



(86) Date de dépôt PCT/PCT Filing Date: 2007/10/22
(87) Date publication PCT/PCT Publication Date: 2008/05/29
(85) Entrée phase nationale/National Entry: 2009/03/19
(86) N° demande PCT/PCT Application No.: US 2007/022423
(87) N° publication PCT/PCT Publication No.: 2008/063340
(30) Priorité/Priority: 2006/10/23 (US60/853,563)

(51) Cl.Int./Int.Cl. *C12N 15/82* (2006.01),
A01H 5/00 (2006.01), *C12N 15/53* (2006.01),
C12N 9/02 (2006.01)
(71) Demandeur/Applicant:
E. I. DU PONT DE NEMOURS AND COMPANY, US
(72) Inventeurs/Inventors:
DAMUDE, HOWARD, US;
ZHU, QUINN QUN, US;
XUE, ZHIXIONG, US
(74) Agent: TORYS LLP

(54) Titre : DELTA-8 DESATURASES ET UTILISATION DE CES DERNIERES DANS LA PREPARATION D'ACIDES
GRAS POLYINSATURES
(54) Title: DELTA-8 DESATURASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

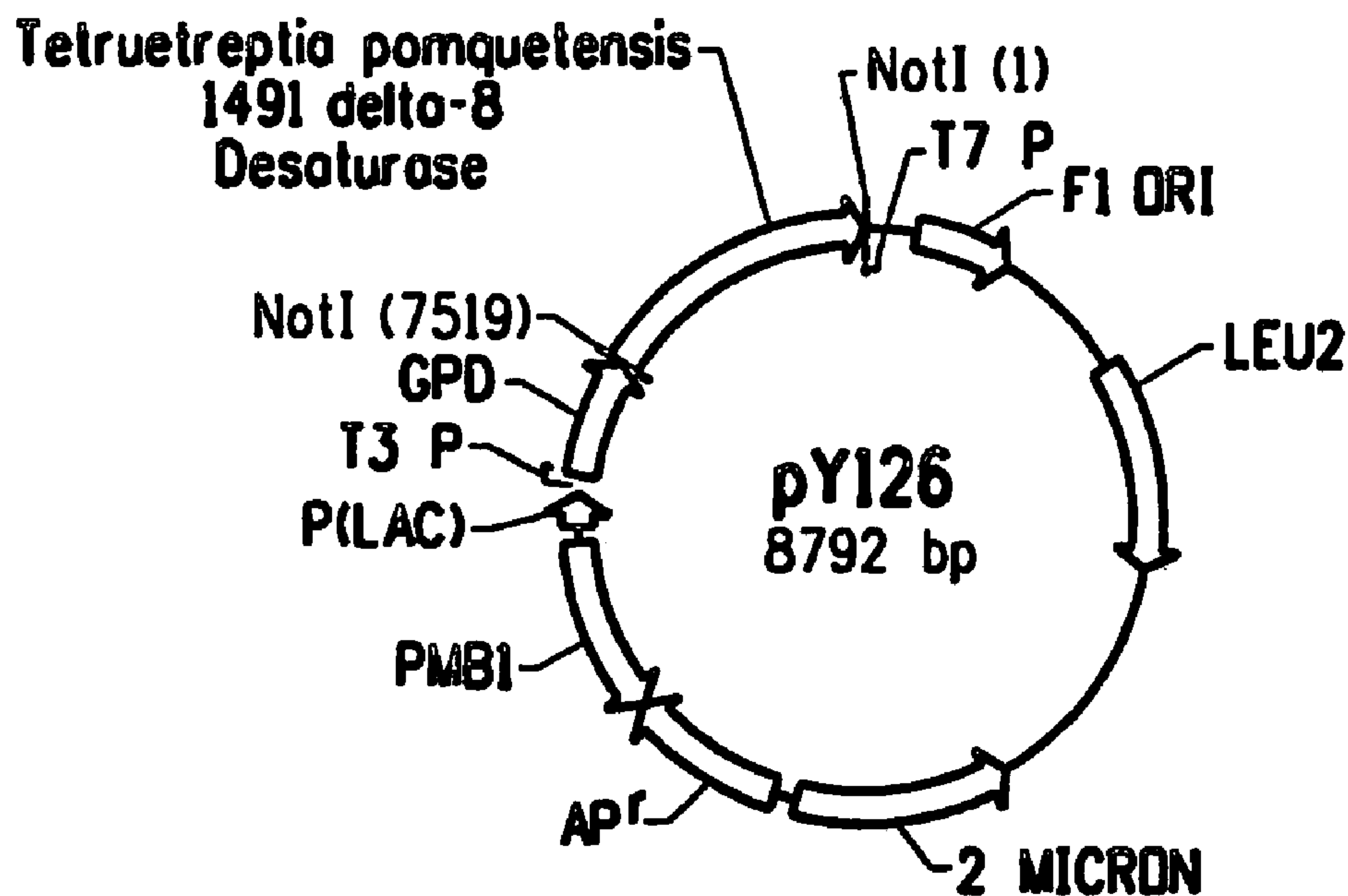


FIG. 1

(57) Abrégé/Abstract:

Isolated nucleic acid fragments and recombinant constructs comprising such fragments encoding delta-8 desaturases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) using these delta-8 desaturases in plants and oleaginous yeast.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

PCT

(43) International Publication Date
29 May 2008 (29.05.2008)(10) International Publication Number
WO 2008/063340 A9

(51) International Patent Classification:

C12N 15/82 (2006.01) *C12N 15/53* (2006.01)
C12N 9/02 (2006.01) *A01H 5/00* (2006.01)

Chester, PA 19382 (US). XUE, Zhixiong [CN/US]; 111
 Harvey Lane, Chadds Ford, PA 19317 (US).

(21) International Application Number:

PCT/US2007/022423

(74) Agent: CHRISTENBURY, Lynne, M.; E.I. du Pont de
 Nemours and Company, Legal Patent Records Center, 4417
 Lancaster Pike, Wilmington, DE 19805 (US).

(22) International Filing Date: 22 October 2007 (22.10.2007)

(81) Designated States (*unless otherwise indicated, for every
 kind of national protection available*): AE, AG, AL, AM,
 AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH,
 CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG,
 ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL,
 IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK,
 LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW,
 MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL,
 PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY,
 TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA,
 ZM, ZW.

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/853,563 23 October 2006 (23.10.2006) US

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

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): DAMUDE, Howard
 [CA/US]; 4 Kenwick Road, Hockessin, DE 19707 (US).
 ZHU, Quinn, Qun [US/US]; 544 Revere Road, West

(84) Designated States (*unless otherwise indicated, for every
 kind of regional protection available*): ARIPO (BW, GH,
 GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
 ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
 European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,

[Continued on next page]

(54) Title: DELTA-8 DESATURASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

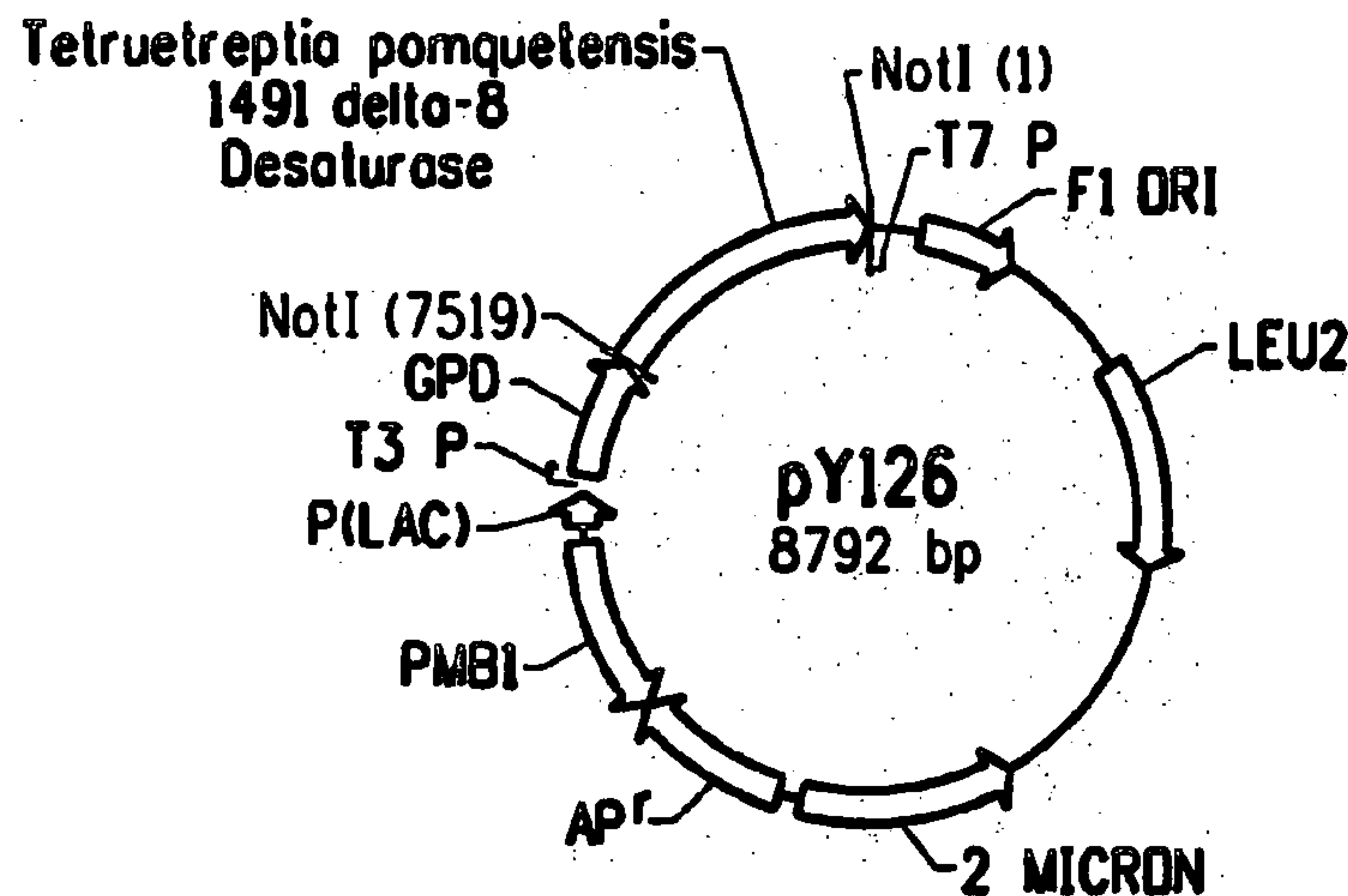


FIG. 1

(57) Abstract: Isolated nucleic acid fragments and recombinant constructs comprising such fragments encoding delta-8 desaturases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) using these delta-8 desaturases in plants and oleaginous yeast.

WO 2008/063340 A9

WO 2008/063340 A9



FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL,
PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM,
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

— *with information concerning authorization of rectification
of an obvious mistake under Rule 91.1*

(88) Date of publication of the international search report:
20 November 2008

(48) Date of publication of this corrected version:
8 January 2009

Published:

- *with international search report*
- *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(15) Information about Correction:
see Notice of 8 January 2009

TITLE

DELTA-8 DESATURASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

This application claims the benefit of U.S. Provisional Application No. 60/853,563, filed October 23, 2006, the entire contents of which are herein incorporated by reference.

FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this invention pertains to polynucleotide sequences encoding delta-8 desaturases and the use of these desaturases 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 elongation and desaturation 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 et al., *Amer. J. Clin. Nutr.* 28:958-966 (1975); Dyerberg et al., *Lancet.* 2(8081):117-119 (1978); Shimokawa, H., *World Rev. Nutr. Diet* 88:100-108 (2001); von Schacky et al., *World Rev. Nutr. Diet* 88:90-99 (2001)). Numerous other studies document wide-ranging health benefits conferred by administration of omega-3 and/or omega-6 PUFAs against a variety of symptoms and diseases (e.g., asthma, psoriasis, eczema, diabetes, cancer).

Today, a variety of different hosts including plants, algae, fungi and yeast are being investigated as means for commercial PUFA production via numerous divergent efforts. Although the natural PUFA-producing abilities of the host organisms are sometimes essential to a given methodology, genetic engineering

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has also proven that the natural abilities of some hosts (even those natively limited to LA and ALA fatty acid production) can be substantially altered to result in high-level production of various long-chain omega-3/omega-6 PUFAs. Whether this effect is the result of natural abilities or recombinant technology, production of arachidonic acid (ARA; 20:4 ω -6), eicosapentaenoic acid (EPA; 20:5 ω -3) and docosahexaenoic acid (DHA; 22:6 ω -3) all require expression of either the delta-9 elongase/delta-8 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) or the delta-6 desaturase/delta-6 elongase pathway (which is predominantly found in algae, mosses, fungi, nematodes and humans and which is characterized by the production of γ -linoleic acid (GLA; 18:3 ω -6) and/or stearidonic acid (STA; 18:4 ω -3) (Figure 6A and 6B). A delta-6 elongase is also known as a $C_{18/20}$ elongase.

The delta-8 desaturase enzymes identified thus far have the ability to convert both EDA to dihomogamma-linolenic acid (DGLA; 20:3) and ETra to eicosatetraenoic acid (ETA; 20:4) (wherein ARA and EPA are subsequently synthesized from DGLA and ETA, respectively, following reaction with a delta-5 desaturase, while DHA synthesis requires subsequent expression of an additional $C_{20/22}$ elongase and a delta-4 desaturase).

Based on the role delta-8 desaturase enzymes play in the synthesis of e.g., ARA, EPA and DHA, there has been considerable effort to identify and characterize these enzymes. Most efforts thus far have focused on the isolation and characterization of delta-8 desaturases from *Euglena gracilis*; and, several sequence variations within the *Euglena gracilis* delta-8 desaturase have been reported (see, e.g., Wallis et al., *Arch. Biochem. and Biophys.* 365(2):307-316 (1999); PCT Publication No. WO 2000/34439; U.S. Patent No. 6,825,017; PCT Publication No. WO 2004/057001). Also, Applicants' Assignee's co-pending applications having U.S. Application Nos. 11/166,003 and 11/166,993 filed June 24, 2005 (Attorney Docket Nos. BB-1547 and CL-3150, respectively (PCT Publication Nos. WO 2006/012325 and WO 2006/012326; both published February 2, 2006)) disclose amino acid and nucleic acid sequences for a *Euglena gracilis* delta-8 desaturase.

More recently, PCT Publication No. WO 2005/103253 (published April 22, 2005) discloses amino acid and nucleic acid sequences for a delta-8 desaturase enzyme from *Pavlova salina* (see also U.S. Publication No. 2005/0273885). Sayanova et al. (*FEBS Lett.* 580:1946-1952 (2006)) describes the isolation and characterization of a cDNA from the free living soil amoeba *Acanthamoeba castellanii* that, when expressed in *Arabidopsis*, encodes a C₂₀ delta-8 desaturase. Also, Applicants' Assignee's co-pending application having Provisional Application No. 60/795,810 filed April 28, 2006 (Attorney Docket No. BB-1566) discloses amino acid and nucleic acid sequences for a delta-8 desaturase enzyme from *Pavlova lutheri* (CCMP459).

Based on the utility of expressing delta-8 desaturases in conjunction with delta-9 elongases, there has also been considerable effort to identify and characterize delta-9 elongases from various sources. A delta-9 elongase from *Isochrysis galbana* has been publicly available (described in GenBank Accession No. AAL37626, as well as PCT Publication No. WO 02/077213). Applicants' Assignee's co-pending application having U.S. Provisional Application No. 60/739,989 filed November 23, 2005 (Attorney Docket No. BB-1562), discloses a delta-9 elongase from *Eulgena gracilis*.

Applicants' Assignee has a number of patent applications concerning the production of PUFAs in oleaginous yeasts (i.e., *Yarrowia lipolytica*), including: PCT Publication Nos. WO 2004/101757 and WO 2004/101753 (both published November 25, 2004); U.S. Application No. 11/265,761 (filed November 2, 2005); U.S. Application No. 11/264,784 (filed November 1, 2005); and U.S. Application No. 11/264,737 (filed November 1, 2005).

Relatedly, PCT Publication No. WO 2004/071467 (published August 26, 2004; Attorney Docket No. BB-1538) concerns the production of PUFAs in plants, while PCT Publication No. WO 2004/071178 (published August 26, 2004) concerns annexin promoters and their use in expression of transgenes in plants; both are Applicants' Assignee's copending applications.

SUMMARY OF THE INVENTION

The present invention concerns an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity,

based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57;

(b) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62;

(c) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence hybridizes under stringent conditions to a nucleotide sequence as set forth in SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62; or

(d) a complement of the nucleotide sequence of (a), (b) or (c), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

In a second embodiment, the invention concerns a recombinant DNA construct comprising any of the isolated polynucleotides of the invention operably linked to at least one regulatory sequence.

In a third embodiment, the invention concerns a cell comprising in its genome the recombinant DNA construct of the invention. Such cells can be plant cells or yeast cells.

In a fourth embodiment, the invention concerns a method for transforming a cell, comprising transforming a cell with a recombinant construct of the invention or an isolated polynucleotide of the invention and selecting those cells transformed with the recombinant construct or the isolated polynucleotide.

In a fifth embodiment, the invention concerns transgenic seed comprising in its genome the recombinant construct of the invention or a transgenic seed obtained from a plant made by a method of the invention. Also of interest is oil or by-products obtained from such transgenic seeds.

In a sixth embodiment, the invention concerns a method for making long-chain polyunsaturated fatty acids in a plant cell comprising:

(a) transforming a cell with the recombinant construct of the invention;
and

(b) selecting those transformed cells that make long-chain

polyunsaturated fatty acids.

In a seventh embodiment, the invention concerns a method for producing at least one polyunsaturated fatty acid in an oilseed plant cell comprising:

(a) transforming an oilseed plant cell with a first recombinant DNA construct comprising an isolated polynucleotide encoding at least one delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence and at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase;

(b) regenerating an oilseed plant from the transformed cell of step (a);
and

(c) selecting those seeds obtained from the plants of step (b) having an altered level of polyunsaturated fatty acids when compared to the level in seeds obtained from a nontransformed oilseed plant.

In an eighth embodiment, the invention concerns an oilseed plant comprising in its genome the recombinant construct of the invention. Suitable oilseed plants include, but are not limited to, soybean, *Brassica* species, sunflower, maize, cotton, flax and safflower.

In a ninth embodiment, the invention concerns an oilseed plant comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding at least one delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase.

Also of interest are transgenic seeds obtained from such oilseed plants as well as oil or by-products obtained from these transgenic seeds. A preferred product is lecithin.

In a tenth embodiment, the invention concerns food or feed incorporating an oil or seed of the invention or food or feed comprising an ingredient derived from the processing of the seeds.

In an eleventh embodiment, the invention concerns a method for making long-chain polyunsaturated fatty acids in a cell having a reduced level of by-product fatty acids, said method comprising:

(a) transforming a host cell with at least one recombinant DNA construct comprising an isolated polynucleotide encoding at least two delta-8 desaturases operably linked to at least one regulatory sequence; and

(b) selecting those transformed host cells obtained having a reduced level of by-product fatty acids, when compared to the level of such metabolic by-product fatty acids in a transformed host cell having at least one recombinant DNA construct comprising an isolated polynucleotide encoding one delta-8 desaturase operably linked to a regulatory sequence.

In a twelfth embodiment, the invention concerns progeny plants obtained from obtained from a plant made by the method of the invention or an oilseed plant of the invention.

BIOLOGICAL DEPOSITS

The following plasmid 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 (Table 1).

TABLE 1
ATCC Deposit

Plasmid	Accession Number	Date of Deposit
pKR72	PTA-6019	May 28, 2004

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

The invention can be more fully understood from the following detailed description and the accompanying drawings and Sequence Listing, which form a part of this application.

FIG. 1 is the yeast expression vector pY126.

FIG. 2 is the soybean expression vector pKR1013.

FIG. 3 is the soybean expression vector pKR1014.

FIG. 4 is the soybean expression vector pKR1005.

FIG. 5A and 5B are the lipid profiles of somatic soybean embryos expressing the *Tetruetreptia pomquetensis* CCMP1491 delta-8 desaturase and the *Isochrysis galbana* delta-9 elongase (see Example 10).

FIG. 6A and 6B is a representative omega-3 and omega-6 fatty acid pathway providing for the conversion of myristic acid through various intermediates to DHA.

FIGs. 7A and 7B show a Clustal V alignment of the delta-8 desaturases from *Tetruetreptia pomquetensis* CCMP1491 (SEQ ID NO:57), *Eutreptiella* sp. CCMP389 (SEQ ID NO:47), *Eutreptiella cf. gymnastica* CCMP1594 (SEQ ID NO:49), *Euglena gracilis* (SEQ ID NO:98; NCBI Accession No. AAD45877 (GI 5639724)) and *Euglena gracilis* (SEQ ID NO:112).

FIG. 8 is a schematic of the *Yarrowia lipolytica* expression vector pFBAIn-MOD1.

FIG. 9 is a schematic of the construct pZKLeuN-29E3.

FIG. 10A and 10B are the lipid profiles of somatic soybean embryos expressing *Tetruetreptia pomquetensis* CCMP1491 (TpomD8) and *Euglena gracilis* delta-9 elongase (EgD9e) for the top 5 events (see Example 12).

FIG. 11 is the soybean expression vector pKR973.

FIG. 12 is the soybean expression vector pKR1084.

FIG. 13 is the soybean expression vector pKR1123.

FIG. 14 shows the lipid profiles of somatic soybean embryos expressing E1594D8 and EgD9e for the top 5 events. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 14 are expressed as a weight percent (wt. %) of total fatty acids. The activity of E1594D8 is expressed as percent desaturation (% desat), calculated according to the following formula:

$([\text{product}]/[\text{substrate} + \text{product}]) \times 100$. The combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat". The individual omega-6 delta-8 desaturation ("EDA % delta-8 desat.") was calculated as: $([\text{DGLA}]/[\text{DGLA} + \text{EDA}]) \times 100$. Similarly, the individual omega-3 delta-8 desaturation ("ERA % delta-8 desat.") was calculated as: $([\text{ETA}]/[\text{ETA} + \text{ERA}]) \times 100$. The ratio of delta-8 desaturation for omega-6 versus omega-3 substrates ("ratio [EDA/ERA] % desat.") was obtained by dividing the EDA % delta-8 desaturation by the ERA % delta-8 desaturation.

FIG. 15 is the soybean expression vector pKR1122.

FIG. 16 shows the lipid profiles of somatic soybean embryos expressing E389D8 and EgD9e for the top 5 events.

FIG. 17 is the Arabidopsis Binary Expression pKR1022R.

