification of the 5'-end of genomic DNA encoding the *Pythium aphanidermatum* Δ17 desaturase.

SEQ ID NOs:40 and 41 correspond to primers PUD17-3-1 and PUD17-3-2, respectively, used for PCR amplification of the 3'-end of cDNA encoding the *Pythium aphanidermatum* Δ17 desaturase.

SEQ ID NOs:54 and 55 correspond to primers PUD17-F and PUD17-R, respectively, used for amplification of the full length cDNA encoding the *Pythium aphanidermatum* Δ17 desaturase.

SEQ ID NOs:96-98 correspond to Δ17 desaturase motif #1, Δ17 desaturase motif #2 and Δ17 desaturase motif #3, respectively.

SEQ ID NOs:99-101 correspond to His-rich motifs that are featured in membrane-bound fatty acid desaturases belonging to a super-family of membrane di-iron proteins.

DETAILED DESCRIPTION OF THE INVENTION

All patents, patent applications, and publications cited herein are incorporated by reference in their entirety. This specifically includes the following Applicants' Assignee's co-pending applications: U.S. Patent 7,125,672, U.S. Patent 7,189,559, U.S. Patent 7,192,762, U.S. Patent 7,198,937, U.S. Patent 7,202,356, U.S. Patent Applications No. 10/840579 and No. 10/840325 (filed May 6, 2004), U.S. Patent Application No. 10/869630 (filed June 16, 2004), U.S. Patent Application No. 10/882760 (filed July 1, 2004), U.S. Patent Applications No. 10/985254 and No. 10/985691 (filed November 10, 2004), U.S. Patent Application No.

11/024544 (filed December 29, 2004), U.S. Patent Application No. 11/166993 (filed June 24, 2005), U.S. Patent Application No. 11/183664 (filed July 18, 2005), U.S. Patent Application No. 11/185301 (filed July 20, 2005), U.S. Patent Application No. 11/190750 (filed July 27, 2005), U.S. Patent Application No. 11/198975 (filed August 8, 2005), U.S. Patent Application No. 11/225354 (filed September 13, 2005), U.S. Patent Application No. 11/253882 (filed October 19, 2005), U.S. Patent Applications No. 11/264784 and No. 11/264737 (filed November 1, 2005), U.S. Patent Application No. 11/265761 (filed November 2, 2005), U.S. Patent Application No. 60/853563 (filed October 23, 2006), U.S. Patent Application No. 60/855177 (filed October 30, 2006), U.S. Patent Applications No. 11/601563 and No. 11/601564 (filed November 16, 2006), U.S. Patent Application No. 11/635258 (filed December 7, 2006), U.S. Patent Application No. 11/613420 (filed December 20, 2006), U.S. Patent Application No. 60/909790 (filed April 3, 2007), U.S. Patent Application No. 60/910831 (filed April 10, 2007), U.S. Patent Application No. 60/911925 (filed April 16, 2007), U.S. Patent Application No. 11/787772 (filed April 18, 2007), U.S. Patent Application No. 11/737772 (filed April 20, 2007), U.S. Patent Application No. 11/740298 (filed April 26, 2007), U.S. Patent Application No. 60/915733 (filed May 3, 2007) and U.S. Patent Applications No. 11/748629 and No. 11/748637 (filed May 15, 2007).

The invention provides a novel Oomycota Δ17 desaturase enzyme and gene encoding the same that may be used for the manipulation of biochemical pathways for the production of healthful PUFAs.

PUFAs, or derivatives thereof, made by the methodology disclosed herein can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous feeding or for preventing or treating malnutrition. Alternatively, the purified PUFAs (or derivatives thereof) may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount for dietary supplementation. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food

products and may find use as anti-inflammatory or cholesterol lowering agents. Optionally, the compositions may be used for pharmaceutical use (human or veterinary).

Supplementation of humans or animals with PUFAs produced by recombinant means can result in increased levels of the added PUFAs, as well as their metabolic progeny. For example, treatment with EPA can result not only in increased levels of EPA, but also downstream products of EPA such as eicosanoids (i.e., prostaglandins, leukotrienes, thromboxanes). Complex regulatory mechanisms can make it desirable to combine various PUFAs, or add different conjugates of PUFAs, in order to prevent, control or overcome such mechanisms to achieve the desired levels of specific PUFAs in an individual.

Definitions

In this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

"American Type Culture Collection" is abbreviated ATCC.

"Polyunsaturated fatty acid(s)" is abbreviated PUFA(s).

"Triacylglycerols" are abbreviated TAGs.

As used herein the term "invention" or "present invention" is intended to refer to all aspects and embodiments of the invention as described in the claims and specification herein and should not be read so as to be limited to any particular embodiment or aspect.

The term "fatty acids" refers to long-chain aliphatic acids (alkanoic acids) of varying chain lengths, from about C_{12} to C_{22} (although both longer and shorter chain-length acids are known). The predominant chain lengths are between C_{16} and C_{22}. The structure of a fatty acid is represented by a simple notation system of "X:Y", where X is the total number of carbon (C) atoms in the particular fatty acid and Y is the number of double bonds. Additional details concerning the differentiation between "saturated fatty acids" versus "unsaturated fatty acids", "monounsaturated fatty acids" versus "polyunsaturated fatty acids" (or

"PUFAs"), and "omega-6 fatty acids" (ω-6 or *n*-6) versus "omega-3 fatty acids" (ω-3 or *n*-3) are provided in PCT Publication No. WO 2004/101757.

Nomenclature used to describe PUFAs in the present disclosure is shown below in Table 2. In the column titled "Shorthand Notation", the omega-reference system is used to indicate the number of carbons, the number of double bonds and the position of the double bond closest to the omega carbon, counting from the omega carbon (which is numbered 1 for this purpose). The remainder of the Table summarizes the common names of ω-3 and ω-6 fatty acids and their precursors, the abbreviations that will be used throughout the specification and each compounds' chemical name.

Table 2

Nomenclature of Polyunsaturated Fatty Acids And Precursors

Common Name	Abbreviation	Chemical Name	Shorthand Notation
Myristic	--	tetradecanoic	14:0
Palmitic	Palmitate	hexadecanoic	16:0
Palmitoleic	--	9-hexadecenoic	16:1
Stearic	--	octadecanoic	18:0
Oleic	--	*cis*-9-octadecenoic	18:1
Linoleic	LA	*cis*-9, 12-octadecadienoic	18:2 ω-6
γ–Linolenic	GLA	*cis*-6, 9, 12-octadecatrienoic	18:3 ω-6
Eicosadienoic	EDA	*cis*-11, 14-eicosadienoic	20:2 ω-6
Dihomo-γ–Linolenic	DGLA	*cis*-8, 11, 14-eicosatrienoic	20:3 ω-6
Arachidonic	ARA	*cis*-5, 8, 11, 14-eicosatetraenoic	20:4 ω-6
α–Linolenic	ALA	*cis*-9, 12, 15-octadecatrienoic	18:3 ω-3
Stearidonic	STA	*cis*-6, 9, 12, 15-octadecatetraenoic	18:4 ω-3
Eicosatrienoic	ETrA	*cis*-11, 14, 17-eicosatrienoic	20:3 ω-3
Eicosa-tetraenoic	ETA	*cis*-8, 11, 14, 17-eicosatetraenoic	20:4 ω-3
Eicosa-pentaenoic	EPA	*cis*-5, 8, 11, 14, 17-eicosapentaenoic	20:5 ω-3
Docosa-pentaenoic	DPA	*cis*-7, 10, 13, 16, 19-docosapentaenoic	22:5 ω-3
Docosa-hexaenoic	DHA	*cis*-4, 7, 10, 13, 16, 19-docosahexaenoic	22:6 ω-3

The terms "triacylglycerol", "oil" and "TAGs" refer to neutral lipids composed of three fatty acyl residues esterified to a glycerol molecule (and such terms will be used interchangeably throughout the present disclosure herein). Such oils can contain long chain PUFAs, as well as shorter saturated and unsaturated fatty acids and longer chain saturated fatty acids. Thus, "oil biosynthesis" generically refers to the synthesis of TAGs in the cell. "Microbial oils" or "single cell oils" are those oils naturally produced by microorganisms during their lifespan.

"Percent (%) PUFAs in the total lipid and oil fractions" refers to the percent of PUFAs relative to the total fatty acids in those fractions. The term "total lipid fraction" or "lipid fraction" both refer to the sum of all lipids (i.e., neutral and polar) within an oleaginous organism, thus including those lipids that are located in the phosphatidylcholine (PC) fraction, phosphatidyletanolamine (PE) fraction and triacylglycerol (TAG or oil) fraction. However, the terms "lipid" and "oil" will be used interchangeably throughout the specification.

A metabolic pathway, or biosynthetic pathway, in a biochemical sense, can be regarded as a series of chemical reactions occurring within a cell, catalyzed by enzymes, to achieve either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many of these pathways are elaborate, and involve a step by step modification of the initial substance to shape it into a product having the exact chemical structure desired.

The term "PUFA biosynthetic pathway" refers to a metabolic process that converts oleic acid to LA, EDA, GLA, DGLA, ARA, ALA, STA, ETrA, ETA, EPA, DPA and DHA. This process is well described in the literature (e.g., see PCT Publication No. WO 2006/052870). Briefly, this process involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds, via a series of special desaturation and elongation enzymes (i.e., "PUFA biosynthetic pathway enzymes") present in the endoplasmic

reticulim membrane. More specifically, "PUFA biosynthetic pathway enzymes" refer to any of the following enzymes (and genes which encode said enzymes) associated with the biosynthesis of a PUFA, including: a Δ4 desaturase, a Δ5 desaturase, a Δ6 desaturase, a Δ12 desaturase, a Δ15 desaturase, a Δ17 desaturase, a Δ9 desaturase, a Δ8 desaturase, a Δ9 elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase and/or a $C_{20/22}$ elongase.

The term "ω-3/ω-6 fatty acid biosynthetic pathway" refers to a set of genes which, when expressed under the appropriate conditions encode enzymes that catalyze the production of either or both ω-3 and ω-6 fatty acids. Typically the genes involved in the ω-3/ω-6 fatty acid biosynthetic pathway encode PUFA biosynthetic pathway enzymes. A representative pathway is illustrated in Figure 1, providing for the conversion of myristic acid through various intermediates to DHA, which demonstrates how both ω-3 and ω-6 fatty acids may be produced from a common source. The pathway is naturally divided into two portions where one portion will generate ω-3 fatty acids and the other portion, only ω-6 fatty acids. That portion that only generates ω-3 fatty acids will be referred to herein as the ω-3 fatty acid biosynthetic pathway, whereas that portion that generates only ω-6 fatty acids will be referred to herein as the ω-6 fatty acid biosynthetic pathway.

The term "functional" as used herein in context with the ω-3/ω-6 fatty acid biosynthetic pathway means that some (or all) of the genes in the pathway express active enzymes, resulting in *in vivo* catalysis or substrate conversion. It should be understood that "ω-3/ω-6 fatty acid biosynthetic pathway" or "functional ω-3/ω-6 fatty acid biosynthetic pathway" does not imply that all the genes in the above paragraph are required, as a number of fatty acid products will only require the expression of a subset of the genes of this pathway.

The term "desaturase" refers to a polypeptide that can desaturate, i.e., introduce a double bond, in one or more fatty acids to produce a fatty acid or precursor of interest. Despite use of the omega-reference system

throughout the specification to refer to specific fatty acids, it is more convenient to indicate the activity of a desaturase by counting from the carboxyl end of the substrate using the delta-system. Of interest herein are: 1.) Δ8 desaturases that will catalyze the conversion of EDA to DGLA and/or ETrA to ETA; 2.) Δ5 desaturases that catalyze the conversion of DGLA to ARA and/or ETA to EPA; 3.) Δ6 desaturases that catalyze the conversion of LA to GLA and/or ALA to STA; 4.) Δ4 desaturases that catalyze the conversion of DPA to DHA; 5.) Δ12 desaturases that catalyze the conversion of oleic acid to LA; 6.) Δ15 desaturases that catalyze the conversion of LA to ALA and/or GLA to STA; and, 7.) Δ9 desaturases that catalyze the conversion of palmitate to palmitoleic acid (16:1) and/or stearate to oleic acid (18:1).

Of particular interest herein are Δ17 desaturases that desaturate a fatty acid between the 17th and 18th carbon atom numbered from the carboxyl-terminal end of the molecule and which, for example, catalyze the conversion of ARA to EPA (and optionally DGLA to ETA). In the art, Δ17 desaturases (and also Δ15 desaturases) are also occasionally referred to as "omega-3 desaturases", "w-3 desaturases", and/or "ω-3 desaturases", based on their ability to convert ω-6 fatty acids into their ω-3 counterparts (e.g., conversion of LA into ALA or DGLA into ETA and ARA into EPA, respectively).

Some desaturases have activity on two or more substrates. Based on this ability, these enzymes can be further classified with respect to their desaturase activities as being either "monofunctional" or "bifunctional". In some embodiments, it is most desirable to empirically determine the specificity of a fatty acid desaturase by transforming a suitable host with the gene for the fatty acid desaturase and determining its effect on the fatty acid profile of the host.

More specifically, Δ17 desaturases are defined herein as those fatty acid desaturases having monofunctional or bifunctional Δ17 desaturase activity, wherein Δ17 desaturase activity is the conversion of ARA to EPA and/or DGLA to ETA. The term "monofunctional Δ17 desaturase",

"monofunctional Δ17 desaturase activity" or "exclusive Δ17 desaturase activity" refers to a Δ17 desaturase that is capable of converting ARA to EPA and/or DGLA to ETA but not LA to ALA. In contrast, "bifunctional Δ17 desaturase", "bifunctional Δ17 desaturase activity" or "primary Δ17 desaturase activity" refers to a Δ17 desaturase that preferentially converts ARA to EPA and/or DGLA to ETA but additionally has limited ability to convert LA into ALA (thus exhibiting primarily Δ17 desaturase activity and limited Δ15 desaturase activity).

It should be noted that Δ17 desaturases can have specificities other than Δ17 and Δ15 desaturation that are not relevant in this classification.

For the purposes herein, the term "PaD17" refers to a Δ17 desaturase enzyme (SEQ ID NO:2) isolated from *Pythium aphanidermatum,* encoded by SEQ ID NO:1. Similarly, the term "PaD17*" refers to a Δ17 desaturase enzyme (SEQ ID NO:3) comprising up to (and including) two conservative amino acid mutations (i.e., 155S to P and 351A to T) with respect to SEQ ID NO:2. In contrast, the term "PaD17S" refers to a synthetic Δ17 desaturase derived from *Pythium aphanidermatum* that is codon-optimized for expression in *Yarrowia lipolytica* (i.e., SEQ ID NOs:4 and 2). Based on analyses described herein, PaD17 and PaD17S are further classified as bifunctional Δ17 desaturases.

For the purposes herein, the term "PsD17" refers to a Δ17 desaturase enzyme (SEQ ID NO:45) isolated from *Phytophthora sojae,* encoded by SEQ ID NO:44. In contrast, the term "PsD17S" refers to a synthetic Δ17 desaturase derived from *Phytophthora sojae* that is codon-optimized for expression in *Yarrowia lipolytica* (i.e., SEQ ID NOs:81 and 82). Based on analyses described herein, PsD17 and PsD17S are further classified as bifunctional Δ17 desaturases.

Similarly, the term "PrD17" refers to a Δ17 desaturase enzyme (SEQ ID NO:47) isolated from *Phytophthora ramorum,* encoded by SEQ ID NO:46. In contrast, the term "PrD17S" refers to a synthetic Δ17 desaturase derived from *Phytophthora ramorum* that is codon-optimized

for expression in *Yarrowia lipolytica* (i.e., SEQ ID NOs:84 and 47). Previous analyses described in U.S. Patent Application No. 11/787772 classified PrD17 and PrD17S as monofunctional Δ17 desaturases; however, based on analyses described herein, PrD17 and PrD17S are now identified as bifunctional Δ17 desaturases.

Relatedly, the term "PiD17" refers to a Δ17 desaturase enzyme (SEQ ID NO:43) isolated from *Phytophthora infestans*, encoded by SEQ ID NO:42.

The terms "conversion efficiency" and "percent substrate conversion" refer to the efficiency by which a particular enzyme (e.g., a desaturase) can convert substrate to product. The conversion efficiency is measured according to the following formula: ([product]/[substrate + product])*100, where 'product' includes the immediate product and all products in the pathway derived from it.

The term "elongase" refers to a polypeptide that can elongate a fatty acid carbon chain to produce an acid that is 2 carbons longer than the fatty acid substrate that the elongase acts upon. This process of elongation occurs in a multi-step mechanism in association with fatty acid synthase, as described in PCT Publication No. WO 2004/101757. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA and EPA to DPA. In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree and type of unsaturation. For example: a $C_{14/16}$ elongase will utilize a C_{14} substrate (e.g., myristic acid); a $C_{16/18}$ elongase will utilize a C_{16} substrate (e.g., palmitate); a $C_{18/20}$ elongase (also known as a Δ6 elongase as the terms can be used interchangeably) will utilize a C_{18} substrate (e.g., GLA, STA); and, a $C_{20/22}$ elongase will utilize a C_{20} substrate (e.g., EPA). In like manner, a Δ9 elongase is able to catalyze the conversion of LA and ALA to EDA and ETrA, respectively. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions (e.g., thereby acting as both a $C_{16/18}$ elongase and a $C_{18/20}$ elongase).

The term "oomycetes" refers to a group of heterotrophic organisms generally known as the water molds and downy mildews. They are filamentous protists that must absorb their food from the surrounding water or soil, or may invade the body of another organism to feed. As such, oomycetes play an important role in the decomposition and recycling of decaying matter. Although oomycetes have similarities to fungi through convergent evolution, they are not fungi (as previously thought); instead, the oomycetes are part of the kingdom Stramenopiles and are thereby distinct from plants, fungi and animals. Diatoms and golden-brown and brown algae (e.g., kelp) are also included within kingdom Stramenopiles.

Pythium is a genus of the oomycetes, comprising about eighty-five species. *Pythium* species are common pathogens causing disease in plants and fishes. The species of this genus are among the most destructive plant pathogens, inflicting serious economic losses of crops by destroying seed, storage organs, roots and other plant tissues. Members of the genus *Pythium* have been described as "aquatic fungi".

The term "oleaginous" refers to those organisms that tend to store their energy source in the form of lipid (Weete, In: Fungal Lipid Biochemistry, 2nd Ed., Plenum, 1980). The term "oleaginous yeast" refers to those microorganisms classified as yeasts that can make oil. Generally, the cellular oil or TAG content of oleaginous microorganisms follows a sigmoid curve, wherein the concentration of lipid increases until it reaches a maximum at the late logarithmic or early stationary growth phase and then gradually decreases during the late stationary and death phases (Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)). It is not uncommon for oleaginous microorganisms to accumulate in excess of about 25% of their dry cell weight as oil. Examples of oleaginous yeast include, but are no means limited to, the following genera: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces*.

The term "amino acid" will refer to the basic chemical structural unit of a protein or polypeptide. Amino acids are identified by either the one-

letter code or the three-letter codes for amino acids, in conformity with the IUPAC-IYUB standards described in *Nucleic Acids Research,* 13:3021-3030 (1985) and in the *Biochemical Journal,* 219(2):345-373 (1984), which are herein incorporated by reference.

The term "conservative amino acid substitution" refers to a substitution of an amino acid residue in a given protein with another amino acid, without altering the chemical or functional nature of that protein. For example, it is well known in the art that alterations in a gene that result in the production of a chemically equivalent amino acid at a given site (but that do not affect the structural and functional properties of the encoded, folded protein) are common. For the purposes of the present invention, "conservative amino acid substitutions" are defined as exchanges within one of the following five groups:

1. Small aliphatic, nonpolar or slightly polar residues: Ala [A], Ser [S], Thr [T] (Pro [P], Gly [G]);
2. Polar, negatively charged residues and their amides: Asp [D], Asn [N], Glu [E], Gln [Q];
3. Polar, positively charged residues: His [H], Arg [R], Lys [K];
4. Large aliphatic, nonpolar residues: Met [M], Leu [L], Ile [I], Val [V] (Cys [C]); and,
5. Large aromatic residues: Phe [F], Tyr [Y], Trp [W].

Conservative amino acid substitutions generally maintain: 1.) the structure of the polypeptide backbone in the area of the substitution; 2.) the charge or hydrophobicity of the molecule at the target site; or 3.) the bulk of the side chain. Additionally, in many cases, alterations of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein.

The term "non-conservative amino acid substitution" refers to an amino acid substitution that is generally expected to produce the greatest change in protein properties. Thus, for example, a non-conservative amino acid substitution would be one whereby: 1.) a hydrophilic residue is substituted for/by a hydrophobic residue (e.g., Ser or Thr for/by Leu, Ile, Val); 2.) a Cys or Pro is substituted for/by any other residue; 3.) a residue

having an electropositive side chain is substituted for/by an electronegative residue (e.g., Lys, Arg or His for/by Asp or Glu); or, 4.) a residue having a bulky side chain is substituted for/by one not having a side chain (e.g., Phe for/by Gly). Sometimes, non-conservative amino acid substitutions between two of the five groups will not affect the activity of the encoded protein.

As used herein, an "isolated nucleic acid fragment" or "isolated nucleic acid molecule" will be used interchangeably and refers to a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

A nucleic acid fragment is "hybridizable" to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA molecule, when a single-stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T. Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for moderately similar fragments (such as homologous sequences from distantly related organisms), to highly similar fragments (such as genes that duplicate functional enzymes from closely related organisms). Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45 °C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50 °C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X

SSC, 0.5% SDS was increased to 60 °C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65 °C. An additional set of stringent conditions include hybridization at 0.1X SSC, 0.1% SDS, 65 °C and washes with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS, for example.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T*m* for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T*m*) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T*m* have been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferably a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment

Search Tool; Altschul, S. F., et al., *J. Mol. Biol.*, 215:403-410 (1993)). In general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial colonies or bacteriophage plaques). In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence. The instant specification teaches the complete amino acid and nucleotide sequence encoding a particular oomycete protein. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another. For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the instant invention also includes isolated nucleic acid fragments that are complementary to the complete sequences as reported in the accompanying Sequence Listing, as well as those substantially similar nucleic acid sequences.

The terms "homology" and "homologous" are used interchangeably and refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms

also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that homologous nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize, under moderately stringent conditions (e.g., 0.5 X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the present nucleotide sequences and which are functionally equivalent to any of the nucleic acid sequences disclosed herein.

"Codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Chemically synthesized", as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures or, automated chemical synthesis can be performed using one of a number of commercially available machines. "Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the

host. Determination of preferred codons can be based on a survey of genes derived from the host cell, where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, and that may refer to the coding region alone or may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. A "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Suitable regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be

derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The terms "3' non-coding sequences" and "transcription terminator" refer to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA that is complementary to, and derived from, mRNA. "Sense" RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. "Antisense RNA" refers to a RNA transcript that is complementary to all or part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. Patent No. 5,107,065; PCT Publication No. WO 99/28508). The complementarity of an antisense RNA may be with

any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that is not translated and yet has an effect on cellular processes.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragments of the invention. Expression may also refer to translation of mRNA into a polypeptide.

"Mature" protein refers to a post-translationally processed polypeptide, i.e., one from which any pre- or propeptides present in the primary translation product have been removed. "Precursor" protein refers to the primary product of translation of mRNA, i.e., with pre- and propeptides still present. Pre- and propeptides may be (but are not limited to) intracellular localization signals.

"Transformation" refers to the transfer of a nucleic acid molecule into a host organism, resulting in genetically stable inheritance. The nucleic acid molecule may be a plasmid that replicates autonomously, for example, or, it may integrate into the genome of the host organism. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA,

derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell. "Expression cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "similarity" can be readily calculated by known methods, including but not limited to those described in: 1.) Computational Molecular Biology (Lesk, A. M., Ed.) Oxford University: NY (1988); 2.) Biocomputing: Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY (1993); 3.) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., Eds.) Humania: NJ (1994); 4.) Sequence Analysis in Molecular Biology (von Heinje, G., Ed.) Academic (1987); and, 5.) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).

Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences is performed using the "Clustal method of alignment" which encompasses several varieties of the algorithm including the "Clustal V method of alignment" corresponding to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS*, 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in

the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program. Additionally the "Clustal W method of alignment" is available and corresponds to the alignment method labeled Clustal W (described by Higgins and Sharp, *CABIOS*, 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). Default parameters for multiple alignment correspond to GAP PENALTY=10, GAP LENGTH PENALTY=0.2, Delay Divergen Seqs(%)=30, DNA Transition Weight=0.5, Protein Weight Matrix=Gonnet Series and DNA Weight Matrix=IUB. After alignment of the sequences using the Clustal W program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

"BLASTN method of alignment" is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare nucleotide sequences using default parameters.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar function or activity. Suitable nucleic acid fragments (isolated polynucleotides of the present invention) encode polypeptides that are at least about 70% identical, preferably at least about 75% identical, and more preferably at least about 80% identical to the amino acid sequences reported herein. Preferred nucleic acid fragments encode amino acid sequences that are at least about 85% identical to the amino acid sequences reported herein.

More preferred nucleic acid fragments encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid fragments that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein. Indeed, any integer amino acid identity from 70% to 100% may be useful in describing the present invention, such as 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least 50 amino acids, preferably at least 100 amino acids, more preferably at least 150 amino acids, still more preferably at least 200 amino acids, and most preferably at least 250 amino acids.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will include, but is not limited to: 1.) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); 2.) BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990)); 3.) DNASTAR (DNASTAR, Inc. Madison, WI); 4.) Sequencher (Gene Codes Corporation, Ann Arbor, MI); and, 5.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

The term "conserved domain" or "motif" means a set of amino acids conserved at specific positions along an aligned sequence of evolutionarily

related proteins. While amino acids at other positions can vary between homologous proteins, amino acids that are highly conserved at specific positions indicate amino acids that are essential in the structure, the stability, or the activity of a protein. Because they are identified by their high degree of conservation in aligned sequences of a family of protein homologues, they can be used as identifiers, or "signatures", to determine if a protein with a newly determined sequence belongs to a previously identified protein family. For the purposes herein, the following Table describes motifs of the present invention which are indicative of a protein having Δ17 desaturase activity.

Table 3

Summary Of Δ17 Desaturase Motifs

Description	Sequence	Protein SEQ ID NO.
Δ17 Desaturase Motif #1	F T X G H D X G H	96
Δ17 Desaturase Motif #2	H R H H H K N T G	97
Δ17 Desaturase Motif #3	I G T H Q X H H L F P	98

The term "His Box" refers to a histidine box having a motif selected from the group consisting of: $H(X)_3H$ (SEQ ID NO:99), $H(X)_2HH$ (SEQ ID NO:100) and $H/Q(X)_2HH$ (SEQ ID NO:101).

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

An Overview: Microbial Biosynthesis Of Fatty Acids And Triacylglycerols

In general, lipid accumulation in oleaginous microorganisms is triggered in response to the overall carbon to nitrogen ratio present in the growth medium. This process, leading to the *de novo* synthesis of free palmitate (16:0) in oleaginous microorganisms, is described in detail in PCT Publication No. WO 2004/101757. Palmitate is the precursor of longer-chain saturated and unsaturated fatty acid derivates, which are formed through the action of elongases and desaturases (Figure 1).

TAGs (the primary storage unit for fatty acids) are formed by a series of reactions that involve: 1.) the esterification of one molecule of acyl-CoA to glycerol-3-phosphate via an acyltransferase to produce lysophosphatidic acid; 2.) the esterification of a second molecule of acyl-CoA via an acyltransferase to yield 1,2-diacylglycerol phosphate (commonly identified as phosphatidic acid); 3.) removal of a phosphate by phosphatidic acid phosphatase to yield 1,2-diacylglycerol (DAG); and, 4.) the addition of a third fatty acid by the action of an acyltransferase to form TAG. A wide spectrum of fatty acids can be incorporated into TAGs, including saturated and unsaturated fatty acids and short-chain and long-chain fatty acids.

Biosynthesis Of Omega Fatty Acids

The metabolic process wherein oleic acid is converted to ω-3/ω-6 fatty acids involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds. This requires a series of special desaturation and elongation enzymes present in the endoplasmic reticulim membrane. However, as seen in Figure 1 and as described below, there are often multiple alternate pathways for production of a specific ω-3/ω-6 fatty acid.