FIG. 18 shows the lipid profiles of T2 bulk seed for 22 events where wild-type Arabidopsis was transformed with pKR1022R (SEQ ID NO:141).

FIG. 19 shows the average fatty acid profiles (average of 10 embryos per event) of soybean embryos transformed with the Ascl fragments of pKR1005 (SEQ ID NO:90; FIG. 4) and pKR973 (SEQ ID NO:125; FIG. 11), for the 10 events having the highest amounts of delta-8 desaturation products. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and are expressed as a weight percent (wt. %) of total fatty acids. Fatty acids listed as "others" include: 18:2 (5,9), STA, 20:0, 20:1(11), 20:2 (7,11) or 20:2 (8,11), and DHA. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula: $([\text{DGLA} + \text{ETA}]/[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]) \times 100$.

FIG. 20 shows the fatty acid profiles for ten individual T1 seeds from 2 plants from event AFS 4882-4-6 (plant #4882-4-6-1 & #4882-4-6-2) having some of the highest amounts of total delta-8 desaturation products

FIG. 21 shows the average fatty acid profiles (average of 10 embryos per event) of soybean embryos transformed with the Ascl fragments of pKR1005 (SEQ ID NO:90; FIG. 4) and pKR1084 (SEQ ID NO:129; FIG. 12), for the 10 events having the highest amounts of delta-8 desaturation products. Fatty acids are

identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and, fatty acid compositions are expressed as a weight percent (wt. %) of total fatty acids. Fatty acids listed as "others" include: 18:2 (5,9), STA, 20:0, 20:1(11), 20:2 (7,11) or 20:2 (8,11), and DHA. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula: $[(DGLA + ETA)/(DGLA + ETA + EDA + ERA)] \times 100$.

FIG. 22 shows the fatty acid profiles for individual T1 seeds from 2 plants from event AFS 5003-1-8 (plant #5003-1-8-1 & #5003-1-8-2) having some of the highest amounts of total delta-8 desaturation products.

The sequence descriptions summarize the Sequences Listing attached hereto. The Sequence Listing contains one letter codes for nucleotide sequence characters and the single and three letter codes for amino acids as defined in the IUPAC-IUB standards described in *Nucleic Acids Research* 13:3021-3030 (1985) and in the *Biochemical Journal* 219(2):345-373 (1984).

A Sequence Listing is provided herewith on Compact Disk. The contents of the Compact Disk containing the Sequence Listing are hereby incorporated by reference in compliance with 37 C.F.R. §1.52(e).

SEQ ID NOs:1-11 are the nucleotide sequences of primers D8F1, D8F2, D8F3, D8F4, D8F5, D8F6, D8F7, D8F8, D8F9, D8R1 and D8R2, respectively.

SEQ ID NO:12 is the amino acid sequence of primers D8F1 and D8F4.

SEQ ID NO:13 is the amino acid sequence of primers D8F2, D8F3, D8F5 and D8F6.

SEQ ID NO:14 is the amino acid sequence of primers D8F7, D8F8 and D8F9.

SEQ ID NO:15 is the amino acid sequence of primers D8R1 and D8R2.

SEQ ID NO:16 is the partial nucleotide sequence of the delta-8 desaturase from *Tetruetrepia pomquetensis* CCMP1491 using the primer combination D8F4/D8R1 (see Example 1).

SEQ ID NO:17 is the partial nucleotide sequence of the delta-8 desaturase from *Eutreptiella* sp. CCMP389 using the primer combination D8F4/D8R1 (see Example 1).

SEQ ID NO:18 is the partial nucleotide sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 using the primer combination D8F4/D8R1 (see Example 1).

SEQ ID NO:19 is the nucleotide sequence of the SMART IV oligonucleotide.

SEQ ID NOs:20-24 are the nucleotide sequences of primers 389D8-3-1, 389D8-3-2, 389D8-5-1, 389D8-5-2 and 389D8-5-3, respectively.

SEQ ID NOs:25-29 are the nucleotide sequences of primers ED8-5-1, ED8-5-2, ED8-5-3, ED8-3-1 and ED8-3-2, respectively.

SEQ ID NO:30 is the nucleotide sequence of CDSIII/3' PCR primer.

SEQ ID NO:31 is the nucleotide sequence of the Adaptor Primer from Invitrogen 3'-RACE kit.

SEQ ID NOs:32-36 are the nucleotide sequences of primers 1594D8-3-1, 1594D8-3-2, 1594D8-5-1, 1594D8-5-2 and 1594D8-5-3, respectively.

SEQ ID NO:37 is the nucleotide sequence of the GenomeWalker adaptor (see also SEQ ID NO:111).

SEQ ID NOs:38 and 39 are the nucleotide sequences of primer AP1 and AP2, respectively.

SEQ ID NO:40 is nucleotide sequence of pCR2.1-TOPO.

SEQ ID NO:41 is the 5'-region nucleotide sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 (see Example 2).

SEQ ID NO:42 is the 5'-region nucleotide sequence of the delta-8 desaturase from *Eutreptiella* sp. CCMP389 (see Example 2).

SEQ ID NO:43 is the 3'-region nucleotide sequence of the delta-8 desaturase from *Eutreptiella* sp. CCMP389 (see Example 2).

SEQ ID NO:44 is a 3'-region nucleotide sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 (1594D8-3'A) (see Example 2).

SEQ ID NO:45 is a 3'-region nucleotide sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 (1594D8-3'B) (see Example 2).

SEQ ID NO:46 is the nucleotide sequence of the delta-8 desaturase from *Eutreptiella* sp. CCMP389 (1963 bp contig).

SEQ ID NO:47 is the amino acid sequence of the delta-8 desaturase from *Eutreptiella* sp. CCMP389 (coding region of SEQ ID NO:46 and SEQ ID NO:92).

SEQ ID NO:48 is the nucleotide sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 (2063 bp contig).

SEQ ID NO:49 is the amino acid sequence of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594 (coding region of SEQ ID NO:48 and SEQ ID NO:93).

SEQ ID NO:50 is the nucleotide sequence of the TOPO linker.

SEQ ID NO:51 is the nucleotide sequence of the LinkAmp primer 1.

SEQ ID NO:52 is the nucleotide sequence of the LinkAmp primer 2.

SEQ ID NO:53 is the 5'-region nucleotide sequence of the delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 (see Example 3).

SEQ ID NO:54 is the nucleotide sequence of primer AUAP.

SEQ ID NO:55 is the 3'-region nucleotide sequence of the delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 (see Example 3).

SEQ ID NO:56 is the nucleotide sequence of the delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 (2233 bp contig).

SEQ ID NO:57 is the amino acid sequence of the delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 (coding region of SEQ ID NO:56 and SEQ ID NO:62).

SEQ ID NOs:58 and 59 are the nucleotide sequences of TpomNot-5 and TpomNot-3, respectively.

SEQ ID NO:60 is the nucleotide sequence of primer T7.

SEQ ID NO:61 is the nucleotide sequence of primer M13-28Rev.

SEQ ID NO:62 is the nucleotide sequence of the coding sequence of *Tetruetreptia pomquetensis* CCMP1491 delta-8 desaturase.

SEQ ID NO:63 is the nucleotide sequence of pLF114-10.

SEQ ID NO:64 is the nucleotide sequence of pY-75.

SEQ ID NO:65 is the nucleotide sequence of pY126 (see FIG. 1).

SEQ ID NO:66 is the nucleotide sequence of pKR123r.

SEQ ID NO:67 is the nucleotide sequence of pKR1007.

SEQ ID NO:68 is the nucleotide sequence of pKR607.

SEQ ID NO:69 is the nucleotide sequence of pKR1013 (see FIG. 2).

SEQ ID NO:70 is the nucleotide sequence of the coding sequence of the *Isochrysis galbana* delta-9 elongase (NCBI Accession No. AAL37626 (GI 17226123), locus AAL37626, CDS AF390174; Qi et al., *FEBS Lett.* 510(3):159-165 (2002)).

SEQ ID NO:71 is the sequence of a portion of the cDNA insert from *Euglena gracilis* clone eeg1c.pk001.n5.f (5' end of cDNA insert).

SEQ ID NO:72 is the sequence of a portion of the cDNA insert from clone eeg1c.pk001.n5.f (3' end of cDNA insert).

SEQ ID NO:73 is the sequence of clone eeg1c.pk001.n5.f (5' and 3' sequences were aligned).

SEQ ID NO:74 is the *Euglena gracilis* delta-9 elongase coding sequence from the cDNA in clone eeg1c.pk001.n5.f.

SEQ ID NO:75 is the amino acid sequence of the *Euglena gracilis* delta-9 elongase from clone eeg1c.pk001.n5.f (coding region of SEQ ID NO:74).

SEQ ID NO:76 is the amino acid sequence of the long-chain PUFA elongation enzyme (delta-9 elongase) from *Isochrysis galbana* (NCBI Accession No. AAL37626 (GI 17226123), locus AAL37626, CDS AF390174) (designated "IgD9e").

SEQ ID NOs:77 and 78 are the nucleotide sequences of oligonucleotide primers oEugEL1-1 and oEugEL1-2, respectively.

SEQ ID NO:79 is the nucleotide sequence of pKR906.

SEQ ID NO:80 is the nucleotide sequence of pKR72 (ATCC Accession No. PTA-6019).

SEQ ID NO:81 is the nucleotide sequence of pK912.

SEQ ID NO:82 is the nucleotide sequence of pKR1014 (see FIG. 3).

SEQ ID NO:83 is the nucleotide sequence of pKR271.

SEQ ID NO:84 is the nucleotide sequence of pKR226.

SEQ ID NO:85 is the nucleotide sequence of pKR886r.

SEQ ID NOs:86 and 87 are the nucleotide sequences of oligonucleotide primers oCon1 and oCon2, respectively.

SEQ ID NO:88 is the nucleotide sequence of pKR179.

SEQ ID NO:89 is the nucleotide sequence of pKR1002.

SEQ ID NO:90 is the nucleotide sequence of pKR1005 (see FIG. 4).

SEQ ID NO:91 is the nucleotide sequence of the M13F universal primer.

SEQ ID NO:92 is the nucleotide sequence of the coding sequence of *Eutreptiella* sp. CCMP389 delta-8 desaturase.

SEQ ID NO:93 is the nucleotide sequence of the coding sequence of *Eutreptiella cf. gymnastica* CCMP1594 delta-8 desaturase.

SEQ ID NO:94 is the nucleotide sequence of *Yarrowia lipolytica* expression vector pFBAIn-MOD1.

SEQ ID NO:95 is the nucleotide sequence of *Yarrowia lipolytica* expression vector pFBAIn-389D8.

SEQ ID NO:96 is the nucleotide sequence of *Yarrowia lipolytica* expression vector pFBAIn-1594D8.

SEQ ID NO:97 is the nucleotide sequence of *Yarrowia lipolytica* expression vector pFBAIn-1491D8.

SEQ ID NO:98 is the amino acid sequence of the *Euglena gracilis* delta-8 fatty acid desaturase gene (NCBI Accession No. AAD45877 (GI 5639724)). SEQ ID NO:98 is the amino acid sequence encoded by nucleotides 14 -1273 of NCBI Accession No. AF139720 (GI 5639723). This delta-8 fatty acid desaturase has been shown to be non-functional.

SEQ ID NOs:99 and 100 are the nucleotide sequences of primers 389D8-F and 389D8-R, respectively.

SEQ ID NOs:101 and 102 are the nucleotide sequences of primers 1491D8-F and 1491D8-R, respectively.

SEQ ID NOs:103 and 104 are the nucleotide sequences of primers 1594D8-F and 1594D8-R, respectively.

SEQ ID NO:105 is the 5' PCR primer used in Example 1.

SEQ ID NO:106 is the nucleotide sequence of plasmid pZKLeuN-29E3 (see FIG. 9).

SEQ ID NO:107 is the nucleotide sequence of a synthetic delta-9 elongase (initially from *Euglena gracilis* – see SEQ ID NO:74) codon-optimized for *Yarrowia lipolytica*; see also U.S. Patent Application No. 60/739,989, filed November 23, 2005 (Attorney Docket No. BB-1562) (designated “EgD9E” or “EgD9S”)

SEQ ID NO:108 is the nucleotide sequence of the LoxP sequence from *Escherichia coli*.

SEQ ID NO:109 is the nucleotide sequence of a synthetic C_{16/18} elongase (initially from *M. alpina*) codon-optimized for *Yarrowia lipolytica*; see also U.S. Patent Application No. 11/253882, filed October 19, 2005.

SEQ ID NO:110 is the nucleotide sequence of a synthetic delta-9 elongase (initially from *Isochrysis galbana*) codon-optimized for *Yarrowia lipolytica* (designated "IgD9eS").

SEQ ID NO:111 is the nucleotide sequence of the GenomeWalker adaptor (see also SEQ ID NO:37).

SEQ ID NO:112 is the amino acid sequence of *Euglena gracilis* delta-8 desaturase (SEQ ID NO:2 of U.S. Publication No. 20050287652).

SEQ ID NO:113 is the nucleotide sequence of pKR132.

SEQ ID NO:114 is the nucleotide sequence of pKR953.

SEQ ID NO:115 is the nucleotide sequence of pKR287.

SEQ ID NO:116 is the nucleotide sequence of *Mortierella alpina* delta-5 desaturase (which is described in U.S. Patent No. 6,075,183).

SEQ ID NO:117 is the nucleotide sequence of pKR277.

SEQ ID NO:118 is the nucleotide sequence of pKR952.

SEQ ID NO:119 is the nucleotide sequence of pKR457.

SEQ ID NO:120 is the nucleotide sequence of the modified Kti-NotI-Kti3'Salb3'cassette.

SEQ ID NO:121 is the nucleotide sequence of the *Pavlova lutheri* Delta-8 Desaturase codon sequence described in U.S. Provisional Application No. 60/795,810 and US patent application No. 11/737,772.

SEQ ID NO:122 is the nucleotide sequence of oligonucleotide primer PvDES5'Not-1.

SEQ ID NO:123 is the nucleotide sequence of oligonucleotide primer PvDES3'Not-1.

SEQ ID NO:124 is the nucleotide sequence of pKR970.

SEQ ID NO:125 is the nucleotide sequence of pKR973.

SEQ ID NO:126 is the nucleotide sequence of pKS129.

SEQ ID NO:127 is the nucleotide sequence of pKR606.

SEQ ID NO:128 is the nucleotide sequence of pKR804.

SEQ ID NO:129 is the nucleotide sequence of pKR1084.

SEQ ID NO:130 is the nucleotide sequence of pKR908.
SEQ ID NO:131 is the nucleotide sequence of pKR1118
SEQ ID NO:132 is the nucleotide sequence of pKR1120.
SEQ ID NO:133 is the nucleotide sequence of pKR1123
SEQ ID NO:134 is the nucleotide sequence of pKR1117.
SEQ ID NO:135 is the nucleotide sequence of pKR1119.
SEQ ID NO:136 is the nucleotide sequence of pKR1122.
SEQ ID NO:137 is the nucleotide sequence of pKR393.
SEQ ID NO:138 is the nucleotide sequence of pKR407.
SEQ ID NO:139 is the nucleotide sequence of pKR1018.
SEQ ID NO:140 is the nucleotide sequence of pKR1020R.
SEQ ID NO:141 is the nucleotide sequence of pKR1022R.

DETAILED DESCRIPTION OF THE INVENTION

The disclosure of each reference set forth herein is hereby incorporated by reference in its entirety.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes a plurality of such plants, reference to "a cell" includes one or more cells and equivalents thereof known to those skilled in the art, and so forth.

The present invention relates to delta-8 desaturase enzymes and nucleic acid for encoding the same isolated from *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594 delta-8. These are useful for, *inter alia*, for the manipulation of biochemical pathways for the production of healthful PUFAs. Thus, the subject invention finds many applications. 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). In this case, the PUFAs are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally (e.g., subcutaneously, intramuscularly or intravenously), rectally, vaginally or topically (e.g., as a skin ointment or lotion).

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.

In the context of 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.

The term “fatty acids” refers to long-chain aliphatic acids (alkanoic acids) of varying chain lengths, from about C₁₂ to C₂₂ (although both longer and shorter chain-length acids are known). The predominant chain lengths are between C₁₆ and C₂₂. 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.

Fatty acids are described herein by a simple notation system of “X:Y”, wherein X is number of carbon (C) atoms in the particular fatty acid and Y is the number of double bonds. The number following the fatty acid designation indicates the position of the double bond from the carboxyl end of the fatty acid with the “c”

affix for the *cis*-configuration of the double bond (e.g., palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, 9c), petroselinic acid (18:1, 6c), LA (18:2, 9c,12c), GLA (18:3, 6c,9c,12c) and ALA (18:3, 9c,12c,15c)). Unless otherwise specified, 18:1, 18:2 and 18:3 refer to oleic, LA and ALA fatty acids, respectively. If not specifically written as otherwise, double bonds are assumed to be of the *cis* configuration. For instance, the double bonds in 18:2 (9,12) would be assumed to be in the *cis* configuration.

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 omega-3 and omega-6 fatty acids and their precursors, the abbreviations that will be used throughout the remainder of 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	PA	hexadecanoic	16:0
palmitoleic	--	9-hexadecenoic	16:1
stearic	--	octadecanoic	18:0
oleic	--	<i>cis</i> -9-octadecenoic	18:1
linoleic	LA	<i>cis</i> -9,12-octadecadienoic	18:2 ω-6
gamma-linolenic	GLA	<i>cis</i> -6,9,12-octadecatrienoic	18:3 ω-6
eicosadienoic	EDA	<i>cis</i> -11,14-eicosadienoic	20:2 ω-6
dihomo-gamma-linolenic	DGLA	<i>cis</i> -8,11,14-eicosatrienoic	20:3 ω-6
sciadonic	SCI	<i>cis</i> -5,11,14-eicosatrienoic	20:3b ω-6
arachidonic	ARA	<i>cis</i> -5,8,11,14-eicosatetraenoic	20:4 ω-6
alpha-linolenic	ALA	<i>cis</i> -9,12,15-octadecatrienoic	18:3 ω-3

stearidonic	STA	<i>cis</i> -6,9,12,15-octadecatetraenoic	18:4 ω -3
eicosatrienoic	ETrA or ERA	<i>cis</i> -11,14,17-eicosatrienoic	20:3 ω -3
eicosa-tetraenoic	ETA	<i>cis</i> -8,11,14,17-eicosatetraenoic	20:4 ω -3
juniperonic	JUP	<i>cis</i> -5,11,14,17-eicosatrienoic	20:4b ω -3
eicosa-pentaenoic	EPA	<i>cis</i> -5,8,11,14,17-eicosapentaenoic	20:5 ω -3
docosa-pentaenoic	DPA	<i>cis</i> -7,10,13,16,19-docosapentaenoic	22:5 ω -3
docosa-hexaenoic	DHA	<i>cis</i> -4,7,10,13,16,19-docosahexaenoic	22:6 ω -3

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). Simplistically, 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 reticulum membrane. More specifically, "PUFA biosynthetic pathway enzyme" refers to any of the following enzymes (and genes which encode said enzymes) associated with the biosynthesis of a PUFA, including: a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 desaturase, a delta-8 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and/or a C_{20/22} elongase.

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The term "omega-3/omega-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 omega-3 and omega-6 fatty acids. Typically the genes involved in the omega-3/omega-6 fatty acid biosynthetic pathway encode PUFA biosynthetic pathway enzymes. A representative pathway is illustrated in FIG. 6A and 6B, providing for the conversion of myristic acid through various intermediates to DHA, which demonstrates how both omega-3 and omega-6 fatty acids may be produced from a common source. The pathway is naturally divided into two portions where one portion will generate omega-3 fatty acids and the other portion, omega-6 fatty acids.

The term "functional" as used herein in context with the omega-3/omega-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 "omega-3/omega-6 fatty acid biosynthetic pathway" or "functional omega-3/omega-6 fatty acid biosynthetic pathway" does not imply that all the PUFA biosynthetic pathway enzyme genes 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 "delta-9 elongase/delta-8 desaturase pathway" refers to a biosynthetic pathway for production of long-chain PUFAs. This pathway, at a minimum, comprises a delta-9 elongase and a delta-8 desaturase, thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized. This pathway may be advantageous in some embodiments, as the biosynthesis of GLA and/or STA is excluded.

The term "intermediate fatty acid" refers to any fatty acid produced in a fatty acid metabolic pathway that can be further converted to an intended product fatty acid in this pathway by the action of other metabolic pathway enzymes. For instance, when EPA is produced using the delta-9 elongase/delta-8 desaturase pathway, EDA, ETrA, DGLA, ETA and ARA can be produced and are considered "intermediate fatty acids" since these fatty acids can be further converted to EPA via action of other metabolic pathway enzymes.

The term “by-product fatty acid” refers to any fatty acid produced in a fatty acid metabolic pathway that is not the intended fatty acid product of the pathway nor an “intermediate fatty acid” of the pathway. For instance, when EPA is produced using the delta-9 elongase/delta-8 desaturase pathway, sciadonic acid (SCI) and juniperonic acid (JUP) also can be produced by the action of a delta-5 desaturase on either EDA or ETrA, respectively. They are considered to be “by-product fatty acids” since neither can be further converted to EPA by the action of other metabolic pathway enzymes.

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.

“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, phosphatidylethanolamine (PE) fraction and triacylglycerol (TAG or oil) fraction. However, the terms “lipid” and “oil” will be used interchangeably throughout the specification.

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: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$, where ‘product’ includes the immediate product and all products in the pathway derived from it.

“Desaturase” is 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 particular interest herein are delta-8 desaturases that will desaturate a fatty acid between the eighth and ninth carbon atom numbered from the carboxyl-terminal end

of the molecule and that can, for example, catalyze the conversion of EDA to DGLA and/or ETrA to ETA. Other useful fatty acid desaturases include, for example: (1) delta-5 desaturases that catalyze the conversion of DGLA to ARA and/or ETA to EPA; (2) delta-6 desaturases that catalyze the conversion of LA to GLA and/or ALA to STA; (3) delta-4 desaturases that catalyze the conversion of DPA to DHA; (4) delta-12 desaturases that catalyze the conversion of oleic acid to LA; (5) delta-15 desaturases that catalyze the conversion of LA to ALA and/or GLA to STA; (6) delta-17 desaturases that catalyze the conversion of ARA to EPA and/or DGLA to ETA; and (7) delta-9 desaturases that catalyze the conversion of palmitic acid to palmitoleic acid (16:1) and/or stearic acid to oleic acid (18:1). In the art, delta-15 and delta-17 desaturases are also occasionally referred to as “omega-3 desaturases”, “w-3 desaturases”, and/or “ ω -3 desaturases”, based on their ability to convert omega-6 fatty acids into their omega-3 counterparts (e.g., conversion of LA into ALA and ARA into EPA, respectively). In some embodiments, it is most desirable to empirically determine the specificity of a particular 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.

For the purposes herein, the term “TpomD8” refers to a delta-8 desaturase enzyme (SEQ ID NO:57) isolated from *Tetruetrepia pomquetensis* CCMP1491, encoded by SEQ ID NO:62 herein. The term “E389D8” refers to a delta-8 desaturase enzyme (SEQ ID NO:47) isolated from *Eutrepitiella* sp. CCMP389, encoded by SEQ ID NO:92 herein. Likewise, the term “E1594D8” refers to a delta-8 desaturase enzyme (SEQ ID NO:49) isolated from *Eutrepitiella cf. gymnastica* CCMP1594, encoded by SEQ ID NO:93 herein.

Similarly, the term “EgD8” refers to a delta-8 desaturase enzyme (SEQ ID NO:112) isolated from *Euglena gracilis*. EgD8 is 100% identical and functionally equivalent to “Eg5”, as described in PCT Publication Nos. WO 2006/012325 and WO 2006/012326 (SEQ ID NO:2 of U.S. Publication No. 20050287652-A1).

The term “elongase system” refers to a suite of four enzymes that are responsible for elongation of a fatty acid carbon chain to produce a fatty acid that is two carbons longer than the fatty acid substrate that the elongase system acts upon. More specifically, the process of elongation occurs in association with fatty acid synthase, whereby CoA is the acyl carrier (Lassner et al., *Plant Cell* 8:281-292

(1996)). In the first step, which has been found to be both substrate-specific and also rate-limiting, malonyl-CoA is condensed with a long-chain acyl-CoA to yield carbon dioxide (CO₂) and a β -ketoacyl-CoA (where the acyl moiety has been elongated by two carbon atoms). Subsequent reactions include reduction to β -hydroxyacyl-CoA, dehydration to an enoyl-CoA and a second reduction to yield the elongated acyl-CoA. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA, LA to EDA, ALA to ETRA and EPA to DPA.

For the purposes herein, an enzyme catalyzing the first condensation reaction (i.e., conversion of malonyl-CoA and long-chain acyl-CoA to β -ketoacyl-CoA) will be referred to generically as an “elongase”. In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree of unsaturation. Accordingly, elongases can have different specificities. For example, a C_{14/16} elongase will utilize a C₁₄ substrate (e.g., myristic acid), a C_{16/18} elongase will utilize a C₁₆ substrate (e.g., palmitate), a C_{18/20} elongase will utilize a C₁₈ substrate (e.g., GLA, STA) and a C_{20/22} elongase will utilize a C₂₀ substrate (e.g., EPA). Similarly, a “delta-9 elongase” may be able to catalyze the conversion of LA to EDA and/or ALA to ETrA. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions. Thus, for example, a delta-9 elongase may also act as a C_{16/18} elongase, C_{18/20} elongase and/or C_{20/22} elongase and may have alternate, but not preferred, specificities for delta-5 and delta-6 fatty acids such as EPA and/or GLA, respectively.

For the purposes herein, the term “IgD9e” refers to a delta-9 elongase (SEQ ID NO:76; NCBI Accession No. AAL37626 [GI 17226123], locus AAL37626, CDS AF390174; GenBank Accession No. AF390174) isolated from *Isochrysis galbana*, encoded by SEQ ID NO:70. In contrast, the term “IgD9eS” refers to a synthetic (codon-optimized) delta-9 elongase (SEQ ID NO:110) derived from the DNA sequence of the *Isochrysis galbana* delta-9 elongase (SEQ ID NO:70) which can be used for expression in *Yarrowia lipolytica*.

Similarly for the purposes herein, the term “EgD9e” refers to a delta-9 elongase (SEQ ID NO:75) isolated from *Euglena gracilis*, encoded by SEQ ID NO:74 (see Example 11 herein).

As used herein, “nucleic acid” means a polynucleotide and includes single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms “polynucleotide”, “nucleic acid sequence”, “nucleotide sequence” or “nucleic acid fragment” are used interchangeably and is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: “A” for adenylate or deoxyadenylate (for RNA or DNA, respectively), “C” for cytidylate or deoscytidylate, “G” for guanylate or deoxyguanylate, “U” for uridate, “T” for deosythymidylate, “R” for purines (A or G), “Y” for pyrimidiens (C or T), “K” for G or T, “H” for A or C or T, “I” for inosine, and “N” for any nucleotide.

The terms “subfragment that is functionally equivalent” and “functionally equivalent subfragment” are used interchangeably herein. These terms refer to a portion or subsequence of an isolated nucleic acid fragment in which the ability to alter gene expression or produce a certain phenotype is retained whether or not the fragment or subfragment encodes an active enzyme. For example, the fragment or subfragment can be used in the design of chimeric genes to produce the desired phenotype in a transformed plant. Chimeric genes can be designed for use in suppression by linking a nucleic acid fragment or subfragment thereof, whether or not it encodes an active enzyme, in the sense or antisense orientation relative to a plant promoter sequence.

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.

The terms “homology”, “homologous”, “substantially similar” and “corresponding substantially” are used interchangeably herein. They 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 substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize (under moderately stringent conditions, e.g., 0.5X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. 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.

The term “selectively hybridizes” includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 90% sequence identity, up to and including 100% sequence identity (*i.e.*, fully complementary) with each other.

The term “stringent conditions” or “stringent hybridization conditions” includes reference to conditions under which a probe will selectively hybridize to its target sequence. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or

washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 to 65 °C.

Specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth et al., *Anal. Biochem.* 138:267-284 (1984): $T_m = 81.5\text{ °C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m , hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq 90\%$ identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent

conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution) it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Hybridization and/or wash conditions can be applied for at least 10, 30, 60, 90, 120, or 240 minutes.

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

Thus, "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. Useful examples of percent sequence identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from

50% to 100%. These identities can be determined using any of the programs described herein.

Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). 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 “Clustal V method of alignment” corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS*. 5:151-153 (1989); Higgins, D.G. et al. (1992) *Comput. Appl. Biosci.* 8:189-191) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). 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 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.

“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. Useful examples of percent identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 50% to 100%. Indeed, any integer amino acid identity from 50% to 100% may be useful in describing the present invention, such as 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%,

61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 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%. Also, of interest is any full-length or partial complement of this isolated nucleotide fragment.

“Gene” refers to a nucleic acid fragment that expresses a specific protein, including 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. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure.

The term “genome” as it applies to a plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondrial, plastid) of the cell.

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.

An “allele” is one of several alternative forms of a gene occupying a given locus on a chromosome. When all the alleles present at a given locus on a chromosome are the same that plant is homozygous at that locus. If the alleles present at a given locus on a chromosome differ that plant is heterozygous at that locus.

“Coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence. “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, but are not limited to: 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. The promoter sequence consists of proximal and more distal upstream elements, the latter elements often referred to as enhancers. Accordingly, an “enhancer” is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. 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 conditions. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of some variation may have identical promoter activity. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro, J. K., and Goldberg, R. B. *Biochemistry of Plants* 15:1-82 (1989).

“Translation leader sequence” refers to a polynucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D., *Mol. Biotechnol.* 3:225-236 (1995)).

“3' non-coding sequences”, “transcription terminator” or “termination sequences” refer to DNA sequences located downstream of a coding sequence and include 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 use of different

3' non-coding sequences is exemplified by Ingelbrecht, I. L., et al. *Plant Cell* 1:671-680 (1989).

"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. A RNA transcript is referred to as the mature RNA when it is a RNA sequence derived from post-transcriptional processing of the primary transcript. "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 DNA that is complementary to, and synthesized from, a mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into double-stranded form using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. "Antisense RNA" refers to an 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). 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, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

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 regulated by the other. For example, a promoter is operably linked with a coding sequence when it is capable of regulating 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 a sense or antisense orientation. In another example, the complementary RNA regions of the invention can be operably linked, either directly or indirectly, 5' to the target mRNA, or 3' to the target mRNA, or within the target mRNA, or a first complementary region is 5' and its complement is 3' to the target mRNA.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989). Transformation methods are well known to those skilled in the art and are described *infra*.

“PCR” or “polymerase chain reaction” is a technique for the synthesis of large quantities of specific DNA segments and consists of a series of repetitive cycles (Perkin Elmer Cetus Instruments, Norwalk, CT). Typically, the double-stranded DNA is heat denatured, the two primers complementary to the 3' boundaries of the target segment are annealed at low temperature and then extended at an intermediate temperature. One set of these three consecutive steps is referred to as a “cycle”.

The term “recombinant” refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

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. “Transformation cassette” refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host 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 (i.e., to a discrete nucleic acid fragment into which a nucleic acid sequence or fragment can be moved.)

The terms “recombinant construct”, “expression construct”, “chimeric construct”, “construct”, and “recombinant DNA construct” are used interchangeably

herein. A recombinant construct comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not found together in nature. For example, a chimeric construct 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. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events will result in different levels and patterns of expression (Jones et al., *EMBO J.* 4:2411-2418 (1985); De Almeida et al., *Mol. Gen. Genetics* 218:78-86 (1989)), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

The term "expression", as used herein, refers to the production of a functional end-product (e.g., a mRNA or a protein [either precursor or mature]).

The term "introduced" means providing a nucleic acid (e.g., expression construct) or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. Introduced includes reference to stable or transient transformation methods, as well as sexually crossing. Thus, "introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct/expression construct) into a cell, means "transfection" or "transformation" or "transduction" and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome,

plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

"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.

"Stable transformation" refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in genetically stable inheritance. In contrast, "transient transformation" refers to the transfer of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host organism resulting in gene expression without integration or stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms.

As used herein, "transgenic" refers to a plant or a cell which comprises within its genome a heterologous polynucleotide. Preferably, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of an expression construct. Transgenic is used herein to include any cell, cell line, callus, tissue, plant part or plant, the genotype of which has been altered by the presence of heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

"Antisense inhibition" refers to the production of antisense RNA transcripts capable of suppressing the expression of the target protein. "Co-suppression" refers to the production of sense RNA transcripts capable of suppressing the expression of identical or substantially similar foreign or endogenous genes (U.S. Patent No. 5,231,020). Co-suppression constructs in plants previously have been

designed by focusing on overexpression of a nucleic acid sequence having homology to an endogenous mRNA, in the sense orientation, which results in the reduction of all RNA having homology to the overexpressed sequence (Vaucheret et al., *Plant J.* 16:651-659 (1998); Gura, *Nature* 404:804-808 (2000)). The overall efficiency of this phenomenon is low, and the extent of the RNA reduction is widely variable. More recent work has described the use of "hairpin" structures that incorporate all, or part, of an mRNA encoding sequence in a complementary orientation that results in a potential "stem-loop" structure for the expressed RNA (PCT Publication No. WO 99/53050, published October 21, 1999; PCT Publication No. WO 02/00904, published January 3, 2002). This increases the frequency of co-suppression in the recovered transgenic plants. Another variation describes the use of plant viral sequences to direct the suppression, or "silencing", of proximal mRNA encoding sequences (PCT Publication No. WO 98/36083, published August 20, 1998). Both of these co-suppressing phenomena have not been elucidated mechanistically, although genetic evidence has begun to unravel this complex situation (Elmayan et al., *Plant Cell* 10:1747-1757 (1998)).

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). A class of plants identified as oleaginous are commonly referred to as "oilseed" plants. Examples of oilseed plants include, but are not limited to: soybean (*Glycine* and *Soja* sp.), flax (*Linum* sp.), rapeseed (*Brassica* sp.), maize, cotton, safflower (*Carthamus* sp.) and sunflower (*Helianthus* sp.).

Within oleaginous microorganisms the cellular oil or TAG content generally 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)). The term "oleaginous yeast" refers to those microorganisms classified as yeasts that make oil. 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*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

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The term "Euglenophyceae" refers to a group of unicellular colorless or photosynthetic flagellates ("euglenoids") found living in freshwater, marine, soil, and parasitic environments. The class is characterized by solitary unicells, wherein most are free-swimming and have two flagella (one of which may be nonemergent) arising from an anterior invagination known as a reservoir. Photosynthetic euglenoids contain one to many grass-green chloroplasts, which vary from minute disks to expanded plates or ribbons. Colorless euglenoids depend on osmotrophy or phagotrophy for nutrient assimilation. About 1000 species have been described and classified into about 40 genera and 6 orders. Examples of Euglenophyceae include, but are no means limited to, the following genera: *Eutreptiella* and *Tetruetreptia*.

The term "plant" refers to whole plants, plant organs, plant tissues, seeds, plant cells, seeds and progeny of the same. Plant cells include, without limitation, cells from seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen and microspores.

"Progeny" comprises any subsequent generation of a plant.

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 derivatives, which are formed through the action of elongases and desaturases (FIG. 6A and 6B).

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.

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Biosynthesis of Omega Fatty Acids

The metabolic process wherein oleic acid is converted to long chain omega-3/omega-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 reticulum membrane. However, as seen in FIG. 6A and 6B and as described below, there are often multiple alternate pathways for production of a specific long chain omega-3/omega-6 fatty acid.

Specifically, all pathways require the initial conversion of oleic acid to LA, the first of the omega-6 fatty acids, by a delta-12 desaturase. Then, using the "delta-9 elongase/delta-8 desaturase pathway", long chain omega-6 fatty acids are formed as follows: (1) LA is converted to EDA by a delta-9 elongase; (2) EDA is converted to DGLA by a delta-8 desaturase; and (3) DGLA is converted to ARA by a delta-5 desaturase. Alternatively, the "delta-9 elongase/delta-8 desaturase pathway" can be utilized for formation of long chain omega-3 fatty acids as follows: (1) LA is converted to ALA, the first of the omega-3 fatty acids, by a delta-15 desaturase; (2) ALA is converted to ETrA by a delta-9 elongase; (3) ETrA is converted to ETA by a delta-8 desaturase; (4) ETA is converted to EPA by a delta-5 desaturase; (5) EPA is converted to DPA by a C_{20/22} elongase; and (6) DPA is converted to DHA by a delta-4 desaturase. Optionally, omega-6 fatty acids may be converted to omega-3 fatty acids; for example, ETA and EPA are produced from DGLA and ARA, respectively, by delta-17 desaturase activity.

Alternate pathways for the biosynthesis of omega-3/omega-6 fatty acids utilize a delta-6 desaturase and C_{18/20} elongase (also known as delta-6 elongase, the terms can be used interchangeably) (i.e., the "delta-6 desaturase/delta-6 elongase pathway"). More specifically, LA and ALA may be converted to GLA and STA, respectively, by a delta-6 desaturase; then, a C_{18/20} elongase converts GLA to DGLA and/or STA to ETA.

It is contemplated that the particular functionalities required to be introduced into a specific host organism for production of omega-3/omega-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). For example, expression of the delta-9 elongase/delta-8 desaturase pathway may be preferred in

some embodiments, as opposed to expression of the delta-6 desaturase/delta-6 elongase pathway, since PUFAs produced via the former pathway are devoid of GLA.

One skilled in the art will be able to identify various candidate genes encoding each of the enzymes desired for omega-3/omega-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, 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; and/or (4) co-factors required by the polypeptide. 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 unpurified oils produced in a host cell will typically be a mixture of various PUFAs consisting of the desired omega-3/omega-6 fatty acid, as well as various upstream intermediary PUFAs. Thus, consideration of each enzyme's conversion efficiency is also a variable when optimizing biosynthesis of a desired fatty acid that must be considered in light of the final desired lipid profile of the product.

With each of the considerations above in mind, candidate genes having the appropriate desaturase and elongase activities (e.g., delta-6 desaturases, C_{18/20} elongases, delta-5 desaturases, delta-17 desaturases, delta-15 desaturases, delta-9 desaturases, delta-12 desaturases, C_{14/16} elongases, C_{16/18} elongases, delta-9 elongases, delta-8 desaturases, delta-4 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.

Sequence Identification of Novel Delta-8 Desaturases

In the present invention, nucleotide sequences encoding delta-8 desaturases have been isolated from *Tetruetreptia pomquetensis* CCMP1491 (designated herein as "TpomD8"), *Eutreptiella* sp. CCMP389 (designated herein as "E389D8") and *Eutreptiella cf. gymnastica* CCMP1594 (designated herein as "E1594D8").

Thus, the present invention concerns an isolated polynucleotide comprising:

- (a) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:47 [E389D8], SEQ ID NO:49 [E1594D8] or SEQ ID NO:57 [TpomD8];
- (b) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:92 [E389D8], SEQ ID NO:93 [E1594D8] or SEQ ID NO:62 [TpomD8]; or,
- (c) a complement of the nucleotide sequence of (a) or (b), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

In still another aspect, this invention concerns an isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence has at least 90% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62.

In alternate embodiments, the instant E389D8, E1594D8 or TpomD8 desaturase sequences 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 embodiment of the invention herein, E389D8, E1594D8 and/or TpomD8 could be codon-optimized for expression in *Yarrowia lipolytica*, as taught in PCT Publication No. WO 04/101757. In alternate embodiments, it may be desirable to modify a portion of the codons encoding E389D8, E1594D8 and/or TpomD8 (as set forth in SEQ ID NOs:92, 93 and 62, respectively) to enhance expression of the gene in a host organism including, but not limited to, a plant or plant part.

One skilled in the art would be able to use the teachings herein to create various other codon-optimized delta-8 desaturase proteins suitable for optimal expression in alternate hosts, based on the wildtype E389D8, E1594D8 and/or TpomD8 sequences. Accordingly, the instant invention relates to any codon-optimized delta-8 desaturase protein that is derived from the wildtype E389D8 (i.e., encoded by SEQ ID NO:47), the wildtype E1594D8 (i.e., encoded by SEQ ID NO:49) or the wildtype TpomD8 (i.e., encoded by SEQ ID NO:57).

Identification and Isolation of Homologs

Any of the instant desaturase sequences (i.e., E389D8, E1594D8 or TpomD8) or portions thereof may be used to search for delta-8 desaturase homologs in the same or other bacterial, algal, fungal, euglenoid 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 delta-8 desaturase 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 delta-8 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, euglenoid 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 et al., *Proc. Acad. Sci. USA* 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 delta-8 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 e.g., any desired yeast or fungus using methodology well known to those skilled in the art (wherein those organisms producing DGLA and/or ETA 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. Humana: Totowa, NJ).

Generally two short segments of the instant 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., *PNAS USA* 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., *PNAS USA* 86:5673 (1989); Loh et al., *Science* 243:217 (1989)).

In other embodiments, any of the delta-8 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 delta-8 desaturase nucleic acid

fragments described herein are exchanged with a functional domain in an alternate desaturase gene to thereby result in a novel protein. As used herein, "domain" or "functional domain" refer to nucleic acid sequence(s) that are capable of eliciting a biological response in plants.

Methods for Production of Various Omega-3 and/or Omega-6 Fatty Acids

It is expected that introduction of chimeric genes encoding the delta-8 desaturases described herein (i.e., E389D8, E1594D8, TpomD8 or other mutant enzymes, codon-optimized enzymes or homologs thereof), under the control of the appropriate promoters will result in increased production of DGLA and/or ETA 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., EDA and/or ETrA) to the desaturase enzymes described herein (e.g., E389D8, E1594D8, TpomD8), such that the substrate is converted to the desired fatty acid product (i.e., DGLA and/or ETA).

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

- (a) a recombinant construct encoding a delta-8 desaturase polypeptide selected from the group consisting of SEQ ID NO:47, SEQ ID NO:49 and SEQ ID NO:57; and,
- (b) a source of EDA;

wherein the host cell is grown under conditions such that the delta-8 desaturase is expressed and the EDA is converted to DGLA, and wherein the DGLA is optionally recovered.

In alternate embodiments of the present invention, the delta-8 desaturase may be used for the use of the enzyme for the conversion of ETrA to ETA. Accordingly the invention provides a method for the production of ETA, wherein the host cell comprises:

- (a) a recombinant construct encoding a delta-8 desaturase polypeptide selected from the group consisting of SEQ ID NO:47, SEQ ID NO:49 and SEQ ID NO:57; and,
- (b) a source of ETrA;

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wherein the host cell is grown under conditions such that the delta-8 desaturase is expressed and the ETrA is converted to ETA, and wherein the ETA is optionally recovered.

Alternatively, each delta-8 desaturase gene and its corresponding enzyme product described herein can be used indirectly for the production of various omega-6 and omega-3 PUFAs, including e.g., DGLA, ETA, ARA, EPA, DPA and/or DHA (FIG. 6A and 6B; see PCT Publication No. WO 2004/101757). Indirect production of omega-3/omega-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 delta-8 desaturases described herein (i.e., E389D8, E1594D8, TpomD8, 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., delta-6 desaturases, C_{18/20} elongases, delta-17 desaturases, delta-8 desaturases, delta-15 desaturases, delta-9 desaturases, delta-12 desaturases, C_{14/16} elongases, C_{16/18} elongases, delta-9 elongases, delta-5 desaturases, delta-4 desaturases, C_{20/22} elongases) to result in higher levels of production of longer-chain omega-3/omega-6 fatty acids (e.g., ARA, EPA, DPA and DHA).

In preferred embodiments, the delta-8 desaturases of the present invention will minimally be expressed in conjunction with a delta-9 elongase (e.g., a delta-9 elongase as set forth in SEQ ID NO:75 or a codon-optimized delta-9 elongase as set forth in SEQ ID NO:110). However, 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).

At times, it may be desirable to minimize by-product fatty acids. The relative abundance of by-product fatty acids could be decreased by increasing total delta-8 desaturase activity. One approach to minimize by-product fatty acids would be to express more than one delta-8 desaturase (i.e., the same or different delta-8 desaturase). For instance, the presence of sciadonic acid (SCI) and/or juniperonic acid (JUP) [commonly found in the seed lipids of gymnosperms (Wolff et al., *Lipids* 35(1):1-22 (2000)), such as those in the *Pinaceae* family (pine)] might be considered by-product fatty acids of a delta-6 desaturase/delta-6 elongase pathway or delta-9-

elongase/delta-8 desaturase pathway. Although these fatty acids are considered to have various health-enhancing properties themselves (Nakane et al., *Biol. Pharm. Bull.* 23: 758-761 (2000)), their presence as by-product fatty acids in an engineered PUFA pathway, such as in an oilseed crop, may not be desirable depending on the application.