Specifically, all pathways require the initial conversion of oleic acid to LA, the first of the ω-6 fatty acids, by a Δ12 desaturase. Then, using the "Δ6 desaturase/Δ6 elongase pathway", ω-6 fatty acids are formed as follows: (1) LA is converted to GLA by a Δ6 desaturase; (2) GLA is converted to DGLA by a $C_{18/20}$ elongase; and (3) DGLA is converted to ARA by a Δ5 desaturase. Alternatively, the "Δ6 desaturase/Δ6 elongase

pathway" can be utilized for formation of ω-3 fatty acids as follows: (1) LA is converted to ALA, the first of the ω-3 fatty acids, by a Δ15 desaturase; (2) ALA is converted to STA by a Δ6 desaturase; (3) STA is converted to ETA by a $C_{18/20}$ elongase; (4) ETA is converted to EPA by a Δ5 desaturase;(5) EPA is converted to DPA by a $C_{20/22}$ elongase; and, (6) DPA is converted to DHA by a Δ4 desaturase. Optionally, ω-6 fatty acids may be converted to ω-3 fatty acids; for example, ETA and EPA are produced from DGLA and ARA, respectively, by Δ17 desaturase activity.

Alternate pathways for the biosynthesis of ω-3/ω-6 fatty acids utilize the Δ9 elongase/Δ8 desaturase biosynthetic pathway. More specifically, LA and ALA may be converted to EDA and ETrA, respectively, by a Δ9 elongase; then, a Δ8 desaturase converts EDA to DGLA and/or ETrA to ETA.

It is contemplated that the particular functionalities required to be expressed in a specific host organism for production of ω-3/ω-6 fatty acids will depend on the host cell (and its native PUFA profile and/or desaturase/elongase profile), the availability of substrate, and the desired end product(s). One skilled in the art will be able to identify various candidate genes encoding each of the enzymes desired for ω-3/ω-6 fatty acid biosynthesis. Useful desaturase and elongase sequences may be derived from any source, e.g., isolated from a natural source (from bacteria, algae, fungi, oomycetes, plants, animals, etc.), produced via a semi-synthetic route or synthesized *de novo*. Although the particular source of the desaturase and elongase genes introduced into the host is not critical, considerations for choosing a specific polypeptide having desaturase or elongase activity include: 1.) the substrate specificity of the polypeptide; 2.) whether the polypeptide or a component thereof is a rate-limiting enzyme; 3.) whether the desaturase or elongase is essential for synthesis of a desired PUFA; 4.) co-factors required by the polypeptide; and/or, 5.) whether the polypeptide is modified after its production (e.g., by a kinase). The expressed polypeptide preferably has parameters

compatible with the biochemical environment of its location in the host cell (see PCT Publication No. WO 2004/101757 for additional details).

In additional embodiments, it will also be useful to consider the conversion efficiency of each particular desaturase and/or elongase. More specifically, since each enzyme rarely functions with 100% efficiency to convert substrate to product, the final lipid profile of un-purified oils produced in a host cell will typically be a mixture of various PUFAs consisting of the desired ω-3/ω-6 fatty acid, as well as various upstream intermediary PUFAs. Thus, consideration of each enzyme's conversion efficiency is also a variable to consider when optimizing biosynthesis of a desired fatty acid.

With each of the considerations above in mind, candidate genes having the appropriate desaturase and elongase activities (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ5 desaturases, Δ17 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ8 desaturases, Δ4 desaturases and $C_{20/22}$ elongases) can be identified according to publicly available literature (e.g., GenBank), the patent literature, and experimental analysis of organisms having the ability to produce PUFAs. These genes will be suitable for introduction into a specific host organism, to enable or enhance the organism's synthesis of PUFAs.

Identification Of A Novel Δ17 Desaturase

In the present invention, a nucleotide sequence has been isolated from *Pythium aphanidermatum* encoding a Δ17 desaturase, designated herein as "PaD17".

Comparison of the PaD17 nucleotide base and deduced amino acid sequences to public databases reveals that the most similar known sequences are about 75.3% identical to the amino acid sequence of PaD17 reported herein over a length of 359 amino acids using the Clustal W method of alignment algorithms. More preferred amino acid fragments are at least about 70%-85% identical to the sequences herein, where those sequences that are at least about 85%-90% identical are particularly suitable and those sequences that are at least about 90%-95% identical

are most preferred. Similarly, preferred PaD17 encoding nucleic acid sequences corresponding to the instant Δ17 desaturase ORF are those encoding active proteins and which are at least about 70%-85% identical to the nucleic acid sequences of PaD17 reported herein, where those sequences that are at least about 85%-90% identical are particularly suitable and those sequences that are at least about 90%-95% identical are most preferred.

In alternate embodiments, the instant PaD17 sequence can be codon-optimized for expression in a particular host organism. As is well known in the art, this can be a useful means to further optimize the expression of the enzyme in the alternate host, since use of host-preferred codons can substantially enhance the expression of the foreign gene encoding the polypeptide. In general, host-preferred codons can be determined within a particular host species of interest by examining codon usage in proteins (preferably those expressed in the largest amount) and determining which codons are used with highest frequency. Then, the coding sequence for a polypeptide of interest having e.g., desaturase activity can be synthesized in whole or in part using the codons preferred in the host species.

In one preferred embodiment of the invention, PaD17 was codon-optimized for expression in *Yarrowia lipolytica*. This was possible by first determining the *Y. lipolytica* codon usage profile (see PCT Publication No. WO 04/101757; U.S. Patent 7,125,672) and identifying those codons that were preferred. Further optimization of gene expression in *Y. lipolytica* was achieved by determining the consensus sequence around the 'ATG' initiation codon. This optimization resulted in modification of 188 bp of the 1080 bp coding region (17.4%) and optimization of 175 codons (48.6%). None of the modifications in the codon-optimized gene ("PaD17S"; SEQ ID NO:4) changed the amino acid sequence of the encoded protein (SEQ ID NO:2). As described in Example 10, the codon-optimized gene was more efficient desaturating ARA to EPA than the wildtype gene, when expressed in *Y. lipolytica*.

One skilled in the art would be able to use the teachings herein to create various other codon-optimized Δ17 desaturase proteins suitable for optimal expression in alternate hosts (i.e., other than *Yarrowia lipolytica*), based on the wildtype PaD17 sequence (i.e., SEQ ID NO:2) or a variant thereof as set forth in SEQ ID NO:3. Accordingly, the instant invention relates to any codon-optimized Δ17 desaturase protein that is derived from either SEQ ID NO:2 or SEQ ID NO:3. This includes, but is not limited to, the nucleotide sequence set forth in SEQ ID NO:4, which encodes a synthetic Δ17 desaturase protein (i.e., PaD17S) that was codon-optimized for expression in *Yarrowia lipolytica*.

Upon identification of the Oomycete polypeptide described above, the activity of the wildtype and codon-optimized fatty acid desaturase was determined by transformation into a suitable host (i.e., *Yarrowia lipolytica*) and determination of its effect on the fatty acid profile of the host (Examples 7, 10 and 17). As expected, PaD17 and PaD17S both possessed Δ17 desaturase activity, such that the enzyme was capable of catalyzing conversion of ARA to EPA. Specifically, the ARA to EPA conversion efficiency of PaD17 ranged from 18.4-19.5%, while the ARA to EPA conversion efficiency of PaD17S ranged from 54.1-55.8% (based on determination in two different strains of *Y. lipolytica* and under different growth conditions). Conversion efficiency was measured according to the following formula: ([product]/[substrate+product])*100, where 'product' includes the immediate product and all products in the pathway derived from it.

Unexpectedly, however, PaD17S additionally possessed limited Δ15 desaturase activity (i.e., the LA to ALA conversion efficiency was 34.6%) (Example 17). Thus, the *Pythium aphanidermatum* desaturase is defined herein as a bifunctional Δ17 desaturase.

Further analysis with PaD17S revealed that the enzyme demonstrated broad catalytic promiscuity, based on greater than 25% conversion efficiency using the ω–6 fatty acid substrates EDA and DGLA (Example 17). Thus, the ω–6 fatty acid substrate specificity of PaD17S is

similar to that of the synthetic Δ17 desaturase derived from *Phytopthora sojae* and codon-optimized for expression in *Yarrowia lipolytica* (i.e., PsD17S; U.S. Patent Application No. 11/787772 and Example 17 herein) and the synthetic Δ17 desaturase derived from *Phytopthora ramorum* and codon-optimized for expression in *Yarrowia lipolytica* (i.e., PrD17S; U.S. Patent Application No. 11/787772 and Example 17 herein). These results are in contrast to those demonstrated for the related ω–3 desaturase of *Saprolegnia diclina,* which has been reported to function exclusively on C20 ω–6 fatty acid substrates as a monofunctional Δ17 desaturase (Pereira, S.L. et. al., *Biochem. J.*, 378:665 (2004))

In another aspect this invention concerns an isolated nucleic acid fragment comprising a nucleic acid sequence encoding a Δ17 desaturase, excluding SEQ ID NO:43 (i.e., "PiD17", the ω–3 desaturase from *Phytophthora infestans* (GenBank Accession No. CAJ30870)) and SEQ ID NO:95 (i.e., "SdD17", the Δ17 desaturase from *Saprolegnia diclina* (GenBank Accession No. AAR20444)), wherein the amino acid sequence comprising said Δ17 desaturase contains at least one of the following amino acid sequence motifs selected from the group consisting of:

a) F T X G H D X G H (Δ17 Desaturase Motif #1; SEQ ID NO:96);

b) H R H H H K N T G (Δ17 Desaturase Motif #2; SEQ ID NO:97); and,

c) I G T H Q X H H L F P (Δ17 Desaturase Motif #3; SEQ ID NO:98);

wherein X can be any amino acid.

The underlined amino acids represent histidine residues that are part of the desaturase His Box motifs. The His Box motifs are described as: $H(X)_3H$ (SEQ ID NO:99), $H(X)_2HH$ (SEQ ID NO:100) and $H/Q(X)_2HH$ (SEQ ID NO:101). Figure 14 sets forth a comparison of the Δ17 desaturase of the present invention with other publicly disclosed Δ17 desaturases using a Clustal V alignment (with default parameters). Specifically, SEQ ID NO:2 (PaD17), SEQ ID NO:43 (PiD17), SEQ ID NO:47 (PrD17), SEQ ID NO:82 (PsD17S) and SEQ ID NO:95 (SdD17) were compared. Regions comprising the motifs of the invention (i.e., Δ17

Desaturase Motif #1, Δ17 Desaturase Motif #2 and Δ17 Desaturase Motif #3, respectively) are shown in boxes.

Identification And Isolation Of Homologs

Any of the instant desaturase sequences (i.e., PaD17, PaD17*, PaD17S) or portions thereof (i.e., Δ17 Desaturase Motif #1, Δ17 Desaturase Motif #2 and/or Δ17 Desaturase Motif #3) may be used to search for Δ17 desaturase homologs in the same or other bacterial, algal, fungal, Oomycete or plant species using sequence analysis software. In general, such computer software matches similar sequences by assigning degrees of homology to various substitutions, deletions and other modifications.

Alternatively, any of the instant desaturase sequences or portions thereof may also be employed as hybridization reagents for the identification of Δ17 homologs. The basic components of a nucleic acid hybridization test include a probe, a sample suspected of containing the gene or gene fragment of interest and a specific hybridization method. Probes of the present invention are typically single-stranded nucleic acid sequences that are complementary to the nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected. Although the probe length can vary from 5 bases to tens of thousands of bases, typically a probe length of about 15 bases to about 30 bases is suitable. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base.

Hybridization methods are well defined. Typically the probe and sample must be mixed under conditions that will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between

the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added (e.g., guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide, cesium trifluoroacetate). If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1 M sodium chloride, about 0.05 to 0.1 M buffers (e.g., sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9)), about 0.05 to 0.2% detergent (e.g., sodium dodecylsulfate), or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kdal), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA (e.g., calf thymus or salmon sperm DNA, or yeast RNA), and optionally from about 0.5 to 2% wt/vol glycine. Other additives may also be included, such as volume exclusion agents that include a variety of polar water-soluble or swellable agents (e.g., polyethylene glycol), anionic polymers (e.g., polyacrylate or polymethylacrylate) and anionic saccharidic polymers (e.g., dextran sulfate).

Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the sequence.

In additional embodiments, any of the Δ17 desaturase nucleic acid fragments described herein (or any homologs identified thereof) may be used to isolate genes encoding homologous proteins from the same or other bacterial, algal, fungal, oomycete or plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to: 1.) methods of nucleic acid hybridization; 2.) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Patent 4,683,202; ligase chain reaction (LCR), Tabor, S. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 82:1074 (1985); or strand displacement amplification (SDA), Walker, et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89:392 (1992)]; and 3.) methods of library construction and screening by complementation.

For example, genes encoding similar proteins or polypeptides to the Δ17 desaturases described herein could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from any desired yeast, fungus or oomycete using methodology well known to those skilled in the art (wherein those yeast or fungus producing EPA [or derivatives thereof] would be preferred). Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis, *supra*). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan (e.g., random primers DNA labeling, nick translation or end-labeling techniques), or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of (or full-length of) the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full-length DNA fragments under conditions of appropriate stringency.

Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending

on the desired test conditions, the sequences of the primers should be designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art (Thein and Wallace, "The use of oligonucleotide as specific hybridization probes in the Diagnosis of Genetic Disorders", in *Human Genetic Diseases: A Practical Approach*, K. E. Davis Ed., (1986) pp 33-50, IRL: Herndon, VA; and Rychlik, W., In Methods in Molecular Biology, White, B. A. Ed., (1993) Vol. 15, pp 31-39, PCR Protocols: Current Methods and Applications. Humania: Totowa, NJ).

Generally two short segments of the instant desaturase sequences may be used in PCR protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. PCR may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding eukaryotic genes.

Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., *Proc. Natl. Acad. Sci. U.S.A.*, 85:8998 (1988)) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (Gibco/BRL, Gaithersburg, MD), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., *Proc. Natl. Acad. Sci. U.S.A.*, 86:5673 (1989); Loh et al., *Science*, 243:217 (1989)).

In other embodiments, any of the Δ17 desaturase nucleic acid fragments described herein (or any homologs identified thereof) may be used for creation of new and improved fatty acid desaturases. As is well known in the art, *in vitro* mutagenesis and selection, chemical mutagenesis, "gene shuffling" methods or other means can be employed to obtain mutations of naturally occurring desaturase genes. Alternatively,

improved fatty acids may be synthesized by domain swapping, wherein a functional domain from any of the Δ17 desaturase nucleic acid fragments described herein are exchanged with a functional domain in an alternate desaturase gene to thereby result in a novel protein.

Methods For Production Of Various ω-3 And/Or ω-6 Fatty Acids

It is expected that introduction of chimeric genes encoding the Δ17 desaturases described herein (i.e., PaD17, PaD17*, PaD17S or other mutant enzymes, codon-optimized enzymes or homologs thereof), under the control of the appropriate promoters will result in increased production of EPA in the transformed host organism, respectively. As such, the present invention encompasses a method for the direct production of PUFAs comprising exposing a fatty acid substrate (i.e., ARA) to the desaturase enzymes described herein (e.g., PaD17, PaD17*, PaD17S), such that the substrate is converted to the desired fatty acid product (i.e., EPA).

More specifically, it is an object of the present invention to provide a method for the production of EPA in a host cell (e.g., oleaginous yeast), wherein the host cell comprises:

a.) an isolated nucleotide molecule encoding a Δ17 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

b) a source of ARA;

c.) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the Δ17 desaturase polypeptide is expressed and the ARA is converted to EPA; and,

d.) optionally recovering the EPA of step (c).

The person of skill in the art will recognize that the broad substrate range of the Δ17 desaturase will allow for the use of the enzyme for the conversion of DGLA to ETA. Accordingly, the invention provides a method for the production of ETA in a host cell, wherein the host cell comprises:

a.) an isolated nucleotide molecule encoding a Δ17 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

b.) a source of DGLA;

c.) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the Δ17 desaturase polypeptide is expressed and the DGLA is converted to ETA; and,

d.) optionally recovering the ETA of step (c).

In an alternate embodiment, based on the bifunctionality of the *Pythium aphanidermatum* Δ17 desaturases, it is an object of the present invention to provide a method for the production of polyunsaturated fatty acids in a host cell (e.g., oleaginous yeast), wherein the host cell comprises:

a.) an isolated nucleotide molecule encoding a bifunctional Δ17 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

b.) a source of fatty acid selected from the group consisting of: linoleic acid and eicosadienoic acid;

wherein the host cell is grown under conditions wherein the nucleic acid molecule encoding the bifunctional Δ17 desaturase polypeptide is expressed and the linoleic acid is converted to α-linolenic acid and the eicosadienoic acid is converted to eicosatrienoic acid; and, said fatty acid is then optionally recovered.

Substrate feeding may be required in any of the methods described above.

Alternatively, the Δ17 desaturase gene and its corresponding enzyme product described herein can be used indirectly for the production

of ω-3 fatty acids (see PCT Publications No. WO 2004/101757 and No. WO 2006/052870). Indirect production of ω-3/ω-6 PUFAs occurs wherein the fatty acid substrate is converted indirectly into the desired fatty acid product, via means of an intermediate step(s) or pathway intermediate(s). Thus, it is contemplated that the Δ17 desaturases described herein (e.g., PaD17, PaD17*, PaD17S or other mutant enzymes, codon-optimized enzymes or homologs thereof) may be expressed in conjunction with additional genes encoding enzymes of the PUFA biosynthetic pathway (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ5 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ8 desaturases, Δ4 desaturases, $C_{20/22}$ elongases) to result in higher levels of production of longer-chain ω-3 fatty acids (e.g., EPA, DPA and DHA). The particular genes included within a particular expression cassette will depend on the host cell (and its PUFA profile and/or desaturase/elongase profile), the availability of substrate and the desired end product(s).

In alternative embodiments, it may be useful to disrupt a host organism's native Δ17 desaturase, based on the complete sequences described herein, the complement of those complete sequences, substantial portions of those sequences, codon-optimized desaturases derived therefrom and those sequences that are substantially homologous thereto. For example, the targeted disruption of the Δ17 desaturase (and optionally a Δ15 desaturase) in a host organism produces a mutant strain that has diminished ability to synthesize ω-3 fatty acids. This mutant strain could be useful for the production of "pure" ω-6 fatty acids (without co-synthesis of ω-3 fatty acids).

Expression Systems, Cassettes And Vectors

The genes and gene products of the instant sequences described herein may be expressed in heterologous host cells. Expression in recombinant hosts may be useful for the production of various PUFA pathway intermediates, or for the modulation of PUFA pathways already

existing in the host for the synthesis of new products heretofore not possible using the host.

Expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct chimeric genes for production of any of the gene products of the instant sequences. These chimeric genes could then be introduced into appropriate host cells via transformation to provide high-level expression of the encoded enzymes.

Vectors or DNA cassettes useful for the transformation of suitable host cells are well known in the art. The specific choice of sequences present in the construct is dependent upon the desired expression products (*supra*), the nature of the host cell and the proposed means of separating transformed cells versus non-transformed cells. Typically, however, the vector or cassette contains sequences directing transcription and translation of the relevant gene(s), a selectable marker and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene that controls transcriptional initiation (e.g., a promoter) and a region 3' of the DNA fragment that controls transcriptional termination (i.e., a terminator). It is most preferred when both control regions are derived from genes from the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Initiation control regions or promoters which are useful to drive expression of the instant Δ17 desaturase ORFs in the desired host cell are numerous and familiar to those skilled in the art. Virtually any promoter capable of directing expression of these genes in the selected host cell is suitable for the present invention. Expression in a host cell can be accomplished in a transient or stable fashion. Transient expression can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest. Stable expression can be achieved by the use of a constitutive promoter operably linked to the gene

of interest. As an example, when the host cell is yeast, transcriptional and translational regions functional in yeast cells are provided, particularly from the host species (e.g., see PCT Publication No. WO 2006/052870 [Patent Publication US 2006-0115881-A1] for preferred transcriptional initiation regulatory regions for use in *Yarrowia lipolytica*). Any one of a number of regulatory sequences can be used, depending upon whether constitutive or induced transcription is desired, the efficiency of the promoter in expressing the ORF of interest, the ease of construction and the like.

The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and function satisfactorily in a variety of hosts (when utilized both in the same and different genera and species from where they were derived). The termination region usually is selected more as a matter of convenience rather than because of any particular property. Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

As one of skill in the art is aware, merely inserting a gene into a cloning vector does not ensure that it will be successfully expressed at the level needed. In response to the need for a high expression rate, many specialized expression vectors have been created by manipulating a number of different genetic elements that control aspects of transcription, translation, protein stability, oxygen limitation, and secretion from the host cell. More specifically, some of the molecular features that have been manipulated to control gene expression include: 1.) the nature of the relevant transcriptional promoter and terminator sequences; 2.) the number of copies of the cloned gene and whether the gene is plasmid-borne or integrated into the genome of the host cell; 3.) the final cellular location of the synthesized foreign protein; 4.) the efficiency of translation and correct folding of the protein in the host organism; 5.) the intrinsic stability of the mRNA and protein of the cloned gene within the host cell;

and, 6.) the codon usage within the cloned gene, such that its frequency approaches the frequency of preferred codon usage of the host cell. Each of these types of modifications are encompassed in the present invention, as means to further optimize expression of the Δ17 desaturases described herein.

Transformation Of Host Cells

Once the DNA encoding a polypeptide suitable for expression in an appropriate host cell has been obtained, it is placed in a plasmid vector capable of autonomous replication in the host cell, or it is directly integrated into the genome of the host cell. Integration of expression cassettes can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination with the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of selection and should lack homology to the other construct(s) to maintain stable expression and prevent reassortment of elements among constructs. Judicious choice of regulatory regions, selection means and method of propagation of the introduced construct(s) can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

Constructs comprising the gene of interest may be introduced into a host cell by any standard technique. These techniques include transformation (e.g., lithium acetate transformation [*Methods in Enzymology*, 194:186-187 (1991)]), protoplast fusion, biolistic impact, electroporation, microinjection, or any other method that introduces the gene of interest into the host cell.

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence (e.g., an expression cassette) will be referred to as "transformed" or "recombinant" herein. The transformed

host will have at least one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers. The transformed host cell can be identified by various selection techniques, as described in PCT Publications No. WO 2004/101757, No. WO 2005/003310 and No. WO 2006/052870.

Following transformation, substrates suitable for the instant Δ17 desaturases (and, optionally other PUFA enzymes that are co-expressed within the host cell) may be produced by the host either naturally or transgenically, or they may be provided exogenously.

Metabolic Engineering Of ω-3 And/Or ω-6 Fatty Acid Biosynthesis

Knowledge of the sequences of the present Δ17 desaturases will be useful for manipulating ω-3 and/or ω-6 fatty acid biosynthesis in various host cells. This may require metabolic engineering directly within the PUFA biosynthetic pathway or additional manipulation of pathways that contribute carbon to the PUFA biosynthetic pathway. Methods useful for up-regulating desirable biochemical pathways and down-regulating undesirable biochemical pathways are well known to those skilled in the art. For example, biochemical pathways competing with the ω-3 and/or ω-6 fatty acid biosynthetic pathways for energy or carbon, or native PUFA biosynthetic pathway enzymes that interfere with production of a particular PUFA end-product, may be eliminated by gene disruption or down-regulated by other means (e.g., antisense mRNA and zinc-finger targeting technologies).

Detailed discussion of manipulations within the PUFA biosynthetic pathway as a means to increase ARA, EPA or DHA (and associated techniques thereof) are presented in PCT Publication No. WO 2006/055322 [Patent Publication No. US 2006-0094092-A1], PCT Publication No. WO 2006/052870 [Patent Publication No. US 2006-0115881-A1] and PCT Publication No. WO 2006/052871 [Patent Publication No. US 2006-0110806-A1], respectively, as are desirable manipulations in the TAG biosynthetic pathway and the TAG degradation pathway (and associated techniques thereof).

Preferred Hosts For Recombinant Expression Of Δ17 Desaturases

Host cells for expression of the instant genes and nucleic acid fragments may include hosts that grow on a variety of feedstocks, including simple or complex carbohydrates, fatty acids, organic acids, oils and alcohols, and/or hydrocarbons over a wide range of temperature and pH values. Based on the needs of the Applicants' Assignee, the genes described in the instant invention were initially isolated for expression in an oleaginous yeast (and in particular *Yarrowia lipolytica*); however, it is contemplated that because transcription, translation and the protein biosynthetic apparatus are highly conserved, any plant, bacteria, yeast, algae, oomycete and/or filamentous fungus will be a suitable host for expression of the present nucleic acid fragments.

Preferred hosts are oleaginous organisms, such as oleaginous yeast. These oleaginous organisms are naturally capable of oil synthesis and accumulation, wherein the oil can comprise greater than about 25% of the cellular dry weight, more preferably greater than about 30% of the cellular dry weight, and most preferably greater than about 40% of the cellular dry weight. Genera typically identified as oleaginous yeast include, but are not limited to: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces*. More specifically, illustrative oil-synthesizing yeast include: *Rhodosporidium toruloides, Lipomyces starkeyii, L. lipoferus, Candida revkaufi, C. pulcherrima, C. tropicalis, C. utilis, Trichosporon pullans, T. cutaneum, Rhodotorula glutinus, R. graminis* and *Yarrowia lipolytica* (formerly classified as *Candida lipolytica*).

Most preferred is the oleaginous yeast *Yarrowia lipolytica;* and, in a further embodiment, most preferred are the *Y. lipolytica* strains designated as ATCC #76982, ATCC #20362, ATCC #8862, ATCC #18944 and/or LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.*, 82(1):43-9 (2002)).

Specific teachings applicable for engineering EPA and DHA in *Y. lipolytica* are provided in U.S. Patent Applications No. 11/265761 (PCT Publication No. WO 2006/052870; Patent Publication No. US 2006-

0115881-A1) and No. 11/264737 (PCT Publication No. WO 2006/052871; Patent Publication No. US 2006-0110806-A1), respectively. Detailed means for the synthesis and transformation of expression vectors comprising Δ17 desaturases in oleaginous yeast (i.e., *Yarrowia lipolytica*) are provided in PCT Publications No. WO 2004/101757 and No. WO 2006/052870. The preferred method of expressing genes in this yeast is by integration of linear DNA into the genome of the host; and, integration into multiple locations within the genome can be particularly useful when high level expression of genes are desired [e.g., in the *Ura3* locus (GenBank Accession No. AJ306421), the *Leu2* gene locus (GenBank Accession No. AF260230), the *Lys5* gene locus (GenBank Accession No. M34929), the *Aco2* gene locus (GenBank Accession No. AJ001300), the *Pox3* gene locus (Pox3: GenBank Accession No. XP_503244; or, Aco3: GenBank Accession No. AJ001301), the Δ12 desaturase gene locus (PCT Publication No. WO 2004/104167), the *Lip1* gene locus (GenBank Accession No. Z50020) and/or the *Lip2* gene locus (GenBank Accession No. AJ012632)].

Preferred selection methods for use in *Yarrowia lipolytica* are resistance to kanamycin, hygromycin and the amino glycoside G418, as well as ability to grow on media lacking uracil, leucine, lysine, tryptophan or histidine. In alternate embodiments, 5-fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; "5-FOA") is used for selection of yeast *Ura*⁻ mutants. The compound is toxic to yeast cells that possess a functioning *URA3* gene encoding orotidine 5'-monophosphate decarboxylase (OMP decarboxylase); thus, based on this toxicity, 5-FOA is especially useful for the selection and identification of *Ura*⁻ mutant yeast strains (Bartel, P.L. and Fields, S., Yeast 2-Hybrid System, Oxford University: New York, v. 7, pp 109-147, 1997).

Other preferred microbial hosts include oleaginous bacteria, algae, Oomycetes and other fungi; and, within this broad group of microbial hosts, of particular interest are microorganisms that synthesize ω-3/ω-6 fatty acids (or those that can be genetically engineered for this purpose [e.g., other yeast such as *Saccharomyces cerevisiae*]). Thus, for

example, transformation of *Mortierella alpina* (which is commercially used for production of ARA) with any of the present Δ17 desaturase genes under the control of inducible or regulated promoters could yield a transformant organism capable of synthesizing EPA. The method of transformation of *M. alpina* is described by Mackenzie et al. (*Appl. Environ. Microbiol.*, 66:4655 (2000)). Similarly, methods for transformation of Thraustochytriales microorganisms are disclosed in U.S. Patent 7,001,772.

No matter what particular host is selected for expression of the Δ17 desaturases described herein, it is preferable if multiple transformants are screened in order to obtain a strain displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA blots (Southern, *J. Mol. Biol.*, 98:503 (1975)), Northern analysis of mRNA expression (Kroczek, *J. Chromatogr. Biomed. Appl.*, 618 (1-2):133-145 (1993)), Western and/or Elisa analyses of protein expression, phenotypic analysis or GC analysis of the PUFA products.

Fermentation Processes For Omega Fatty Acid Production

The transformed host cell is grown under conditions that optimize expression of chimeric desaturase genes and produce the greatest and most economical yield of desired PUFAs. In general, media conditions that may be optimized include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the amount of different mineral ions, the oxygen level, growth temperature, pH, length of the biomass production phase, length of the oil accumulation phase and the time and method of cell harvest. *Yarrowia lipolytica* are generally grown in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)) or a defined minimal media that lacks a component necessary for growth and thereby forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI)).