The term "delta-6 desaturase/delta-6 elongase pathway" also refers to a biosynthetic pathway for production of long-chain PUFAs. This pathway, at a minimum, comprises a delta-6 desaturase and a delta-6 elongase, thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized. Occasionally, a delta-6 elongase may elongate fatty acids other than the intended fatty acid. For instance, delta-6 elongases generally convert GLA to DGLA but some delta-6 elongases may also convert unintended substrates such as LA or ALA to EDA or ETrA, respectively. In a delta-6 desaturase/delta-6 elongase pathway, EDA and ETrA would be considered "by-product fatty acids" as defined below. Addition of a delta-8 desaturase to a delta-6 desaturase/delta-6 elongase pathway would provide a means to convert the "by-product fatty acids" EDA and ETrA back into the "intermediate fatty acids" (as defined below) DGLA and ETA, respectively.

Plant Expression Systems, Cassettes and Vectors, and Transformation

In one embodiment, this invention concerns a recombinant construct comprising any one of the delta-8 desaturase polynucleotides of the invention operably linked to at least one regulatory sequence suitable for expression in a plant. A promoter is a DNA sequence that directs cellular machinery of a plant to produce RNA from the contiguous coding sequence downstream (3') of the promoter. The promoter region influences the rate, developmental stage, and cell type in which the RNA transcript of the gene is made. The RNA transcript is processed to produce mRNA which serves as a template for translation of the RNA sequence into the amino acid sequence of the encoded polypeptide. The 5' non-translated leader sequence is a region of the mRNA upstream of the protein coding region that may play a role in initiation and translation of the mRNA. The 3' transcription termination/polyadenylation signal is a non-translated region downstream of the protein coding region that functions in the plant cell to cause

termination of the RNA transcript and the addition of polyadenylate nucleotides to the 3' end of the RNA.

The origin of the promoter chosen to drive expression of the delta-8 desaturase coding sequence is not important as long as it has sufficient transcriptional activity to accomplish the invention by expressing translatable mRNA for the desired nucleic acid fragments in the desired host tissue at the right time. Either heterologous or non-heterologous (i.e., endogenous) promoters can be used to practice the invention. For example, suitable promoters include, but are not limited to: the alpha prime subunit of beta conglycinin promoter, the Kunitz trypsin inhibitor 3 promoter, the annexin promoter, the glycinin Gy1 promoter, the beta subunit of beta conglycinin promoter, the P34/Gly Bd m 30K promoter, the albumin promoter, the Leg A1 promoter and the Leg A2 promoter.

The annexin, or P34, promoter is described in PCT Publication No. WO 2004/071178 (published August 26, 2004). The level of activity of the annexin promoter is comparable to that of many known strong promoters, such as: (1) the CaMV 35S promoter (Atanassova et al., *Plant Mol. Biol.* 37:275-285 (1998); Battraw and Hall, *Plant Mol. Biol.* 15:527-538 (1990); Holtorf et al., *Plant Mol. Biol.* 29:637-646 (1995); Jefferson et al., *EMBO J.* 6:3901-3907 (1987); Wilmink et al., *Plant Mol. Biol.* 28:949-955 (1995)); (2) the *Arabidopsis* oleosin promoters (Plant et al., *Plant Mol. Biol.* 25:193-205 (1994); Li, Texas A&M University Ph.D. dissertation, pp. 107-128 (1997)); (3) the *Arabidopsis* ubiquitin extension protein promoters (Callis et al., *J Biol. Chem.* 265(21):12486-93 (1990)); (4) a tomato ubiquitin gene promoter (Rollfinke et al., *Gene*. 211(2):267-76 (1998)); (5) a soybean heat shock protein promoter (Schoffl et al., *Mol Gen Genet.* 217(2-3):246-53 (1989)); and, (6) a maize H3 histone gene promoter (Atanassova et al., *Plant Mol Biol.* 37(2):275-85 (1989)).

Another useful feature of the annexin promoter is its expression profile in developing seeds. The annexin promoter is most active in developing seeds at early stages (before 10 days after pollination) and is largely quiescent in later stages. The expression profile of the annexin promoter is different from that of many seed-specific promoters, e.g., seed storage protein promoters, which often provide highest activity in later stages of development (Chen et al., *Dev. Genet.* 10:112-122 (1989); Ellerstrom et al., *Plant Mol. Biol.* 32:1019-1027 (1996); Keddle

et al., *Plant Mol. Biol.* 24:327-340 (1994); Plant et al., (*supra*); Li, (*supra*)). The annexin promoter has a more conventional expression profile but remains distinct from other known seed specific promoters. Thus, the annexin promoter will be a very attractive candidate when overexpression, or suppression, of a gene in embryos is desired at an early developing stage. For example, it may be desirable to overexpress a gene regulating early embryo development or a gene involved in the metabolism prior to seed maturation.

Following identification of an appropriate promoter suitable for expression of a specific delta-8 desaturase coding sequence, the promoter is then operably linked in a sense orientation using conventional means well known to those skilled in the art.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J. et al., In *Molecular Cloning: A Laboratory Manual*; 2nd ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 1989 (hereinafter "Sambrook et al., 1989") or Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K., Eds.; In *Current Protocols in Molecular Biology*; John Wiley and Sons: New York, 1990 (hereinafter "Ausubel et al., 1990").

Once the recombinant construct has been made, it may then be introduced into a plant cell of choice by methods well known to those of ordinary skill in the art (e.g., transfection, transformation and electroporation). Oilseed plant cells are the preferred plant cells. The transformed plant cell is then cultured and regenerated under suitable conditions permitting expression of the long-chain PUFA which is then optionally recovered and purified.

The recombinant constructs of the invention may be introduced into one plant cell; or, alternatively, each construct may be introduced into separate plant cells.

Expression in a plant cell may be accomplished in a transient or stable fashion as is described above.

The desired long-chain PUFAs can be expressed in seed. Also within the scope of this invention are seeds or plant parts obtained from such transformed plants.

Plant parts include differentiated and undifferentiated tissues including, but not limited to the following: roots, stems, shoots, leaves, pollen, seeds, tumor tissue

and various forms of cells and culture (e.g., single cells, protoplasts, embryos and callus tissue). The plant tissue may be in plant or in a plant organ, tissue or cell culture.

The term "plant organ" refers to plant tissue or a group of tissues that constitute a morphologically and functionally distinct part of a plant. The term "genome" refers to the following: (1) the entire complement of genetic material (genes and non-coding sequences) that is present in each cell of an organism, or virus or organelle; and/or (2) a complete set of chromosomes inherited as a (haploid) unit from one parent.

Thus, this invention also concerns a method for transforming a cell, comprising transforming a cell with the recombinant construct of the invention and selecting those cells transformed with the recombinant construct of Claim 8.

Also of interest is a method for producing a transformed plant comprising transforming a plant cell with the delta-8 desaturase polynucleotides of the instant invention and regenerating a plant from the transformed plant cell.

Methods for transforming dicots (primarily by use of *Agrobacterium tumefaciens*) and obtaining transgenic plants have been published, among others, for: cotton (U.S. Patent No. 5,004,863; U.S. Patent No. 5,159,135); soybean (U.S. Patent No. 5,569,834; U.S. Patent No. 5,416,011); *Brassica* (U.S. Patent No. 5,463,174); peanut (Cheng et al. *Plant Cell Rep.* 15:653-657 (1996); McKently et al. *Plant Cell Rep.* 14:699-703 (1995)); papaya (Ling, K. et al. *Bio/technology* 9:752-758 (1991)); and pea (Grant et al. *Plant Cell Rep.* 15:254-258 (1995)). For a review of other commonly used methods of plant transformation see Newell, C.A. (*Mol. Biotechnol.* 16:53-65 (2000)). One of these methods of transformation uses *Agrobacterium rhizogenes* (Tepfler, M. and Casse-Delbart, F. *Microbiol. Sci.* 4:24-28 (1987)). Transformation of soybeans using direct delivery of DNA has been published using PEG fusion (PCT Publication No. WO 92/17598), electroporation (Chowrira, G.M. et al., *Mol. Biotechnol.* 3:17-23 (1995); Christou, P. et al., *Proc. Natl. Acad. Sci. U.S.A.* 84:3962-3966 (1987)), microinjection and particle bombardment (McCabe, D.E. et. al., *Bio/Technology* 6:923 (1988); Christou et al., *Plant Physiol.* 87:671-674 (1988)).

There are a variety of methods for the regeneration of plants from plant tissue. The particular method of regeneration will depend on the starting plant

tissue and the particular plant species to be regenerated. The regeneration, development and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach and Weissbach, In: Methods for Plant Molecular Biology, (Eds.), Academic: San Diego, CA (1988)). This regeneration and growth process typically includes the steps of selection of transformed cells and culturing those individualized cells through the usual stages of embryonic development through the rooted plantlet stage.

Transgenic embryos and seeds are similarly regenerated. The resulting transgenic rooted shoots are thereafter planted in an appropriate plant growth medium such as soil. Preferably, the regenerated plants are self-pollinated to provide homozygous transgenic plants. Otherwise, pollen obtained from the regenerated plants is crossed to seed-grown plants of agronomically important lines. Conversely, pollen from plants of these important lines is used to pollinate regenerated plants. A transgenic plant of the present invention containing a desired polypeptide is cultivated using methods well known to one skilled in the art.

In addition to the above discussed procedures, practitioners are familiar with the standard resource materials which describe specific conditions and procedures for: the construction, manipulation and isolation of macromolecules (e.g., DNA molecules, plasmids, etc.); the generation of recombinant DNA fragments and recombinant expression constructs; and, the screening and isolating of clones. See, for example: Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor: NY (1989); Maliga et al., Methods in Plant Molecular Biology, Cold Spring Harbor: NY (1995); Birren et al., Genome Analysis: Detecting Genes, Vol.1, Cold Spring Harbor: NY (1998); Birren et al., Genome Analysis: Analyzing DNA, Vol.2, Cold Spring Harbor: NY (1998); Plant Molecular Biology: A Laboratory Manual, eds. Clark, Springer: NY (1997).

Examples of oilseed plants include, but are not limited to: soybean, *Brassica* species, sunflower, maize, cotton, flax and safflower.

Examples of PUFAs having at least twenty carbon atoms and five or more carbon-carbon double bonds include, but are not limited to, omega-3 fatty acids such as EPA, DPA and DHA. Seeds obtained from such plants are also within the scope of this invention as well as oil obtained from such seeds.

Thus, in one embodiment this invention concerns an oilseed plant comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding a delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-9 desaturase, a delta-9 elongase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase.

Such additional desaturases are discussed, for example, in U.S. Patent Nos. 6,075,183, 5,968,809, 6,136,574, 5,972,664, 6,051,754, 6,410,288 and PCT Publication Nos. WO 98/46763, WO 98/46764, WO 00/12720 and WO 00/40705.

The choice of combination of cassettes used depends in part on the PUFA profile and/or desaturase/elongase profile of the oilseed plant cells to be transformed and the long-chain PUFA which is to be expressed.

In another aspect, this invention concerns a method for making long-chain PUFAs in a plant cell comprising:

- (a) transforming a cell with the recombinant construct of the invention; and,
- (b) selecting those transformed cells that make long-chain PUFAs.

In still another aspect, this invention concerns a method for producing at least one PUFA in a soybean cell comprising:

- (a) transforming a soybean cell with a first recombinant DNA construct comprising:
 - (i) an isolated polynucleotide encoding a delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,
 - (ii) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the

- group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-9 desaturase, a delta-9 elongase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase;
- (b) regenerating a soybean plant from the transformed cell of step (a); and,
- (c) selecting those seeds obtained from the plants of step (b) having an altered level of PUFAs when compared to the level in seeds obtained from a nontransformed soybean plant.

In other preferred embodiments, the at least one additional recombinant DNA construct encodes a polypeptide having delta-9 elongase activity, e.g., the delta-9 elongase isolated or derived from *Isochrysis galbana* (GenBank Accession No. AF390174; IgD9e) as set forth in SEQ ID NO:76 or the delta-9 elongase isolated or derived from *Euglena gracilis* as set forth in SEQ ID NO:75.

Microbial Expression Systems, Cassettes and Vectors, and Transformation

The delta-8 elongase genes and gene products described herein (i.e., E389D8, E1594D8, TpomD8, or other mutant enzymes, codon-optimized enzymes or homologs thereof) may also be produced in heterologous microbial host cells, particularly in the cells of oleaginous yeasts (e.g., *Yarrowia lipolytica*).

Microbial 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 microorganisms via transformation to provide high-level expression of the encoded enzymes.

Vectors or DNA cassettes useful for the transformation of suitable microbial 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 microbial 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 delta-8 desaturase ORFs in the desired microbial 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 microbial 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 Nos. WO 2004/101757 and WO 2006/052870 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.

Nucleotide sequences surrounding the translational initiation codon 'ATG' have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. For expression in yeast, this can be done by site-directed mutagenesis of an inefficiently expressed gene by fusing it in-frame to an endogenous yeast gene, preferably a highly expressed gene. Alternatively, one can determine the consensus translation initiation sequence in the host and engineer this sequence into heterologous genes for their optimal expression in the host of interest.

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. Preferably, when the microbial host is a yeast cell, the termination region is derived from a yeast gene (particularly *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Yarrowia* or *Kluyveromyces*). The 3'-regions of mammalian genes encoding γ -interferon and α -2 interferon are also known to function in yeast. 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. Although not intended to be limiting, termination regions useful in the disclosure herein include: ~100 bp of the 3' region of the *Yarrowia lipolytica* extracellular protease (XPR; GenBank Accession No. M17741); the acyl-coA oxidase (Aco3: GenBank Accession No. AJ001301 and No. CAA04661; Pox3: GenBank Accession No. XP_503244) terminators; the Pex20 (GenBank Accession No. AF054613) terminator; the Pex16 (GenBank Accession No. U75433) terminator; the *Lip1* (GenBank Accession No. Z50020) terminator; the *Lip2* (GenBank Accession No. AJ012632) terminator; and the 3-oxoacyl-coA thiolase (OCT; GenBank Accession No. X69988) terminator.

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 microbial 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 delta-8 desaturases described herein.

Once the DNA encoding a polypeptide suitable for expression in an appropriate microbial host cell (e.g., oleaginous yeast) has been obtained (e.g., a chimeric gene comprising a promoter, ORF and terminator), it is placed in a plasmid vector capable of autonomous replication in a 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 within 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.

In the present invention, the preferred method of expressing genes in *Yarrowia lipolytica* 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 (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 delta-12 desaturase gene locus (PCT Publication No. WO2004/104167), the *Lip1* gene locus (GenBank Accession No. Z50020) and/or the *Lip2* gene locus (GenBank Accession No. AJ012632)].

Advantageously, the *Ura3* gene can be used repeatedly in combination with 5-fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; "5-FOA") selection (*infra*), to readily permit genetic modifications to be integrated into the *Yarrowia* genome in a facile manner.

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 microbial host cell by any standard technique. These techniques include transformation (e.g., lithium acetate transformation [*Methods in Enzymology*, 194:186-187 (1991)]), protoplast fusion, bolistic impact, electroporation, microinjection, or any other method that introduces the gene of interest into the host cell. More specific teachings applicable for oleaginous yeasts (i.e., *Yarrowia lipolytica*) include U.S. 4,880,741 and U.S. 5,071,764 and Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)).

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 Publication Nos. WO2004/101757 and WO 2006/052870. Preferred selection methods for use herein 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-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). More specifically, one can first knockout the native Ura3 gene to produce a strain having a Ura⁻ phenotype, wherein selection occurs based on 5-FOA resistance. Then, a cluster of multiple chimeric genes and a new Ura3 gene can be integrated into a different locus of the *Yarrowia* genome to thereby produce a new strain having a Ura⁺ phenotype. Subsequent integration produces a new Ura3⁻ strain (again identified using 5-FOA selection), when the introduced Ura3 gene is knocked

out. Thus, the Ura3 gene (in combination with 5-FOA selection) can be used as a selection marker in multiple rounds of transformation.

Following transformation, substrates suitable for the instant delta-8 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.

Microbial 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 will be expressed in an oleaginous yeast (and in particular *Yarrowia lipolytica*); however, it is contemplated that because transcription, translation and the protein biosynthetic apparatus is highly conserved, any bacteria, yeast, algae and/or fungus will be a suitable microbial host for expression of the present nucleic acid fragments.

Preferred microbial hosts, however, are oleaginous yeasts. These 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*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*. More specifically, illustrative oil-synthesizing yeasts include: *Rhodospiridium 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 #20362, ATCC #8862, ATCC #18944, ATCC #76982 and/or LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.* 82(1):43-9 (2002)).

Historically, various strains of *Y. lipolytica* have been used for the manufacture and production of: isocitrate lyase; lipases; polyhydroxyalkanoates; citric acid; erythritol; 2-oxoglutaric acid; γ -decalactone; γ -dodecalatone; and pyruvic

acid. Specific teachings applicable for engineering ARA, EPA and DHA production in *Y. lipolytica* are provided in U.S. Patent Application No. 11/264784 (WO 2006/055322), U.S. Patent Application No. 11/265761 (WO 2006/052870) and U.S. Patent Application No. 11/264737 (WO 2006/052871), respectively.

Other preferred microbial hosts include oleaginous bacteria, algae and other fungi; and, within this broad group of microbial hosts, of particular interest are microorganisms that synthesize omega-3/omega-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 delta-8 desaturase genes under the control of inducible or regulated promoters could yield a transformant organism capable of synthesizing increased quantities of DGLA. 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. 7,001,772.

Based on the teachings described above, in one embodiment this invention is drawn to a method of producing either DGLA or ETA, respectively, comprising:

- (a) providing an oleaginous yeast comprising:
 - (i) a first recombinant DNA construct comprising an isolated polynucleotide encoding a delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,
 - (ii) a source of desaturase substrate consisting of either EDA or ETrA, respectively; and,
- (b) growing the yeast of step (a) in the presence of a suitable fermentable carbon source wherein the gene encoding the delta-8 desaturase polypeptide is expressed and EDA is converted to DGLA or ETrA is converted to ETA, respectively; and,
- (c) optionally recovering the DGLA or ETA, respectively, of step (b).

Substrate feeding may be required.

Of course, since naturally produced PUFAs in oleaginous yeast are limited to 18:2 fatty acids (i.e., LA), and less commonly, 18:3 fatty acids (i.e., ALA), in more preferred embodiments of the present invention the oleaginous yeast will be genetically engineered to express multiple enzymes necessary for long-chain PUFA

biosynthesis (thereby enabling production of e.g., ARA, EPA, DPA and DHA), in addition to the delta-8 desaturases described herein.

Specifically, in one embodiment this invention concerns an oleaginous yeast comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding a delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and,

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of: a delta-4 desaturase, a delta-5 desaturase, delta-6 desaturase, a delta-9 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase.

In particularly preferred embodiments, the at least one additional recombinant DNA construct encodes a polypeptide having delta-9 elongase activity, e.g., the delta-9 elongase isolated or derived from *Isochrysis galbana* (GenBank Accession No. AF390174; IgD9e or IgD9eS) as set forth in SEQ ID NO:76 or the delta-9 elongase isolated or derived from *Euglena gracilis* as set forth in SEQ ID NO:75.

Metabolic Engineering of Omega-3 and/or Omega-6 Fatty Acid Biosynthesis in Microbes

Methods for manipulating biochemical pathways are well known to those skilled in the art; and, it is expected that numerous manipulations will be possible to maximize omega-3 and/or omega-6 fatty acid biosynthesis in oleaginous yeasts, and particularly, in *Yarrowia lipolytica*. This manipulation may require metabolic engineering directly within the PUFA biosynthetic pathway or additional coordinated manipulation of various other metabolic pathways.

In the case of manipulations within the PUFA biosynthetic pathway, it may be desirable to increase the production of LA to enable increased production of omega-6 and/or omega-3 fatty acids. Introducing and/or amplifying genes encoding delta-9 and/or delta-12 desaturases may accomplish this. To maximize production of omega-6 unsaturated fatty acids, it is well known to one skilled in the art that production is favored in a host microorganism that is substantially free of ALA; thus, preferably, the host is selected or obtained by removing or inhibiting delta-15 or

omega-3 type desaturase activity that permits conversion of LA to ALA.

Alternatively, it may be desirable to maximize production of omega-3 fatty acids (and minimize synthesis of omega-6 fatty acids). In this example, one could utilize a host microorganism wherein the delta-12 desaturase activity that permits conversion of oleic acid to LA is removed or inhibited; subsequently, appropriate expression cassettes would be introduced into the host, along with appropriate substrates (e.g., ALA) for conversion to omega-3 fatty acid derivatives of ALA (e.g., STA, ETrA, ETA, EPA, DPA, DHA).

In alternate embodiments, biochemical pathways competing with the omega-3 and/or omega-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).

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 Nos. WO 2006/055322, WO 2006/052870 and WO 2006/052871, respectively, as are desirable manipulations in the TAG biosynthetic pathway and the TAG degradation pathway (and associated techniques thereof).

Within the context of the present invention, it may be useful to modulate the expression of the fatty acid biosynthetic pathway by any one of the strategies described above. For example, the present invention provides methods whereby genes encoding key enzymes in the delta-9 elongase/delta-8 desaturase biosynthetic pathway are introduced into oleaginous yeasts for the production of omega-3 and/or omega-6 fatty acids. It will be particularly useful to express the present the delta-8 desaturase genes in oleaginous yeasts that do not naturally possess omega-3 and/or omega-6 fatty acid biosynthetic pathways and coordinate the expression of these genes, to maximize production of preferred PUFA products using various means for metabolic engineering of the host organism.

Microbial Fermentation Processes for PUFA Production

The transformed host cell is grown under conditions that optimize expression of chimeric desaturase genes and produce the greatest and the 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., $(\text{NH}_4)_2\text{SO}_4$) 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., 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 oleaginous yeast (e.g., *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.

Purification and Processing of PUFA Oils

PUFAs may be found in the host microorganisms and plants as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cells through a variety of means well-known in the art. One review of extraction techniques, quality analysis and acceptability standards for yeast lipids is that of Z. Jacobs (*Critical Reviews in Biotechnology*, 12(5/6):463-491 (1992)). A brief review of downstream processing is also available by A. Singh and O. Ward (*Adv. Appl. Microbiol.*, 45:271-312 (1997)).

In general, means for the purification of PUFAs may include extraction with organic solvents, sonication, supercritical fluid extraction (e.g., using carbon dioxide), saponification and physical means such as presses, or combinations thereof. One is referred to the teachings of PCT Publication No. WO 2004/101757 for additional details. Methods of isolating seed oils are well known in the art: (Young et al., Processing of Fats and Oils, In *The Lipid Handbook*, Gunstone et al., eds., Chapter 5 pp 253-257; Chapman & Hall: London (1994)). For example, soybean oil is produced using a series of steps involving the extraction and purification of an edible oil product from the oil-bearing seed. Soybean oils and soybean byproducts are produced using the generalized steps shown in Table 3.

TABLE 3

Generalized Steps for Soybean Oil and Byproduct Production

Process Step	Process	Impurities Removed and/or By-Products Obtained
# 1	soybean seed	
# 2	oil extraction	meal
# 3	degumming	lecithin
# 4	alkali or physical refining	gums, free fatty acids, pigments

# 5	water washing	soap
# 6	bleaching	color, soap, metal
# 7	(hydrogenation)	
# 8	(winterization)	stearine
# 9	deodorization	free fatty acids, tocopherols, sterols, volatiles
# 10	oil products	

More specifically, soybean seeds are cleaned, tempered, dehulled and flaked, thereby increasing the efficiency of oil extraction. Oil extraction is usually accomplished by solvent (e.g., hexane) extraction but can also be achieved by a combination of physical pressure and/or solvent extraction. The resulting oil is called crude oil. The crude oil may be degummed by hydrating phospholipids and other polar and neutral lipid complexes that facilitate their separation from the nonhydrating, triglyceride fraction (soybean oil). The resulting lecithin gums may be further processed to make commercially important lecithin products used in a variety of food and industrial products as emulsification and release (i.e., antisticking) agents. Degummed oil may be further refined for the removal of impurities (primarily free fatty acids, pigments and residual gums). Refining is accomplished by the addition of a caustic agent that reacts with free fatty acid to form soap and hydrates phosphatides and proteins in the crude oil. Water is used to wash out traces of soap formed during refining. The soapstock byproduct may be used directly in animal feeds or acidulated to recover the free fatty acids. Color is removed through adsorption with a bleaching earth that removes most of the chlorophyll and carotenoid compounds. The refined oil can be hydrogenated, thereby resulting in fats with various melting properties and textures. Winterization (fractionation) may be used to remove stearine from the hydrogenated oil through crystallization under carefully controlled cooling conditions. Deodorization (principally via steam distillation under vacuum) is the last step and is designed to remove compounds which impart odor or flavor to the oil. Other valuable byproducts such as tocopherols and sterols may be removed during the deodorization process. Deodorized distillate containing these byproducts may be

sold for production of natural vitamin E and other high-value pharmaceutical products. Refined, bleached, (hydrogenated, fractionated) and deodorized oils and fats may be packaged and sold directly or further processed into more specialized products. A more detailed reference to soybean seed processing, soybean oil production and byproduct utilization can be found in Erickson, Practical Handbook of Soybean Processing and Utilization, The American Oil Chemists' Society and United Soybean Board (1995). Soybean oil is liquid at room temperature because it is relatively low in saturated fatty acids when compared with oils such as coconut, palm, palm kernel and cocoa butter.

Plant and microbial oils containing PUFAs that have been refined and/or purified can be hydrogenated, to thereby result in fats with various melting properties and textures. Many processed fats (including spreads, confectionary fats, hard butters, margarines, baking shortenings, etc.) require varying degrees of solidity at room temperature and can only be produced through alteration of the source oil's physical properties. This is most commonly achieved through catalytic hydrogenation.

Hydrogenation is a chemical reaction in which hydrogen is added to the unsaturated fatty acid double bonds with the aid of a catalyst such as nickel. For example, high oleic soybean oil contains unsaturated oleic, LA and linolenic fatty acids and each of these can be hydrogenated. Hydrogenation has two primary effects. First, the oxidative stability of the oil is increased as a result of the reduction of the unsaturated fatty acid content. Second, the physical properties of the oil are changed because the fatty acid modifications increase the melting point resulting in a semi-liquid or solid fat at room temperature.

There are many variables which affect the hydrogenation reaction, which in turn alter the composition of the final product. Operating conditions including pressure, temperature, catalyst type and concentration, agitation and reactor design are among the more important parameters that can be controlled. Selective hydrogenation conditions can be used to hydrogenate the more unsaturated fatty acids in preference to the less unsaturated ones. Very light or brush hydrogenation is often employed to increase stability of liquid oils. Further hydrogenation converts a liquid oil to a physically solid fat. The degree of hydrogenation depends on the desired performance and melting characteristics designed for the particular end

product. Liquid shortenings (used in the manufacture of baking products, solid fats and shortenings used for commercial frying and roasting operations) and base stocks for margarine manufacture are among the myriad of possible oil and fat products achieved through hydrogenation. A more detailed description of hydrogenation and hydrogenated products can be found in Patterson, H. B. W., *Hydrogenation of Fats and Oils: Theory and Practice*. The American Oil Chemists' Society (1994).

Hydrogenated oils have become somewhat controversial due to the presence of *trans*-fatty acid isomers that result from the hydrogenation process. Ingestion of large amounts of *trans*-isomers has been linked with detrimental health effects including increased ratios of low density to high density lipoproteins in the blood plasma and increased risk of coronary heart disease.

PUFA-Containing Oils for Use in Foodstuffs

The market place currently supports a large variety of food and feed products, incorporating omega-3 and/or omega-6 fatty acids (particularly ARA, EPA and DHA). It is contemplated that the plant/seed oils, altered seeds and microbial oils of the invention comprising PUFAs will function in food and feed products to impart the health benefits of current formulations. Compared to other vegetable oils, the oils of the invention are believed to function similarly to other oils in food applications from a physical standpoint (for example, partially hydrogenated oils such as soybean oil are widely used as ingredients for soft spreads, margarine and shortenings for baking and frying).

Plant/seed oils, altered seeds and microbial oils containing omega-3 and/or omega-6 fatty acids as described herein will be suitable for use in a variety of food and feed products including, but not limited to: food analogs, meat products, cereal products, baked foods, snack foods and dairy products. Additionally, the present plant/seed oils, altered seeds and microbial oils may be used in formulations to impart health benefit 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 plant and microbial 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.

Food analogs can be made using processes well known to those skilled in the art. There can be mentioned meat analogs, cheese analogs, milk analogs and the like. Meat analogs made from soybeans contain soy protein or tofu and other ingredients mixed together to simulate various kinds of meats. These meat alternatives are sold as frozen, canned or dried foods. Usually, they can be used the same way as the foods they replace. Meat alternatives made from soybeans are excellent sources of protein, iron and B vitamins. Examples of meat analogs include, but are not limited to: ham analogs, sausage analogs, bacon analogs, and the like.

Food analogs can be classified as imitation or substitutes depending on their functional and compositional characteristics. For example, an imitation cheese need only resemble the cheese it is designed to replace. However, a product can generally be called a substitute cheese only if it is nutritionally equivalent to the cheese it is replacing and meets the minimum compositional requirements for that cheese. Thus, substitute cheese will often have higher protein levels than imitation cheeses and be fortified with vitamins and minerals.

Milk analogs or nondairy food products include, but are not limited to, imitation milks and nondairy frozen desserts (e.g., those made from soybeans and/or soy protein products).

Meat products encompass a broad variety of products. In the United States "meat" includes "red meats" produced from cattle, hogs and sheep. In addition to the red meats there are poultry items which include chickens, turkeys, geese, guineas, ducks and the fish and shellfish. There is a wide assortment of seasoned and processed meat products: fresh, cured and fried, and cured and cooked. Sausages and hot dogs are examples of processed meat products. Thus, the term "meat products" as used herein includes, but is not limited to, processed meat products.

A cereal food product is a food product derived from the processing of a cereal grain. A cereal grain includes any plant from the grass family that yields an edible grain (seed). The most popular grains are barley, corn, millet, oats, quinoa, rice, rye, sorghum, triticale, wheat and wild rice. Examples of a cereal food product

include, but are not limited to: whole grain, crushed grain, grits, flour, bran, germ, breakfast cereals, extruded foods, pastas, and the like.

A baked goods product comprises any of the cereal food products mentioned above and has been baked or processed in a manner comparable to baking (i.e., to dry or harden by subjecting to heat). Examples of a baked good product include, but are not limited to: bread, cakes, doughnuts, bars, pastas, bread crumbs, baked snacks, mini-biscuits, mini-crackers, mini-cookies, and mini-pretzels. As was mentioned above, oils of the invention can be used as an ingredient.

A snack food product comprises any of the above or below described food products.

A fried food product comprises any of the above or below described food products that has been fried.

A health food product is any food product that imparts a health benefit. Many oilseed-derived food products may be considered as health foods.

A beverage can be in a liquid or in a dry powdered form.

For example, there can be mentioned non-carbonated drinks such as fruit juices, fresh, frozen, canned or concentrate; flavored or plain milk drinks, etc. Adult and infant nutritional formulas are well known in the art and commercially available (e.g., Similac[®], Ensure[®], Jevity[®], and Alimentum[®] from Ross Products Division, Abbott Laboratories).

Infant formulas are liquids or reconstituted powders fed to infants and young children. "Infant formula" is defined herein as an enteral nutritional product which can be substituted for human breast milk in feeding infants and typically is composed of a desired percentage of fat mixed with desired percentages of carbohydrates and proteins in an aqueous solution (e.g., see U.S. Patent No. 4,670,285). Based on the worldwide composition studies, as well as levels specified by expert groups, average human breast milk typically contains about 0.20% to 0.40% of total fatty acids (assuming about 50% of calories from fat); and, generally the ratio of DHA to ARA would range from about 1:1 to 1:2 (see, e.g., formulations of Enfamil LIPIL[™] (Mead Johnson & Company) and Similac Advance[™] (Ross Products Division, Abbott Laboratories)). Infant formulas have a special role to play in the diets of infants because they are often the only source of nutrients for infants;

and, although breast-feeding is still the best nourishment for infants, infant formula is a close enough second that babies not only survive but thrive.

A dairy product is a product derived from milk. A milk analog or nondairy product is derived from a source other than milk, for example, soymilk as was discussed above. These products include, but are not limited to: whole milk, skim milk, fermented milk products such as yogurt or sour milk, cream, butter, condensed milk, dehydrated milk, coffee whitener, coffee creamer, ice cream, cheese, etc.

Additional food products into which the PUFA-containing oils of the invention could be included are, for example, chewing gums, confections and frostings, gelatins and puddings, hard and soft candies, jams and jellies, white granulated sugar, sugar substitutes, sweet sauces, toppings and syrups, and dry-blended powder mixes.

PUFA-Containing Oils For Use in Health Food Products and Pharmaceuticals

A health food product is any food product that imparts a health benefit and include functional foods, medical foods, medical nutritionals and dietary supplements. Additionally, the plant/seed oils, altered seeds and microbial oils of the invention may be used in standard pharmaceutical compositions (e.g., the long-chain PUFA containing oils could readily be incorporated into the any of the above mentioned food products, to thereby produce a functional or medical food). More concentrated formulations comprising PUFAs include capsules, powders, tablets, softgels, gelcaps, liquid concentrates and emulsions which can be used as a dietary supplement in humans or animals other than humans.

PUFA-Containing Oils For Use in Animal Feeds

Animal feeds are generically defined herein as products intended for use as feed or for mixing in feed for animals other than humans. The plant/seed oils, altered seeds and microbial oils of the invention can be used as an ingredient in various animal feeds.

More specifically, although not limited therein, it is expected that the oils of the invention can be used within pet food products, ruminant and poultry food products and aquacultural food products. Pet food products are those products intended to be fed to a pet (e.g., dog, cat, bird, reptile, rodent). These products can include the cereal and health food products above, as well as meat and meat byproducts, soy protein products, grass and hay products (e.g., alfalfa, timothy, oat

or brome grass, vegetables). Ruminant and poultry food products are those wherein the product is intended to be fed to an animal (e.g., turkeys, chickens, cattle, swine). As with the pet foods above, these products can include cereal and health food products, soy protein products, meat and meat byproducts, and grass and hay products as listed above. Aquacultural food products (or "aquafeeds") are those products intended to be used in aquafarming, i.e., which concerns the propagation, cultivation or farming of aquatic organisms and/or animals in fresh or marine waters.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. 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. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

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. Bannan, 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 (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.

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. 5,366,860; EP 272,007) using a combination of vector and insert-specific primers. Sequence editing was performed in Sequencher (Gene Codes Corporation, Ann Arbor, MI). All sequences represent coverage at least two times in both directions. Comparisons of genetic sequences were accomplished using DNASTAR software (DNA Star, Inc.).

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 strains with ATCC Accession Nos. were purchased from the American Type Culture Collection (Rockville, MD). *Yarrowia lipolytica* strains were typically grown at 28 °C on YPD agar (1% yeast extract, 2% bactopectone, 2% glucose, 2% agar).

Transformation of *Yarrowia lipolytica* was performed according to the method of Chen 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 h. 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; 0.125 mL of 2 M DTT; and 50 μg sheared salmon sperm DNA. Then, approximately 500 ng of linearized

plasmid DNA was incubated in 100 μ L of resuspended cells, and maintained at 39 °C for 1 h 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 and/or uracil were added as appropriate to a final concentration of 0.01% (thereby producing "MMLe" 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, Detroit, MI) 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.

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 μ L 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 μ L hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.

EXAMPLE 1

Identification of Delta-8 Desaturase Enzyme Homologs From *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594

The present Example describes the identification of cDNA fragments (SEQ ID NOs:16, 17 and 18) encoding portions of delta-8 desaturases from *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594, respectively. This work included the generation of genomic DNA and RNA, synthesis of cDNA, and then the identification of portions of the genes encoding delta-8 desaturase, by use of primers derived from the *Euglena gracilis* delta-8 desaturase.

Preparation of Euglenoid RNA and Genomic DNA

Tetruetreptia pomquetensis CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594 cells (each from 1 liter of culture) were purchased from the Provasoli-Guillard National Center for Culture of Marine Phytoplakton (CCMP) (Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine). Total RNA and genomic DNA were isolated from each strain using the trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. Cell pellet from each strain was individually resuspended in 0.75 mL of trizol reagent, mixed with 0.5 mL of 0.5 mm glass beads, and homogenized in a Biospec mini beadbeater (Bartlesville, OK) at the highest setting for 3 min. The mixtures were centrifuged in an Eppendorf centrifuge for 30 sec at 14,000 rpm to remove debris and glass beads. Supernatant from each sample was extracted with 150 µL of 24:1 chloroform:isoamyl alcohol. The upper aqueous phase was used for RNA isolation and lower organic phase for DNA isolation.

For RNA isolation, the aqueous phase from each sample 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 8,000 rpm and 4 °C for 5 min. The pellet was washed once with 0.7 mL of 80% ethanol and air dried. Thus, 360 µg of total RNA was obtained from *Eutreptiella* sp. CCMP389, 95 µg from *Tetruetreptia pomquetensis* CCMP1491 and 720 µg from *Eutreptiella cf. gymnastica* CCMP1594.

For genomic DNA isolation, the lower organic phase of each sample was mixed with 75 µL of ethanol and incubated at room temperature for 5 min. The

samples were then centrifuged at 5,000 rpm for 2 min in an Eppendorf centrifuge. Each pellet was washed with 0.75 mL of 0.1 M sodium citrate:10% ethanol twice. Each time, samples were incubated for 15 min at room temperature in the wash solution, followed by centrifugation at 5,000 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 each sample was adjusted to 7.5 with 1 M HEPES. Each sample was then further purified with the Qiagen PCR purification kit according to the manufacturer's protocol. In this way, 40 µg of genomic DNA was isolated from *Eutreptiella* sp. CCMP389, 15 µg from *Tetruetreptia pomquetensis* CCMP1491 and 45 µg from *Eutreptiella cf. gymnastica* CCMP1594.

Preparation of Euglenoid cDNA

Total RNA (1.2 µg from *Eutreptiella* sp. CCMP389 and 2.4 µg from *Eutreptiella cf. gymnastica* CCMP1594) was used as template to synthesize double stranded cDNA. The CreatorTM SMARTTM cDNA Library Construction Kit from BD Bioscience Clontech (Palo Alto, CA) was used. Each total RNA sample (1 µL) was mixed individually with 1 µL of SMART IV oligonucleotide (SEQ ID NO:19), 1 µL CDSIII/3' PCR primer (SEQ ID NO:30) and 2 µL of water. The mixtures were heated to 75 °C for 5 min and then cooled on ice for 5 min. To each sample were added 2 µL of 5X first strand buffer, 1 µL 20 mM DTT, 1 µL of dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP) and 1 µL of PowerScript reverse transcriptase. The samples were incubated at 42 °C for 1 h. The resulting first strand cDNAs were then used as template for amplification. Each 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:105), 2 µL CDSIII/3' PCR primer (SEQ ID NO:30) and 2 µL 50X Advantage 2 polymerase mix. PCR amplification was performed using the following conditions: 95 °C for 1 min, followed by 20 cycles of 95 °C for 10 sec and 68 °C for 6 min. Amplification products were purified with Qiagen PCR purification kits according to the manufacturer's protocol. Purified products were eluted with 50 µL of water.

For *Tetruetreptia pomquetensis* CCMP1491, 0.95 µg of total RNA in 1 µL was used as template. The procedure used to synthesize cDNA was the same as

above except that CDSIII/3' PCR primer (SEQ ID NO:30) was replaced with the Adaptor Primer from Invitrogen 3'-RACE kit (SEQ ID NO:31).

Identification of cDNA Fragments Encoding Partial Putative Delta-8 Desaturases

Each of the above three cDNA samples were used as template for degenerate PCR using primers based on the amino acid sequence of the *Euglena gracilis* delta-8 fatty acid desaturase (EgD8; SEQ ID NO:112). The 9 forward and 2 reverse primers used are shown in Table 4:

TABLE 4

Degenerate Oligonucleotides Used to Amplify Portions of the Delta-8 Desaturase Genes From *Eutreptiella* sp. CCMP389, *Eutreptiella cf. gymnastica* CCMP1594 and *Tetruetreptia pomquetensis* CCMP1491

Primer	Nucleotide Sequence	Amino Acid Sequence
D8F1	GAYGCNACNGAYGCNTTCATG (SEQ ID NO:1)	DATDAFM (SEQ ID NO:12)
D8F2	GAYGCNACNGAYGCNGTTATG (SEQ ID NO:2)	DATDAVM (SEQ ID NO:13)
D8F3	GAYGCNACNGAYGCNGTGATG (SEQ ID NO:3)	DATDAVM (SEQ ID NO:13)
D8F4	GAYGCNACNGAYGCNTTTATG (SEQ ID NO:4)	DATDAFM (SEQ ID NO:12)
D8F5	GAYGCNACNGAYGCNGTAATG (SEQ ID NO:5)	DATDAVM (SEQ ID NO:13)
D8F6	GAYGCNACNGAYGCNGTGATG (SEQ ID NO:6)	DATDAVM (SEQ ID NO:13)
D8F7	TNGGNTGGTTRGGNGAYGA (SEQ ID NO:7)	GWLGD(D/E) (SEQ ID NO:14)
D8F8	TNGGNTGGCTRGGNGAYGA (SEQ ID NO:8)	GWLGD(D/E) (SEQ ID NO:14)
D8F9	TNGGNTGGCTYGGNGAYGA (SEQ ID NO:9)	GWLGD(D/E) (SEQ ID NO:14)
D8R1	TGRTGYTCDATYTGRTARTT (SEQ ID NO:10)	NYQIEH (SEQ ID NO:15)
D8R2	TGRTGYTCDATYTGCA TRTT (SEQ ID NO:11)	NYQIEH (SEQ ID NO:15)

A total of 18 reactions were set up for each cDNA sample, using all the possible combinations of the 9 forward and 2 reverse primers. The reaction mixture

contained 1 μ L of cDNA, 1 μ L each of the forward and reverse primers (20 μ M), 22 μ L water and 25 μ L of TaKaRa ExTaq 2X premix (TaKaRa Bio, Mountain View, CA). PCR amplification was performed using the following conditions: 94 °C for 1 min, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 1 min, followed by 7 min at 72 °C.

Agarose gel analysis of the PCR products showed that, with several primer combinations, a ~ 1 kb fragment was amplified from each cDNA sample. The fragments from the primer combination D8F4/D8R1 were cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced to afford partial sequences of the putative delta-8 desaturases from *Tetruetreptia pomquetensis* CCMP1491 (SEQ ID NO:16; 977 bp), *Eutreptiella* sp. CCMP389 (SEQ ID NO:17; 968 bp) and *Eutreptiella cf. gymnastica* CCMP1594 (SEQ ID NO:18; 968 bp).

EXAMPLE 2

Isolation of the Full-length Delta-8 Desaturases from *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594

Primers were designed (see Table 5), based on the partial sequences of the putative delta-8 desaturases from *Eutreptiella* sp. CCMP389 (SEQ ID NO:17) and *Eutreptiella cf. gymnastica* CCMP1594 (SEQ ID NO:18), to isolate the 5' and 3' ends of each gene from cDNA and genomic DNA samples.

TABLE 5

Primers Used to Clone the Full-Length Delta-8 Desaturase Genes From *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594

Organism	Primer Name	Primer Sequence	SEQ ID NO:
<i>Eutreptiella</i> sp. CCMP389	389D8-3-1	CAACGCCAGTACGCAAAGGAG	20
	389D8-3-2	CTCTGCATTGGATTCTGAAAGG	21
	389D8-5-1	AATCATGTCCTTTTGAAGCTTG	22
	389D8-5-2	GTCCTCAGCAACCTCGTCGTTG	23
	389D8-5-3	CTTGGGGCTTCGTGGCGAAGTG	24
<i>Eutreptiella cf. gymnastica</i> CCMP1594	1594D8-3-1	GAGCGTTTTCTTGTTCTGTTAC	32
	1594D8-3-2	CGTTTTTCCTTATCTCGGAGTG	33
	1594D8-5-1	GATTTGTACACATAAAACAGAG	34
	1594D8-5-2	ACCCTTCTCAACCATACTGTTG	35
	1594D8-5-3	CTTGGGAGTAAGTGGTGAAGAG	36

Isolation of the 5'-End Sequences of the *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594 Delta-8 Desaturase Genes

The full 5'-end sequences of the putative delta-8 desaturases from *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594 were obtained by genome walking using the Universal GenomeWalker™ kit (BD Biosciences Clontech, Palo Alto, CA) following the manufacturer's protocol (Prot # PT3042-1). First, genomic DNA from *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594 were digested with *DraI*, *EcoRV*, *PvuII* and *StuI* individually as described in the manufacturer's protocol. Genomic DNA (2 µg) was used for each digestion. Digested DNA samples were purified with a Qiagen enzyme reaction clean-up kit according to the manufacturer's protocol. Each sample was eluted with 20 µL of water.

The digested genomic DNA samples were ligated with the GenomeWalker adaptor (SEQ ID NO:37 and SEQ ID NO:111). Specifically, 4 µL each of the digested DNA was mixed with 1.9 µL of 25 µM GenomeWalker adaptor (SEQ ID NO:37 and SEQ ID NO:111), 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 templates for PCR amplification.