Fermentation media in the present invention must contain a suitable carbon source. Suitable carbon sources are taught in PCT Publication No. WO 2004/101757. Although it is contemplated that the source of carbon

utilized in the present invention may encompass a wide variety of carbon-containing sources, preferred carbon sources are sugars, glycerol and/or fatty acids. Most preferred is glucose and/or fatty acids containing between 10-22 carbons.

Nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source. In addition to appropriate carbon and nitrogen sources, the fermentation media must also contain suitable minerals, salts, cofactors, buffers, vitamins and other components known to those skilled in the art suitable for the growth of the oleaginous host and promotion of the enzymatic pathways necessary for PUFA production. Particular attention is given to several metal ions (e.g., Fe^{+2}, Cu^{+2}, Mn^{+2}, Co^{+2}, Zn^{+2}, Mg^{+2}) that promote synthesis of lipids and PUFAs (Nakahara, T. et al., *Ind. Appl. Single Cell Oils,* D. J. Kyle and R. Colin, eds. pp 61-97 (1992)).

Preferred growth media in the present invention are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the transformant host cells will be known by one skilled in the art of microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to pH 8.0, wherein pH 5.5 to pH 7.5 is preferred as the range for the initial growth conditions. The fermentation may be conducted under aerobic or anaerobic conditions, wherein microaerobic conditions are preferred.

Typically, accumulation of high levels of PUFAs in oleaginous yeast cells requires a two-stage process, since the metabolic state must be "balanced" between growth and synthesis/storage of fats. Thus, most preferably, a two-stage fermentation process is necessary for the production of PUFAs in *Yarrowia lipolytica*. This approach is described in PCT Publication No. WO 2004/101757, as are various suitable fermentation process designs (i.e., batch, fed-batch and continuous) and considerations during growth.

Oils For Use In Foodstuffs, Health Food Products, Pharmaceuticals And Animal Feeds

The market place currently supports a large variety of food and feed products, incorporating ω-3 and/or ω-6 fatty acids (particularly ALA, GLA, ARA, EPA, DPA and DHA). It is contemplated that the oils of the invention comprising long-chain PUFAs will function in food and feed products to impart the health benefits of current formulations. More specifically, oils of the invention containing ω-3 and/or ω-6 fatty acids will be suitable for use in a variety of food and feed products including, but not limited to: food analogs, drinks, meat products, cereal products, baked foods, snack foods and dairy products (see Patent Publication No. US 2006/0094092 for details).

Additionally the present oils may be used in formulations to impart health benefits in medical foods including medical nutritionals, dietary supplements, infant formula as well as pharmaceutical products. One of skill in the art of food processing and food formulation will understand how the amount and composition of the present oils may be added to the food or feed product. Such an amount will be referred to herein as an "effective" amount and will depend on the food or feed product, the diet that the product is intended to supplement or the medical condition that the medical food or medical nutritional is intended to correct or treat.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by:

1.) Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (Maniatis); 2.) T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and, 3.) Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, Eds), American Society for Microbiology: Washington, D.C. (1994)); or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of microbial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified. *E. coli* strains were typically grown at 37 °C on Luria Bertani (LB) plates.

General molecular cloning was performed according to standard methods (Sambrook et al., *supra*). DNA sequence was generated on an ABI Automatic sequencer using dye terminator technology (U.S. Patent 5,366,860; EP 272,007) using a combination of vector and insert-specific primers. Comparisons of genetic sequences were accomplished using DNASTAR software (DNA Star, Inc.).

Unless otherwise specified, BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., *J. Mol. Biol.*, 215:403-410 (1993) and *Nucleic Acids Res.*, 25:3389-3402 (1997)) searches were conducted to identity isolated sequences having similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein

Data Bank, the SWISS-PROT protein sequence database, EMBL and DDBJ databases). Query sequences were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). Sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database, using the BLASTX algorithm (Gish, W. and States, D. J. *Nature Genetics,* 3:266-272 (1993)) provided by the NCBI. The results of BLAST comparisons summarizing the sequence to which a query sequence had the most similarity are reported according to the % identity, % similarity, and Expectation value. "% Identity" is defined as the percentage of amino acids that are identical between the two proteins. "% Similarity" is defined as the percentage of amino acids that are identical or conserved between the two proteins. "Expectation value" estimates the statistical significance of the match, specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely by chance.

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" mean micromole(s), "g" means gram(s), "µg" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s) and "kB" means kilobase(s).

Transformation And Cultivation Of *Yarrowia lipolytica*

Yarrowia lipolytica strain ATCC #20362 was purchased from the American Type Culture Collection (Rockville, MD). *Y. lipolytica* strains were usually grown at 28 °C on YPD agar (1% yeast extract, 2% bactopeptone, 2% glucose, 2% agar).

Transformation of *Y. lipolytica* was performed according to the method of Chen, D. C. et al. (*Appl. Microbiol Biotechnol.*, 48(2):232-235 (1997)), unless otherwise noted. Briefly, *Yarrowia* was streaked onto a YPD plate and grown at 30 °C for approximately 18 hr. Several large

loopfuls of cells were scraped from the plate and resuspended in 1 mL of transformation buffer containing: 2.25 mL of 50% PEG, average MW 3350; 0.125 mL of 2 M Li acetate, pH 6.0; and, 0.125 mL of 2 M DTT. Then, approximately 500 ng of linearized plasmid DNA was incubated in 100 µl of resuspended cells, and maintained at 39 °C for 1 hr with vortex mixing at 15 min intervals. The cells were plated onto selection media plates and maintained at 30 °C for 2 to 3 days.

For selection of transformants, minimal medium ("MM") was generally used; the composition of MM is as follows: 0.17% yeast nitrogen base (DIFCO Laboratories, Detroit, MI) without ammonium sulfate or amino acids, 2% glucose, 0.1% proline, pH 6.1. Supplements of leucine, lysine and/or uracil were added as appropriate to a final concentration of 0.01% (thereby producing "MMLeu", "MMLys" and "MMU" selection media, each prepared with 20 g/L agar).

Alternatively, transformants were selected on 5-fluoroorotic acid ("FOA"; also 5-fluorouracil-6-carboxylic acid monohydrate) selection media, comprising: 0.17% yeast nitrogen base (DIFCO Laboratories) without ammonium sulfate or amino acids, 2% glucose, 0.1% proline, 75 mg/L uracil, 75 mg/L uridine, 900 mg/L FOA (Zymo Research Corp., Orange, CA) and 20 g/L agar.

Finally, High Glucose Media ("HGM") was prepared as follows, as a means to promote conditions of oleaginy: 6.3 g/L KH_2PO_4, 27 g/L K_2HPO_4 and 80 g/L glucose (pH 7.5).

The methodology used to create the strains identified herein as Y4001U1, Y4036U and L38 relied on site-specific recombinase systems. Briefly, the site-specific recombination system consists of two elements: (1) a recombination site having a characteristic DNA sequence [e.g., LoxP]; and, (2) a recombinase enzyme that binds to the DNA sequence specifically and catalyzes recombination (i.e., excision) between DNA sequences when two or more of the recombination sites are oriented in the same direction at a given interval on the same DNA molecule [e.g., Cre]. For the purposes herein, an integration construct was created comprising a target gene that was desirable to insert into the host genome

(i.e., a first selection marker [i.e., Ura3 or Leu2]) that was flanked by recombination sites. Following transformation and selection of the transformants, the first selection marker was excised from the chromosome by the introduction of a replicating plasmid carrying a second selection marker (i.e., Leu2 or sulfonylurea resistance [AHAS]) and a recombinase suitable to recognize the site-specific recombination sites introduced into the genome (i.e., Cre). Upon selection of those transformants carrying the second marker, the replicating plasmid was then cured from the host in the absence of selection and excision of the first selection marker from the cured strain's host genome was confirmed by loss of *Ura* or *Leu* prototrophy. This produced a transformant that possessed neither the first nor second selection marker, and thus the cured strain was available for another round of transformation using the first selection marker. Additional details concerning site-specific recombinase based methodology for use in *Yarrowia lipolytica* is described in PCT Publication No. WO 2006/052870.

The second selection marker gene utilized in pY117 (Example 16) was a native *Yarrowia lipolytica* acetohydroxyacid synthase (AHAS or acetolactate synthase; E.C. 4.1.3.18; GenBank Accession No. XM_501277) containing a single amino acid change (W497L) that confers sulfonyl urea herbicide resistance (SU^R; described in PCT Publication No. WO 2006/052870). AHAS is the first common enzyme in the pathway for the biosynthesis of branched-chain amino acids and it is the target of the sulfonylurea and imidazolinone herbicides.

Fatty Acid Analysis Of *Yarrowia lipolytica*

For fatty acid analysis, cells were collected by centrifugation and lipids were extracted as described in Bligh, E. G. & Dyer, W. J. (*Can. J. Biochem. Physiol.*, 37:911-917 (1959)). Fatty acid methyl esters were prepared by transesterification of the lipid extract with sodium methoxide (Roughan, G., and Nishida I., *Arch Biochem Biophys.*, 276(1):38-46 (1990)) and subsequently analyzed with a Hewlett-Packard 6890 GC fitted with a 30-m X 0.25 mm (i.d.) HP-INNOWAX (Hewlett-Packard) column.

The oven temperature was from 170 °C (25 min hold) to 185 °C at 3.5 °C/min.

For direct base transesterification, *Yarrowia* culture (3 mL) was harvested, washed once in distilled water, and dried under vacuum in a Speed-Vac for 5-10 min. Sodium methoxide (100 µl of 1 %) was added to the sample, and then the sample was vortexed and rocked for 20 min. After adding 3 drops of 1 M NaCl and 400 µl hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.

EXAMPLE 1

Pythium aphanidermatum Lipid Profile, Total RNA Isolation And Genomic DNA Isolation

A *Pythium aphanidermatum* strain was obtained from Lisa Hoffman (E.I. duPont de Nemours, Inc., Wlimington, DE).

The strain was grown on malt extract agar medium (Difco Laboratories, Detroit, MI) at room temperature for 3 days. Cells were scraped off the plate and resuspended in 600 µl of sodium methoxide dissolved in methanol. The sample was shaken for 20 min, and 50 µl of 1 M NaCl was added. After mixing, 600 µl of heptane was added. The sample was vortexed and centrifuged in an Eppendorf microfuge for 1 min. The upper layer was carefully separated from the lower layer and placed in a glass vial for GC analysis. The results of the analysis are shown below in Table 4. Fatty acids are identified as 16:0 (palmitate), 16:1 (palmitoleic acid), 18:0, 18:1 (oleic acid), 18:2, GLA, 20:1, 20:2, DGLA, ARA, EPA and DHA; and the composition of each is presented as a % of the total fatty acids.

Table 4

Lipid Profile Of *Pythium aphanidermatum* Cells

Fatty Acid	16:0	16:1	18:0	18:1	18:2	GLA
% of Total Fatty Acids	15.8	7.1	0	30.0	11.2	0.5

Fatty Acid	20:1	20:2	DGLA	ARA	EPA	DHA
% of Total Fatty Acids	1.3	0.5	0.7	7.8	13.4	0.3

Based on the presence of ARA and EPA, it was concluded that the *P. aphanidermatum* strain likely had both a Δ5 desaturase (capable of converting DGLA to ARA) and a Δ17 desaturase (capable of converting ARA to EPA).

Total RNA and genomic DNA were isolated from cells scraped off a malt extract agar plate using the Trizol reagent (Invitrogen, Carlsbad, CA). Specifically, scraped cells were resuspended in 1 mL water and centrifuged for 30 sec in an Eppendorf microfuge. The cell pellet was resuspended in 0.75 mL Trizol reagent, mixed with 0.75 mL of 0.5 mm glass beads, and homogenized in a Biospec mini beadbeater (Bartlesville, OK) at the highest setting for 3 min. The mixture was centrifuged in an Eppendorf centrifuge for 30 sec at 14,000 rpm to remove debris and glass beads. The supernatant was extracted with 150 µl of 24:1 chloroform:isoamyl alcohol (Invitrogen). The upper aqueous phase was used for RNA isolation and the lower organic phase for DNA isolation.

For RNA isolation, the aqueous phase was mixed with 0.375 mL of isopropyl alcohol and allowed to incubate at room temperature for 5 min. Precipitated RNA was collected by centrifugation at 8000 rpm and 4 °C for 5 min. The pellet was washed once with 0.7 mL of 80% ethanol and air-dried. Total RNA (59 µg) was obtained (i.e., 200 µl of sample at 29.5 µg/µl).

For genomic DNA isolation, the lower organic phase of the sample was mixed with 225 µl of ethanol and incubated at room temperature for 5 min. The sample was then centrifuged at 5000 rpm for 2 min in an

Eppendorf centrifuge. The pellet was washed with 0.75 mL of 0.1 M sodium citrate/10% ethanol twice. Each time the sample was incubated for 15 min at room temperature in the wash solution, followed by centrifugation at 5000 rpm for 5 min at 4 °C in an Eppendorf centrifuge. The pellet was air dried and re-dissolved in 300 µl of 8 mM NaOH. The pH of the sample was adjusted to 7.5 with 1 M HEPES, and then further purified with a Qiagen PCR purification kit exactly as described in the manufacturer's protocol. A total of 7.2 µg of *P. aphanidermatum* genomic DNA was obtained.

EXAMPLE 2

Pythium aphanidermatum cDNA Synthesis

Double-stranded cDNA was synthesized directly from the *Pythium aphanidermatum* total RNA using the BD-Clontech Creator™ Smart™ cDNA library kit (Mississauga, ON, Canada). Specifically, 3 µl of total RNA sample (0.9 µg) was mixed with 1 µl of SMART™ IV oligonucleotide (SEQ ID NO:9) and 1 µl CDSIII/3' PCR primer (SEQ ID NO:10). The mixture was heated to 75 °C for 5 min, and cooled on ice for 5 min. Two (2) µl of 5X first strand buffer, 1 µl of 20 mM DTT, 1 µl of dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP) and 1 µl of PowerScript reverse transcriptase were added to the mixture. The sample was incubated at 42 °C for 1 hr.

The resulting first strand cDNA synthesis mixture was then used as template for PCR amplification. The reaction mixture contained 2 µl of the above first strand cDNA sample, 80 µl of water, 10 µl of 10X Advantage 2 PCR buffer, 2 µl 50X dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 2 µl of 5' PCR primer (SEQ ID NO:11), 2 µl CDSIII/3' PCR primer (SEQ ID NO:10) and 2 µl 50X Advantage 2 polymerase mix. The thermocycler conditions were set for 95 °C for 1 min and then 20 cycles of 95 °C for 10 sec and 68 °C for 6 min.

Amplification product was purified with a Qiagen PCR purification kit following the manufacturer's protocol exactly. Purified cDNA product was eluted with 50 µl of water.

EXAMPLE 3

Isolation Of A Portion Of The Coding Region Of The *Pythium aphanidermatum* Δ17 Desaturase Gene

The present Example describes the identification of a portion of the *Pythium aphanidermatum* gene encoding Δ17 desaturase (designated herein as "PaD17" (SEQ ID NOs:1 and 2)), by use of primers derived from conserved regions of other known Δ17 desaturase sequences.

The *P. aphanidermatum* cDNA sample from Example 2 was used as template for PCR using degenerated primers designed to amplify portions of the potential Δ17 desaturase gene, based on the Δ17 fatty acid desaturase sequences of *Phytophthora sojae* (SEQ ID NO:45; U.S. Patent Application No. 11/787772, filed April 18, 2007; see also Example 11, *infra*) and *Phytophthora ramorum* (SEQ ID NO:47; U.S. Patent Application No. 11/787772, filed April 18, 2007; see also Example 13, *infra*). Based on the alignment provided herein as Figure 2, degenerate primers were designed as shown in Table 5 (location of primers with respect to SEQ ID NOs:45 and 47 are shown as dotted boxes on Figure 2).

Table 5

Degenerate Oligonucleotides Used To Amplify The Δ17 Desaturase Gene From *Pythium aphanidermatum*

Primer	Nucleotide Sequence	Amino Acid Sequence
PD17-F1	TTYTGGGGNTTYTTYACNGT (SEQ ID NO:12)	FWGFFTY (SEQ ID NO:13)
PD17-F2	TTCTTYACNGTNGGNCAYGA (SEQ ID NO:14)	FFTVGHD (SEQ ID NO:16)
PD17-F3	TTTTTYACNGTNGGNCAYGA (SEQ ID NO:15)	FFTVGHD (SEQ ID NO:16)
PD17-F4	ACNCAYCGNCAYCAYCAYAA (SEQ ID NO:17)	THRHHHK (SEQ ID NO:19)
PD17-F5	ACNCAYAGRCAYCAYCAYAA (SEQ ID NO:18)	THRHHHK (SEQ ID NO:19)
PD17-F6	AARAAYACNGGNAAYATYGA (SEQ ID NO:20)	KNTGNID (SEQ ID NO:22)
PD17-F7	AARAAYACNGGNAAYATAGA (SEQ ID NO:21)	KNTGNID (SEQ ID NO:22)
PD17-R1	TCRTCRTTRTGRTGNAGRAA	FLHHNDE

	(SEQ ID NO:23)	(SEQ ID NO:25)
PD17-R2	TCRTCRTTRTGRTGYAARAA (SEQ ID NO:24)	FLHHNDE (SEQ ID NO:25)
PD17-R3	AARAARGCYTTDATDATNGG (SEQ ID NO:26)	PIIKAFF (SEQ ID NO:28)
PD17-R4	AARAAYGCYTTDATDATNGG (SEQ ID NO:27)	PIIKAFF (SEQ ID NO:28)
PD17-R5	TTRTGNGTNCCDATRTTATG (SEQ ID NO:29)	HNIGTHQ (SEQ ID NO:31)
PD17-R6	TTRTGNGTNCCDATRTTGTG (SEQ ID NO:30)	HNIGTHQ (SEQ ID NO:31)
PD17-R7	CCYTTNACRTANGTCCAYTC (SEQ ID NO:32)	EWTYVKG (SEQ ID NO:33)

[Note: The nucleic acid degeneracy code used for SEQ ID NOs:12, 14, 15, 17, 18, 20, 21, 23, 24, 26, 27, 29, 30 and 32 was as follows: R= A/G; Y=C/T; D=A/G/T; and N=A/C/T/G.]

A total of 49 different PCR amplification reactions were performed, using all possible combinations of the 7 forward and 7 reverse primers. Each reaction mixture contained 1 µl of 1:10 diluted *P. aphanidermatum* cDNA, 5 µl each of the forward and reverse primers (20 µM), 14 µl water and 25 µl of TaKaRa ExTaq 2X premix (TaKaRa Bio, Mountain View, CA). The thermocycler conditions were set for 94 °C for 1 min, then 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. PCR products were analyzed by electrophoresis on standard agarose gels, and putative Δ17 desaturase fragments were detected as shown below in Table 6.

Table 6

Detected Putative Δ17 Desaturase Fragments

Product	Forward Primer	Reverse Primer
~460 bp fragment	PD17-F1	PD17-R5
~400 bp fragment	PD17-F4	PD17-R2
~350 bp fragment	PD17-F6	PD17-R2

Each of the fragments described above in Table 6 were purified with a Qiagen PCR purification kit (Valencia, CA), cloned into pCR2.1-TOPO (Invitrogen) and sequenced.

BLAST sequence analysis showed that each of the fragments were from a single gene that showed extensive homology to the known Δ17 desaturases from other organisms. The sequences were assembled into a 614 bp contig (SEQ ID NO:5), which was assumed to encode a putative Δ17 desaturase from *P. aphanidermatum*.

EXAMPLE 4

Isolation Of The Full-Length Δ17 Desaturase From *Pythium aphanidermatum*

Primers were designed to isolate the 5' and 3' ends of the putative Δ17 desaturase gene from cDNA and genomic DNA samples of *P. aphanidermatum*, based on the partial sequence set forth in SEQ ID NO:5 and described in Example 3.

The 5' region of the putative Δ17 desaturase from *P. aphanidermatum* was isolated by genome walking using the Universal GenomeWalker™ kit (BD Biosciences Clonetech, Palo Alto, CA), according to the manufacturer's protocol. First, genomic DNA from *P. aphanidermatum* (1 μg per digestion) was digested with *Dra*I, *Eco*RV, *Pvu*II and *Stu*I separately. Digested DNA samples were purified with Qiagen enzyme reaction clean-up kits according to the manufacturer's protocol and each sample was eluted with 20 μl of water.

The digested genomic DNA samples were ligated with Universal GenomeWalker™ adaptor (SEQ ID NOs:34 [top strand] and 35 [bottom strand]), as shown below:

5'-GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
3'-H2N-CCCGACCA-5'

Specifically, 4 μl each of the digested DNA was mixed with 1.9 μl of 25 μM GenomeWalker™ adaptor, 1.6 μl of 10X ligation buffer and 0.5 μl of T4 DNA ligase. The reaction was carried out overnight at 16 °C. After heating at 70 °C for 5 min, 72 μl of 10 mM Tris, 1 mM EDTA, pH 7.4 buffer was added to each reaction mixture. These reaction mixtures were then used as template for PCR amplification.

For the first round of PCR, primers PUD17-5-1 (SEQ ID NO:36) and Universal GenomeWalker™ primer AP1 (SEQ ID NO:37) from the kit were used. The reaction mixture contained 1 μl of each primer at 10 μM, 2 μl of the purified ligation products as template, 21 μl water and 25 μl of TaKaRa ExTaq 2X premix. The thermocycler conditions were set for 94 °C for 90 sec, then 30 cycles at 94 °C for 20 sec, 55 °C for 20 sec and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min.

PCR products were diluted 1:20, and 1 μl of diluted PCR product was used as template for a second round of PCR using primers PUD17-5-3 (SEQ ID NO:38) and Universal GenomeWalker™ primer AP2 (SEQ ID NO:39). PCR components and amplification conditions were as described above.

A ~750 bp DNA fragment was generated from the second-round of PCR. This fragment was purified with a Qiagen PCR purification kit, cloned into pCR2.1-TOPO (Invitrogen) and sequenced. Subsequent sequence analysis showed that this fragment contained the 5' end of the putative Δ17 desaturase gene, including the translation initiation codon and 387 bp of additional untranslated 5' sequence. The 5' fragment (SEQ ID NO:6) shared significant homology to the *Saprolegnia diclina* Δ17 desaturase (GenBank Accession No. AAR20444; SEQ ID NO:95).

The 3' region of the putative Δ17 desaturase was isolated by PCR amplification using *P. aphanidermatum* cDNA as template. Primers PUD17-3-1 (SEQ ID NO:40) and CDSIII/3' PCR primer (SEQ ID NO:10; from BD-Clontech Creator™ Smart™ cDNA library construction kit, see Example 1) were used for the first round of amplification. The reaction mixture contained 1 μl of each primer (10 μM), 1 μl of *P. aphanidermatum* cDNA, 22 μl water and 25 μl TaKaRa ExTaq 2X premix. The thermocycler conditions were set for 94 °C for 90 sec, then 30 cycles at 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec, followed by a final extension at 72 °C for 5 min.

PCR product was diluted 1:20, and 1 μl of the diluted product was used as template for a second round of PCR using PUD17-3-2 (SEQ ID

NO:41) and CDSIII/3' PCR primer (SEQ ID NO:10), using components and amplification conditions as described above. The second round PCR generated a ~550 bp DNA fragment. This was purified with a Qiagen PCR purification kit, cloned into pCR2.1-TOPO and sequenced. Sequence analysis showed that this fragment contained the 3'-region of the putative Δ17 desaturase cDNA, including the polyA tail. The 3' fragment (SEQ ID NO:7) shared significant homology to the *Saprolegnia diclina* Δ17 desaturase (GenBank Accession No. AAR20444; SEQ ID NO:95).

Assembly of the 5' genomic region (SEQ ID NO:6), the original partial cDNA sequence (SEQ ID NO:5) and the 3' cDNA sequence (SEQ ID NO:7) resulted in a 1533 bp contig (SEQ ID NO:8), comprising the complete sequence of the putative Δ17 desaturase from *P. aphanidermatum* and additional untranslated 5' and 3' ends. The coding region of SEQ ID NO:8, which is set forth as SEQ ID NO:1, is 1080 bp long (corresponding to bases 388-1467 of SEQ ID NO:8) and encodes a peptide of 359 amino acids (SEQ ID NO:2). The coding sequence of *Pythium aphanidermatum* was designated herein as "PaD17".

The results of BLAST searches using the full length PaD17 gene (i.e., SEQ ID NO:1) as the query sequence showed that it shared 58% identity and 71% similarity with the amino acid sequence of the Δ17 desaturase of *Saprolegnia diclina* (GenBank Accession No. AAR20444), with an Expectation value of e-121; additionally, it shared identity and similarity with other omega-3 desaturases.

Similarly, pairwise comparison between and among Δ17 desaturase proteins from *Phytophthora infestans* ("PiD17"; SEQ ID NO:43), *Phytophthora sojae* ("PsD17"; SEQ ID NO:45), *Phytophthora ramorum* ("PrD17"; SEQ ID NO:47) and *Pythium aphanidermatum* ("PaD17"; SEQ ID NO:2) using a Clustal W analysis (MegAlign™ program of DNASTAR software) resulted in the following percent similarities: 74.5% between PiD17 and PaD17; 75.0% between PrD17 and PaD17; and 75.3% between PsD17 and PaD17.

EXAMPLE 5

Generation Of *Yarrowia lipolytica* Expression Vectors Comprising The *Pythium aphanidermatum* Δ17 Desaturase ("PaD17")

The present Example describes the construction of plasmids pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4, each comprising a chimeric FBAINm::PaD17*::XPR gene, wherein PaD17* (SEQ ID NO:3) comprises up to (and including) 2 amino acid mutations with respect to SEQ ID NO:2. Plasmids pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4 were utilized to test functional expression of PaD17*, as described in Example 7, *infra*.

Plasmids pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4 were constructed by three-way ligation using fragments from plasmid pFmD8S, a 5' portion of PaD17 and a 3' portion of PaD17. Plasmid pFmD8S (SEQ ID NO:51; Figure 3D) was constructed by three-way ligation using fragments from plasmids pKUNFmkF2, pDMW287F and pDMW214.

Plasmid pKUNFmkF2

pKUNFmkF2 (SEQ ID NO:48; Figure 3A; PCT Publication No. WO 2006/012326) is a construct comprising a chimeric FBAINm::F.D12::Lip2 gene (wherein "FBAINmK" is the *Yarrowia lipolytica* FBAINm promoter [PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356], "F.D12" is the *Fusarium moniliforme* Δ12 desaturase [PCT Publication No. WO 2005/047485], and "Lip2" is the *Yarrowia lipolytica* Lip2 terminator sequence (GenBank Accession No. AJ012632)).

Plasmid pDMW287F

pDMW287F (SEQ ID NO:49; Figure 3B; PCT Publication No. WO 2006/012326) is a construct comprising a synthetic Δ8 desaturase ("EgD8S"; SEQ ID NO:52 herein), derived from wildtype *Euglena gracilis,* and codon-optimized for expression in *Yarrowia lipolytica* (wherein EgD8S is identified as "D8SF" in the Figure). The desaturase gene is flanked by a *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356; identified as "FBA1+intron" in the Figure) and a Pex16 terminator sequence of the *Yarrowia Pex16* gene (GenBank Accession No. U75433).

Plasmid pDMW214

pDMW214 (SEQ ID NO:50; Figure 3C; PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356) is a shuttle plasmid that replicates both in *E. coli* and *Yarrowia lipolytica*. It contained the following components:

Table 7

Description Of Plasmid pDMW214 (SEQ ID NO:50)

RE Sites And Nucleotides Within SEQ ID NO:50	Description Of Fragment And Chimeric Gene Components
1150-270	ColE1 plasmid origin of replication
2080-1220	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
2979-4256	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
PmeI/SphI 6501-4256	*Yarrowia* Leu2 gene (GenBank Accession No. AF260230)
6501-1	FBA1+intron::GUS::XPR, comprising: • FBA1+intron: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • GUS: *E. coli* gene encoding β-glucuronidase (Jefferson, R.A., *Nature*, 342:837-838 (1989)); • XPR: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)

Plasmid pFmD8S

The *PmeI/NcoI* fragment of plasmid pKUNFmkF2 (Figure 3A; comprising the FBAINm promoter) and the *NcoI/NotI* fragment of plasmid pDMW287F (Figure 3B; comprising the synthetic Δ8 desaturase gene "EgD8S") were used directionally to replace the *PmeI/Not* I fragment of pDMW214 (Figure 3C). This resulted in generation of pFmD8S (SEQ ID NO:51; Figure 3D), comprising a chimeric FBAINm::EgD8S::XPR gene. Thus, the components of pFmD8S are as described in Table 8 below.

Table 8

Components Of Plasmid pFmD8S (SEQ ID NO:51)

RE Sites And Nucleotides Within SEQ ID NO:51	Description Of Fragment And Chimeric Gene Components
Swa I/Sac II (7988-1461)	FBAINm::EgD8S::XPR, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • EgD8S: codon-optimized Δ8 desaturase gene (SEQ ID NO:52, identified as "D8-corrected" in Figure 3D), derived from *E. gracilis* (PCT Publication No. WO 2006/012326); • XPR: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)
2601-1721	ColE1 plasmid origin of replication
3531-2671	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
4430-5734	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
7942-5741	*Yarrowia Leu2* gene (GenBank Accession No. AF260230)

Generation Of Plasmids pFmD17-1, pFmD17-2, pFmD17-3 And pFmD17-4

The *P. aphanidermatum* Δ17 desaturase was amplified from cDNA via a reaction mixture that contained: 1 μl of 20 μM forward primer PUD17-F (SEQ ID NO:54), 1 μl of 20 μM reverse primer PUD17-R (SEQ ID NO:55), 1 μl *P. aphanidermatum* cDNA, 10 μl 5X PCR buffer, 1 μl dNTP mix (10 μM each), 35 μl water and 1 μl Phusion polymerase (New England Biolabs). The thermocycler conditions were set for 98 °C for 1 min, then 30 cycles at 98 °C for 10 sec, 55 °C for 10 sec and 72 °C for 30 sec, followed by a final extension at 72 °C for 5 min.

The PCR product was cloned into pCR2.1-TOPO (Invitrogen) and 8 individual clones were sequenced. Based on the sequence results, 2 clones (i.e., clone 2 and clone 4) were used to construct the final expression plasmid. Clone 2 contained a 351A to T mutation with respect to SEQ ID NO:2, while clone 4 contained a 155S to P mutation with respect to SEQ ID NO:2; thus, they differed from one another by two

conservative amino acid substitutions and they each differed from the wildtype cDNA PaD17 sequence set forth in SEQ ID NO:2 by one conservative amino acid substitution.

Each clone was digested with *Nco*I and *Bgl*II to generate a ~370 bp fragment that contained the 5' region of the Δ17 desaturase cDNA; and, each clone was also digested with *Bgl*II and *Not*I to generate a 710 bp fragment that contained the 3' region of the cDNA. The ~370 bp fragment comprising the 5' region of the Δ17 desaturase and the 710 bp fragment comprising the 3' region of the Δ17 desaturase were ligated into pFmD8S predigested with *Nco*I and *Not*I (such that the codon-optimized Δ8 desaturase gene ["EgD8S"] was excised from the plasmid) in a three-way ligation reaction. The reaction mixture contained 10 μl 2X ligation buffer and 1 μl T4 DNA ligase (Promega), 4 μl each of the 5' and the 3' Δ17 desaturase fragments (~300 ng each) and 1 μl pFmD8S (~150 ng).

Using the above methodology, the components of the newly created expression plasmids pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4 are identical to those described in Table 8 for pFmD8S (SEQ ID NO:51), with the exception that the pFmD17 vectors possessed chimeric FBAINm::PaD17*::XPR genes instead of the chimeric FBAINm::EgD8S::XPR gene within pFmD8S. The notation of "PaD17*" corresponds to the below mutations with respect to SEQ ID NO:2 (i.e., the amino acid of PaD17 as described in Example 4). The null mutation, 155S to P mutation, 351A to T mutation, and 155S to P and 351A to T mutations are each encompassed in SEQ ID NO:3, hereinafter referred to as PaD17*. Based on the combination of the two clones, the four variant expression plasmids contained the following mutations, as shown below in Table 9.

Table 9

Variant pFmD17 *Yarrowia lipolytica* Expression Vectors Comprising Chimeric FBAINm::PaD17*::XPR Genes

Plasmid	5' Fragment	3' Fragment	Mutation With Respect To SEQ ID NO:2
pFmD17-1	clone 2	clone 2	351A to T
pFmD17-2	clone 4	clone 4	155S to P
pFmD17-3	clone 2	clone 4	None
pFmD17-4	clone 4	clone 2	155S to P, 351A to T

Each reaction mixture was incubated at room temperature for 2 hrs and used to transform *E. coli* Top10 competent cells. Plasmid DNA from transformants was recovered with Qiagen Miniprep kits.

EXAMPLE 6

Generation Of *Yarrowia lipolytica* Strain Y2047 To Produce About 11% ARA Of Total Lipids Via The Δ6 Desaturase/Δ6 Elongase Pathway

The present Example describes the construction of strain Y2047, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing 11% ARA relative to the total lipids via expression of a Δ6 desaturase/Δ6 elongase pathway (Figure 4A). This strain was utilized to test the functional expression of PaD17* in Example 7, *infra*.

Yarrowia lipolytica strain Y2047 has been deposited under the terms of the Budapest Treaty and bears the ATCC number PTA-7186. Additionally, construction of Y2047 has been described in co-pending U.S. Patent Application No. 11/265761 (Patent Publication No. US 2006-0115881 A1 and PCT Publication No. WO 2006/052870), herein incorporated by reference.

The development of strain Y2047 first required the construction of strain M4 (producing 8% DGLA).

Generation Of M4 Strain To Produce About 8% DGLA Of Total Lipids

Construct pKUNF12T6E (Figure 4B; SEQ ID NO:56) was generated to integrate four chimeric genes (comprising a Δ12 desaturase, a Δ6 desaturase and two $C_{18/20}$ elongases) into the *Ura3* loci of wild type

Yarrowia strain ATCC #20362, to thereby enable production of DGLA.

The pKUNF12T6E plasmid contained the following components:

Table 10

Description Of Plasmid pKUNF12T6E (SEQ ID NO:56)

RE Sites And Nucleotides Within SEQ ID NO:56	Description Of Fragment And Chimeric Gene Components
AscI/BsiWI (9420-8629)	784 bp 5' portion of *Yarrowia Ura3* gene (GenBank Accession No. AJ306421)
SphI/PacI (12128-1)	516 bp 3' portion of *Yarrowia Ura3* gene (GenBank Accession No. AJ306421)
SwaI/BsiWI (6380-8629)	FBAIN::EL1S::Pex20, comprising: • FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • EL1S: codon-optimized elongase 1 gene (PCT Publication No. WO 2004/101753), derived from *Mortierella alpina* (GenBank Accession No. AX464731); • Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613)
BglII/SwaI (4221-6380)	TEF::Δ6S::Lip1, comprising: • TEF: *Yarrowia lipolytica* TEF promoter (GenBank Accession No. AF054508); • Δ6S: codon-optimized Δ6 desaturase gene (PCT Publication No. WO 2004/101753; U.S. Patent 7,125,672), derived from *Mortierella alpina* (GenBank Accession No. AF465281); • Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020)
PmeI/ClaI (4207-1459)	FBA::F.Δ12::Lip2, comprising: • FBA: *Yarrowia lipolytica* FBA promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • F.Δ12: *Fusarium moniliforme* Δ12 desaturase gene (PCT Publication No. WO 2005/047485); • Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632)
ClaI/PacI (1459-1)	TEF::EL2S::XPR, comprising: • TEF: TEF promoter (GenBank Accession No. AF054508); • EL2S: codon-optimized elongase gene (SEQ ID NO:57), derived from *Thraustochytrium aureum* (U.S. Patent 6,677,145);

	• XPR: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)

The pKUNF12T6E plasmid was digested with *AscI*/*SphI*, and then used for transformation of wild type Y. *lipolytica* ATCC #20362 according to the General Methods. The transformant cells were plated onto FOA selection media plates and maintained at 30 °C for 2 to 3 days. The FOA resistant colonies were picked and streaked onto MM and MMU selection plates. The colonies that could grow on MMU plates but not on MM plates were selected as *Ura*- strains. Single colonies of *Ura*- strains were then inoculated into liquid MMU at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of DGLA in the transformants containing the 4 chimeric genes of pKUNF12T6E, but not in the wild type *Yarrowia* control strain. Most of the selected 32 *Ura*⁻ strains produced about 6% DGLA of total lipids. There were 2 strains (i.e., strains M4 and 13-8) that produced about 8% DGLA of total lipids.

Generation Of Y2047 Strain To Produce About 11% ARA Of Total Lipids

Construct pDMW271 (Figure 4C; SEQ ID NO:59) was generated to integrate three Δ5 chimeric genes into the *Leu2* gene of *Yarrowia* strain M4. Plasmid pDMW271 contained the following components, as described in Table 11:

Table 11

Description Of Plasmid pDMW271 (SEQ ID NO:59)

RE Sites And Nucleotides Within SEQ ID NO:59	Description Of Fragment And Chimeric Gene Components
AscI/*BsiWI* (5520-6315)	788 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)
SphI/*PacI* (2820-2109)	703 bp 3' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)

SwaI/BsiWI (8960-6315)	FBAIN::MAΔ5::Pex20, comprising: • FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • MAΔ5: *Mortierella alpina* Δ5 desaturase gene (GenBank Accession No. AF067654); • Pex20: Pex20 terminator sequence of *Yarrowia Pex20* gene (GenBank Accession No. AF054613)
SwaI/ClaI (8960-11055)	TEF::MAΔ5::Lip1, comprising: • TEF: TEF promoter (GenBank Accession No. AF054508); • MAΔ5: *Mortierella alpina* Δ5 desaturase gene (GenBank Accession No. AF067654); • Lip1: Lip1 terminator sequence of *Yarrowia Lip1* gene (GenBank Accession No. Z50020)
PmeI/ClaI (12690-11055)	*Yarrowia Ura3* gene (GenBank Accession No. AJ306421)
ClaI/PacI (1-2109)	TEF::HΔ5S::Pex16, comprising: • TEF: TEF promoter (GenBank Accession No. AF054508); • HΔ5S: codon-optimized Δ5 desaturase gene (SEQ ID NO:60), derived from *Homo sapiens* (GenBank Accession No. NP_037534); • Pex16: Pex16 terminator sequence of *Yarrowia Pex16* gene (GenBank Accession No. U75433)

Plasmid pDMW271 was digested with *AscI/SphI*, and then used to transform strain M4 according to the General Methods. Following transformation, the cells were plated onto MMLeu plates and maintained at 30 °C for 2 to 3 days. The individual colonies grown on MMLeu plates were picked and streaked onto MM and MMLeu plates. Those colonies that could grow on MMLeu plates but not on MM plates were selected as *Leu2*⁻ strains. Single colonies of *Leu2*⁻ strains were then inoculated into liquid MMLeu media at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of ARA in pDMW271 transformants, but not in the parental M4 strain. Specifically, among the 48 selected *Leu2*⁻ transformants with pDMW271, there were 35 strains that produced less than 5% ARA of total lipids, 12 strains that produced 6-

8% ARA, and 1 strain that produced about 11% ARA of total lipids in the engineered *Yarrowia*. The strain that produced 11% ARA was named "Y2047".

EXAMPLE 7

Functional Analysis Of The *Pythium aphanidermatum* Δ17 Desaturase ("PaD17*") In *Yarrowia lipolytica* Strain Y2047

The present Example describes functional analysis of PaD17* in *Yarrowia lipolytica* strain Y2047 (Example 6). Thus, following transformation of the variant pFmD17 plasmids comprising PaD17* (from Example 5), lipid profiles within the transformant organisms were compared.

Transformation Of *Yarrowia lipolytica*

Plasmids pFmD17-1, pFmD17-2, pFm17-3 and pFmD17-4 (comprising the chimeric FBAINm::PaD17*::XPR genes) were transformed into *Yarrowia lipolytica* strain Y2047 as described in the General Methods. The transformant cells were plated onto MM plates lacking uracil and maintained at 30 °C for 2 to 3 days. Then, single colonies of transformant *Yarrowia lipolytica* were patched onto fresh MM plates lacking uracil and allowed to grow at 30 °C for 1 day. The patches were then used to inoculate 3 mL MM liquid medium. Cells were grown for 2 days in MM medium and then 4 days in HGM medium. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC, as described in the General Methods.

As shown in Table 12, GC analyses demonstrated conversion of ARA to EPA in each of the clones comprising pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4, respectively. Composition of ARA and EPA are presented as a % of the total fatty acids. The conversion efficiency ("Conv. Effic.") was measured according to the following formula: ([product]/[substrate+product])*100, where 'product' includes the immediate product and all products in the pathway derived from it.

Table 12

Comparison Of Fatty Acid Composition In *Yarrowia* Strain Y2047 Transformed With pFmD17-1, pFmD17-2, pFmD17-3 and pFmD17-4

Clone	Plasmid	Mutation With Respect To SEQ ID NO:2	% ARA	% EPA	Conv. Effic
1	pFmD17-1	351A to T	3.99	1.09	21.46
2	pFmD17-1	351A to T	3.98	1.2	23.17
3	pFmD17-2	155S to P	4.22	1.06	20.08
4	pFmD17-2	155S to P	4.22	1.07	20.23
5	pFmD17-2	155S to P	4.22	1.07	20.23
6	pFmD17-3	None	4.17	0.94	18.40
7	pFmD17-3	None	4.04	0.98	19.52
8	pFmD17-3	None	4.04	0.92	18.55
9	pFmD17-4	155S to P, 351A to T	4.01	1.22	23.33
10	pFmD17-4	155S to P, 351A to T	4.01	1.31	24.62
11	pFmD17-4	155S to P, 351A to T	3.99	1.09	21.46

The conversion efficiency whereby PaD17* converted ARA to EPA ranged from 18.4 to 24.6%. More specifically, the experimental data demonstrated that the cloned cDNA from *P. aphanidermatum* (SEQ ID NO:2; PaD17) that was present in vector pFmD17-3 functioned as a Δ17 desaturase, efficiently desaturating ARA to EPA (conversion efficiency ranged from 18.4% to 19.52%); however, neither the Ser at amino acid position 155 of SEQ ID NO:2 nor the Ala at amino acid position 351 of SEQ ID NO:2 were required for enzyme activity. The PaD17* variants encoded by SEQ ID NO:3 comprising the 155S to P mutation, the 351A to T mutation, or both mutations (expressed in pFmD17-2, pFmD17-1 and pFmD17-4, respectively) all had greater conversion efficiency than that of PaD17 (SEQ ID NO:2) in pFmD17-3. Transformant cells demonstrating the highest Δ17 desaturase conversion efficiency were those expressing vector pFmD17-4, comprising the PaD17* variant with the S155 to P and A351 to T mutations (SEQ ID NO:3).

EXAMPLE 8

Synthesis Of A Codon-Optimized Δ17 Desaturase Gene Of *Pythium aphanidermatum* ("PaD17S") For *Yarrowia lipolytica*

The codon usage of the Δ17 desaturase gene of *Pythium aphanidermatum* (SEQ ID NOs:1 and 2) was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in PCT Publication No. WO 2004/101753 and U.S. Patent 7,125,672. Specifically, a codon-optimized Δ17 desaturase gene of *Pythium aphanidermatum* (designated "PaD17S", SEQ ID NO:4) was designed based on the coding sequence of PaD17, according to the *Yarrowia* codon usage pattern (PCT Publication No. WO 2004/101753), the consensus sequence around the 'ATG' translation initiation codon, and the general rules of RNA stability (Guhaniyogi, G. and J. Brewer, *Gene*, 265(1-2):11-23 (2001)). In addition to modification of the translation initiation site, 188 bp of the 1080 bp coding region (including the stop codon) were modified (17.4%; Figure 5A and 5B) and 175 codons were optimized (48.6%). The GC content was reduced from 61.8% within the wild type gene (i.e., PaD17) to 54.5% within the synthetic gene (i.e., PaD17S). A *NcoI* site and a *NotI* site were incorporated around the translation initiation codon and after the stop codon of PaD17S, respectively. None of the modifications in the codon-optimized gene changed the amino acid sequence of the encoded protein (SEQ ID NO:2). The designed PaD17S gene (SEQ ID NO:4) was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pPaD17S (SEQ ID NO:62).

EXAMPLE 9

Generation Of *Yarrowia lipolytica* Strain Y4070 To Produce About 12% ARA Of Total Lipids *Via* The Δ9 Elongase/Δ8 Desaturase Pathway

The present Example describes *Yarrowia lipolytica* strain Y4070, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing about 12% ARA relative to the total lipids via expression of a Δ9 elongase/Δ8 desaturase pathway (Figure 6A). Strain Y4070 was utilized to test the functional expression of PaD17S in Example 10, *infra*.

The development of strain Y4070 required the construction of strain Y2224 (a FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362), strain Y4001 (producing 17% EDA with a *Leu-* phenotype), strain Y4001U (producing 17% EDA with a *Leu-* and *Ura-* phenotype), strain Y4036 (producing 18% DGLA with a *Leu-* phenotype) and strain Y4036U (producing 18% DGLA with a *Leu-* and *Ura-* phenotype).

Generation Of Strain Y2224

Strain Y2224 was isolated in the following manner: *Yarrowia lipolytica* ATCC #20362 cells from a YPD agar plate (1% yeast extract, 2% bactopeptone, 2% glucose, 2% agar) were streaked onto a MM plate (75 mg/L each of uracil and uridine, 6.7 g/L YNB with ammonia sulfate, without amino acid, and 20 g/L glucose) containing 250 mg/L 5-FOA (Zymo Research). Plates were incubated at 28 °C and four of the resulting colonies were patched separately onto MM plates containing 200 mg/mL 5-FOA and MM plates lacking uracil and uridine to confirm uracil *Ura3* auxotrophy.

Generation Of Strain Y4001 To Produce About 17% EDA Of Total Lipids

Strain Y4001 was created via integration of construct pZKLeuN-29E3 (Figure 6B). This construct, comprising four chimeric genes (i.e., a Δ12 desaturase, a $C_{16/18}$ elongase and two Δ9 elongases), was integrated into the *Leu2* loci of strain Y2224 to thereby enable production of EDA.

Construct pZKLeuN-29E3 contained the components shown in Table 13.

TABLE 13

Description of Plasmid pZKLeuN-29E3 (SEQ ID NO:63)

RE Sites And Nucleotides Within SEQ ID NO:63	Description Of Fragment And Chimeric Gene Components
BsiW I/Asc I (7797-7002)	788 bp 3' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)
Sph I/Pac I (4302-3591)	703 bp 5' portion of *Yarrowia Leu2* gene (GenBank Accession No. AF260230)

Swa I/BsiW I (10500-7797)	GPD::F.D12::Pex20, comprising: • GPD: *Yarrowia lipolytica* GPD promoter (PCT Publication No. WO 2005/003310); • F.D12: *Fusarium moniliforme* Δ12 desaturase gene (PCT Publication No. WO 2005/047485); • Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613)
Bgl II/Swa I (12526-10500)	Exp pro::EgD9E::Lip1, comprising: • Exp pro: *Yarrowia lipolytica* export protein (EXP1) promoter (PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761); • EgD9E: codon-optimized Δ9 elongase (SEQ ID NO:64), derived from *Euglena gracilis* ("EgD9eS"; U.S. Patent Applications No. 11/601563 and No. 11/601564); • Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020)
Pme I/Cla I (12544-1)	FBAINm::EgD9S::Lip2, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805); • EgD9S: codon-optimized Δ9 elongase gene (SEQ ID NO:64), derived from *Euglena gracilis* ("EgD9eS"; U.S. Patent Applications No. 11/601563 and No. 11/601564); • Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632)
Cla I/EcoR I (1-1736)	LoxP::*Ura3*::LoxP, comprising: • LoxP sequence (SEQ ID NO:66); • *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421); • LoxP sequence (SEQ ID NO:66)
EcoR I/Pac I (1736-3591)	YAT::ME3S::Pex16, comprising: • NT: *Yarrowia lipolytica* YAT1 promoter (Patent Publication No. US 2006/0094102-A1); • ME3S: codon-optimized $C_{16/18}$ elongase gene (SEQ ID NO:67), derived from *M. alpina* (U.S. Patent Application No. 11/253882 and also PCT Publication No. WO 2006/052870); • Pex16: Pex16 terminator sequence of *Yarrowia Pex 16* gene (GenBank Accession No. U75433)

Plasmid pZKLeuN-29E3 was digested with *Asc I/Sph* I, and then used for transformation of *Y. lipolytica* strain Y2224 (i.e., ATCC #20362 *Ura3*-) according to the General Methods. The transformant cells were plated onto MMLeu media plates and maintained at 30 °C for 2 to 3 days. The colonies were picked and streaked onto MM and MMLeu selection

plates. The colonies that could grow on MMLeu plates but not on MM plates were selected as *Leu-* strains. Single colonies of *Leu-* strains were then inoculated into liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of EDA in the transformants containing the 4 chimeric genes of pZKLeuN-29E3, but not in the *Yarrowia* Y2224 control strain. Most of the selected 36 *Leu-* strains produced about 12 to 16.9% EDA of total lipids. There were 3 strains (i.e., strains #11, #30 and #34) that produced about 17.4%, 17% and 17.5% EDA of total lipids; they were designated as strains Y4001, Y4002 and Y4003, respectively.

Generation Of Strain Y4001U (*Leu-*, *Ura-*) To Produce About 17% EDA Of Total Lipids

Strain Y4001U was created via temporary expression of the *Cre* recombinase enzyme in plasmid pY116 (Figure 6C) within strain Y4001 to produce a *Leu-* and *Ura-* phenotype. Construct pY116 contained the following components:

Table 14

Description of Plasmid pY116 (SEQ ID NO:69)

RE Sites And Nucleotides Within SEQ ID NO:69	Description Of Fragment And Chimeric Gene Components
1328-448	ColE1 plasmid origin of replication
2258-1398	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
3157-4461	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
PacI/SawI 6667-4504	*Yarrowia* Leu2 gene (GenBank Accession No. AF260230)
Swa I/Pme I (6667-218	GPAT::Cre::XPR2, comprising: • GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937); • Cre: Enterobacteria phage P1 Cre gene for recombinase protein (GenBank Accession No. X03453); • XPR2: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)

Plasmid pY116 was used for transformation of freshly grown Y4001 cells according to the General Methods. The transformants were plated onto MMLeu + Ura plates (MMU plus Leucine) containing 280 μg/mL sulfonylurea and maintained at 30 °C for 3 to 4 days. Four colonies were picked, inoculated into 3 mL liquid YPD media at 30 °C and shaken at 250 rpm/min for 1 day. The cultures were diluted to 1:50,000 with liquid MMLeu + Ura media, and 100 μL was plated onto new YPD plates and maintained at 30 °C for 2 days. Colonies were picked and streaked onto MMLeu and MMLeu+Ura selection plates. The colonies that could grow on MMLeu+Ura plates but not on MMLeu plates were selected and analyzed by GC to confirm the presence of C20:2 (EDA). One strain, having a *Leu-* and *Ura-* phenotype, produced about 17% EDA of total lipids and was designated as Y4001U.

Generation Of Y4036 Strain To Produce About 18% DGLA Of Total Lipids

Construct pKO2UF8289 (Figure 7A; SEQ ID NO:70) was generated to integrate four chimeric genes (comprising a Δ12 desaturase, one Δ9 elongase and two mutant Δ8 desaturases) into the Δ12 loci of strain Y4001U1, to thereby enable production of DGLA. Construct pKO2UF8289 contained the following components:

Table 15

Description of Plasmid pKO2UF8289 (SEQ ID NO:70)

RE Sites And Nucleotides Within SEQ ID NO:70	Description Of Fragment And Chimeric Gene Components
AscI/*BsiWI* (10304-9567)	5' portion of *Yarrowia* Δ12 desaturase gene (PCT Publication No. WO 2004/104167)
EcoRI/*SphI* (13568-13012)	3' portion of *Yarrowia* Δ12 desaturase gene (PCT Publication No. WO 2004/104167)

SwaI/BsiWI (7055-9567)	FBAINm::EgD8M::Pex20, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356); • EgD8M: Synthetic mutant Δ8 desaturase ("EgD8S-23"; SEQ ID NO:71; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326); • Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613)
SwaI/PmeI (7055-4581)	YAT::F.D12::OCT, comprising: • YAT: *Yarrowia lipolytica* YAT1 promoter (Patent Publication No. US 2006/0094102-A1); • F.D12: *Fusarium moniliforme* Δ12 desaturase gene (PCT Publication No. WO 2005/047485); • OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988)
PmeI/PacI (4581-2124)	EXP::EgD8M::Pex16, comprising: • EXP: *Yarrowia lipolytica* export protein (EXP1) promoter (PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761); • EgD8M: Synthetic mutant Δ8 desaturase ("EgD8S-23"; SEQ ID NO:71; U.S. Patent Application No. 11/635258), derived from *Euglena gracilis* ("EgD8S"; PCT Publication No. WO 2006/012326); • Pex16: Pex16 terminator of *Yarrowia Pex16* gene (GenBank Accession No. U75433)
PmeI/ClaI (2038-1)	GPAT::EgD9e::Lip2, comprising: • GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937); • EgD9e: *Euglena gracilis* Δ9 elongase gene (SEQ ID NO:73) (U.S. Patent Applications No. 11/601563 and No. 11/601564); • Lip2: Lip2 terminator sequence from *Yarrowia Lip2* gene (GenBank Accession No. AJ012632)
ClaI/EcoRI (13568-1)	LoxP::*Ura3*::LoxP, comprising: • LoxP sequence (SEQ ID NO:66); • *Yarrowia* Ura3 gene (GenBank Accession No. AJ306421); • LoxP sequence (SEQ ID NO:66)

The pKO2UF8289 plasmid was digested with *AscI/SphI*, and then used for transformation of strain Y4001U1 according to the General Methods. The transformants were plated onto MMLeu plates and maintained at 30 °C for 2 to 3 days. The colonies were picked and

streaked onto MMLeu selection plates at 30 °C for 2 days. These cells were then inoculated into liquid MMLeu at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of DGLA in the transformants containing the 4 chimeric genes of pKO2UF8289, but not in the parent Y4001U1 strain. Most of the selected 96 strains produced between 7 and 13% DGLA of total lipids. There were 6 strains (i.e., #32, #42, #60, #68, #72 and #94) that produced about 15%, 13.8%, 18.2%, 13.1%, 15.6% and 13.9% DGLA of total lipids. These six strains were designated as Y4034, Y4035, Y4036, Y4037, Y4038 and Y4039, respectively.

Generation Of Strain Y4036U (*Leu-, Ura3-*) To Produce About 18% DGLA Of Total Lipids

Construct pY116 (Figure 6C; SEQ ID NO:69) was utilized to temporarily express a *Cre* recombinase enzyme in strain Y4036. This released the LoxP sandwiched *Ura3* gene from the genome.

Plasmid pY116 was used to transform strain Y4036 according to the General Methods. Following transformation, the cells were plated onto MMLeu+Ura plates (MMU plus Leucine) and maintained at 30 °C for 2 to 3 days. The individual colonies grown on MMLeu+Ura plates were picked, and streaked into YPD liquid media at 30 °C and shaken at 250 rpm/min for 1 day to cure the pY116 plasmid. The grown cultures were streaked on MMLeu+Ura u plates . After two days at 30 °C, the individual colonies were re-streaked on MMLeu+Ura, MMU and MMLeu plates. Those colonies that could grow on MMLeu+Ura, but not on MMU or MMLeu plates were selected. One of these strains with *Leu-* and *Ura-* phenotypes was designated as Y4036U (*Ura-, Leu-*).

Generation Of Y4070 Strain To Produce About 12% ARA Of Total Lipids

Construct pZKSL-555R (Figure 7B; SEQ ID NO:74) was generated to integrate three Δ5 desaturase genes into the *Lys* loci of strain Y4036U,

to thereby enable production of ARA. The pZKSL-555R plasmid contained the following components:

Table 16

Description of Plasmid pZKSL-555R (SEQ ID NO:74)

RE Sites And Nucleotides Within SEQ ID NO:74	Description Of Fragment And Chimeric Gene Components
AscI/*BsiWI* (3321-2601)	720 bp 5' portion of *Yarrowia Lys5* gene (GenBank Accession No. M34929)
PacI/*SphI* (6716-6029)	687 bp 3' portion of *Yarrowia Lys5* gene (GenBank Accession No. M34929)
BglII/*BsiWI* (15-2601)	EXP::EgD5S::Pex20, comprising: • EXP: *Yarrowia lipolytica* export protein (EXP1) promoter (PCT Publication No. WO 2006/052870 and U.S. Patent Application No. 11/265761); • EgD5S: codon-optimized Δ5 desaturase (SEQ ID NO:75), derived from *Euglena gracilis* (U.S. Patent Application No. 11/748629); • Pex20: Pex20 terminator sequence from *Yarrowia Pex20* gene (GenBank Accession No. AF054613)
ClaI/*PmeI* (11243-1)	YAT::RD5S::OCT, comprising: • YAT: *Yarrowia lipolytica* YAT1 promoter (Patent Publication No. US 2006/0094102-A1); • RD5S: codon-optimized Δ5 desaturase (SEQ ID NO:77), derived from *Peridinium* sp. CCMP626 (U.S. Patent Application No. 11/748637); • OCT: OCT terminator sequence of *Yarrowia OCT* gene (GenBank Accession No. X69988)
EcoRI/*PacI* (9500-6716)	FBAIN::EgD5WT::Aco, comprising: • FBAIN: *Yarrowia lipolytica* FBAIN promoter (PCT Publication No. WO 2005/049805); • EgD5WT: *Euglena gracilis* Δ5 desaturase (SEQ ID NO:79; U.S. Patent Application No. 11/748629) with elimination of internal *BglII*, *HindIII* and *NcoI* restriction enzyme sites; • Aco: Aco terminator of *Yarrowia Aco* gene (GenBank Accession No. AJ001300)
EcoRI/*ClaI* (9500-11243)	*Yarrowia Leu2* gene (GenBank Accession No. M37309)

The pZKSL-555R plasmid was digested with *AscI*/*SphI*, and then used for transformation of strain Y4036U according to the General

Methods. The transformant cells were plated onto MMLeuLys plates (MMLeu plus Lysine) and maintained at 30 °C for 2 to 3 days. Single colonies were then re-streaked onto MMLeuLys plates, and then inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of ARA in the transformants containing the 3 chimeric genes of pZKSL-555R, but not in the parent Y4036U strain. Most of the selected 96 strains produced ~10% ARA of total lipids. There were 4 strains (i.e., #57, #58, #69 and #75) that produced about 11.7%, 11.8%, 11.9% and 11.7% ARA of total lipids. These four strains were designated as Y4068, Y4069, Y4070 and Y4071, respectively. Further analyses showed that the three chimeric genes of pZKSL-555R were not integrated into the *Lys5* site in the Y4068, Y4069, Y4070 and Y4071 strains. All strains possessed a *Lys+* phenotype.