For the first round of PCR, primers 389D8-5-1 (SEQ ID NO:22) and Universal GenomeWalker™ primer AP1 (SEQ ID NO:38) from the kit were used to amplify from *Eutreptiella* sp. CCMP389 samples, while primers 1594D8-5-1 (SEQ ID NO:34) and AP1 (SEQ ID NO:38) were used for *Eutreptiella cf. gymnastica* CCMP1594 samples. Each 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 PCR reaction conditions used were as follows: 94 °C for 30 sec, 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec, and 72 °C for 2 min, followed by 5 min at 72 °C.

The PCR products were diluted 1:100, and 1 µL of each diluted PCR product was used as template for a second round of PCR using primers 389D8-5-3 (SEQ ID NO:24) and Universal GenomeWalker™ primer AP2 (SEQ ID NO:39) for *Eutreptiella* sp. CCMP389 samples, and primers 1594D8-5-3 (SEQ ID NO:36) and

Universal GenomeWalker™ primer AP2 (SEQ ID NO:39) for *Eutreptiella cf_gymnastica* CCMP1594 samples. Amplification was conducted as described above.

The second-round PCR products were purified by Qiagen PCR purification kit, cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced. A 694 bp PCR fragment generated from *Eutreptiella cf_gymnastica* CCMP1594 samples and a 648 bp fragment generated from *Eutreptiella* sp. CCMP389 samples were shown to contain the 5' end of the putative delta-8 desaturase genes, including parts of the non-translated region (SEQ ID NO:41 and SEQ ID NO:42, respectively).

Isolation of the 3'-End Sequences of the *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594 Delta-8 Desaturase Genes

The full 3'-end sequences of the putative delta-8 desaturases from *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594 were obtained by PCR amplification using cDNA samples as templates.

389D8-3-1 (SEQ ID NO:19) and CDSIII/3' PCR primer (SEQ ID NO:30; supplied with the Creator™ SMART™ cDNA Library Construction Kit of Example 1) were used as primers for first round amplification, using *Eutreptiella* sp. CCMP389 cDNA as template. 1594D8-3-1 (SEQ ID NO:32) and CDSIII/3' PCR primer (SEQ ID NO:30) were used as primers for amplification with *Eutreptiella cf_gymnastica* CCMP1594 cDNA as template. The reaction mixtures contained: 1 µL of each primer (10 µM), 1 µL of cDNA from Example 1, 22 µL water and 25 µL TaKaRa ExTaq 2X premix. The PCR reaction conditions used were as follows: 94 °C for 90 sec, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, followed by 5 min at 72 °C.

The PCR product was diluted 1:50, and 1 µL of the diluted product was used as template for a second round of PCR using either 389D8-3-2 (SEQ ID NO:21) or 1594D8-3-2 (SEQ ID NO:33) with the CDSIII/3' PCR primer (SEQ ID NO:30) under the conditions described above. The second-round PCR products were purified with Qiagen PCR purification kit, cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced. A fragment amplified from *Eutreptiella* sp. CCMP389 cDNA was shown to contain the 3'-end of the cDNA of putative delta-8 desaturase, including the polyA tail (SEQ ID NO:43; 717 bp).

Two different fragments were obtained and shown to contain the 3' end of the delta-8 desaturase from *Eutreptiella cf_gymnastica* CCMP1594. One of them, 1594D8-3'A (SEQ ID NO:44), was 1164 bp long and contained a long 3' untranslated region of 760 bp and a polyA tail. The other, 1594D8-3'B (SEQ ID NO:45), was 435 bp long and had a short 3' untranslated region of 30 bp. The sequences of the coding region of both fragments were the same.

Assembly of the Full-Length Sequences of the *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594 Delta-8 Desaturase Genes

Assembly of the 5' genomic region, the original partial cDNA sequence and the 3'-cDNA sequence resulted in the complete sequence of the delta-8 desaturases from *Eutreptiella* sp. CCMP389 and *Eutreptiella cf_gymnastica* CCMP1594 (SEQ ID NO:46 (1963 bp) and SEQ ID NO:48 (2063 bp), respectively; each sequence also contained untranslated 5' and 3' ends). Each coding region is 1254 bp long and each encodes a peptide of 417 amino acids (SEQ ID NO:47 and SEQ ID NO:49, respectively). SEQ ID NO:92 is the nucleotide sequence of the coding sequence of *Eutreptiella* sp. CCMP389 delta-8 desaturase (designated herein as "E389D8"), while SEQ ID NO:93 is the nucleotide sequence of the coding sequence of *Eutreptiella cf_gymnastica* CCMP1594 delta-8 desaturase (designated herein as "E1594D8").

EXAMPLE 3

Isolation of the Full-length Delta-8 Desaturase From
Tetruetreptia pomquetensis CCMP1491

Primers were designed (see Table 6), based on the partial sequence of the putative delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 (SEQ ID NO:16), to isolate the 5' and 3' end of the gene from cDNA and genomic DNA samples.

TABLE 6

Primers Used to Clone the Full-Length Delta-8 Desaturase Gene From
Tetruetreptia pomquetensis CCMP1491

Primer Name	Primer Sequence	SEQ ID NO:
ED8-5-1	CTCGAACATACCCTTGGAGATG	25
ED8-5-2	CCCGCAACTTGCGGAAATCCTC	26
ED8-5-3	GGGCTCATCACGCTTAGGCTTG	27
ED8-3-1	CACTTTCTATTGCAGTGCCATG	28
ED8-3-2	CTTTGCCACCGGTTTGGGATGC	29

Isolation of the 5'-End Sequence of the *Tetruetreptia pomquetensis* CCMP1491
Delta-8 Desaturase Gene

The Invitrogen TOPO walker kit was used for isolating the 5' end of the putative delta-8 desaturase gene from *Tetruetreptia pomquetensis* CCMP1491, following the manufacturer's protocol. Genomic DNA (0.3 µg) from *Tetruetreptia pomquetensis* CCMP1491 (see Example 1) was digested with *Apal*. The reaction mixture contained 10 µL genomic DNA (~0.3 µg), 4 µL of 10X restriction buffer, 2 µL restriction enzyme (*Apal* or *KpnI*) and 24 µL water. The reaction was carried out at 37 °C for 2 h. Then, 50 µL of water, 6 µL of dephosphorylation buffer and 4 µL of kit-supplied CIP were added to the mixture, and the reaction was allowed to continue for 1 h at 37 °C. The reaction mixture was then purified with Qiagen reaction purification kit according to the manufacturer's protocol. DNA was eluted in 40 µL of water.

For primer extension, 15 µL of the purified DNA was mixed with 2 µL of 10X PCR buffer (Invitrogen Corporation), 1 µL of 2.5 mM each dNTPs, 1 µL of primer ED8-5-1 (SEQ ID NO:25) (20 µM) and 1 µL of Advantage 2 cDNA polymerase mix (BD Biosciences Clontech, Palo Alto, CA). The PCR reaction conditions used were as follows: 94 °C for 4 min, 56 °C for 1 min, and 72 °C for 20 min. The primer extension reaction product (8 µL) was then used as substrate for TOPO linker in a mixture additionally comprising 1 µL TOPO linker (SEQ ID NO:50) and 1 µL 10X PCR buffer (Invitrogen Corporation). The mixture was incubated at 37 °C for 10 min and used directly as PCR template.

PCR amplification of the 5' end was carried out in a 50 µL reaction mix that contained 2 µL of TOPO linked genomic DNA, 1 µL of primer ED8-5-2 (SEQ ID NO:26) (10 µM), 1 µL of LinkAmp primer 1 (SEQ ID NO:51) (10 µM), 21 µL water and 25 µL TaKaRa ExTaq 2X premix. The PCR reaction conditions used were as follows: 94 °C for 90 sec, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min, followed by 7 min at 72 °C. The PCR product was diluted 1:50, and 1 µL of the diluted product was used as template for a second round of PCR under the same conditions, except that primers ED8-5-3 (SEQ ID NO:27) and LinkAmp primer 2 (SEQ ID NO:52) replaced ED8-5-2 (SEQ ID NO:26) and LinkAmp primer 1 (SEQ ID NO:51).

A ~600 bp PCR product was purified with a Qiagen PCR purification kit, cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced. Comparison of the partial delta-8 desaturase sequence of SEQ ID NO:16 with the 5' extension product (SEQ ID NO:53; 601 bp) showed that SEQ ID NO:53 extended upstream of the 'ATG' initiation codon of the delta-8 desaturase.

Isolation of the 3'-End Sequence of the *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase Gene

The full 3'-end sequence of the putative delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491 was obtained by PCR amplification using a cDNA sample as template. Specifically, primers ED8-3-1 (SEQ ID NO:28) and AUAP (SEQ ID NO:54; supplied in Invitrogen's 3'-RACE kit) were used as primers. The reaction mixture contained 1 µL of each primer (10 µM), 1 µL of *Tetruetreptia pomquetensis* CCMP1491 cDNA from Example 1, 22 µL water and 25 µL TaKaRa ExTaq 2X premix. The PCR reaction conditions used were as follows: 94 °C for 90 sec, 30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 30 sec, followed by 5 min at 72 °C.

The PCR product was diluted 1:50, and 1 µL of the diluted product was used as template for a second round of PCR using ED8-3-2 (SEQ ID NO:29) and AUAP (SEQ ID NO:54) as primers under the same conditions as described above. The second round PCR generated a ~1 kb fragment, which was purified with Qiagen PCR purification kit, cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced. The result of sequence analysis showed that this fragment (SEQ ID NO:55; 1028 bp) contained the 3' end of the putative delta-8 desaturase, including the polyA tail.

Assembly of the Full-Length Sequence of the *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase Gene

Assembly of the 5' genomic region, the original partial cDNA fragment and 3'-cDNA fragment resulted in the complete sequence of the delta-8 desaturase from *Tetruetreptia pomquetensis* CCMP1491, plus 358 bp of 5' untranslated region and 612 bp of 3' untranslated region (SEQ ID NO:56; 2233 bp). The coding region is 1263 bp long and encodes a protein of 420 amino acids (SEQ ID NO:57). SEQ ID NO:62 is the nucleotide sequence of the coding sequence of *Tetruetreptia pomquetensis* CCMP1491 delta-8 desaturase (designated herein as "TpomD8").

EXAMPLE 4Comparison of the Delta-8 Desaturase Sequences of *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594 to a Delta-8 Desaturase Sequence of *Euglena gracilis*

The delta-8 desaturase sequences of *Tetruetreptia pomquetensis* CCMP1491 (i.e., TpomD8), *Eutreptiella* sp. CCMP389 (i.e., E389D8) and *Eutreptiella cf. gymnastica* CCMP1594 (i.e., E1594D8) were analyzed for similarity to all publicly available protein sequences contained in the "nr" database provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins. TpomD8, E389D8 and E1594D8 each shared the greatest identity and similarity with the delta-8 desaturase of *Euglena gracilis* set forth as SEQ ID NO:98 (corresponding to NCBI Accession No. AAD45877 (GI 5639724)).

The delta-8 desaturase sequences of *Tetruetreptia pomquetensis* CCMP1491 (i.e., TpomD8), *Eutreptiella* sp. CCMP389 (i.e., E389D8) and *Eutreptiella cf. gymnastica* CCMP1594 (i.e., E1594D8) were also analyzed for similarity to the *Euglena gracilis* delta-8 desaturase (SEQ ID NO:112 of the instant application) in Applicants' Assignee's co-pending applications having U.S. Application Nos. 11/166,003 and 11/166,993 filed June 24, 2005 (Attorney Docket Nos. BB-1547 and CL-3150, respectively (PCT Publication Nos. WO 2006/012325 and WO 2006/012326; both published February 2, 2006)).

FIGs. 7A and 7B show a Clustal V alignment of the delta-8 desaturases from *Tetruetreptia pomquetensis* CCMP1491 (SEQ ID NO:57), *Eutreptiella* sp. CCMP389 (SEQ ID NO:47), *Eutreptiella cf. gymnastica* CCMP1594 (SEQ ID NO:49), *Euglena gracilis* (SEQ ID NO:98; NCBI Accession No. AAD45877 (GI 5639724)) and *Euglena gracilis* (SEQ ID NO:112). SEQ ID NO:57 has 70.5%, 71.7%, 57.5% and 61.8% identity to SEQ ID NO:47, SEQ ID NO:49, SEQ ID NO:98 and SEQ ID NO:112, respectively. SEQ ID NO:47 has 83.0%, 58.3% and 63% identity to SEQ ID NO:49, SEQ ID NO:98 and SEQ ID NO:112, respectively. SEQ ID NO:49 has 58.0% and

62.7% identity to SEQ ID NO:98 and SEQ ID NO:112, respectively. SEQ ID NO:98 has 95% identity to SEQ ID NO:112.

More specifically, TpomD8, E389D8 and E1594D8 were evaluated by BLASTP, yielding a pLog value versus EgD8 (SEQ ID NO:112). Then, the % identity of TpomD8, E389D8 and E1594D8 was determined with respect to EgD8 using the Jotun Hein method. Sequence percent identity calculations performed by the Jotun Hein method (Hein, J. J., *Meth. Enz.* 183:626-645 (1990)) were done using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=2). As discussed above, the % identity of TpomD8, E389D8 and E1594D8 was determined with respect to EgD8 using the Clustal V method. Sequence percent identity calculations performed by the Clustal V method (Higgins, D.G. and Sharp, P.M., *Comput. Appl. Biosci.* 5:151-153 (1989); Higgins et al., *Comput. Appl. Biosci.* 8:189-191 (1992)) were done using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5 and GAP LENGTH PENALTY=10). These results are summarized in Table 7.

TABLE 7
Sequence Comparison of TpomD8, E389D8 and E1594D8
to EgD8 (SEQ ID NO:112)

Desaturase	pLog value versus EgD8 by BLASTP	% Identity to EgD8 by the Jotun Hein Method	% Identity to EgD8 by the Clustal V Method
TpomD8 (SEQ ID NO:57)	155 (E value of 1e-155)	63.5%	61.8%
E389D8 (SEQ ID NO:47)	164 (E value of 1e-164)	63.3%	63.0%
E1594D8 (SEQ ID NO:49)	163 (E value of 1e-163)	64.2%	62.7%

BLAST scores and probabilities indicate that the nucleic acid fragments set forth in SEQ ID NO:57, SEQ ID NO:47 and SEQ ID NO:49 each encode an entire delta-8 desaturase.

EXAMPLE 5

Functional Analysis of the *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) In *Saccharomyces cerevisiae*

The present Example describes functional analysis of TpomD8 in *Saccharomyces cerevisiae*. This work included the following steps: (1) cloning of TpomD8 from a *Tetruetreptia pomquetensis* CCMP1491 cDNA library; (2) cloning of TpomD8 into yeast expression vector pY-75 to produce pY126; and, (3) comparison of lipid profiles within transformant organisms comprising pY-75 and pY126, after substrate feeding.

Cloning TpomD8 From a cDNA Library

Tetruetreptia pomquetensis CCMP1491 cDNA (1 μ L; synthesized as described in Example 1) was combined with 50 pmol of TpomNot-5 (SEQ ID NO:58), 50 pmol of TpomNot-3 (SEQ ID NO:59), 1 μ L of PCR nucleotide mix (10 mM, Promega, Madison, WI), 5 μ L of 10X PCR buffer (Invitrogen Corporation), 1.5 μ L of MgCl₂ (50 mM, Invitrogen Corporation), 0.5 μ L of Taq polymerase (Invitrogen Corporation) and water to 50 μ L. The reaction conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 1 min. The PCR was finished at 72 °C for 7 min and then held at 4 °C. The PCR reaction was analyzed by agarose gel electrophoresis on 5 μ L and a DNA band with molecular weight around 1.3 kb was observed.

The remaining 45 μ L of product was separated by agarose gel electrophoresis and the DNA purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA) following the manufacturer's protocol. The resulting DNA was cloned into the pGEM®-T Easy Vector (Promega) following the manufacturer's protocol. Multiple clones were sequenced using the T7 (SEQ ID NO:60) and M13-28Rev (SEQ ID NO:61) oligonucleotides to verify that the TpomD8 sequence was identical to the previously deduced coding sequence of Example 3 (i.e., SEQ ID NOs:62 and 57). Clone pLF114-10 (SEQ ID NO:63) was chosen for further expression studies.

Construction of Plasmids pY-75 (Control) and pY126, Comprising TpomD8

The yeast episomal plasmid (YE_p)-type vector pRS425 (Christianson et al., *Gene* 110:119-122 (1992)) contains sequences from the *Saccharomyces cerevisiae* 2 μ endogenous plasmid, a LEU2 selectable marker and sequences based on the

backbone of a multifunctional phagemid, pBluescript II SK(+). The *Saccharomyces cerevisiae* strong, constitutive glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter was cloned between the *SacI* and *SpeI* sites of pRS425 in the same way as described by Jia et al. (*Physiol. Genom.* 3:83-92 (2000)) to produce pGPD-425. A *NotI* site was introduced into the *Bam*HI site of pGPD-425, thus giving a *NotI* site flanked by *Bam*HI sites, and this plasmid was called pY-75 (SEQ ID NO:64), which was previously described in PCT Publication No. WO 2006/012325 (published February 2, 2006; the contents of which are hereby incorporated by reference) .

TpomD8 was released from pLF114-10 (*supra*) by digestion with *NotI* and cloned into the *NotI* site of pY75 to produce pY126 (SEQ ID NO:65; FIG. 1).

Functional Analysis of TpomD8

Expression plasmids pY75 (control) and pY126 were transformed into *Saccharomyces cerevisiae* INVSC1 (Invitrogen Corporation) using standard lithium acetate transformation procedures. Transformants were selected on DOBA media supplemented with CSM-leu (Qbiogene, Carlsbad, CA). Transformants were evaluated for delta-8 desaturase activities in the following way. Transformants from each plate were inoculated into 2 mL of DOB medium supplemented with CSM-leu (Qbiogene) and 0.2% tergitol. Cells were grown for 1 day at 30 °C after which 0.1 mL was transferred to 3 mL of the same medium supplemented with either EDA [20:2(11,14)] or ETrA [20:3(11,14,17)] to 0.175 mM. These cells were incubated for 16 h at 30 °C, 250 rpm and then pellets were obtained by centrifugation. Cells were washed once with water, pelleted by centrifugation and air dried. Pellets were transesterified (Roughan, G. and Nishida, I., *Arch. Biochem. Biophys.* 276(1):38-46 (1990)) with 500 µL of 1% sodium methoxide for 30 min at 50 °C after which 500 µL of 1 M sodium chloride and 100 µL of heptane were added. After thorough mixing and centrifugation, fatty acid methyl esters (FAMES) were analyzed by GC as described in Example 10.

Results for 3 individual clones of pY126 (i.e., clones 6, 7 and 10) as well as the vector control are shown in Table 8. Fatty acid compositions are expressed as a weight percent of total fatty acids. The activity of the delta-8 desaturase is expressed as "percent desaturation", where % Desat. was calculated according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$.

TABLE 8**Comparison of Lipid Profiles of Yeast Expressing TpomD8**

Vector	Fatty Acid	16:0	16:1	18:0	18:1	EDA	DGLA	ETrA	ETA	% Desat.
pY75	EDA	13.3	37.4	4.0	34.2	11.1	0.0	0.0	0.0	0.0
pY126-6	EDA	14.4	38.6	4.0	32.9	10.0	0.2	0.0	0.0	1.5
pY126-7	EDA	13.6	36.3	4.4	34.3	11.2	0.2	0.0	0.0	1.9
pY126-10	EDA	11.7	37.9	3.9	34.5	11.5	0.4	0.1	0.0	3.5
pY75	ETrA	11.8	33.5	3.1	24.3	0.1	0.0	27.2	0.0	0.1
pY126-6	ETrA	13.4	35.3	3.4	25.3	0.1	0.0	22.3	0.2	1.0
pY126-7	ETrA	12.2	32.8	3.4	24.8	0.1	0.0	26.2	0.4	1.6
pY126-10	ETrA	11.1	29.5	3.4	25.0	0.1	0.0	30.0	0.9	2.9

When feeding the cells EDA, the product of the TpomD8 delta-8 desaturation is DGLA; in contrast, substrate feeding with ETrA results in production of ETA by TpomD8 desaturation.

EXAMPLE 6**Generation of *Yarrowia lipolytica* Strain Y4001 to Produce About 17% EDA of Total Lipids**

The present Example describes the construction of strain Y4001, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing 17% EDA (C20:2) relative to the total lipids. The strain was engineered to test functional expression of TpomD8, E389D8 and E1594D8; specifically, it was necessary to construct a host strain capable of producing the delta-8 desaturase substrate, EDA.

The development of strain Y4001 required the construction of strain Y2224 (a FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362).

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% bactopectone, 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 17% EDA of Total Lipids

Strain Y4001 was created via integration of construct pZKLeuN-29E3 (Figure 9; comprising four chimeric genes - a delta-12 desaturase, a C_{16/18} elongase and two delta-9 elongases) into the *Leu2* loci of Y2224 strain to thereby enable production of EDA.

Construct pZKLeuN-29E3 (FIG. 9) contained the components shown in Table 9.