The final genotype of strain Y4070 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was *Ura3-, Leu+, Lys+*, GPD::F.D12::Pex20, YAT::F.D12::OCT, YAT::ME3S::Pex16, GPAT::EgD9e::Lip2, Exp::EgD9eS::Lip1, FBAINm::EgD9eS::Lip2, FBAINm::EgD8M::Pex20, EXP::EgD8M::Pex16, FBAIN::EgD5WT::Aco, EXP::EgD5S::Pex20, YAT::RD5S::OCT.

EXAMPLE 10

Generation Of Construct pFBAINPaD17S (Comprising The Codon-Optimized Δ17 Desaturase Gene "PaD17S") And Expression In *Yarrowia lipolytica*

The present Example describes functional analysis of PaD17S in *Yarrowia lipolytica* strain Y4070 (Example 9). Thus, following construction of plasmid pFBAINPaD17S (SEQ ID NO:102) comprising a chimeric FBAINm::PaD17S::Pex20 gene and transformation, lipid profiles within the transformant organisms were compared.

Specifically, plasmid pFBAINPaD17S was constructed by three-way ligation using 5' PaD17S and 3' PaD17S fragments from plasmid

pPaD17S (Example 8; wherein the 5' PaD17S fragment was generated by *Nco*I and *Bgl*II digestion and wherein the 3' PaD17S fragment was generated by *Bgl*II and *Not*I digestion, as described in Example 5) and plasmid pFBAIN-MOD-1 (SEQ ID NO:80; Figure 8A) predigested with *Nco*I and *Not*I. Thus, PaD17S was operably linked with the *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805; U.S. Patent 7,202,356) and the PEX20-3' terminator region of the *Yarrowia Pex20* gene (GenBank Accession No. AF054613).

Plasmid pFBAINPaD17S (SEQ ID NO:102) was transformed into *Yarrowia lipolytica* strain Y4070 and transformants were selected on SD-Ura plates (comprising: 20 g/L agar; 6.7 g/L YNB without amino acids but with ammonium sulfate; 20 g/L glucose; 20 mg/L each of adenine sulfate, L-tryptophan, L-histidine-HCl, L-arginine-HCl, L-methionine; 30 mg/L each of L-tyrosine, L- leucine, L-isoleucine, L-lysine-HCl; 50 mg/L L-phenylalanine; 100 mg/mL each of L-glutamic acid, L-aspartic acid; 150 mg/L L-valine; 200 mg/L L-threonine; and 400 mg/L L-serine).

The fatty acid profile and conversion efficiency of four transformants were determined as described in Example 7. The results of GC analysis are shown in Table 17; composition of ARA and EPA are presented as a % of the total fatty acids.

Table 17

Comparison Of Fatty Acid Composition In *Yarrowia* Strain Y4070 Transformed With pFBAINPaD17S, Comprising PaD17S

Clone	Plasmid	% ARA	% EPA	Conver. Effic. (%)
1	pFBAIN-MOD-1	13.23	0	0
2	pFBAIN-MOD-1	13.20	0	0
3	pFBAINPaD17S	6.22	7.34	54.1
4	pFBAINPaD17S	6.15	7.73	54.7
5	pFBAINPaD17S	6.04	7.34	54.9
6	pFBAINPaD17S	6.02	7.53	55.6

The GC results demonstrated production of ARA and EPA in the transformants carrying pFBAINPaD17S, but only production of ARA in

transformants carrying the control plasmid pFBAIN-MOD-1 (Figure 8A, vector only). The conversion efficiency of the codon-optimized *P. aphanidermatum* Δ17 desaturase (PaD17S; SEQ ID NO:4) ranged between 54.1% to 55.6%, compared with 18.4 to 19.5% conversion efficiency for the wild-type PaD17 (SEQ ID NO:2).

EXAMPLE 11

Identification Of A *Phytophthora sojae* Gene Encoding Δ17 Desaturase

The present Example, disclosed in U.S. Patent Application No. 11/787772, describes the identification of a Δ17 desaturase from *Phytophthora sojae* (SEQ ID NOs:44 and 45).

The U.S. Department of Energy's Joint Genome Institute ("JGI"; Walnut Creek, CA) created version 1.0 of the *Phytophthora sojae* genome (estimated genome size is 95 Mbp). This genomic sequence was generated using a whole genome shotgun strategy and comprises a total of 19,276 gene models.

Using the amino acid sequence of the Δ17 desaturase of *Phytophthora infestans* (GenBank Accession No. CAJ30870; designated as "PiD17" herein and corresponding to SEQ ID NO:43) as a query sequence, a TBLASTN (BLAST protein versus translated nucleotide) search was conducted against JGI's *Phytophthora sojae* database (using the default parameters available from JGI). One *P. sojae* ORF located on scaffold 17:338148-339167 was found to share extensive homology with PiD17 (i.e., 91.8% identity and 95.6% similarity, with an Expectation value of 0). Based on this homology, the *P. sojae* ORF was tentatively identified as a Δ17 desaturase and was designated as "PsD17". When the 1092 bp DNA sequence of PsD17 (SEQ ID NO:44) was retrieved from the database, it was found to encode a polypeptide of 363 amino acids in length (SEQ ID NO:45). Amino acid sequence alignment using a Clustal W analysis (MegAlign™ program of DNASTAR software) showed that there was 90.9% identity between PiD17 and PsD17; in contrast, the nucleotide sequences shared only 86.6% identity.

The sequence homology of PsD17 to all publicly available protein sequences contained in the "nr" database (see General Methods) was

also determined by conducting protein-protein BLAST searches using PsD17 (SEQ ID NO:45) as the query sequence. Based on this analysis, PsD17 was found to share the most homology with the omega-3 fatty acid desaturase of *Saprolegnia diclina* (GenBank Accession No. AAR20444); specifically, PsD17 had 60% identity and 74% similarity with the amino acid sequence of GenBank Accession No. AAR20444 with an Expectation value of 7E-117. Additionally, PsD17 had 39% identity and 57% similarity with the amino acid sequence of the fatty acid desaturase of *Anabaena variabilis* ATCC #29413 (GenBank Accession No. ABA23809), with an Expectation value of 4E-57.

EXAMPLE 12

Synthesis Of A Codon-Optimized Δ17 Desaturase Gene ("PsD17S") For *Yarrowia lipolytica*

The present Example, disclosed in U.S. Patent Application No. 11/787772, describes the creation of a synthetic Δ17 desaturase, derived from *Phytophthora sojae* (SEQ ID NOs:44 and 45) and codon-optimized for expression in *Yarrowia lipolytica* (SEQ ID NOs:81 and 82).

The codon usage of the Δ17 desaturase gene of *Phytophthora sojae* was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in U.S. Patent 7,125,672. Specifically, a codon-optimized Δ17 desaturase gene (designated "PsD17S", SEQ ID NOs:81 and 82) was designed based on the coding sequence of PsD17 (SEQ ID NOs:44 and 45), according to the *Yarrowia* codon usage pattern (PCT Publication No. WO 2004/101753), the consensus sequence around the 'ATG' translation initiation codon, and the general rules of RNA stability (Guhaniyogi, G. and J. Brewer, *Gene*, 265(1-2):11-23 (2001)). In addition to modification of the translation initiation site, 175 bp of the 1092 bp coding region were modified (16.0%) and 168 codons were optimized (46.2%). The GC content was reduced from 65.1% within the wild type gene (i.e., PsD17) to 54.5% within the synthetic gene (i.e., PsD17S). A *Nco*I site and *Not*I sites were incorporated around the translation initiation codon and after the stop codon of PsD17S (SEQ ID NO:81), respectively. Figure 9 shows a comparison of the nucleotide sequences of PsD17 and

PsD17S. At the amino acid level, PsD17S lacked the third and forth amino acid, as compared with the wild type PsD17; thus, the total length of PsD17S is 361 amino acids (SEQ ID NO:82). The designed PsD17S gene was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pPsD17S (SEQ ID NO:83).

EXAMPLE 13

Identification Of A *Phytophthora ramorum* Gene Encoding Δ17 Desaturase

The present Example, disclosed in U.S. Patent Application No. 11/787772, describes the identification of a Δ17 desaturase from *Phytophthora ramorum* (SEQ ID NOs:46 and 47).

The U.S. Department of Energy's Joint Genome Institute ("JGI"; Walnut Creek, CA) created version 1.0 of the *Phytophthora ramorum* genome (estimated genome size is 65 Mbp). This genomic sequence was generated using a whole genome shotgun strategy and comprises a total of 16,066 gene models.

In a manner similar to that described in Example 11, the amino acid sequence of PiD17 (SEQ ID NO:43) was used as a query sequence to perform a TBLASTN search against JGI's *Phytophthora ramorum* database (using the default parameters available from JGI).

Two ORFs were found to share extensive homology with PiD17 in the genome sequence of *Phytophthora ramorum*. Specifically, ORF 80222 shared 89% identity and 94% similarity with SEQ ID NO:43, with an Expectation value of 0. Similarly, ORF48790 shared up to 40% identity and 61% similarity with SEQ ID NO:43, with an Expectation value of 6E-44. Based on these results, ORF 80222 was tentatively identified as a Δ17 desaturase and was designated as "PrD17".

When the 1086 bp DNA sequence of PrD17 (SEQ ID NO:46) was retrieved from the database, it was found to encode a polypeptide of 361 amino acids in length (SEQ ID NO:47). Amino acid sequence alignment using a Clustal W analysis (MegAlign™ program of DNASTAR software) showed that there was 89.5% identity between PiD17 and PrD17; in contrast, the nucleotide sequences shared only 85.7% identity.

The sequence homology of PrD17 was in turn compared with all publicly available protein sequences contained in the "nr" database (see General Methods) by conducting protein-protein BLAST searches using PrD17 (SEQ ID NO:47) as the query sequence. The sequence that showed the highest degree of similarity was that of the omega-3 fatty acid desaturase of *Saprolegnia diclina* (GenBank Accession No. AAR20444), sharing 59% identity and 74% similarity, with an Expectation value of E-124. Additionally, PrD17 had 38% identity and 57% similarity with the amino acid sequence of the fatty acid desaturase of *Anabaena variabilis* ATCC #29413 (GenBank Accession No. ABA23809), with an Expectation value of 6E-61.

EXAMPLE 14

Synthesis Of A Codon-Optimized Δ17 Desaturase Gene ("PrD17S") For *Yarrowia lipolytica*

The present Example, disclosed in U.S. Patent Application No. 11/787772, describes the creation of a synthetic Δ17 desaturase, derived from *Phytophthora ramorum* (SEQ ID NOs:46 and 47) and codon-optimized for expression in *Yarrowia lipolytica* (SEQ ID NOs:84 and 47).

The codon usage of the Δ17 desaturase gene of *Phytophthora ramorum* was optimized for expression in *Yarrowia lipolytica,* in a manner similar to that described in U.S. Patent 7,125,672. Specifically, a codon-optimized Δ17 desaturase gene (designated "PrD17S", SEQ ID NO:84) was designed based on the coding sequence of PrD17 (SEQ ID NOs:46 and 47), according to the *Yarrowia* codon usage pattern (PCT Publication No. WO 2004/101753), the consensus sequence around the 'ATG' translation initiation codon, and the general rules of RNA stability (Guhaniyogi, G. and J. Brewer, *Gene,* 265(1-2):11-23 (2001)). In addition to modification of the translation initiation site, 168 bp of the 1086 bp coding region were modified (15.5%) and 160 codons were optimized (44.2%). The GC content was reduced from 64.4% within the wild type gene (i.e., PrD17) to 54.5% within the synthetic gene (i.e., PrD17S). A *Nco*I site and *Not*I sites were incorporated around the translation initiation codon and after the stop codon of PrD17S (SEQ ID NO:84), respectively.

Figure 10 shows a comparison of the nucleotide sequences of PrD17 and PrD17S. None of the modifications in the codon-optimized gene changed the amino acid sequence of the encoded protein (SEQ ID NO:47). The designed PrD17S gene was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pPrD17S (SEQ ID NO:85).

EXAMPLE 15

Generation Of Constructs pY130, pY138, pY139 And pY140 (Comprising A *Fusarium moniliforme* Δ15 Desaturase, PrD17S, PsD17S And PaD17S) For Comparison Of Omega-6 Fatty Acid Substrate Specificity

The present Example, and related Examples 16 and 17 (*infra*) describe comparison of the substrate specificity of a *Fusarium moniliforme* Δ15 desaturase (FmD15; SEQ ID NOs:86 and 87) to that of PaD17S (SEQ ID NOs:4 and 2), PrD17S (SEQ ID NOs:84 and 47) and PsD17S (SEQ ID NOs:81 and 82) in *Yarrowia lipolytica*.

This work included the following steps: (1) construction of *Yarrowia* expression vectors pY130 (comprising FmD15), pY138 (comprising PrD17S), pY139 (comprising PsD17S) and pY140 (comprising PaD17S), as described in Example 15 herein; (2) construction of a Δ12 desaturase-disrupted strain of *Yarrowia lipolytica* ATCC #76982, identified as strain L38, as described in Example 16; 3.) transformation of pY130, pY138, pY139 and pY140 into wildtype *Yarrowia* and *Yarrowia* strain L38, as described in Example 17; and, 4.) comparison of lipid profiles within transformant organisms comprising of pY130, pY138, pY139 or pY140 after feeding fatty acid substrates, as described in Example 17.

Experimental Basis

Omega–3 desaturases, which include both Δ15 desaturases that act on C18 fatty acids substrates and Δ17 desaturases that act on C20 fatty acids substrates, play an important role in the biosynthesis of long chain PUFAs by converting ω–6 fatty acids into their ω–3 counterparts (Figure 1). It is well known that some fungal ω–3 desaturases show broad catalytic promiscuity. For example, the Δ15 desaturases of *Fusarium moniliforme* (GenBank Accession No. DQ272516.1) and *Magnaporthe*

grisea (GenBank Accession No. XP_362963) both additionally have limited Δ17 desaturase activity (PCT Publications No. WO 2005/047485 and No. WO 2005/047480; U.S. Patent Application No. 11/740298).

Similarly, the synthetic Δ17 desaturase derived from *Phytopthora sojae* and codon-optimized for expression in *Yarrowia lipolytica* (i.e., PsD17S) was previously demonstrated in U.S. Patent Application No. 11/787772 to have both Δ17 and Δ15 desaturase activities. More specifically, PsD17S displayed "bifunctional Δ17 desaturase activity" or "primary Δ17 desaturase activity", wherein the desaturase preferentially converts ARA to EPA and/or DGLA to ETA but additionally has limited ability to convert LA into ALA (thus exhibiting primarily Δ17 desaturase activity and limited Δ15 desaturase activity).

Despite the broad catalytic promiscuity described above, not all ω–3 desaturases possess bifunctional activity. For example, the *Saprolegnia diclina* Δ17 desaturase functions exclusively on C20 ω6 fatty acid substrates (Pereira, S.L. et. al., *Biochem. J.*, 378:665 (2004)).

The purpose of the following Examples was to compare the relative ω–6 fatty acid substrate specificities of Δ17 desaturases from *Phytopthora sojae* (PsD17S; SEQ ID NOs:81 and 82), *Phytopthora ramorum* (PrD17S; SEQ ID NOs:84 and 47) and *Pythium aphanidermatum* (PaD17S; SEQ ID NOs:4 and 2) with that of the previously characterized *Fusarium moniliforme* Δ15 desaturase (FmD15; SEQ ID NOs:86 and 87). In contrast to previous work performed with PsD17S and PrD17S in U.S. Patent Application No. 11/787772, the ω–3 desaturases were expressed herein in *Yarrowia lipolytica* strains lacking desaturases and elongases involved in converting LA to EPA, since their presence allows alternative routes for long-chain PUFA biosynthesis (Figure 1). As a result, interpretation concerning ω–6 substrate specificity in PrD17S, PsD17S and PaD17S is much clearer than in previous work.

Construction Of *Yarrowia* Expression Vector pY130, Comprising FmD15

Plasmid pY6.GPD.Leu2 (SEQ ID NO:88) is a shuttle plasmid that can replicate both in *E. coli* and *Yarrowia lipolytica*, containing the

following: a *Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. M91600); a ColE1 plasmid origin of replication; an *E. coli* f1 origin of replication; an ampicillin-resistance gene (AmpR) for selection in *E. coli*; a *Yarrowia* Leu2 gene (GenBank Accession No. AF260230) for selection in *Yarrowia;* and, a chimeric GPD::*Nco*I/*Not*I::XPR cassette. The *Yarrowia* "GPD promoter" refers to the 5' upstream untranslated region in front of the 'ATG' translation initiation codon of a protein encoded by the *Yarrowia lipolytica* glyceraldehyde-3-phosphate dehydrogenase (GPD) gene and that is necessary for expression (PCT Publication No. WO 2005/003310). "XPR" refers to ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741). Although the construction of plasmid pY6.GPD.Leu2 is not described herein in detail, it was derived from pY28 GPD.YID12d (previously described in U.S. Patent Application No. 11/740298, filed April 26, 2007, and comprising a chimeric GPD::*Yarrowia lipolytica* Δ12 desaturase (Yld12d)::Lip1 gene cassette).

The *Fusarium moniliforme* Δ15 desaturase was derived from plasmid pY34 which was previously described in PCT Publication No. WO 2005/047485 (the contents of which are hereby incorporated by reference), first by a single bp substitution at position 180 of the FmD15 desaturase ORF. This C180T "silent" mutation resulted in the loss of the *Nco*I site in the ORF for cloning convenience. Then, the modified sequence was used to PCR the ORF using 5' and 3' PCR primers with *Nco*I and *Not*I restriction sites, and the resultant *Nco*I-*Not*I fragment containing the FmD15 desaturase ORF (SEQ ID NO:86) was used to replace the Yld12d ORF in plasmid pY28 described *supra* using *Nco*I and *Not* I sites to produce pY130 (SEQ ID NO:89; Figure 11A [labeled as "pY130.GPD.Fmd15" therein]).

The 9048 bp sequence of expression vector pY130 containing the chimeric GPD::FmD15::Lip1 gene is disclosed in SEQ ID NO:89 and described in the table below.

Table 18

Description of Plasmid pY130 (SEQ ID NO:89)

RE Sites And Nucleotides Within SEQ ID NO:89	Description Of Fragment And Chimeric Gene Components
*BsiW*I- *Sph*I	Contains: • ColE1 plasmid origin of replication (157-1037 bp); • ampicillin resistance gene (Amp^R) for selection in *E. coli* (1107-1967 bp); • *E. coli* f1 origin of replication (2147-2537 bp); • *Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608) (2866-4143 bp)
*Sph*I-*Nco*I	Contains: • *Yarrowia* LEU2 gene (GenBank Accession No. AF260230) (4152-6379 bp); • *Yarrowia* GPD promoter (corresponding to 825835-826763 bp in GenBank Accession No. CR382129, except for a single bp change (C826238T) made to destroy the *Nco*I for cloning convenience and two unexpected changes: a single A insertion at position 826161 and a 37 bp direct repeat of nucleotides 825884-825922) (6382-7346 bp)
*Nco*I-*Not*I	Contains *Fusarium moniliforme* (*Gibberella fujikuroi*) Δ15 desaturase ORF (SEQ ID NO:86) (GenBank Accession No. DQ272516.1; PCT Publication No. WO 2005/047480; except for a single silent bp change (C180A) to destroy the *Nco*I site for cloning convenience) (7350-8558 bp)
*Not*I- *BsiW*I	Contains Lip1: Lip1 terminator sequence from *Yarrowia Lip1* gene (GenBank Accession No. Z50020) (8567-8888 bp)

Construction Of *Yarrowia* Expression Vectors pY138 (Comprising PrD17S), pY139 (Comprising PsD17S) And pY140 (Comprising PaD17S)

The *Nco*I-*Not*I fragment comprising FmD15 in pY130 was replaced by similarly digested fragments comprising the synthetic Δ17 desaturase ORFs of *Phytopthora ramorum* and *Phytopthora sojae* that had been codon-optimized for expression in *Yarrowia* (i.e., PrD17S and PsD17S, respectively) from the source plasmids pPrD17S (SEQ ID NO:85; Example 14, *supra*) and pPsD17S (SEQ ID NO:83; Example 12, *supra*). This produced plasmids pY138 (SEQ ID NO:90; Figure 11B [labeled as "pY138 GPD-PrD17" therein]) and pY139 (SEQ ID NO:91; Figure 11C [labeled as "pY139 GPD PsD17" therein]), respectively.

A similar strategy was used to substitute the FmD15 ORF in pY130 with the synthetic Δ17 desaturase ORF of *Pythium aphanidermatum* from the source plasmid pPaD17S (SEQ ID NO:62; Example 8, *supra*); however, since PaD17S contained an internal *Nco*I site, this was achieved by a three-way ligation of the *Nco*I-*Bgl*II and *Bgl*II-*Not*I fragments of PaD17S into the pY130 vector backbone. This resulted in formation of plasmid pY140 (SEQ ID NO:92), as shown in Figure 11D (labeled as "pY140 GPD-PaD17" therein).

EXAMPLE 16

Generation Of *Yarrowia lipolytica* Δ12 Knockout Strain L38

The present Example, disclosed in U.S. Patent Application No. 11/740298, describes the creation of a Δ12 desaturase-disrupted [Δ12 knockout (KO)] strain of *Yarrowia lipolytica* ATCC #76982, identified as strain L38 and referred to generically as a "d12KO" strain. The only native Δ12 desaturase gene of this strain was disrupted by replacement with a disrupted version via homologous recombination.

The methodology used to create the d12KO strain identified herein as L38 relied on site-specific recombinase systems, as described in the General Methods.

Experimental Methodology

Yarrowia lipolytica ATCC #76982 was transformed with *Sph*I and *Asc*I linearized plasmid pY137. The sequence of plasmid pY137 (labeled as pY137.YlD12ko.Leu2 in Figure 12A) is disclosed as SEQ ID NO:93 and pY137 Is described in the table below.

Table 19

Description of pY137 (SEQ ID NO:93)

RE Sites And Nucleotides Within SEQ ID NO:93	Description Of Fragment And Chimeric Gene Components
*Pac*I- *Bgl*II [digestion with *Pac*I-*Sal*I releases LoxP::Leu2]	Contains LoxP::Leu2::LoxP, comprising: • LoxP sequence (SEQ ID NO:66) (28-61 bp); • *Yarrowia* LEU2 gene (GenBank Accession No. AF260230) (68-2228 bp); • LoxP sequence (SEQ ID NO:66) (2308-2341)

BglII- AscI	Contains 3' portion of *Yarrowia lipolytica* Δ12 desaturase gene (2357-2950 bp) that corresponds to 661-1254 bp of GenBank Accession No. XM_500707
AscI-SphI	Contains: • ColE1 plasmid origin of replication (3003-3883); • ampicillin resistance gene (Amp^R) for selection in *E. coli* (3941-4801); • *E. coli* f1 origin of replication (5009-5409)
SphI-PacI	Contains 5' portion of *Yarrowia lipolytica* Δ12 desaturase gene (5662-6262 bp) that corresponds to 1-601 bp of GenBank Accession No. XM_500707

Eleven LEU prototrophic pY137 transformants were analyzed by GC and four were identified as Δ12 knockout (d12KO) strains by the absence of detectable 18:2 (LA) upon GC analysis. One of these was designated strain L37.

The LEU2 gene in d12KO strain L37 was excised by transient expression of Cre recombinase under the control of the *Yarrowia* glycerol-3-phosphate acyltransferase (GPAT) promoter. Specifically, strain L37 was transformed with plasmid pY117. The mutated *Yarrowia* AHAS enzyme in plasmid pY117 conferred SU^R, which was used as a positive screening marker.

Plasmid pY117 was derived from plasmid pY116 (described in Table 14 herein and in U.S. Patent Application No. 11/635258) by inserting the mutant AHAS gene flanked by *PacI-SwaI* sites into *PacI-SwaI* digested pY116 thereby replacing the LEU selectable marker with the sulfonylurea marker. Plasmid pY117 (SEQ ID NO:94) is represented in Figure 12B (labeled therein as pY117.Cre.AHASw497L) and is described in Table 20 below.

Table 20

Description of pY117 (SEQ ID NO:94)

RE Sites And Nucleotides Within SEQ ID NO:94	Description Of Fragment And Chimeric Gene Components
BsiWI- Eco RI	Contains: • ColE1 plasmid origin of replication (448-1328); • ampicillin resistance gene (Amp^R) for selection

	in *E. coli* (1328-2258, complementary); • *E. coli* f1 origin of replication (2438-2838)
Eco RI- PacI	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608) (3157-4461 bp)
PacI-SwaI	*Yarrowia lipolytica* AHAS gene (corresponding to 27040-30026 bp [complementary] in Genbank Accession No. CR382129) comprising a W497L mutation (3157-4461 bp)
Swa I/BsiWI [digestion with *SwaI-NotI* releases GPAT::Cre]	Contains GPAT::Cre::XPR2 comprising: • GPAT: *Yarrowia lipolytica* GPAT promoter (PCT Publication No. WO 2006/031937) (7498-8535 bp); • Cre: Enterobacteria phage P1 Cre gene for recombinase protein (GenBank Accession No. X03453) (8537-9570 bp) except for single base change (T4G) resulting in a single amino acid change (S2A) to create a *NcoI* site for cloning convenience; • XPR2: ~170 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)

L37 transformed by pY117 were plated on minimal plates containing Leu and 280 μg/mL sulfonyurea (chlorimuron ethyl, E. I. duPont de Nemours & Co., Inc., Wilmington, DE). To cure the strains of pY117, two SU^R colonies were used to inoculate 3 mL YPD. After overnight growth at 30 °C, 100 μl of 1:250,000 diluted cultures were plated on YPD plates. After overnight growth at 30 °C, 6 single colonies were streaked on both YPD and MM plates. All grew on YPD but not on MM plates, confirming their *Leu* auxotrophy. One of these was designated as strain L38.

Example 17

Expression Of Constructs pY130, pY138, pY139 And pY140 (Comprising FmD15, PrD17S, PsD17S And PaD17S) In *Yarrowia lipolytica* Strains For Comparison Of Omega-6 Fatty Acid Substrate Specificity

The present Example describes transformation of expression plasmids pY130, pY138, pY139 and pY140 into *Yarrowia lipolytica* ATCC #76982, followed by comparison of lipid profiles within transformant organisms.

Transformation

The following expression plasmids were transformed into wild type (WT) *Yarrowia lipolytica* ATCC #76982 and its Δ12 desaturase-disrupted derivative (Δ12 KO) strain L38 (Example 16), as described in the General Methods: 1.) plasmid pY130 (comprising FmD15); 2.) plasmid pY138 (comprising PrD17S); 3.) plasmid pY139 (comprising PsD17S); 4.) plasmid pY140 (comprising PaD17S); and, 5.) plasmid pY6.GPD.Leu2 (empty vector control lacking any desaturase ORF; also referred to as plasmid "pY6").

Comparison Of Lipid Profiles Without Substrate Feeding

Three independent transformants from each transformation were streaked on MM plates. Fresh cultures were used to separately inoculate 3 mL MM in triplicate. After growth in a shaker at 30 °C for 2 days, cells from 2 mL aliquots of each were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

The fatty acid profiles for *Yarrowia lipolytica* expressing pY6 (SEQ ID NO:88), pY130 (SEQ ID NO:89), pY138 (SEQ ID NO:90), pY139 (SEQ ID NO:91) and pY140 (SEQ ID NO:92) are shown below in Table 21. In Table 21, fatty acids are identified as 16:0 (palmitate), 16:1, 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (LA) and ALA. Fatty acid compositions were expressed as the weight percent (wt. %) of total fatty acids. The conversion efficiency ("CE") was measured according to the following formula: ([product]/[substrate+product])*100, where 'product' includes the immediate product and all products in the pathway derived from it. Thus, Δ12 activity (i.e., "d12d CE") was calculated according to the following formula: ([LA]/ [oleic acid+LA])*100 and represents percent substrate conversion to LA. "Δ15 Activity" (i.e., "d15d CE") was calculated according to the following formula: ([ALA]/[LA+ALA])*100 and represents percent substrate conversion to ALA. Standard deviation is abbreviated "SD", while "nd" is not detected.

Table 21

Comparison Of Fatty Acid Composition In Wild Type and Δ12 Knockout *Yarrowia* Transformed With pY130, pY138, pY139 And pY140 (Comprising FmD15, PrD17S, PsD17S And PaD17S)

Strain	Plasmid	16:0	16:1	18:0	18:1	18:2	ALA	d12d CE	d15d CE
WT	pY6 (vector ctrl)	9.2	12.2	1.5	28.9	39.6	nd	57.8	nd
	SD	0.3	0.2	0.1	0.3	0.6	0.0	0.6	nd
WT	pY130 (FmD15)	8.5	12.3	2.1	33.7	6.5	29.1	51.4	81.7
	SD	0.3	0.3	0.3	1.1	0.2	0.8	1.5	0.1
WT	pY138 (PrD17S)	9.2	13.8	1.6	30.4	29.1	9.5	56.0	24.6
	SD	0.3	0.3	0.2	0.7	0.4	0.2	0.9	0.2
WT	pY139 (PsD17S)	9.2	14.1	1.5	30.8	26.5	11.8	55.4	30.8
	SD	0.2	0.3	0.1	0.1	0.5	0.0	0.3	0.5
WT	pY140 (PaD17S)	9.0	13.3	1.7	33.6	23.1	12.2	51.2	34.6
	SD	0.2	0.3	0.2	0.3	0.3	0.3	0.5	0.7
d12 KO	pY6 (vector ctrl)	6.7	10.8	2.1	71.4	nd	nd	nd	nd
	SD	0.3	0.3	0.3	1.2	0.0	0.0	nd	nd
d12 KO	pY130 (FmD15)	7.1	10.6	2.5	55.0	0.6	15.7	22.8	96.6
	SD	0.1	0.1	0.2	0.2	0.0	0.3	0.4	0.0
d12 KO	pY138 (PrD17S)	6.8	11.7	2.2	69.5	nd	nd	nd	nd
	SD	0.0	0.1	0.0	0.2	0.0	0.0	nd	nd
d12 KO	pY139 (PsD17S)	7.0	11.9	2.1	70.2	nd	nd	nd	nd
	SD	0.3	0.1	0.2	0.1	0.0	0.0	nd	nd
d12 KO	pY140 (PaD17S)	7.7	11.4	2.6	69.5	nd	nd	nd	nd
	SD	0.1	0.1	0.0	0.3	0.0	0.0	nd	nd

Comparison Of Lipid Profiles With Substrate Feeding

To study the relative substrate specificities of the different ω–3 desaturases on ω6 substrates other than LA, d12 KO strains transformed with the different plasmids (i.e., pY6, pY130, pY138, pY139 and pY140) were fed a mixture of different FAs, For this, the strains were streaked onto MM plates and fresh cultures were used to inoculate 3 mL MM. After

overnight growth at 30 °C, all cultures were diluted to an OD_{600} of 0.5 before aliquoting them into three 3-mL cultures. After growth for another 6 hrs, the cultures were harvested and resuspended in 3 mL MM containing 1% Tergitol and 0.5 mM each of GLA, EDA and ARA and allowed to grow for 24 hr at which time they were harvested, washed once with 12 mL 0.5% Triton X-100, and once with 12 mL distilled water. The pellets were analyzed for fatty acid composition, as described above.

The fatty acid profiles for d12 KO *Yarrowia lipolytica* expressing pY6 (SEQ ID NO:88), pY130 (SEQ ID NO:89), pY138 (SEQ ID NO:90), pY139 (SEQ ID NO:91) and pY140 (SEQ ID NO:92) are shown below in Table 22. In the Table, fatty acids are identified as GLA (ω–6), EDA (ω–6), DGLA (ω–6), ARA (ω–6), ALA (ω–3), STA (ω–3), ETrA (ω–3), ETA (ω–3) and EPA (ω–3). Fatty acid compositions were expressed as the weight percent (wt. %) of total fatty acids. The ω–3 desaturase conversion efficiency ("Conv. Effic.") of the ω–6 substrates GLA, EDA, DGLA, and ARA to their ω–3 products, STA, ETrA, ETA, and EPA, respectively, was calculated according to the following formula: [product/(substrate+product)]*100. Standard deviation is abbreviated "SD", while "nd" is not detected.

Table 22

Comparison Of Fatty Acid Composition In Δ12 Knockout *Yarrowia* Transformed With pY130, pY138, pY139 And pY140 (Comprising FmD15, PrD17S, PsD17S And PaD17S)

		Fatty acid composition (% total fatty acid)									ω3 desaturase Conv. Effic. on			
Host	**Plasmid**	**GLA**	**EDA**	**DGLA**	**ARA**	**ALA**	**STA**	**ETrA**	**ETA**	**EPA**	**GLA**	**EDA**	**DGLA**	**ARA**
d12 KO	pY6 (control)	9.0	3.5	6.9	2.3	nd	nd	nd	nd	nd	nd	nd	nd	nd
	SD	0.3	0.1	0.3	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
d12 KO	pY130 (FmD15)	13.1	4.9	9.6	4.6	8.2	2.7	1.2	1.1	0.2	17.3	19.3	10.1	3.3
	SD	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.1	0.2	0.3
d12 KO	pY138 (PrD17S)	12.5	3.3	6.2	2.2	1.0	0.6	1.3	2.7	1.3	4.5	27.7	30.1	36.5
	SD	0.3	0.2	0.2	0.3	0.0	0.0	0.0	0.0	0.1	0.1	1.0	0.9	1.2
d12 KO	pY139 (PsD17S)	11.8	3.0	5.9	1.6	1.2	0.8	1.6	3.1	1.5	6.0	34.6	34.3	47.5
	SD	0.3	0.0	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.2	0.3
d12 KO	pY140 (PaD17S)	9.8	2.5	5.3	1.2	1.1	0.6	1.2	2.1	1.5	5.5	33.2	28.3	55.8
	SD	0.4	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.1	0.3	1.1	0.8	1.7

Results concerning ω–6 fatty acid substrate specificity of FmD15, PsD17S, PrD17S and PaD17S are visually summarized in Figure 13. Specifically, data relating to LA is from wild type *Y. lipolytica* transformants, as shown in Table 21; all other data are from Δ12-desaturase disrupted (d12KO) *Yarrowia lipolytica* strains fed different ω–6 fatty acid substrates, as shown in Table 22. The fatty acid DGLA is abbreviated as "HGLA" in the Figure.

Based on the data presented herein, FmD15 had the highest Δ15 desaturase activity as compared to PsD17S, PrD17S and PaD17S (Table 21, Figure 13). Unlike FmD15 (which has bifunctional Δ12/Δ15 desaturase activity), however, none of the tested three Δ17 desaturases possessed any detectable Δ12 desaturase activity on oleate (Table 21). Growth in the presence of ω–6 fatty acid substrates showed that all Δ17 desaturases had the strongest preference for ARA, relatively lower activities on EDA and DGLA, and least activity on GLA. PaD17S had the strongest activity on ARA. The Δ17 desaturase had significant Δ15 desaturase activity on the C18 substrate LA, wherein the activity was comparable to the Δ17 desaturase activity on the C20 substrates EDA and DGLA (PsD17S and PrD17S also displayed significant Δ15 desaturase activity on LA, although activity was slightly diminished with respect to the Δ17 desaturase activity on C20 substrates). The broad catalytic promiscuity of the three Δ17 desaturases distinguishes them from the *Saprolegnia diclina* Δ17 desaturase that works exclusively on C20 ω–6 fatty acid substrates.

CLAIMS

What is claimed is:

1. An isolated nucleic acid molecule selected from the group consisting of:

a.) an isolated nucleotide molecule encoding a Δ17 desaturase enzyme, selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3;

b.) an isolated nucleotide molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS; or,

an isolated nucleotide molecule that is completely complementary to (a) or (b).

2. An isolated nucleic acid molecule which encodes a Δ17 desaturase enzyme selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4.

3. A polypeptide encoding a Δ17 desaturase enzyme encoded by the isolated nucleic acid molecule of Claim 1.

4. A polypeptide encoding a Δ17 desaturase enzyme selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

5. An isolated nucleic acid molecule which encodes a Δ17 desaturase enzyme as set forth in SEQ ID NO:2, wherein at least 175 codons are codon-optimized for expression in *Yarrowia*.

6. An isolated nucleic acid molecule comprising a first nucleotide sequence encoding a Δ17 desaturase enzyme of at least 359 amino acids that has at least 75.3% identity based on Clustal W algorithms when compared to a polypeptide having the sequence as set forth in SEQ ID NO:2;

or a second nucleotide sequence comprising the complement of the first nucleotide sequence.

7. A chimeric gene comprising the isolated nucleic acid molecule of Claim 1 operably linked to suitable regulatory sequences.

8. A transformed host cell comprising the isolated nucleic acid molecule of Claim 1.

9. The transformed host cell of Claim 8 selected from the group consisting of algae, bacteria, yeast, oomycetes and fungi.

10. The transformed host cell of Claim 9 wherein the yeast is an oleaginous yeast.

11. The transformed host cell of Claim 10 wherein the oleaginous yeast is selected from the group consisting of: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces.*

12. A method for the production of eicosapentaenoic acid comprising:

a.) providing a host cell comprising:

(i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase

polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

(ii) a source of arachidonic acid;

b.) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the a bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the arachidonic acid is converted to eicosapentaenoic acid; and,

c.) optionally recovering the eicosapentaenoic acid of step (b).

13. A method for the production of eicosatetraenoic acid comprising:

a.) providing a host cell comprising:

(i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

(ii) a source of dihomo-γ–linolenic acid;

b.) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the a bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the dihomo-γ–linolenic acid is converted to eicosatetraenoic acid; and,

c.) optionally recovering the eicosatetraenoic acid of step (c).

14. A method for the production of polyunsaturated fatty acids comprising:

a) providing a host cell comprising:

i) an isolated nucleotide molecule encoding a bifunctional Δ17/ Δ15 desaturase polypeptide having at least 75.3% identity when compared to a polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, based on the Clustal W method of alignment; and,

ii) a source of fatty acid selected from the group consisting of: linoleic acid and eicosadienoic acid;

b) growing the host cell of step (a) under conditions wherein the nucleic acid molecule encoding the bifunctional Δ17/ Δ15 desaturase polypeptide is expressed and the linoleic acid is converted to α-linolenic acid and the eicosadienoic acid is converted to eicosatrienoic acid; and,

c) optionally recovering the fatty acid of step (b).

15. A method according to any of Claims 12, 13 or 14 wherein the isolated nucleic acid molecule encodes a bifunctional Δ17/ Δ15 desaturase polypeptide having the amino acid sequence as set forth in SEQ ID NO:2, wherein at least 175 codons are codon-optimized for expression in *Yarrowia*.

16. A method according to any of Claims 12, 13 or 14 wherein the isolated nucleic acid molecule encodes a bifunctional Δ17/ Δ15 desaturase polypeptide having the amino acid sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:3.

17. A method according to any of Claims 12, 13 or 14 wherein:

a.) the isolated nucleic acid molecule has a nucleic acid sequence selected from the group consisting of SEQ ID NO:1 and SEQ ID NO:4; and,

b.) the host cell is *Yarrowia lipolytica*.

18. A method according to any of Claims 12, 13 or 14, wherein the host cell is selected from the group consisting of: algae, bacteria, yeast, oomycetes and fungi.

19. A method according to Claim 18 wherein the host cell is a fungus selected from the group consisting of: *Thraustochytrium* sp., *Schizochytrium* sp. and *Mortierella* sp.

20. A method according to Claim 18 wherein the yeast is an oleaginous yeast.

21. A method according to Claim 20 wherein the oleaginous yeast is selected from the group consisting of: *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces*.

22. An isolated nucleic acid fragment comprising a nucleic acid sequence encoding a Δ17 desaturase polypeptide comprising at least one amino acid sequence motifs selected from the group consisting of:

a) F T X G H D X G H (SEQ ID NO:96);

b) H R H H H K N T G (SEQ ID NO:97); and,

c) I G T H Q X H H L F P (SEQ ID NO:98);

wherein X can be any amino acid, and

wherein the Δ17 desaturase polypeptide does not have the amino acid sequence as set forth in SEQ ID NOs:43 and 95.

23. A Δ17 desaturase polypeptide comprising at least one amino acid motif selected from the group consisting of SEQ ID NO:96-98.

24. An isolated nucleic acid molecule encoding useful for the identification of a Δ17 desaturase polypeptide encoding an amino acid motif selected from the group consisting of SEQ ID NO:96-98.

25. A method for the identification and isolation of a Δ17 desaturase polypeptide comprising:

a) probing a genomic library with:

i) an isolated nucleic acid fragment encoding an amino

acid sequence selected from the group consisting of SEQ ID NO:96-98; or,

ii) an isolated nucleic acid fragment that is complementary to (i);

b) identifying a DNA clone that hybridizes with the nucleic acid fragment of step (a); and,

c) sequencing the genomic fragment that comprises the clone identified in step (b);

wherein the sequenced genomic fragment encodes a Δ17 desaturase polypeptide.

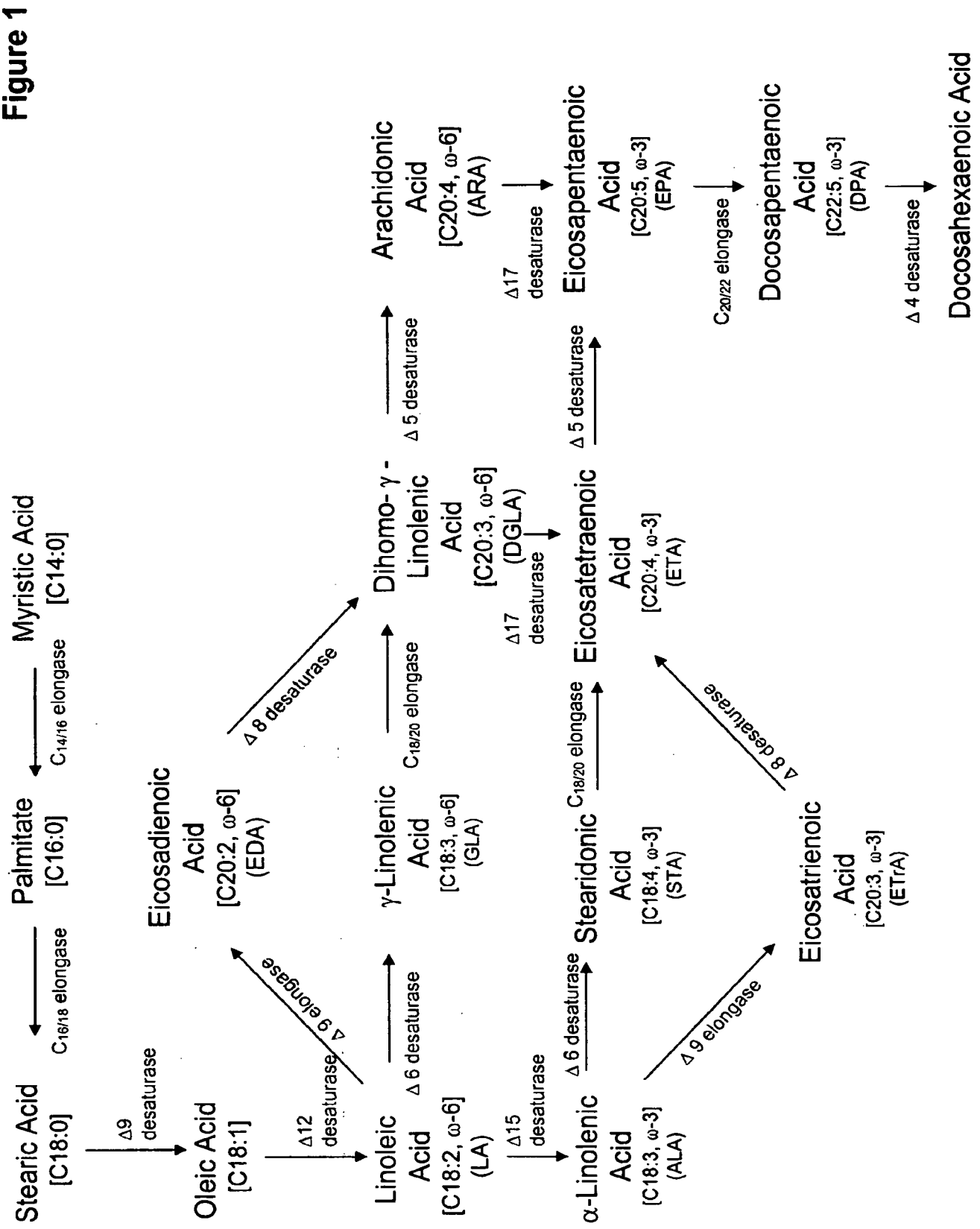
26. A method for the identification and isolation of a Δ17 desaturase polypeptide comprising:

a) synthesizing at least one oligonucleotide primer corresponding to a portion of an isolated nucleic acid sequence encoding an amino acid motift selected from the group consisting of SEQ ID NOs 96-98; and,

b) amplifying an insert present in a cloning vector using the oligonucleotide primer of step (a);

wherein the amplified insert encodes a portion of an amino acid sequence encoding a Δ17 desaturase enzyme.

Figure 1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Palmitate [C16:0]
C14/16 elongase
Myristic Acid [C14:0]
Δ9 desaturase
Oleic Acid [C18:1]
Δ12 desaturase
Linoleic Acid [C18:2, ω-6] (LA)
Δ15 desaturase
α-Linolenic Acid [C18:3, ω-3] (ALA)
Δ 9 elongase
Eicosadienoic Acid [C20:2, ω-6] (EDA)
Δ 8 desaturase
Eicosatrienoic Acid [C20:3, ω-3] (ETrA)
Δ 6 desaturase
γ-Linolenic Acid [C18:3, ω-6] (GLA)
C18/20 elongase
Dihomo- γ - Linolenic Acid [C20:3, ω-6] (DGLA)
Δ17 desaturase
Stearidonic Acid [C18:4, ω-3] (STA)
C18/20 elongase
Eicosatetraenoic Acid [C20:4, ω-3] (ETA)
Δ 8 desaturase
Arachidonic Acid [C20:4, ω-6] (ARA)
Δ 5 desaturase
Eicosapentaenoic Acid [C20:5, ω-3] (EPA)
Δ17 desaturase
C20/22 elongase
Docosapentaenoic Acid [C22:5, ω-3] (DPA)
Δ 5 desaturase
Docosahexaenoic Acid [C22:6, ω-3] (DHA)
Δ 4 desaturase

Figure 2

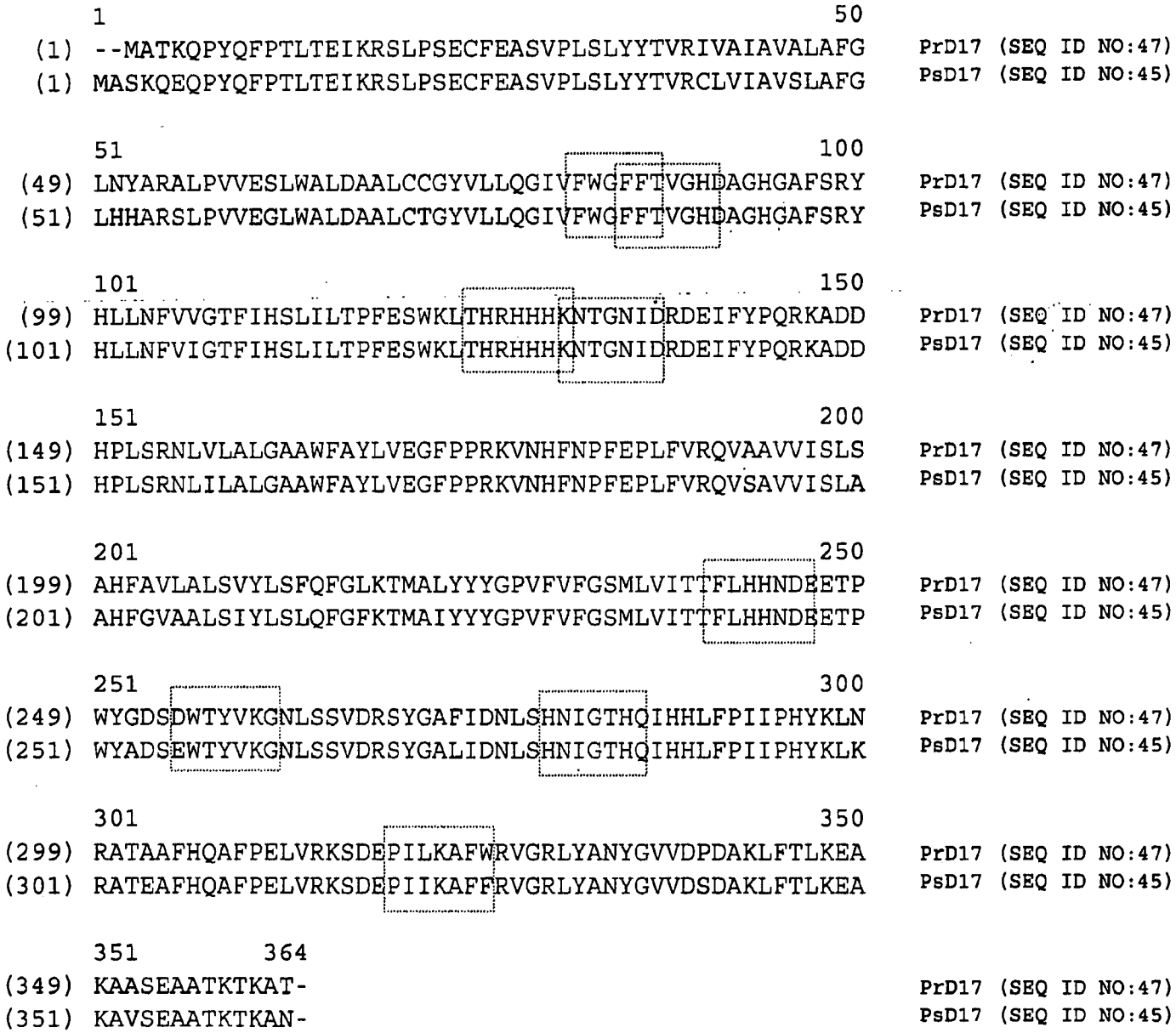
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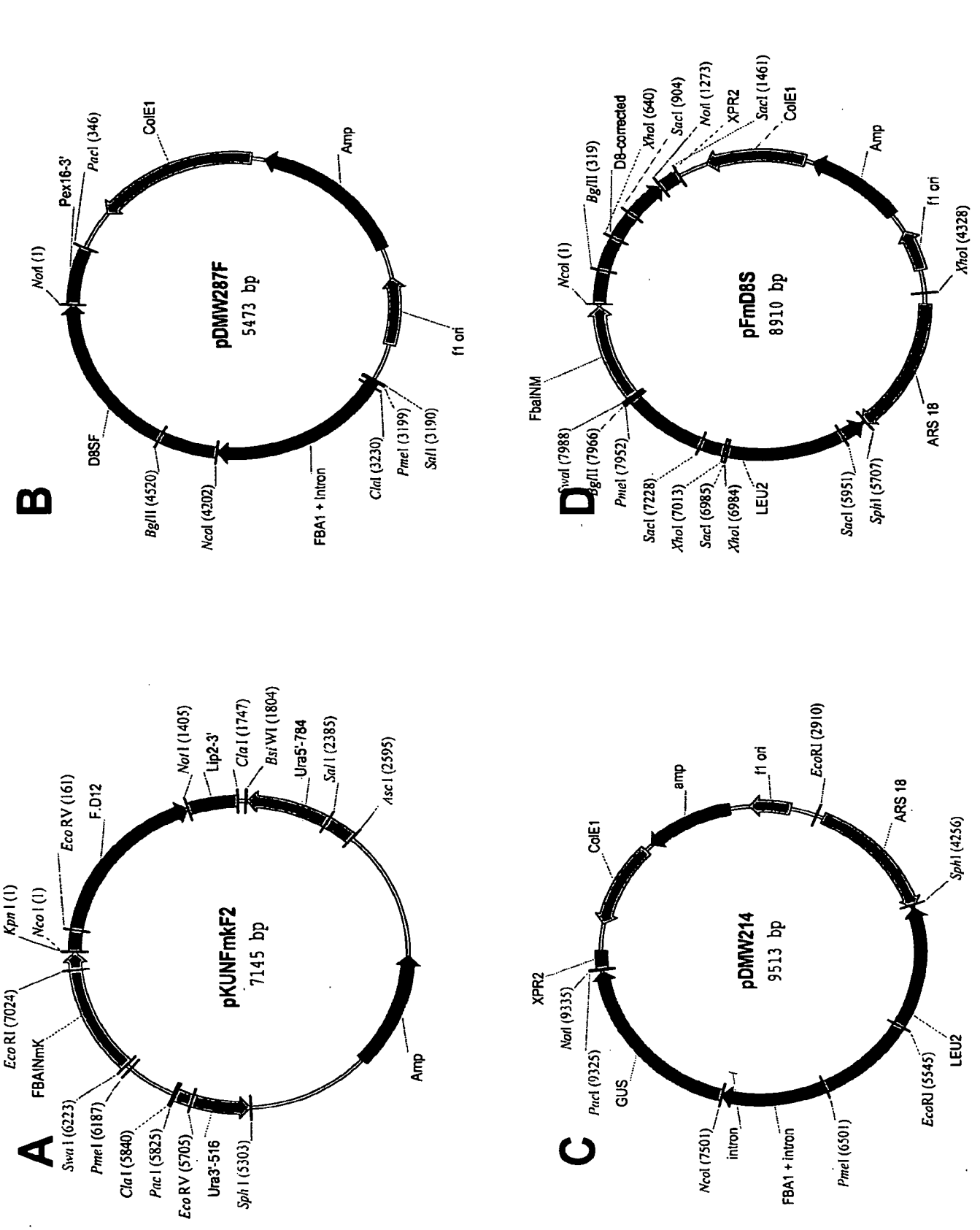
Figure 3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intron
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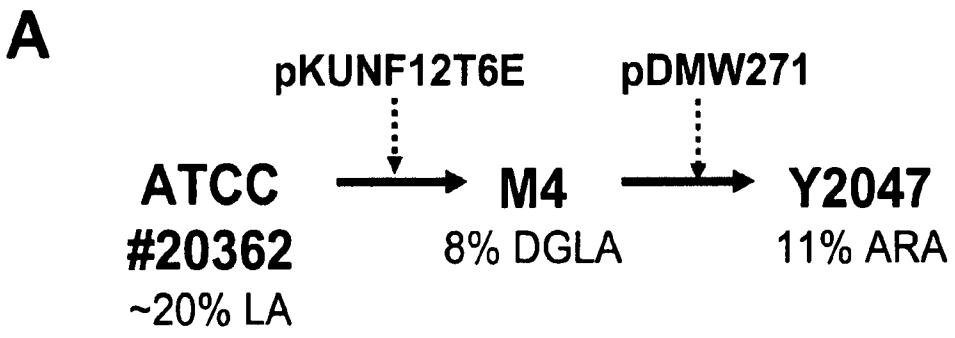
Figure 4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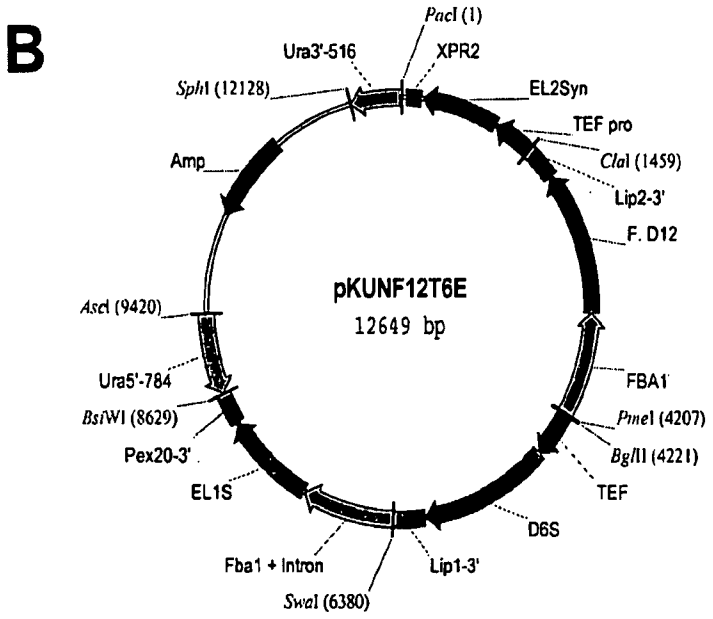
Y2047
11% ARA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PmeI (4207)
BglII (4221)
TEF
D6S
Lip1-3'
SwaI (6380)
Fba1 + Intron
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SphI (12128)
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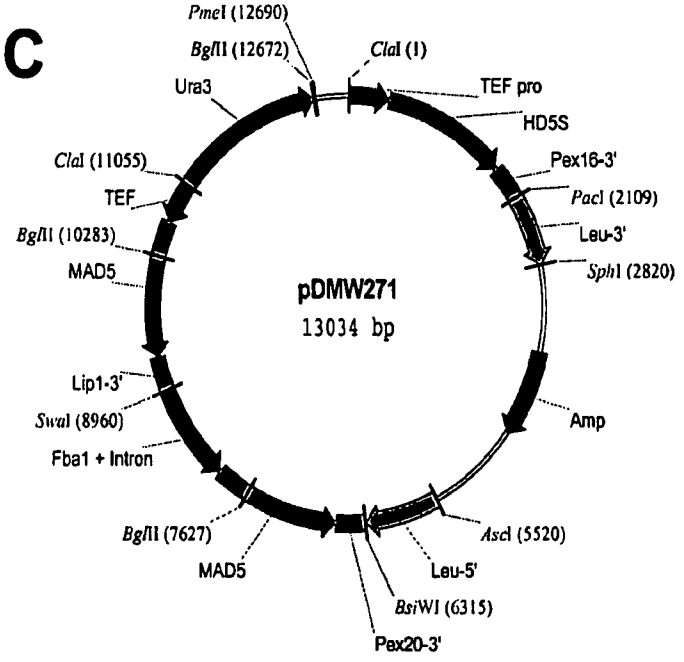
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SphI (2820)
Amp
AscI (5520)
Leu-5'
BsiWI (6315)
Pex20-3'
MAD5
BglII (7627)
Fba1 + Intron
SwaI (8960)
Lip1-3'
MAD5
BglII (10283)
TEF
ClaI (11055)
Ura3