TABLE 9

Description of Plasmid pZKLeuN-29E3 (SEQ ID NO:106)

RE Sites And Nucleotides Within SEQ ID NO:106	Description Of Fragment And Chimeric Gene Components
<i>Bsi</i> W I/Asc I (7797-7002)	795 bp 3' part of <i>Yarrowia Leu2</i> gene (GenBank Accession No. AF260230)
<i>Sph</i> I/Pac I (4302-3591)	703 bp 5' part of <i>Yarrowia Leu2</i> gene (GenBank Accession No. AF260230)
<i>Swa</i> I/ <i>Bsi</i> W I (10500-7797)	GPD::F.D12::Pex20, comprising: • GPD: <i>Yarrowia lipolytica</i> GPD promoter (WO 2005/003310) • F.D12: <i>Fusarium moniliforme</i> delta-12 desaturase gene (WO 2005/047485) • Pex20: Pex20 terminator sequence from <i>Yarrowia Pex20</i> gene (GenBank Accession No. AF054613)
<i>Bgl</i> II/ <i>Swa</i> I (12526-10500)	Exp pro::EgD9E::Lip1, comprising: • Exp pro: <i>Yarrowia lipolytica</i> export protein (EXP1) promoter (WO 2006/052870 and U.S. Patent Application No. 11/265761) • EgD9E: (same as EgD9S, see <i>infra</i>): codon-optimized delta-9 elongase gene (SEQ ID NO:107), derived from <i>Euglena gracilis</i> (SEQ ID NOs:74 and 75 (see also U.S. Provisional Application No. 60/739989) • Lip1: Lip1 terminator sequence from <i>Yarrowia Lip1</i> gene (GenBank Accession No. Z50020)
<i>Pme</i> I/ <i>Cla</i> I (12544-1)	FBAINm::EgD9S::Lip2, comprising: • FBAINm: <i>Yarrowia lipolytica</i> FBAINm promoter (WO 2005/049805)

	• EgD9S: codon-optimized delta-9 elongase gene (SEQ ID NO:107), derived from <i>Euglena gracilis</i> (SEQ ID NOs:74 and 75 (see also U.S. Provisional Application No. 60/739989) • Lip2: Lip2 terminator sequence from <i>Yarrowia Lip2</i> gene (GenBank Accession No. AJ012632)
<i>Cla</i> I/ <i>EcoR</i> I (1-1736)	LoxP:: <i>Ura3</i> ::LoxP, comprising: • LoxP sequence (SEQ ID NO:108) • <i>Yarrowia Ura3</i> gene (GenBank Accession No. AJ306421) • LoxP sequence (SEQ ID NO:108)
<i>EcoR</i> I/ <i>Pac</i> I (1736-3591)	NT::ME3S::Pex16, comprising: • NT: <i>Yarrowia lipolytica</i> YAT1 promoter (Patent Publication No. U.S. 2006/0094102-A1) • ME3S: codon-optimized C_{16/18} elongase gene (SEQ ID NO:109), derived from <i>M. alpina</i> (see U.S. Patent Application No. 11/253882 and also WO 2006/052870) • Pex16: Pex16 terminator sequence of <i>Yarrowia Pex 16</i> 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.

EXAMPLE 7Functional Analysis of the *Tetruetreptia pomquetensis* CCMP1491 (TpomD8), *Eutreptiella* sp. CCMP389 (E389D8) and *Eutreptiella cf. gymnastica* CCMP1594 (E1594D8) Delta-8 Desaturases in *Yarrowia lipolytica* Strain Y4001

The present Example describes functional analysis of TpomD8, E389D8 and E1594D8 in *Yarrowia lipolytica* strain Y4001. This work included the following steps: (1) cloning of E389D8 from a *Eutreptiella* sp. CCMP389 cDNA library, E1594D8 from a *Eutreptiella cf. gymnastica* CCMP1594 cDNA library, and TpomD8 from a *Tetruetreptia pomquetensis* CCMP1491 cDNA library; (2) cloning of E389D8, E1594D8 and TpomD8 into yeast expression vector pFBAIn-MOD1 (SEQ ID NO:94); and, (3) comparison of lipid profiles within transformant organisms of *Yarrowia lipolytica* strain Y4001 that were additionally comprising each desaturase.

Cloning E389D8, E1594D8 and TpomD8 From cDNA Libraries

The Phusion polymerase from New England Biolab was used for amplification of E389D8 and E1594D8 cDNAs. Primers 389D8-F (SEQ ID NO:99) and 389D8-R (SEQ ID NO:100) were used for amplification of E389D8; in contrast, primers 1594D8-F (SEQ ID NO:103) and 1594D8-R (SEQ ID NO:104) were used for amplification of E1584D8. Each reaction mixture contained 1 µL each of 20 µM forward and reverse primers, 1 µL cDNA, 10 µL 5X PCR buffer, 1 µL dNTP mix (10 mM each), 35 µL water and 1 µL Phusion polymerase. The PCR reaction conditions used were as follows: 98 °C for 1 min, 30 cycles of 98 °C for 10 sec, 55 °C for 10 sec, and 72 °C for 40 sec, followed by 5 min at 72 °C. The PCR product was digested with *NcoI* and *NotI*, and cloned into pFBAIn-MOD1 (SEQ ID NO:94) predigested with the same enzymes. The resulting plasmids were named pFBAIn-389D8 (SEQ ID NO:95) and pFBAIn-1594D8 (SEQ ID NO:96).

For amplification of TpomD8, the TaKaRa ExTaq 2X premix was used for PCR instead of the Phusion polymerase. The reaction mixture contained 1 µL of *Tetruetreptia pomquetensis* CCMP1491 cDNA, 1 µL each of 20 µM primers 1491D8-F (SEQ ID NO:101) and 1491D8-R (SEQ ID NO:102), 22 µL water and 25 µL ExTaq premix. The PCR reaction conditions used were as follows: 94 °C for 30 sec, 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec, and 72 °C for 1 min 30 sec, followed by 7 min at 72 °C. The PCR products were cloned into pCR2.1-TOPO (SEQ ID NO:40) and sequenced. One clone with the correct sequence was

digested with *NcoI* and *NotI*, and the 1.3 kb fragment containing *TpomD8* was excised from agarose gel and purified with Qiagen gel purification kit. The purified fragment was then cloned into pFBAln-MOD1 (SEQ ID NO:94; see FIG. 8) pre-digested with *NcoI* and *NotI*. The resulting plasmid was named pFBAln-1491D8 (SEQ ID NO:97). Construct pFBAln-MOD1 (SEQ ID NO:94; FIG. 8) contained the components shown in Table 10.

TABLE 10

Components of Plasmid pFBAln-MOD1 (SEQ ID NO:94)

RE Sites and Nucleotides Within SEQ ID NO:94	Description of Fragment and Chimeric Gene Components
<i>BglII</i> - <i>BsiWI</i> (6278-539)	FBAln promoter:: PEX20 terminator region, comprising: • FBAln: <i>Yarrowia lipolytica</i> FBAln promoter (WO 2005/049805) • Stuffer DNA fragment derived from pDNR-LIB • _PEX20_ terminator sequence of <i>Yarrowia</i> PEX20 gene (GenBank Accession No. AF054613.)
<i>PacI</i> - <i>BglII</i> (4768-6278)	<i>Y. lipolytica</i> URA3 (GenBank Accession No. AJ306421)
(3361-4725)	ARS18, (GenBank Accession No. A17608)
(2702-3102)	<i>f1</i> origin
(1662-2522)	<i>AmpR</i> gene (for selection in media containing ampicilin)
(712-1592)	<i>ColE1</i> <i>E. coli</i> origin of replication

Functional Analysis of *TpomD8*, E389D8 and E1594D8

Plasmids pFBAln-389D8 (SEQ ID NO:95), pFBAln-1491D8 (SEQ ID NO:97), and pFBAln-1594D8 (SEQ ID NO:96) were transformed into *Yarrowia lipolytica* strain Y4001 according to the General Methods.

The cells were plated onto MM plates (lacking uracil) and maintained at 30 °C for 2 to 3 days. Single colonies of transformants were then patched onto fresh MM plates (lacking uracil) and allowed to grow at 30 °C for 1 day. After this step, cells were scraped off the patches and transferred into 1.5 mL microfuge tubes. They were transesterified as described in the General Methods. FAMES from cells containing each plasmid were analyzed by GC.

Lipid profiles of the transformant cells are shown below in Table 11. Fatty acids are identified as 16:0 (palmitic acid), 16:1 (palmitoleic acid), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (linoleic acid), 20:1 (eicosenoic acid), 20:2 (eicosadienicoic acid) and DGLA (20:3; dihomo- γ -linolenic acid); and the composition of each is presented as a % of the total fatty acids.

The conversion efficiency was measured according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$, where 'product' includes the immediate product and all products in the pathway derived from it. As shown in Table 11, the results demonstrated that each delta-8 desaturase was able to convert EDA (20:2) to DGLA (20:3); this confirmed that TpomD8, E389D8 and E1594D8 indeed were delta-8 desaturases. The substrate conversion efficiency for E389D8 and E1594D8 was about 6%, and for that of TpomD8 was 2.89%. Although not included within the data herein, expression of pFBAln-MOD (control) in strain Y4001 under comparable conditions resulted in c.a. 0% C20:2 (on average), wherein the conversion efficiency was c.a. 0% (on average).

TABLE 11

Comparison of Lipid Profiles of *Yarrowia lipolytica*
Expressing TpomD8, E389D8 and E1594D8

Plasmid (Desaturase)	C16:0	C16:1	C18:0	C18:1	C18:2	C20:1	C20:2	DGLA	Conv. efficiency %
pFBAln-389D8 (E389D8)	11.71	7.74	2.06	13.89	40.93	0.58	14.34	0.89	5.84
pFBAln-389D8 (E389D8)	11.64	7.74	2.06	14.57	39.95	0.57	14.69	0.98	6.25
PFBAIn-1491D8 (TpomD8)	11.68	7.91	2.01	14.16	40.27	0.54	14.81	0.44	2.89
PFBAIn-1594D8 (E1594D8)	12.03	7.71	2.3	15.18	38.95	0.57	14.97	0.9	5.67

EXAMPLE 8

Construction of Soybean Expression Vector pKR1013 For Co-Expression of the
Tetruetrepia pomquetensis CCMP1491 Delta-8 Desaturase (TpomD8) With a
Delta-9 Elongase Derived From *Isochrysis galbana* (IgD9eS)

The present Example describes construction of a soybean vector for co-expression of TpomD8 with IgD9eS (a synthetic delta-9 elongase derived from *Isochrysis galbana* and codon-optimized for expression in *Yarrowia lipolytica*). As

demonstrated in Examples 9 and 10 (*infra*), high concentrations of DGLA and/or ETA could readily be produced via expression of this vector in soybean.

Vector pKR123r (SEQ ID NO:66), which was previously described in PCT Publication No. WO 2004/071467 (published August 26, 2004; the contents of which are hereby incorporated by reference), contains a *NotI* site flanked by the Kunitz soybean Trypsin Inhibitor (KTI3) promoter (Jofuku et al., *Plant Cell* 1:1079-1093 (1989)) and the KTI 3' termination region, the isolation of which is described in U.S. Patent No. 6,372,965 (KTI3/*NotI*/KTI3' cassette). *TpomD8* (SEQ ID NO:57) was released from pLF114-10 (SEQ ID NO:63; Example 5) by digestion with *NotI* and cloned into the *NotI* site of pKR123r to produce pKR1007 (SEQ ID NO:67).

Plasmid pKR607 (SEQ ID NO:68), previously described in PCT Publication No. WO 2006/012325 (the contents of which are hereby incorporated by reference), contained a chimeric construct comprising the α' subunit of β -conglycinin ("BCON Pro"; Beachy et al., *EMBO J.* 4:3047-3053 (1985)), IgD9eS (identified as "IG syel1" on FIG. 2 herein) and the 3' transcription termination region of the phaseolin gene (Doyle et al., *J. Biol. Chem.* 261:9228-9238 (1986)). The synthesis of IgDeS is similarly described in PCT Publication No. WO 2006/012325. Briefly, the codon usage of the delta-9 elongase gene of *Isochrysis galbana* was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in PCT Publication No. WO 2004/101753. Thus, a codon-optimized delta-9 elongase gene (designated "IgD9eS", SEQ ID NO:110) was designed based on the coding sequence of IgD9e (SEQ ID NO:70) 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, 127 bp of the 792 bp coding region were modified (16.0%), and 122 codons were optimized. None of the modifications in the codon-optimized gene changed the amino acid sequence of the encoded protein (SEQ ID NO:76).

Plasmid pKR1007 (SEQ ID NO:67) was digested with *PstI* and the fragment containing *TpomD8* was cloned into the *SbfI* site of plasmid pKR607 (SEQ ID NO:68) to produce pKR1013 (SEQ ID NO:69). In this way, *TpomD8* is co-expressed with IgD9eS behind strong, seed-specific promoters. A schematic depiction of pKR1013 is shown in FIG. 2.

EXAMPLE 9

Transformation of Somatic Soybean Embryo Cultures With Soybean Expression Vector pKR1013, For Co-Expression of TpomD8 And IgD9eS

Culture Conditions:

Soybean embryogenic suspension cultures (cv. Jack) were maintained in 35 mL liquid medium SB196 (described *infra*) on a rotary shaker, 150 rpm, 26 °C with cool white fluorescent lights on 16:8 hr day/night photoperiod at light intensity of 60-85 $\mu\text{E}/\text{m}^2/\text{s}$. Cultures were subcultured every 7 days to two weeks by inoculating approximately 35 mg of tissue into 35 mL of fresh liquid SB196 (the preferred subculture interval is every 7 days).

Soybean embryogenic suspension cultures were transformed with the soybean expression plasmids by the method of particle gun bombardment (Klein et al., *Nature* 327:70 (1987)) using a DuPont Biolistic PDS1000/HE instrument (helium retrofit) for all transformations.

Soybean Embryogenic Suspension Culture Initiation:

Soybean cultures were initiated twice each month with 5-7 days between each initiation. Pods with immature seeds from available soybean plants 45-55 days after planting were picked, removed from their shells and placed into a sterilized magenta box. The soybean seeds were sterilized by shaking them for 15 min in a 5% Clorox solution with 1 drop of ivory soap (i.e., 95 mL of autoclaved distilled water plus 5 mL Clorox and 1 drop of soap, mixed well). Seeds were rinsed using 2 1-liter bottles of sterile distilled water and those less than 4 mm were placed on individual microscope slides. The small end of the seed was cut and the cotyledons pressed out of the seed coat. Cotyledons were transferred to plates containing SB199 medium (25-30 cotyledons per plate) for 2 weeks, then transferred to SB1 for 2-4 weeks. Plates were wrapped with fiber tape. After this time secondary embryos were cut and placed into SB196 liquid media for 7 days.

Preparation of DNA for Bombardment:

Either an intact plasmid or a DNA plasmid fragment containing the genes of interest and the selectable marker gene were used for bombardment. Fragments from soybean expression plasmids pKR1013 (see Example 8) were obtained by gel isolation of digested plasmids. In each case, 100 μg of plasmid DNA was used in 0.5 mL of the specific enzyme mix described below. Plasmids were digested with

Ascl (100 units) in NEBuffer 4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, pH 7.9), 100 µg/mL BSA, and 5 mM beta-mercaptoethanol at 37 °C for 1.5 hr. The resulting DNA fragments were separated by gel electrophoresis on 1% SeaPlaque GTG agarose (BioWhitaker Molecular Applications) and the DNA fragments containing gene cassettes were cut from the agarose gel. DNA was purified from the agarose using the GELase digesting enzyme following the manufacturer's protocol.

A 50 µL aliquot of sterile distilled water containing 1 mg of gold particles was added to 5 µL of a 1 µg/µL DNA solution (either intact plasmid or DNA fragment prepared as described above), 50 µL 2.5M CaCl₂ and 20 µL of 0.1 M spermidine. The mixture was pulsed 5 times on level 4 of a vortex shaker and spun for 5 sec in a bench microfuge. After a wash with 150 µL of 100% ethanol, the pellet was suspended by sonication in 85 µL of 100% ethanol. Five µL of DNA suspension was dispensed to each flying disk of the Biolistic PDS1000/HE instrument disk. Each 5 µL aliquot contained approximately 0.058 mg gold particles per bombardment (i.e., per disk).

Tissue Preparation and Bombardment with DNA:

Approximately 100-150 mg of 7 day old embryonic suspension cultures were placed in an empty, sterile 60 x 15 mm petri dish and the dish was placed inside of an empty 150 x 25 mm Petri dish. Tissue was bombarded 1 shot per plate with membrane rupture pressure set at 650 PSI and the chamber was evacuated to a vacuum of 27-28 inches of mercury. Tissue was placed approximately 2.5 inches from the retaining /stopping screen.

Selection of Transformed Embryos:

Transformed embryos were selected using hygromycin as the selectable marker. Specifically, following bombardment, the tissue was placed into fresh SB196 media and cultured as described above. Six to eight days post-bombardment, the SB196 is exchanged with fresh SB196 containing 30 mg/L hygromycin. The selection media was refreshed weekly. Four to six weeks post-selection, green, transformed tissue was observed growing from untransformed, necrotic embryogenic clusters. Isolated, green tissue was removed and inoculated into multiwell plates to generate new, clonally propagated, transformed embryogenic suspension cultures.

Embryo Maturation:

Transformed embryogenic clusters were cultured for one-three weeks at 26 °C in SB196 under cool white fluorescent (Phillips cool white Econowatt F40/CW/RS/EW) and Agro (Phillips F40 Agro) bulbs (40 watt) on a 16:8 hr photoperiod with light intensity of 90-120 $\mu\text{E}/\text{m}^2\text{s}$. After this time embryo clusters were removed to a solid agar media, SB166, for 1 week and then subcultured to medium SB103 for 3 weeks. Alternatively, embryo clusters were removed from SB196 media to 35 mL of SB228 (described *infra*) (SHaM liquid media; Schmidt et al., *Cell Biology and Morphogenesis* 24:393 (2005)) in a 250 mL Erlenmeyer flask for 2-3 weeks. Tissue cultured in SB228 was maintained on a rotary shaker at 130 rpm and 26 °C with cool white fluorescent lights on a 16:8 hr day/night photoperiod at a light intensity of 60-85 $\mu\text{E}/\text{m}^2\text{s}$. After maturation on plates in SB103 or in flasks on SB228 media, individual embryos were removed from the clusters, dried and screened for alterations in their fatty acid compositions as described *supra*.

Media Recipes:

SB 196 - FN Lite Liquid Proliferation Medium (per liter)

MS FeEDTA - 100x Stock 1	10 mL
MS Sulfate - 100x Stock 2	10 mL
FN Lite Halides - 100x Stock 3	10 mL
FN Lite P, B, Mo - 100x Stock 4	10 mL
B5 vitamins (1 mL/L)	1.0 mL
2,4-D (10 mg/L final concentration)	1.0 mL
KNO ₃	2.83 gm
(NH ₄) ₂ SO ₄	0.463 gm
asparagine	1.0 gm
sucrose (1%)	10 gm
pH 5.8	

FN Lite Stock Solutions

Stock Number		1000 mL	500 mL
1	MS Fe EDTA 100x Stock		
	Na ₂ EDTA*	3.724 g	1.862 g
	FeSO ₄ – 7H ₂ O	2.784 g	1.392 g

*Add first, dissolve in dark bottle while stirring

2	MS Sulfate 100x stock		
	MgSO ₄ - 7H ₂ O	37.0 g	18.5 g
	MnSO ₄ - H ₂ O	1.69 g	0.845 g
	ZnSO ₄ - 7H ₂ O	0.86 g	0.43 g
	CuSO ₄ - 5H ₂ O	0.0025 g	0.00125 g
3	FN Lite Halides 100x Stock		
	CaCl ₂ - 2H ₂ O	30.0 g	15.0 g
	KI	0.083 g	0.0715 g
	CoCl ₂ - 6H ₂ O	0.0025 g	0.00125 g
4	FN Lite P, B, Mo 100x Stock		
	KH ₂ PO ₄	18.5 g	9.25 g
	H ₃ BO ₃	0.62 g	0.31 g
	Na ₂ MoO ₄ - 2H ₂ O	0.025 g	0.0125 g

SB1 Solid Medium (per liter)

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066)
 1 mL B5 vitamins 1000X stock
 31.5 g glucose
 2 mL 2,4-D (20 mg/L final concentration)
 pH 5.7
 8 g TC agar

SB199 Solid Medium (per liter)

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066)
 1 mL B5 vitamins 1000X stock
 30 g Sucrose
 4 mL 2,4-D (40 mg/L final concentration)
 pH 7.0
 2 g Gelrite

SB 166 Solid Medium (per liter)

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066)

1 mL B5 vitamins 1000X stock

60 g maltose

750 mg MgCl₂ hexahydrate

5 g activated charcoal

pH 5.7

2 g gelrite

SB 103 Solid Medium (per liter)

1 package MS salts (Gibco/ BRL – Cat. No. 11117-066)

1 mL B5 vitamins 1000X stock

60 g maltose

750 mg MgCl₂ hexahydrate

pH 5.7

2 g gelrite

SB 71-4 Solid Medium (per liter)

1 bottle Gamborg's B5 salts w/ sucrose (Gibco/ BRL – Cat. No. 21153-036)

pH 5.7

5 g TC agar

2,4-D Stock

Obtain premade from Phytotech Cat. No. D 295 – concentration 1 mg/mL

B5 Vitamins Stock (per 100 mL)

Store aliquots at -20 °C

10 g myo-inositol

100 mg nicotinic acid

100 mg pyridoxine HCl

1 g thiamine

If the solution does not dissolve quickly enough, apply a low level of heat via the hot stir plate.

SB 228- Soybean Histodifferentiation & Maturation (SHaM) (per liter)

DDI H ₂ O	600 mL
FN-Lite Macro Salts for SHaM 10X	100 mL
MS Micro Salts 1000x	1 mL
MS FeEDTA 100x	10 mL
CaCl 100x	6.82 mL
B5 Vitamins 1000x	1 mL
L-Methionine	0.149 g
Sucrose	30 g
Sorbitol	30 g

Adjust volume to 900 mL

pH 5.8

Autoclave

Add to cooled media (≤ 30 °C):

*Glutamine (final concentration 30 mM) 4% 110 mL

*Note: Final volume will be 1010 mL after glutamine addition.

Since glutamine degrades relatively rapidly, it may be preferable to add immediately prior to using media. Expiration 2 weeks after glutamine is added; base media can be kept longer w/o glutamine.

FN-lite Macro for SHAM 10X- Stock #1 (per liter)

(NH ₄) ₂ SO ₄ (ammonium sulfate)	4.63 g
KNO ₃ (potassium nitrate)	28.3 g
MgSO ₄ *7H ₂ O (magnesium sulfate heptahydrate)	3.7 g
KH ₂ PO ₄ (potassium phosphate, monobasic)	1.85 g
Bring to volume	
Autoclave	

MS Micro 1000X- Stock #2 (per 1 liter)

H ₃ BO ₃ (boric acid)	6.2 g
MnSO ₄ *H ₂ O (manganese sulfate monohydrate)	16.9 g
ZnSO ₄ *7H ₂ O (zinc sulfate heptahydrate)	8.6 g

Na ₂ MoO ₄ *2H ₂ O (sodium molybdate dihydrate)	0.25 g
CuSO ₄ *5H ₂ O (copper sulfate pentahydrate)	0.025 g
CoCl ₂ *6H ₂ O (cobalt chloride hexahydrate)	0.025 g
KI (potassium iodide)	0.8300 g

Bring to volume

Autoclave

FeEDTA 100X- Stock #3 (per liter)

Na ₂ EDTA* (sodium EDTA)	3.73 g
FeSO ₄ *7H ₂ O (iron sulfate heptahydrate)	2.78 g

*EDTA must be completely dissolved before adding iron.

Bring to Volume

Solution is photosensitive. Bottle(s) should be wrapped in foil to omit light.