Figure 5A

(1) ATGGCTTCTTCCACTGTTGCTGCGCCGTACGAGTTCCCGACGCTGACGGAGATCAAGCGCTCGCTGCCAGCGC (SEQ ID NO:1)
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(74) ACTGCTTTGAGGCCTCGGTCCCGTGGTCGCTCTACTACACCGTGCGCGCGCTGGGCATCGCCGGCTCGCTCGCGC (SEQ ID NO:1)
(74) ACTGCTTCGAAGCCTCTGTTCCCTGGTCCCTCTACTATACCGTGCGAGCTCTGGGCATTGCCGGTTCCCTTGCTC (SEQ ID NO:4)

(149) TCGGCCTCTACTACGCGCGCGCGCTCGCGATCGTGCAGGAGTTTGCCCTGCTGGATGCGGTGCTCTGCACGGGGT (SEQ ID NO:1)
(149) TCGGACTGTACTATGCTCGAGCCCTTGCTATCGTGCAGGAGTTTGCACTGCTCGATGCCGTCCTTTGCACTGGCT (SEQ ID NO:4)

(224) ACATTCTGCTGCAGGGCATCGTATTCTGGGGGTTCTTCACCATCGGCCATGACTGCGGCCACGGCGCGTTCTCGC (SEQ ID NO:1)
(224) ACATTCTGCTCCAGGGTATCGTGTTCTGGGGATTCTTTACCATCGGTCACGACTGTGGACATGGTGCCTTCTCGC (SEQ ID NO:4)

(299) GTTCGCACCTGCTCAACTTCAGCGTCGGCACGCTCATTCACTCGATCATCCTCACGCCGTACGAGTCATGGAAGA (SEQ ID NO:1)
(299) GATCCCACCTGCTCAACTTCTCTGTTGGCACACTCATTCACTCCATCATTCTGACTCCCTACGAGTCGTGGAAGA (SEQ ID NO:4)

(374) TCTCGCACCGCCACCACCACAAGAACACGGGCAACATCGACAAGGACGAGATTTTCTACCCGCAGCGCGAGGCCG (SEQ ID NO:1)
(374) TCAGCCATCGACACCATCACAAGAACACCGGCAACATCGACAAGGATGAGATCTTCTACCCTCAGCGAGAAGCCG (SEQ ID NO:4)

(499) ACTCGCACCCACTGTCCCGACACATGGTGATCTCGCTCGGCTCGGCCTGGTTCGCGTACCTCGTTGCGGGCTTCC (SEQ ID NO:1)
(499) ACTCTCATCCCCTGTCCCGACACATGGTCATCTCCCTTGGTTCGGCTTGGTTTGCCTACCTCGTTGCTGGATTTC (SEQ ID NO:4)

(524) CTCCTCGCAAGGTGAACCACTTCAACCCTTGGGAACCGTTGTACCTGCGCCGCATGTCTGCCGTCATCATCTCAC (SEQ ID NO:1)
(524) CTCCCCGAAAGGTCAACCACTTCAATCCCTGGGAGCCTCTCTACCTGCGAAGAATGTCTGCCGTCATCATTTCCC (SEQ ID NO:4)

Figure 5B

(599) TCGGCTCGCTCGTGGCGTTCGCGGGCTTGTATGCGTATCTCACCTACGTCTATGGCCTTAAGACCATGGCGCTGT (SEQ ID NO:1)
(599) TCGGCTCTCTCGTGGCCTTTGCTGGTCTGTACGCCTACCTTACCTACGTCTACGGCCTCAAGACCATGGCTCTGT (SEQ ID NO:4)

(674) ACTACTTCGCCCCTCTCTTTGGGTTCGCCACGATGCTCGTGGTCACTACCTTTTTGCACCACAATGACGAGGAAA (SEQ ID NO:1)
(674) ATTACTTCGCACCTCTCTTTGGATTCGCCACCATGCTGGTTGTCACTACCTTCCTCCATCACAACGACGAGGAAA (SEQ ID NO:4)

(749) CGCCATGGTACGCCGACTCGGAGTGGACGTACGTCAAGGGCAACCTCTCGTCCGTGGACCGCTCGTACGGCGCGC (SEQ ID NO:1)
(749) CTCCCTGGTACGCCGATTCGGAGTGGACCTATGTCAAGGGCAACTTGTCCTCTGTGGACCGAAGCTACGGAGCCC (SEQ ID NO:4)

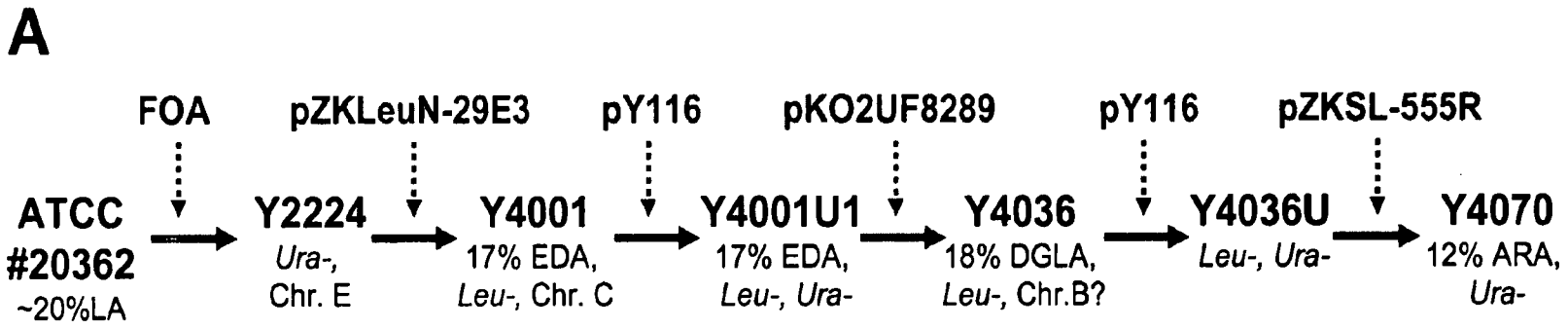
(824) TCATCGACAACCTGAGCCACAACATCGGCACGCACCAGATCCACCACCTGTTTCCGATCATCCCGCACTACAAGC (SEQ ID NO:1)
(824) TCATCGACAACCTGTCCCACAACATTGGTACACATCAGATCCACCATCTGTTTCCCATCATTCCTCACTACAAGC (SEQ ID NO:4)

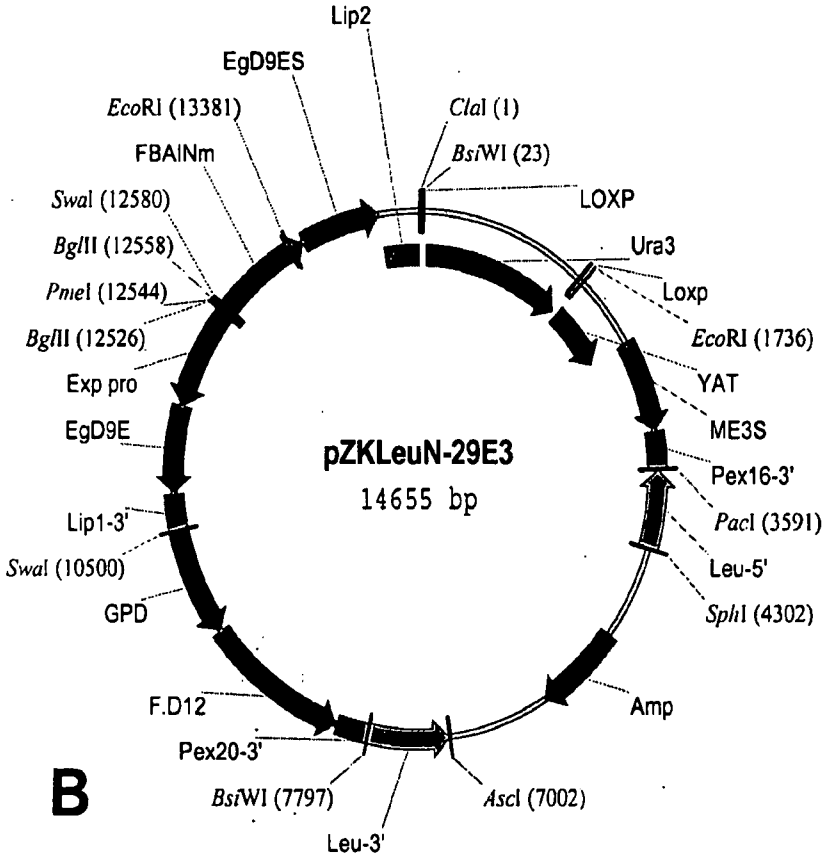
(899) TGAACGAGGCGACGGCAGCGTTCGCGCAGGCGTTCCCGGAGCTCGTGCGCAAGAGCGCGTCGCCGATCATCCCGA (SEQ ID NO:1)
(899) TCAACGAGGCCACTGCTGCCTTCGCTCAGGCCTTTCCCGAACTGGTGCGAAAGTCGGCTTCTCCCATCATTCCCA (SEQ ID NO:4)

(974) CGTTCATCCGCATCGGGCTCATGTACGCCAAGTACGGCGTCGTGGACAAGGACGCCAAGATGTTTACGCTCAAGG (SEQ ID NO:1)
(974) CCTTCATCCGAATTGGTCTTATGTACGCCAAGTACGGCGTGGTCGACAAGGATGCCAAGATGTTTACCCTCAAGG (SEQ ID NO:4)

(1049) AGGCCAAGGCCGCCAAGACCAAGGCCAACTAG (SEQ ID NO:1)
(1049) AGGCCAAGGCTGCCAAGACCAAAGCCAACTAA (SEQ ID NO:4)

Figure 6


A
FOA
pZKLeuN-29E3
pY116
pKO2UF8289
pY116
pZKSL-555R
ATCC #20362 ~20%LA
Y2224 Ura-, Chr. E
Y4001 17% EDA, Leu-, Chr. C
Y4001U1 17% EDA, Leu-, Ura-
Y4036 18% DGLA, Leu-, Chr.B?
Y4036U Leu-, Ura-
Y4070 12% ARA, Ura-


B
pZKLeuN-29E3
14655 bp
Lip2
EgD9ES
EcoRI (13381)
FBAINm
SwaI (12580)
BglII (12558)
PmeI (12544)
BglII (12526)
Exp pro
EgD9E
Lip1-3'
SwaI (10500)
GPD
F.D12
Pex20-3'
BsiWI (7797)
Leu-3'
AscI (7002)
Amp
SphI (4302)
Leu-5'
PacI (3591)
Pex16-3'
ME3S
YAT
EcoRI (1736)
Loxp
Ura3
LOXP
BsiWI (23)
ClaI (1)

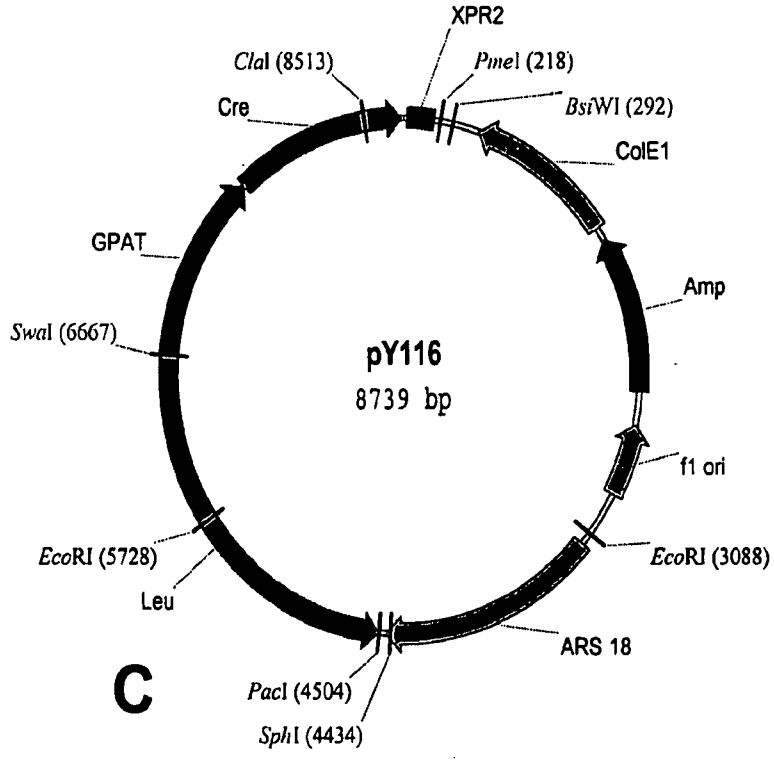

C
pY116
8739 bp
XPR2
ClaI (8513)
PmeI (218)
Cre
BsiWI (292)
ColE1
GPAT
Amp
SwaI (6667)
f1 ori
EcoRI (5728)
EcoRI (3088)
Leu
ARS 18
PacI (4504)
SphI (4434)

Figure 7

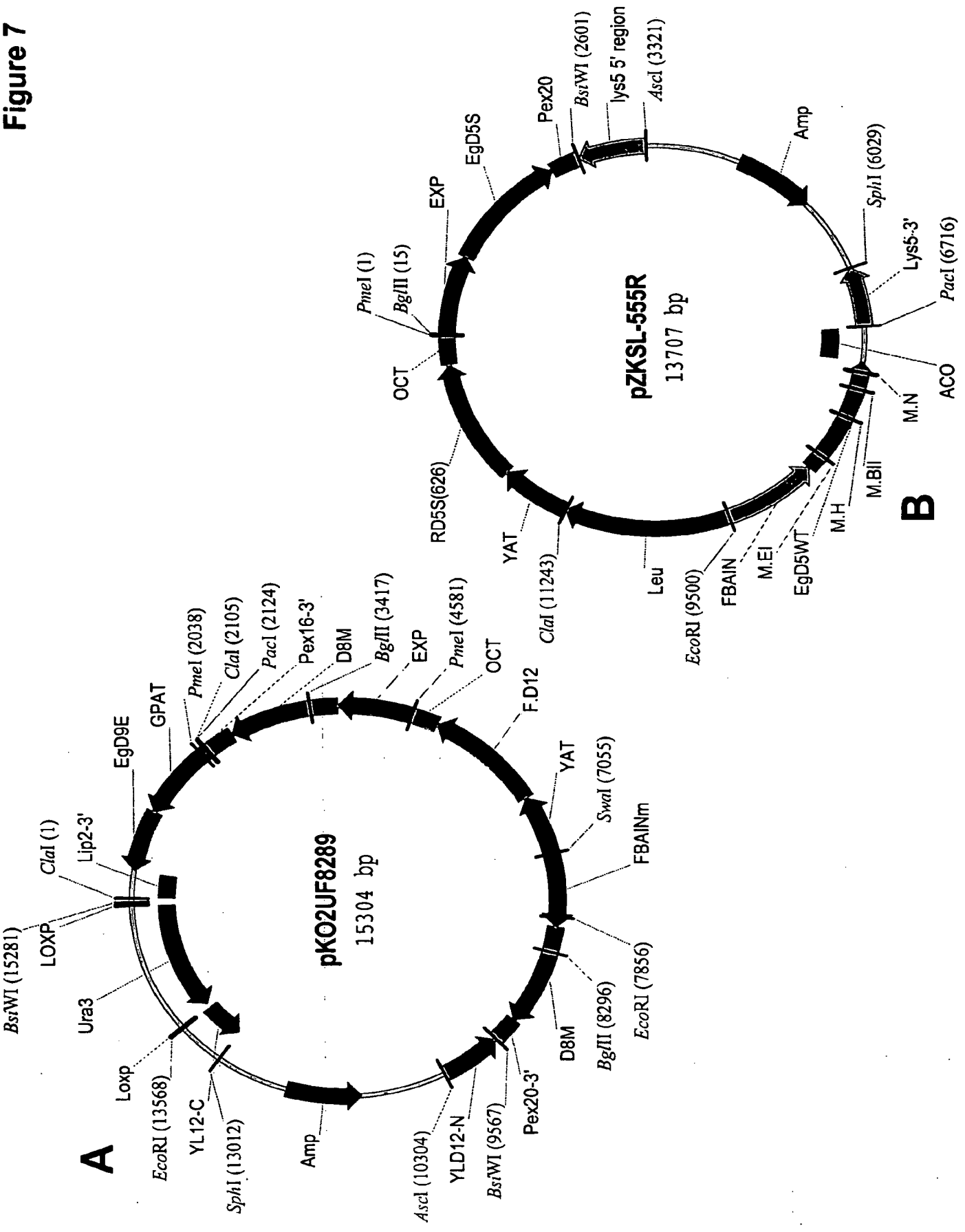

A
pKO2UF8289
15304 bp
BstWI (15281)
LOXP
ClaI (1)
Lip2-3'
EgD9E
GPAT
PmeI (2038)
ClaI (2105)
PacI (2124)
Pex16-3'
D8M
BglII (3417)
EXP
PmeI (4581)
OCT
F.D12
YAT
SwaI (7055)
FBAINm
EcoRI (7856)
BglII (8296)
D8M
Pex20-3'
BstWI (9567)
YLD12-N
AscI (10304)
Amp
SphI (13012)
YL12-C
EcoRI (13568)
Loxp
Ura3
B
pZKSL-555R
13707 bp
PmeI (1)
BglII (15)
OCT
EXP
EgD5S
Pex20
BstWI (2601)
lys5 5' region
AscI (3321)
Amp
SphI (6029)
Lys5-3'
PacI (6716)
ACO
M.N
M.BII
M.H
EgD5WT
M.EI
FBAIN
EcoRI (9500)
Leu
ClaI (11243)
YAT
RD5S(626)

Figure 8

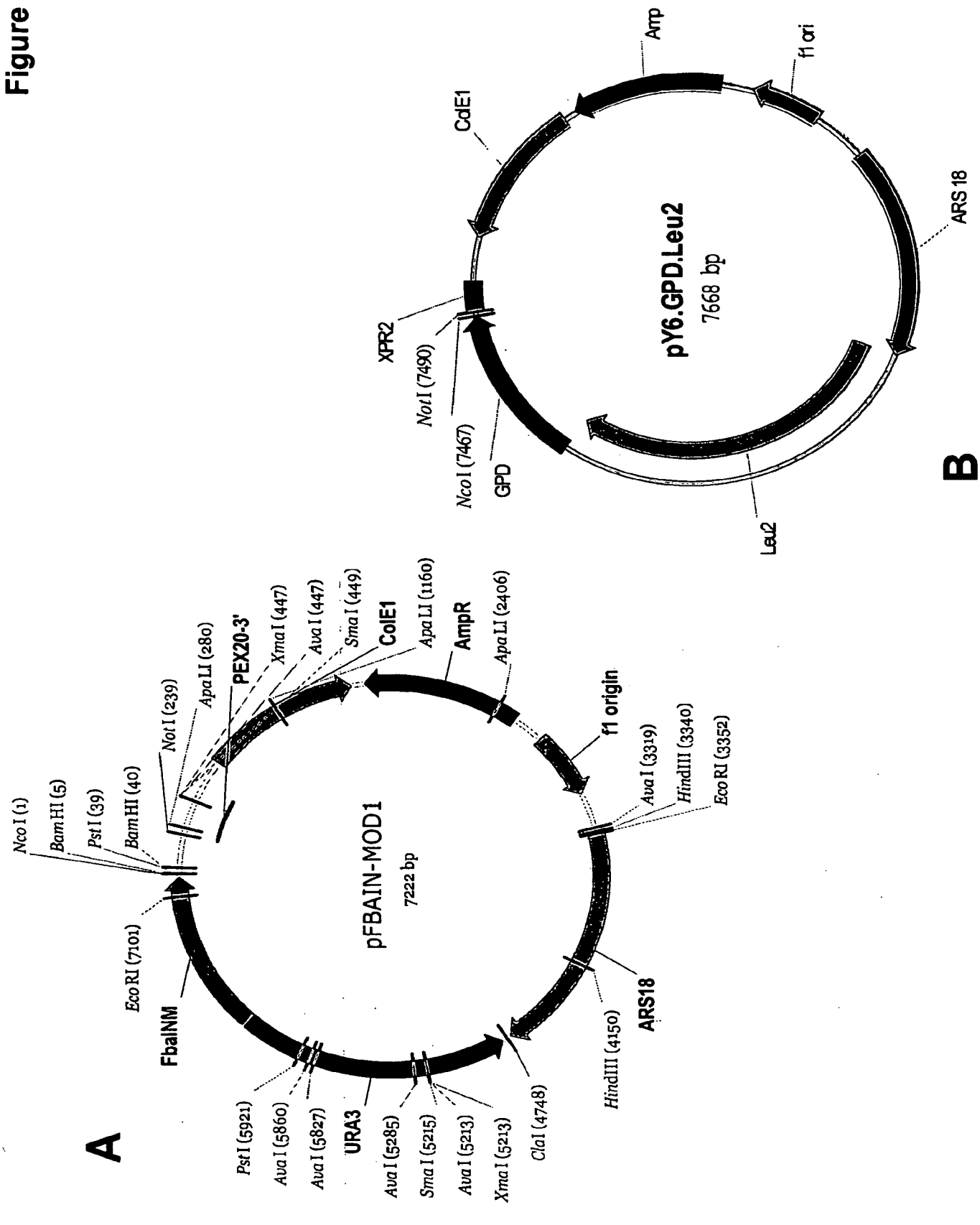

A
pFBAIN-MOD1
7222 bp
FbalNM
URA3
PEX20-3'
ColE1
AmpR
f1 origin
ARS18
Nco I (1)
BamHI (5)
Pst I (39)
BamHI (40)
Not I (239)
Apa LI (280)
Xma I (447)
Ava I (447)
Sma I (449)
Apa LI (1160)
Apa LI (2406)
Ava I (3319)
HindIII (3340)
Eco RI (3352)
HindIII (4150)
Cla I (4748)
Xma I (5213)
Ava I (5213)
Sma I (5215)
Ava I (5285)
Ava I (5827)
Ava I (5860)
Pst I (5921)
Eco RI (7101)
B
pY6.GPD.Leu2
7668 bp
Amp
f1 ori
ARS 18
CdE1
XPR2
Not I (7490)
Nco I (7467)
GPD
Leu2

Figure 9

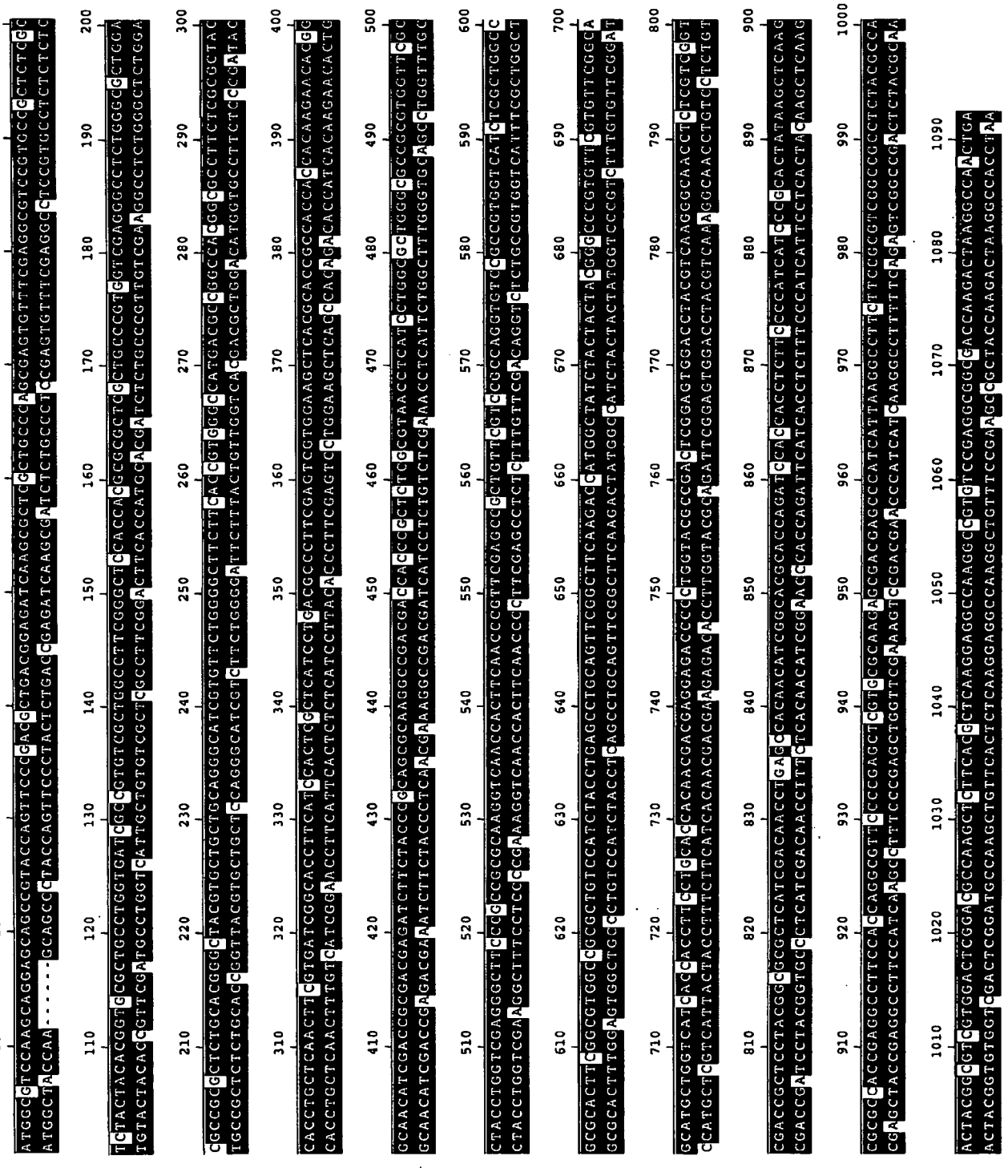


Figure 10

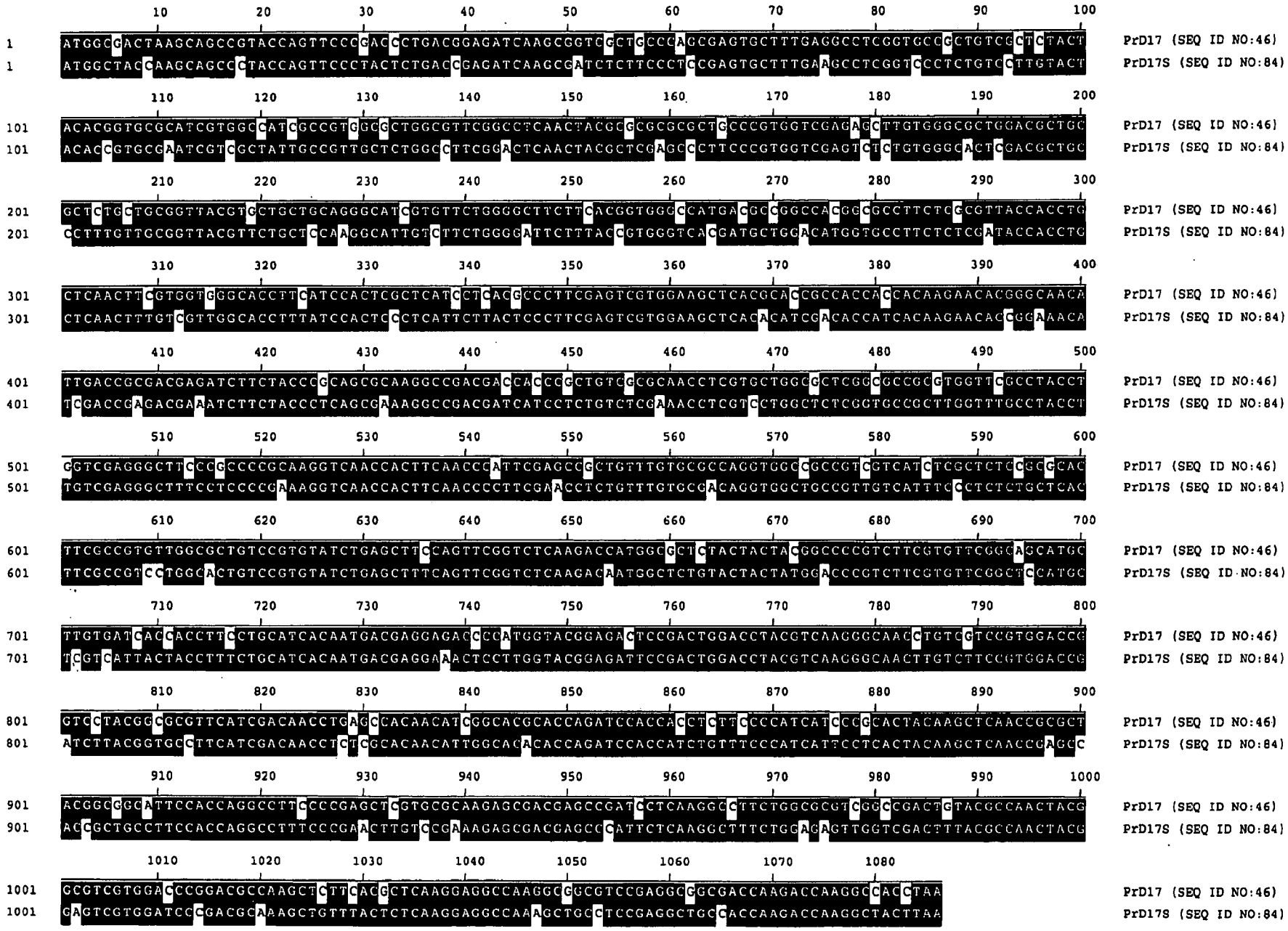


Figure 11

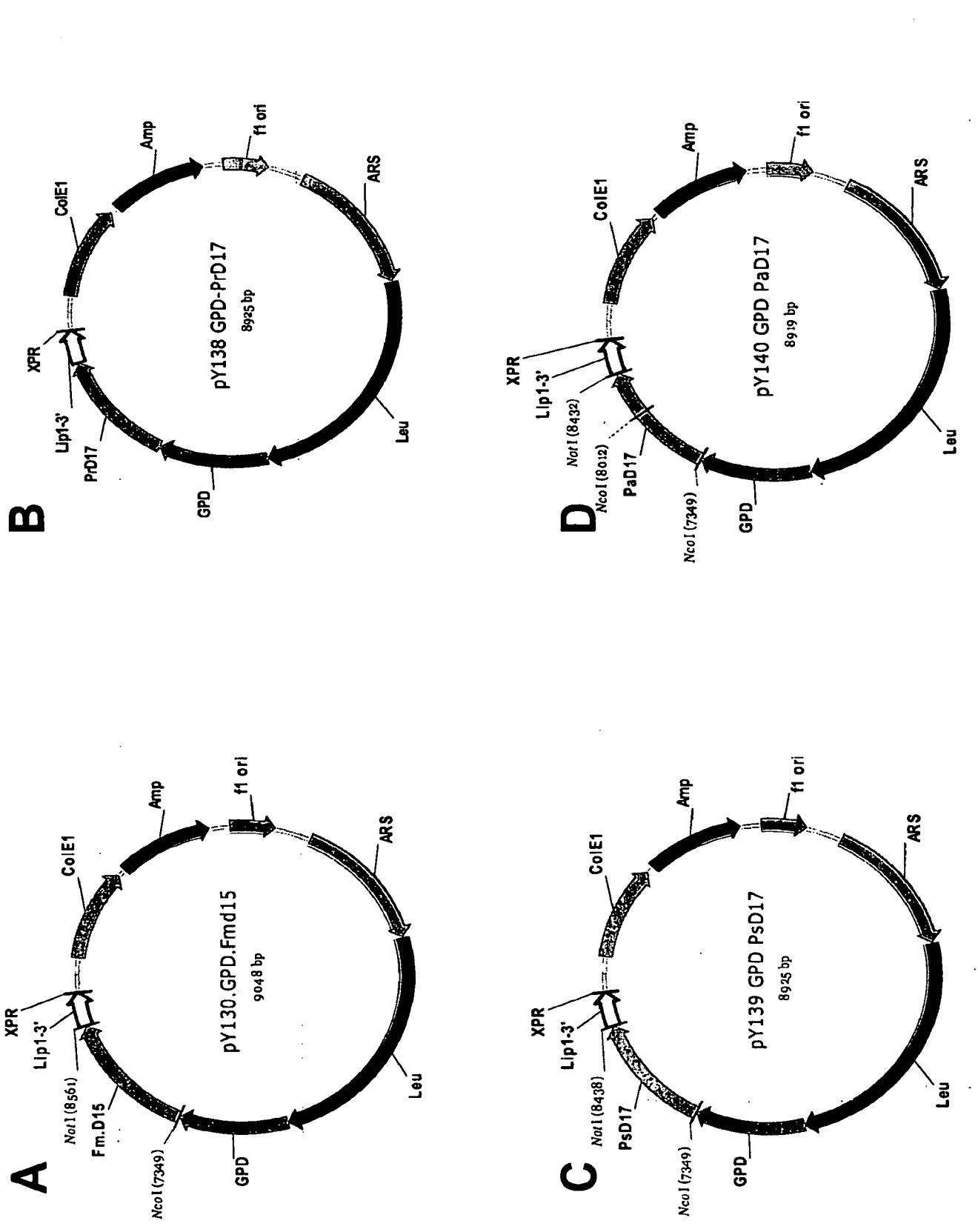

A
pY130.GPD.Fmd15
9048 bp
XPR
Lip1-3'
NotI (8561)
Fm.D15
NcoI (7349)
GPD
Leu
ARS
f1 ori
Amp
ColE1
B
pY138 GPD-PrD17
8925 bp
XPR
Lip1-3'
PrD17
GPD
Leu
ARS
f1 ori
Amp
ColE1
C
pY139 GPD PsD17
8925 bp
XPR
Lip1-3'
NotI (8438)
PsD17
NcoI (7349)
GPD
Leu
ARS
f1 ori
Amp
ColE1
D
pY140 GPD PaD17
8919 bp
XPR
Lip1-3'
NotI (8432)
NcoI (8012)
PaD17
NcoI (7349)
GPD
Leu
ARS
f1 ori
Amp
ColE1

Figure 12

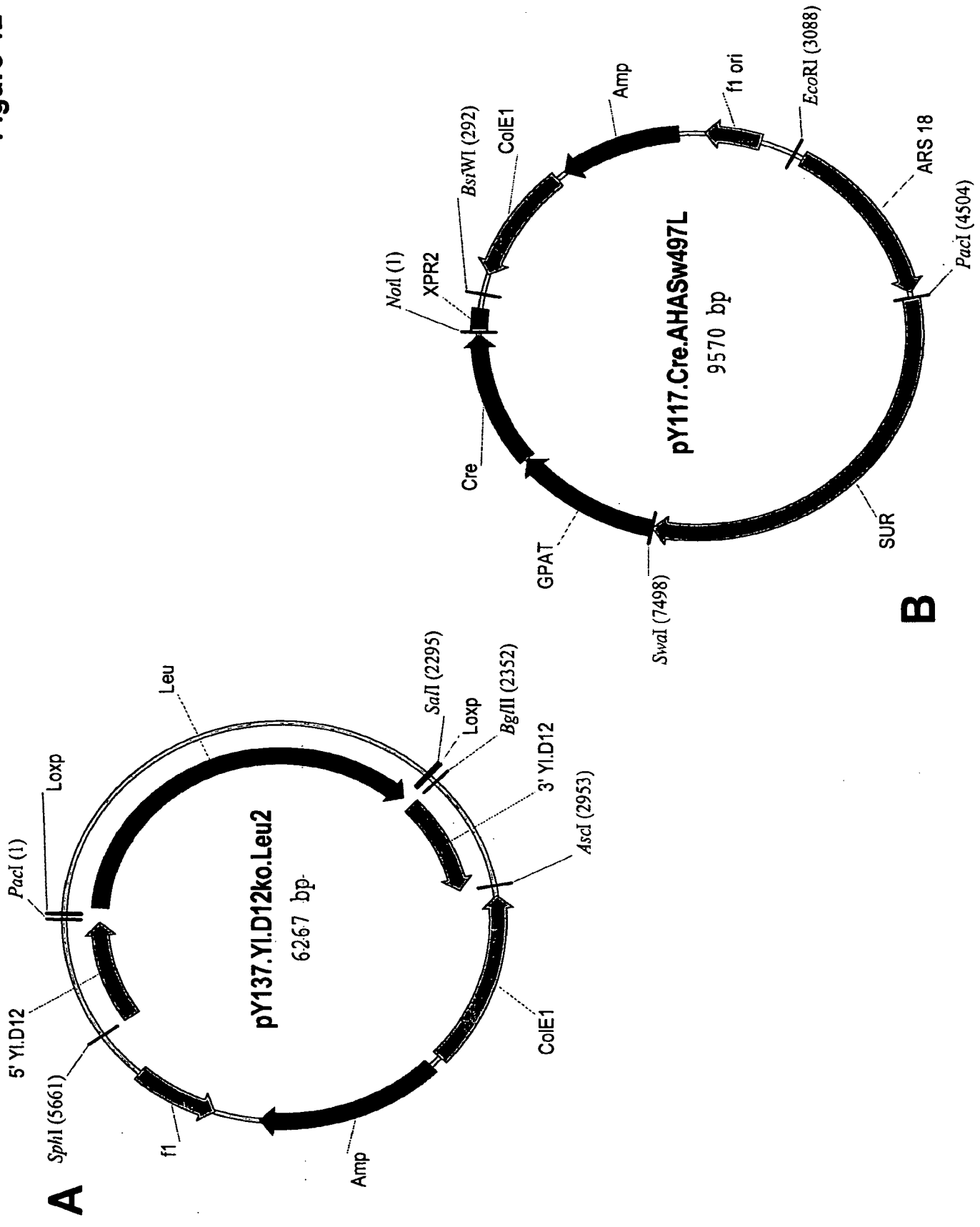

A
pY137.YI.D12ko.Leu2
6267 bp
PacI (1)
Loxp
Leu
SalI (2295)
Loxp
BglII (2352)
3' YI.D12
AscI (2953)
ColE1
Amp
f1
SphI (5661)
5' YI.D12
B
pY117.Cre.AHASw497L
9570 bp
NotI (1)
XPR2
BsiWI (292)
ColE1
Amp
f1 ori
EcoRI (3088)
ARS 18
PacI (4504)
Cre
GPAT
SwaI (7498)
SUR

Figure 13

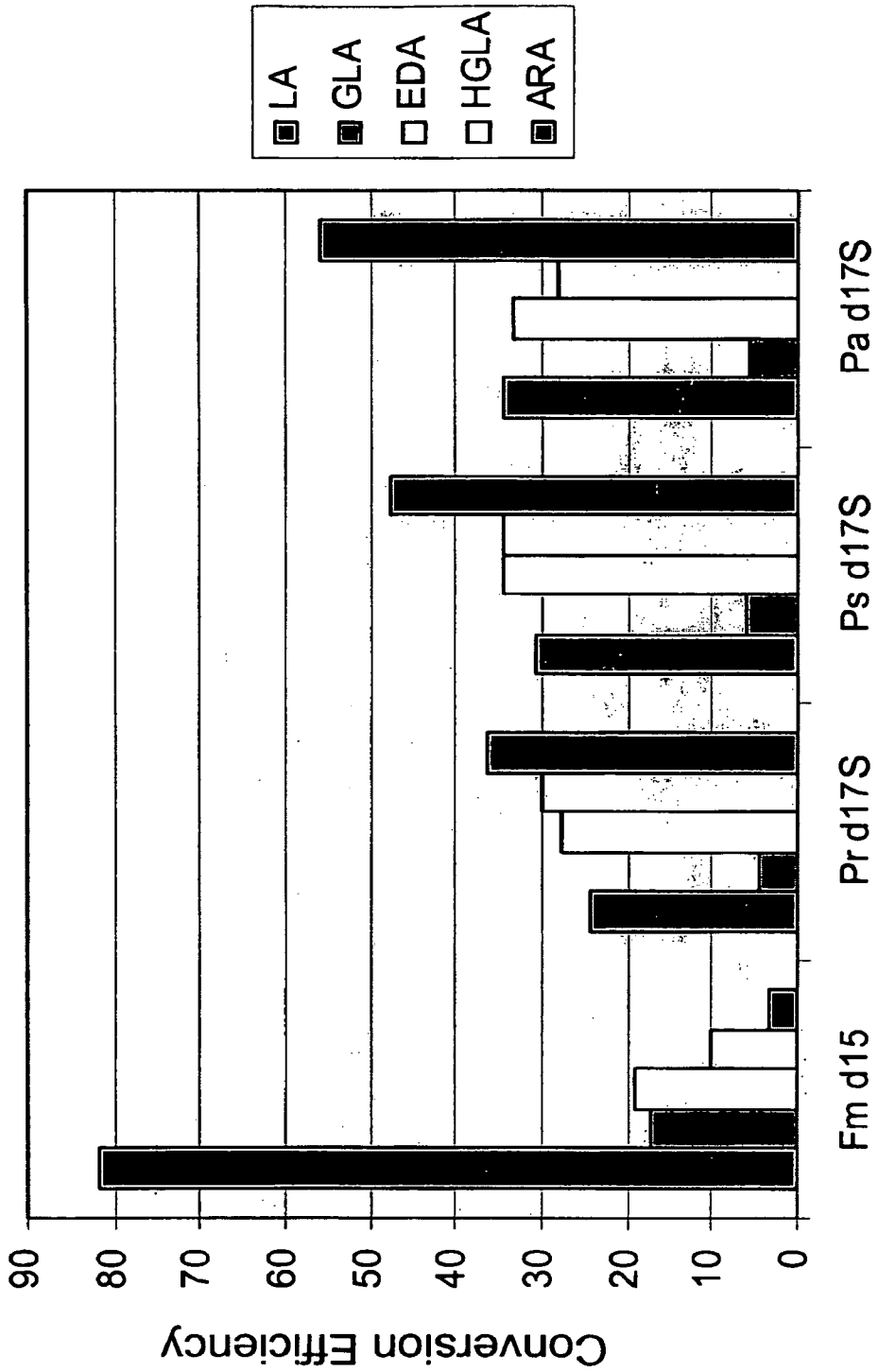
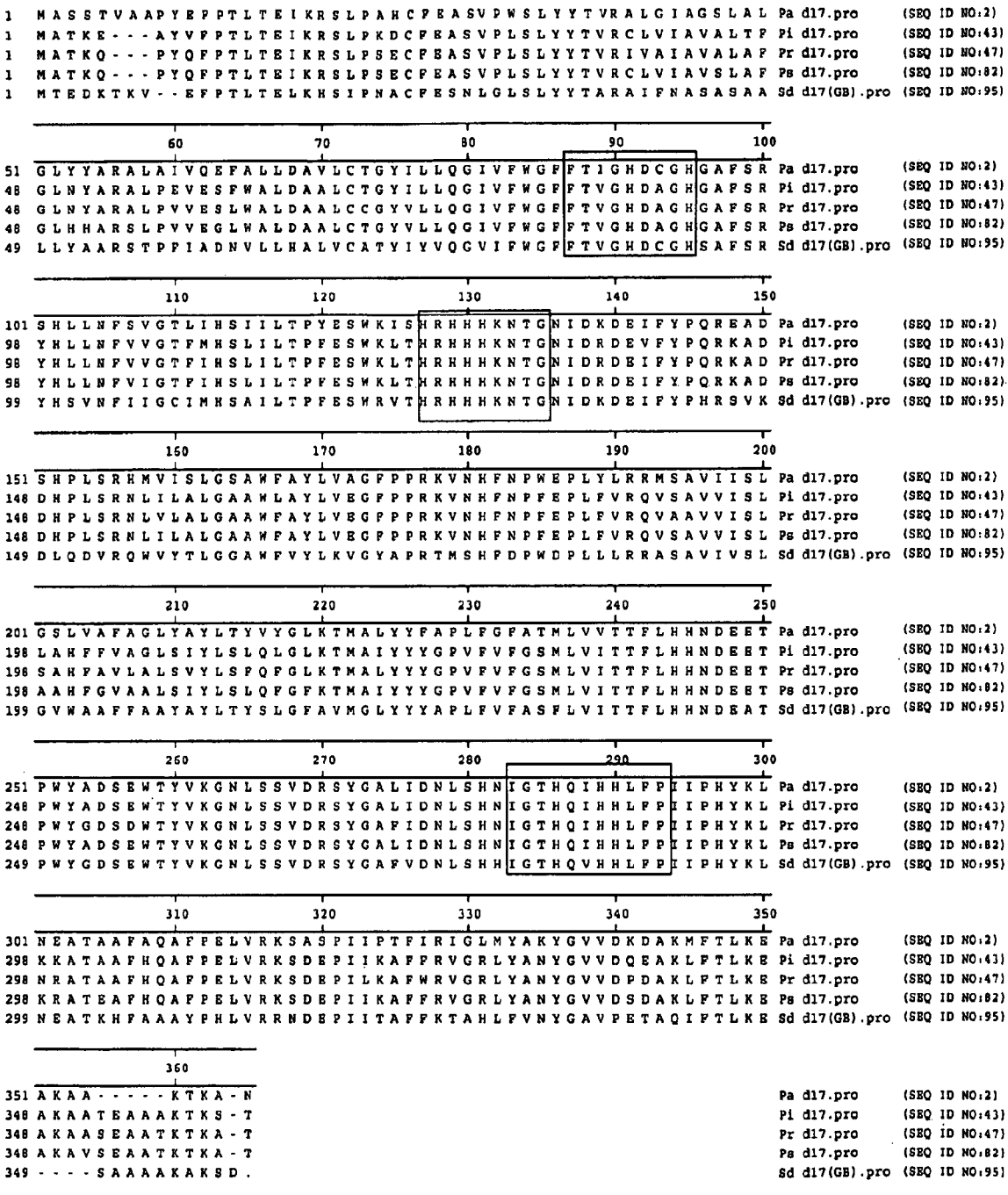

LA
GLA
EDA
HGLA
ARA
Conversion Efficiency
0
10
20
30
40
50
60
70
80
90
Fm d15
Pr d17S
Ps d17S
Pa d17S

Figure 14


1 MASSTVAAPYEPPTLTEIKRSLPAHCFEASVPWSLYYTVRALGIAGSLAL Pa d17.pro (SEQ ID NO:2)
1 MATKE---AYVPPTLTEIKRSLPKDCFEASVPLSLYYTVRCLVIAVALTF Pi d17.pro (SEQ ID NO:43)
1 MATKQ---PYQFPTLTEIKRSLPSECFEASVPLSLYYTVRIVAIAVALAF Pr d17.pro (SEQ ID NO:47)
1 MATKQ---PYQFPTLTEIKRSLPSECFEASVPLSLYYTVRCLVIAVSLAF Ps d17.pro (SEQ ID NO:82)
1 MTEDKTKV--EFPTLTELKHSIPNACFESNLGLSLYYTARAIFNASASAA Sd d17(GB).pro (SEQ ID NO:95)
60 70 80 90 100
51 GLYYARALAIVQEFALLDAVLCTGYILLQGIVFWGFFTIGHDCGHGAFSR Pa d17.pro (SEQ ID NO:2)
48 GLNYARALPEVESFWALDAALCTGYILLQGIVFWGFFTVGHDAGHGAFSR Pi d17.pro (SEQ ID NO:43)
48 GLNYARALPVVESLWALDAALCCGYVLLQGIVFWGFFTVGHDAGHGAFSR Pr d17.pro (SEQ ID NO:47)
48 GLHHARSLPVVEGLWALDAALCTGYVLLQGIVFWGFFTVGHDAGHGAFSR Ps d17.pro (SEQ ID NO:82)
49 LLYAARSTPFIADNVLLHALVCATYIYVQGVIFWGFFTVGHDCGHSAFSR Sd d17(GB).pro (SEQ ID NO:95)
110 120 130 140 150
101 SHLLNFSVGTLIHSIILTPYESWKISHRHHHKNTGNIDKDEIFYPQREAD Pa d17.pro (SEQ ID NO:2)
98 YHLLNFVVGTFMHSLILTPFESWKLTHRHHHKNTGNIDRDEVFYPQRKAD Pi d17.pro (SEQ ID NO:43)
98 YHLLNFVVGTFIHSLILTPFESWKLTHRHHHKNTGNIDRDEIFYPQRKAD Pr d17.pro (SEQ ID NO:47)
98 YHLLNFVIGTFIHSLILTPFESWKLTHRHHHKNTGNIDRDEIFYPQRKAD Ps d17.pro (SEQ ID NO:82)
99 YHSVNFIIGCIMHSAILTPFESWRVTHRHHHKNTGNIDKDEIFYPHRSVK Sd d17(GB).pro (SEQ ID NO:95)
160 170 180 190 200
151 SHPLSRHMVISLGSAWFAYLVAGFPPRKVNHFNPWEPLYLRRMSAVIISL Pa d17.pro (SEQ ID NO:2)
148 DHPLSRNLILALGAAWLAYLVEGFPPRKVNHFNPFEPLFVRQVSAVVISL Pi d17.pro (SEQ ID NO:43)
148 DHPLSRNLVLALGAAWFAYLVEGFPPRKVNHFNPFEPLFVRQVAAVVISL Pr d17.pro (SEQ ID NO:47)
148 DHPLSRNLILALGAAWFAYLVEGFPPRKVNHFNPFEPLFVRQVSAVVISL Ps d17.pro (SEQ ID NO:82)
149 DLQDVRQWVYTLGGAWFVYLKVGYAPRTMSHFDPWDPLLLRRASAVIVSL Sd d17(GB).pro (SEQ ID NO:95)
210 220 230 240 250
201 GSLVAFAGLYAYLTYVYGLKTMALYYFAPLFGFATMLVVTTFLHHNDEET Pa d17.pro (SEQ ID NO:2)
198 LAHFFVAGLSIYLSLQLGLKTMAIYYYGPVFVFGSMLVITTFLHHNDEET Pi d17.pro (SEQ ID NO:43)
198 SAHFAVLALSVYLSFQFGLKTMALYYYGPVFVFGSMLVITTFLHHNDEET Pr d17.pro (SEQ ID NO:47)
198 AAHFGVAALSIYLSLQFGFKTMAIYYYGPVFVFGSMLVITTFLHHNDEET Ps d17.pro (SEQ ID NO:82)
199 GVWAAFFAAYAYLTYSLGFAVMGLYYYAPLFVFASFLVITTFLHHNDEAT Sd d17(GB).pro (SEQ ID NO:95)
260 270 280 290 300
251 PWYADSEWTYVKGNLSSVDRSYGALIDNLSHNIGTHQIHHLFPIIPHYKL Pa d17.pro (SEQ ID NO:2)
248 PWYADSEWTYVKGNLSSVDRSYGALIDNLSHNIGTHQIHHLFPIIPHYKL Pi d17.pro (SEQ ID NO:43)
248 PWYGDSDWTYVKGNLSSVDRSYGAFIDNLSHNIGTHQIHHLFPIIPHYKL Pr d17.pro (SEQ ID NO:47)
248 PWYADSEWTYVKGNLSSVDRSYGALIDNLSHNIGTHQIHHLFPIIPHYKL Ps d17.pro (SEQ ID NO:82)
249 PWYGDSEWTYVKGNLSSVDRSYGAFVDNLSHHIGTHQVHHLFPIIPHYKL Sd d17(GB).pro (SEQ ID NO:95)
310 320 330 340 350
301 NEATAAFAQAFPELVRKSASPIIPTFIRIGLMYAKYGVVDKDAKMFTLKE Pa d17.pro (SEQ ID NO:2)
298 KKATAAFHQAFPELVRKSDEPIIKAFFRVGRLYANYGVVDQEAKLFTLKE Pi d17.pro (SEQ ID NO:43)
298 NRATAAFHQAFPELVRKSDEPILKAFWRVGRLYANYGVVDPDAKLFTLKE Pr d17.pro (SEQ ID NO:47)
298 KRATEAFHQAFPELVRKSDEPIIKAFFRVGRLYANYGVVDSDAKLFTLKE Ps d17.pro (SEQ ID NO:82)
299 NEATKHFAAAYPHLVRRNDEPIITAFFKTAHLFVNYGAVPETAQIFTLKE Sd d17(GB).pro (SEQ ID NO:95)
360
351 AKAA-----KTKA-N Pa d17.pro (SEQ ID NO:2)
348 AKAATEAAAKTKS-T Pi d17.pro (SEQ ID NO:43)
348 AKAASEAATKTKA-T Pr d17.pro (SEQ ID NO:47)
348 AKAVSEAATKTKA-T Ps d17.pro (SEQ ID NO:82)
349 ----SAAAAKAKSD. Sd d17(GB).pro (SEQ ID NO:95)

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/016490

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 C12P7/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, Sequence Search, BIOSIS, EMBASE, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2006/100241 A (BASF PLANT SCIENCE GMBH [DE]; CIRPUS PETRA [DE]; BAUER JOERG [DE]) 28 September 2006 (2006-09-28) cited in the application SEQ ID NO: 23 and 24	1-26
X	WO 2004/101757 A (DU PONT [US]; PICATAGGIO STEPHEN K [US]; YADAV NARENDRA S [US]; ZHU QU) 25 November 2004 (2004-11-25) examples 1-6	1-26
X	WO 2005/083093 A (BASF PLANT SCIENCE GMBH [DE]; CIRPUS PETRA [DE]; BAUER JOERG [DE]; QIU) 9 September 2005 (2005-09-09) sequences 87,88	1-26

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 March 2008	02/04/2008

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Lejeune, Robert

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/016490

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STREDANSKY M ET AL: "Production of polyunsaturated fatty acids by Pythium ultimum in solid-state cultivation" ENZYME AND MICROBIAL TECHNOLOGY, vol. 26, no. 2-4, February 2000 (2000-02), pages 304-307, XP002473542 ISSN: 0141-0229	
A	CHENG M H ET AL: "FUNGAL PRODUCTION OF EICOSAPENTAENOIC AND ARACHIDONIC ACIDS FROM INDUSTRIAL WASTE STREAMS AND CRUDE SOYBEAN OIL" BIORESOURCE TECHNOLOGY, ELSEVIER, GB, vol. 67, no. 2, 1999, pages 101-110, XP001016139 ISSN: 0960-8524	
P,X	DATABASE EMBL [Online] 21 March 2007 (2007-03-21), "PUNA910TV Pythium ultimum ESTs Pythium ultimum DAOM BR144 cDNA clone PUNA910, mRNA sequence." XP002473482 retrieved from EBI accession no. EMBL:EL777858 Database accession no. EL777858 the whole document	1,22,24
E	WO 2007/123999 A (DU PONT [US]; XUE ZHIXIONG [US]; ZHU QUINN QUN [US]) 1 November 2007 (2007-11-01) sequence 4	22-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2007/016490

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] on paper

   [X] in electronic form

   c. time of filing/furnishing

   [X] contained in the international application as filed

   [X] filed together with the international application in electronic form

   [ ] furnished subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2007/016490

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2006100241 A	28-09-2006	AU 2006226338 A1	28-09-2006
		CA 2600286 A1	28-09-2006
		DE 102005013779 A1	28-09-2006
		EP 1866417 A2	19-12-2007
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		CN 1816559 A	09-08-2006
		CN 1816630 A	09-08-2006
		CN 1852986 A	25-10-2006
		EP 1620543 A2	01-02-2006
		JP 2007504839 T	08-03-2007
		KR 20060018832 A	02-03-2006
WO 2005083093 A	09-09-2005	AU 2005217079 A1	09-09-2005
		CA 2559360 A1	09-09-2005
WO 2007123999 A	01-11-2007	NONE	