Autoclave

Ca 100X- Stock #4 (per liter)

CaCl ₂ *2H ₂ O (calcium chloride dihydrate)	44 g
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Bring to Volume

Autoclave

B5 Vitamin 1000X- Stock #5 (per liter)

Thiamine*HCl	10 g
Nicotinic Acid	1 g
Pyridoxine*HCl	1 g
Myo-Inositol	100 g

Bring to Volume

Store frozen

4% Glutamine- Stock #6 (per liter)

DDI water heated to 30 °C	900 mL
L-Glutamine	40 g

Gradually add while stirring and applying low heat.

Do not exceed 35 °C.

Bring to Volume

Filter Sterilize

Store frozen*

*Note: Warm thawed stock in 31 °C bath to fully dissolve crystals.

EXAMPLE 10

Functional Analysis of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) and the *Isochrysis galbana* Delta-9 Elongase (IgD9eS) in Somatic Soybean Embryos Transformed With Soybean Expression Vector pKR1013

Mature somatic soybean embryos are a good model for zygotic embryos. While in the globular embryo state in liquid culture, somatic soybean embryos contain very low amounts of triacylglycerol or storage proteins typical of maturing, zygotic soybean embryos. At this developmental stage, the ratio of total triacylglyceride to total polar lipid (phospholipids and glycolipid) is about 1:4, as is typical of zygotic soybean embryos at the developmental stage from which the somatic embryo culture was initiated. At the globular stage as well, the mRNAs for the prominent seed proteins, α' -subunit of β -conglycinin, kunitz trypsin inhibitor 3, and seed lectin are essentially absent. Upon transfer to hormone-free media to allow differentiation to the maturing somatic embryo state, triacylglycerol becomes the most abundant lipid class. As well, mRNAs for α' -subunit of β -conglycinin, kunitz trypsin inhibitor 3 and seed lectin become very abundant messages in the total mRNA population. On this basis, the somatic soybean embryo system behaves very similarly to maturing zygotic soybean embryos *in vivo*, and is thus a good and rapid model system for analyzing the phenotypic effects of modifying the expression of genes in the fatty acid biosynthesis pathway (see PCT Publication No. WO 2002/00904, Example 3). Most importantly, the model system is also predictive of the fatty acid composition of seeds from plants derived from transgenic embryos.

Fatty Acid Analysis of Transgenic Somatic Soybean Embryos Expressing pKR1013

Individual single, matured, somatic soybean embryos that had been transformed with pKR1013 (as described in Example 9 transformants were matured on SHaM liquid media) were picked into glass GC vials, frozen at minus 80 °C, freeze dried overnight and fatty acid methyl esters were prepared by

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transesterification. For transesterification, 50 μ L of trimethylsulfonium hydroxide (TMSH) and 0.5 mL of hexane were added to the embryos in glass vials and incubated for 30 min at room temperature while shaking. Fatty acid methyl esters (5 μ L injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Catalog #24152, Supelco Inc.). The oven temperature was programmed to hold at 220 °C for 2.6 min, increase to 240 °C at 20 °C/min and then hold for an additional 2.4 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.). Routinely, 5-10 embryos per event were analyzed by GC, using the methodology described above.

Embryo fatty acid profiles for each event (6 embryos each) containing pKR1013 were obtained and the lipid profiles of somatic soybean embryos expressing TpomD8 and IgD9eS for the top 5 events are shown in FIG. 5A and 5B. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 5A and 5B are expressed as a weight percent (wt. %) of total fatty acids. The activity of TpomD8 is expressed as percent desaturation (% desat), calculated according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$.

More specifically, the combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as: $([\text{DGLA} + \text{ETA}]/[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]) \times 100$. This is also referred to as the overall % desaturation. The individual omega-6 delta-8 desaturation ("EDA % delta-8 desat.") was calculated as: $([\text{DGLA}]/[\text{DGLA} + \text{EDA}]) \times 100$. Similarly, the individual omega-3 delta-8 desaturation ("ERA % delta-8 desat.") was calculated as: $([\text{ETA}]/[\text{ETA} + \text{ERA}]) \times 100$. The ratio of delta-8 desaturation for omega-6 versus omega-3 substrates ("ratio [EDA/ERA] % desat.") was obtained by dividing the EDA % delta-8 desaturation by the ERA % delta-8 desaturation.

In summary of FIG. 5A and 5B, TpomD8 worked in soybean to convert both EDA and ERA to DGLA and ETA, respectively. The line with the highest average DGLA content (i.e., 1974-5-6) had embryos with an average DGLA content of 12.9% and an average ETA content of 2.9%. The highest DGLA and ETA content for an individual embryo from this line was 14.6% and 3.4%, respectively. The highest

average overall % desaturation was 50.7% with the highest overall % desaturation for an individual embryo being 55.5%. When broken down into % desaturation for the omega-6 and omega-3 substrates, the highest average % desaturation was 48.3% and 65.0% for EDA and ERA, respectively. The highest % desaturation for an individual embryo was 52.9% and 72.7% for EDA and ERA, respectively. In this example, TpomD8 had a preference for ERA over EDA, with the average desaturation ratio ranging from 0.6 to 0.8. No GLA was found to accumulate in the embryos.

EXAMPLE 11

cDNA Synthesis and PCR of *Euglena gracilis* Delta-9 Elongase

The present Example, disclosed in U.S. Provisional Application No. 60/739,989 (filed November 23, 2005, having Attorney Docket No. BB-1562), describes the isolation of a delta-9 elongase from *Euglena gracilis* ("EgD9e"; SEQ ID NOs:74 and 75). The isolation of this gene allowed co-expression of EgD9e and the delta-8 desaturases of the present invention, to thereby permit expression of the delta-9 elongase/delta-8 desaturase pathway leading to accumulation of DGLA and/or ETA from LA and/or ALA, respectively.

Euglena gracilis Growth Conditions, Lipid Profile and mRNA Isolation

Euglena gracilis was obtained from Dr. Richard Triemer's lab at Michigan State University (East Lansing, MI). From 10 mL of actively growing culture, a 1 mL aliquot was transferred into 250 mL of *Euglena gracilis* (Eg) Medium in a 500 mL glass bottle. Eg medium was made by combining 1 g of sodium acetate, 1 g of beef extract (U126-01, Difco Laboratories, Detroit, MI), 2 g of Bacto® tryptone (0123-17-3, Difco Laboratories), 2 g of Bacto® yeast extract (0127-17-9, Difco Laboratories) in 970 mL of water. After filter sterilizing, 30 mL of soil-water supernatant (15-3790, Carolina Biological Supply Company, Burlington, NC) was aseptically added to give the final Eg medium. *Euglena gracilis* cultures were grown at 23 °C with a 16 h light, 8 h dark cycle for 2 weeks with no agitation.

After 2 weeks, 10 mL of culture was removed for lipid analysis and centrifuged at 1,800 x g for 5 min. The pellet was washed once with water and re-centrifuged. The resulting pellet was dried for 5 min under vacuum, resuspended in 100 µL of trimethylsulfonium hydroxide (TMSH) and incubated at room temperature for 15 min with shaking. After this, 0.5 mL of hexane was added and the vials were

incubated for 15 min at room temperature with shaking. Fatty acid methyl esters (5 μ L injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Supelco Inc., Catalog No. 24152). The oven temperature was programmed to hold at 220 °C for 2.7 min, increase to 240 °C at 20 °C/min and then hold for an additional 2.3 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc. Catalog No. U-99-A).

The remaining 2 week culture (240 mL) was pelleted by centrifugation at 1,800 x g for 10 min, washed once with water and re-centrifuged. Total RNA was extracted from the resulting pellet using the RNA STAT-60™ reagent (TEL-TEST, Inc., Friendswood, TX) and following the manufacturer's protocol provided (use 5 mL of reagent, dissolved RNA in 0.5 mL of water). In this way, 1 mg of total RNA (2 mg/mL) was obtained from the pellet. The mRNA was isolated from 1 mg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol provided. In this way, 85 μ g of mRNA was obtained.

Euglena gracilis cDNA Synthesis, Library Construction And Sequencing

A cDNA library was generated using the Cloneminer™ cDNA Library Construction Kit (Catalog No.18249-029, Invitrogen Corporation, Carlsbad, CA) and following the manufacturer's protocol provided (Version B, 25-0608). Using the non-radiolabeling method, cDNA was synthesized from 3.2 μ g of mRNA (described above) using the Biotin-*att*B2-Oligo(dT) primer. After synthesis of the first and second strand, the *att*B1 adapter was added, ligated and the cDNA was size fractionated using column chromatography. DNA from fractions 7 and 8 (size ranging from ~800-1500 bp) were concentrated, recombined into pDONR™222 and transformed into *E. coli* ElectroMAX™ DH10B™ T1 Phage-Resistant cells (Invitrogen Corporation). The *Euglena gracilis* library was named eeg1c.

For sequencing, clones first were recovered from archived glycerol cultures grown/frozen in 384-well freezing media plates, and replicated with a sterile 384 pin replicator (Genetix, Boston, MA) in 384-well microtiter plates containing LB + 75 μ g/mL Kanamycin (replicated plates). Plasmids then were isolated, using the

Templiphi DNA sequencing template amplification kit method (Amersham Biosciences) following the manufacturer's protocol. Briefly, the Templiphi method uses bacteriophage ϕ 29 DNA polymerase to amplify circular single-stranded or double-stranded DNA by isothermal rolling circle amplification (Dean et al., *Genome Res.*, 11:1095-1099 (2001); Nelson et al., *Biotechniques*, 32:S44-S47 (2002)). After growing 20 h at 37 °C, cells from the replicated plate were added to 5 μ L of dilution buffer and denatured at 95 °C for 3 min to partially lyse cells and release the denatured template. Templiphi premix (5 μ L) was then added to each sample and the resulting reaction mixture was incubated at 30 °C for 16 h, then at 65 °C for 10 min to inactivate the ϕ 29 DNA polymerase activity. DNA quantification with the PicoGreen® dsDNA Quantitation Reagent (Molecular Probes) was performed after diluting the amplified samples 1:3 in distilled water.

The amplified products then were denatured at 95 °C for 10 min and end-sequenced in 384-well plates, using the M13F universal primer (SEQ ID NO:91), and the ABI BigDye version 3.1 Prism Sequencing Kit. For the sequencing reaction, 100-200 ng of templates and 6.4 pmol of primers were used, and the following reaction conditions were repeated 25 times: 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. After ethanol-based cleanup, cycle sequencing reaction products were resolved and detected on Perkin-Elmer ABI 3730xl automated sequencers.

Identification of Long-Chain Polyunsaturated Fatty Acid Elongation Enzyme

Homologs From *Euglena gracilis* cDNA Library eeg1c

cDNA clones encoding long-chain polyunsaturated fatty acid elongation enzyme homologs (i.e., LC-PUFA ELO homologs or delta-9 elongases) were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul et al., *J. Mol. Biol.* 215:403-410 (1993)) searches for 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 last major release of the SWISS-PROT protein sequence database, EMBL and DDBJ databases). The *Euglena gracilis* cDNA sequences obtained above 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). The DNA 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 and States, *Nat. Genet.*, 3:266-272 (1993)) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values (as described in Example 4).

The BLASTX search using the nucleotide sequences from clone eeg1c.pk001.n5.f revealed similarity of the protein encoded by the cDNA to the long-chain PUFA elongation enzyme from *Isochrysis galbana* (IgD9e; SEQ ID NO:76) (GenBank Accession No. AAL37626 (GI 17226123), locus AAL37626, CDS AF390174; Qi et al., *FEBS Lett.* 510(3):159-165 (2002)). The sequence of a portion of the cDNA insert from clone eeg1c.pk001.n5.f is shown in SEQ ID NO:71 (5' end of cDNA insert).

Additional sequence was obtained from the 3' end of the cDNA insert of eeg1c.pk001.n5.1 as described above, but using the poly(A) tail-primed WobbleT oligonucleotides. Briefly, the WobbleT primer is an equimolar mix of 21mer poly(T)A, poly(T)C, and poly(T)G, used to sequence the 3' end of cDNA clones. The 3' end sequence is shown in SEQ ID NO:72.

Both the 5' and 3' sequences were aligned using Sequencher™ (Version 4.2, Gene Codes Corporation, Ann Arbor, MI) and the resulting sequence for the cDNA is shown in SEQ ID NO:73 (1201 bp). Sequence for the coding sequence from the cDNA in eeg1c.pk001.n5.f and the corresponding deduced amino acid sequence is shown in SEQ ID NO:74 (777 bp) and SEQ ID NO:75 (258 amino acids), respectively.

The amino acid sequence set forth in SEQ ID NO:75 was evaluated by BLASTP, yielding a pLog value of 38.70 (E value of 2e-39) versus the *Isochrysis galbana* sequence (SEQ ID NO:76). The *Euglena gracilis* delta-9 elongase is 39.4% identical to IgD9e using the Jotun Hein method (as described in Example 4); similarly, the *Euglena gracilis* delta-9 elongase is 31.8% identical to IgD9e using the Clustal V method (as described in Example 4). BLAST scores and probabilities indicate that the nucleic acid fragment described herein as SEQ ID NO:75 encodes an entire *Euglena gracilis* delta-9 elongase (designated herein as "EgD9e").

EXAMPLE 12

Construction of Soybean Expression Vector pKR1014 For Co-Expression of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) With the Delta-9 Elongase From *Euglena gracilis* (EgD9e)

The present Example describes construction of a soybean vector for co-expression of TpomD8 with EgD9e.

EgD9e (SEQ ID NOs:74 and 75; Example 11) was amplified with oligonucleotide primers oEugEL1-1 (SEQ ID NO:77) and oEugEL1-2 (SEQ ID NO:78) using the VentR[®] DNA Polymerase (Catalog No. M0254S, New England Biolabs Inc., Beverly, MA) following the manufacturer's protocol. The resulting DNA fragment was cloned into the pCR-Blunt[®] cloning vector using the Zero Blunt[®] PCR Cloning Kit (Invitrogen Corporation), following the manufacturer's protocol, to produce pKR906 (SEQ ID NO:79).

A starting plasmid pKR72 (ATCC Accession No. PTA-6019; SEQ ID NO:80, 7085 bp sequence), a derivative of pKS123 which was previously described in PCT Publication No. WO 02/008269 (the contents of which are hereby incorporated by reference), contains the hygromycin B phosphotransferase gene (HPT) (Gritz, L. and Davies, J., *Gene* 25:179-188 (1983)), flanked by the T7 promoter and transcription terminator (T7prom/HPT/T7term cassette), and a bacterial origin of replication (ori) for selection and replication in bacteria (e.g., *E. coli*). In addition, pKR72 also contains HPT, flanked by the 35S promoter (Odell et al., *Nature* 313:810-812 (1985)) and NOS 3' transcription terminator (Depicker et al., *J. Mol. Appl. Genet.* 1:561-570 (1982)) (35S/HPT/NOS3' cassette) for selection in plants such as soybean. pKR72 also contains a *NotI* restriction site, flanked by the promoter for the α' subunit of β -conglycinin (Beachy et al., *EMBO J.* 4:3047-3053 (1985)) and the 3' transcription termination region of the phaseolin gene (Doyle et al., *J. Biol. Chem.* 261:9228-9238 (1986)), thus allowing for strong tissue-specific expression in the seeds of soybean of genes cloned into the *NotI* site.

EgD9e was released from pKR906 by digestion with *NotI* and cloned into the *NotI* site of pKR72 to produce pKR912 (SEQ ID NO:81). In some instances, pKR912 is referred to as pKR1010 but they are identical.

Plasmid pKR1007 (in Example 8, SEQ ID NO:67) was digested with *PstI* and the fragment containing the *Tetruetrepia pomquetensis* delta-8 desaturase was

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cloned into the *Sbf*I site of pKR912 (SEQ ID NO:81), to give pKR1014 (SEQ ID NO:82). In this way, the *Tetruetrepia pomquetensis* delta-8 desaturase is co-expressed with the *Isochrysis galbana* delta-9 elongase behind strong, seed-specific promoters. A schematic depiction of pKR1014 is shown in FIG. 3.

Plasmid pKR1014 was transformed into soybean embryogenic suspension cultures as described in Example 9 and embryos co-expressing of TpomD8 and EgD9e were analyzed as described in Example 10.

Embryo fatty acid profiles for each event (6 embryos each) containing pKR1014 were obtained and the lipid profiles of somatic soybean embryos expressing TpomD8 and EgD9e for the top 5 events are shown in FIG. 10A and 10B. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 10A and 10B are expressed as a weight percent (wt. %) of total fatty acids. The activity of TpomD8 is expressed as percent desaturation (% desat), calculated according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$.

More specifically, the combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as: $([\text{DGLA} + \text{ETA}]/[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]) \times 100$. This is also referred to as the overall % desaturation. The individual omega-6 delta-8 desaturation ("EDA % delta-8 desat.") was calculated as: $([\text{DGLA}]/[\text{DGLA} + \text{EDA}]) \times 100$. Similarly, the individual omega-3 delta-8 desaturation ("ERA % delta-8 desat.") was calculated as: $([\text{ETA}]/[\text{ETA} + \text{ERA}]) \times 100$. The ratio of delta-8 desaturation for omega-6 versus omega-3 substrates ("ratio [EDA/ERA] % desat.") was obtained by dividing the EDA % delta-8 desaturation by the ERA % delta-8 desaturation.

In summary of FIG. 10A and 10B, TpomD8 worked in soybean to convert both EDA and ERA to DGLA and ETA, respectively. The line with the highest average DGLA content (i.e., 2024-3-9) had embryos with an average DGLA content of 14.8% and an average ETA content of 3.8%. The highest DGLA and ETA content for an individual embryo from this line was 16.0% and 3.9%, respectively. The highest average overall % desaturation was 60.9% with the highest overall % desaturation for an individual embryo being 68.7%. When broken down into % desaturation for the omega-6 and omega-3 substrates, the highest average % desaturation was 59.1% and 73.9% for EDA and ERA, respectively. The highest %

9/9

desaturation for

105a

an individual embryo was 66.7% and 80.9% for EDA and ERA, respectively. In this example, TpomD8 had a preference for ERA over EDA, with the average desaturation ratio ranging from 0.8 to 0.9. No GLA was found to accumulate in the embryos.

EXAMPLE 13

Construction of Soybean Expression Vector pKR1005 For Co-Expression of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) With the Delta-17 Desaturase From *Saprolegnia diclina* (SdD17)

The present Example describes construction of a soybean vector for co-expression of TpomD8 with SdD17.

The *Pst*I fragment, containing the Ann/Sdd17/BD30 cassette from pKR271 (SEQ ID NO:83; which is described in PCT Publication No. WO 2004/071467 and the contents of which are hereby incorporated by reference), was cloned into the *Sbf*I site of pKR226 (SEQ ID NO:84, which is also described in PCT Publication No. WO 2004/071467) to produce vector pKR886r (SEQ ID NO:85). In this way, the *Saprolegnia diclina* delta-17 desaturase (SdD17) was cloned behind the annexin promoter which is strong and seed specific.

The β con/*Not*I/Phas3' cassette in plasmid pKR72 (SEQ ID NO:80, having ATCC Accession No. PTA-6019) was amplified using oligonucleotide primers oCon-1 (SEQ ID NO:86) and oCon-2 (SEQ ID NO:87) using the VentR[®] DNA Polymerase (Catalog No. M0254S, New England Biolabs Inc., Beverly, MA) following the manufacturer's protocol. The resulting DNA fragment was digested with *Xba*I and cloned into the *Xba*I site of pUC19, to produce pKR179 (SEQ ID NO:88).

TpomD8 was released from plasmid pLF114-10 (SEQ ID NO:63, Example 5) by digestion with *Not*I and was cloned into the *Not*I site of plasmid pKR179 (SEQ ID NO:88) to produce pKR1002 (SEQ ID NO:89).

Vector pKR1002 was digested with *Pst*I and the fragment containing TpomD8 was cloned into the *Sbf*I site of pKR886r (SEQ ID NO:85) to produce pKR1005 (SEQ ID NO:90). A schematic depiction of pKR1005 is shown in FIG. 4.

One skilled in the art will recognize that pKR1005 could be readily transformed into soybean embryogenic suspension cultures (as described in Example 9) and co-expression of TpomD8 and SdD17 could analyzed (as described in Example 10).

EXAMPLE 14

Construction of Alternate Soybean Expression Vectors For Expression of *Tetruetreptia pomquetensis* CCMP1491 (TpomD8), *Eutreptiella* sp. CCMP389 (E389D8) and/or *Eutreptiella cf. gymnastica* CCMP1594 (E1594D8) Delta-8 Desaturases

In addition to the genes, promoters, terminators and gene cassettes described herein, one skilled in the art can appreciate that other promoter/gene/terminator cassette combinations can be synthesized in a way similar to, but not limited to, that described herein for expression of TpomD8, E389D8 and/or E1594D8. Similarly, it may be desirable to express other PUFA genes (such as those described below in Table 14), for co-expression with any of the delta-8 desaturases of the present invention.

For instance, PCT Publication Nos. WO 2004/071467 and WO 2004/071178 describe the isolation of a number of promoter and transcription terminator sequences for use in embryo-specific expression in soybean. Furthermore, PCT Publication Nos. WO 2004/071467, WO 2005/047479 and WO 2006/012325 describe the synthesis of multiple promoter/gene/terminator cassette combinations by ligating individual promoters, genes and transcription terminators together in unique combinations. Generally, a *NotI* site flanked by the suitable promoter (such as those listed in, but not limited to, Table 12) and a transcription terminator (such as those listed in, but not limited to, Table 13) is used to clone the desired gene. *NotI* sites can be added to a gene of interest such as those listed in, but not limited to, Table 14 using PCR amplification with oligonucleotides designed to introduce *NotI* sites at the 5' and 3' ends of the gene. The resulting PCR product is then digested with *NotI* and cloned into a suitable promoter/*NotI*/terminator cassette.

In addition, PCT Publication Nos. WO 2004/071467, WO 2005/047479 and WO 2006/012325 describe the further linking together of individual gene cassettes in unique combinations, along with suitable selectable marker cassettes, in order to obtain the desired phenotypic expression. Although this is done mainly using different restriction enzymes sites, one skilled in the art can appreciate that a number of techniques can be utilized to achieve the desired promoter/gene/transcription terminator combination. In so doing, any combination of embryo-specific promoter/gene/transcription terminator cassettes can be

achieved. One skilled in the art can also appreciate that these cassettes can be located on individual DNA fragments or on multiple fragments where co-expression of genes is the outcome of co-transformation of multiple DNA fragments.

TABLE 12
Seed-specific Promoters

Promoter	Organism	Promoter Reference
β -conglycinin α' -subunit	soybean	Beachy et al., <i>EMBO J.</i> 4:3047-3053 (1985)
kunitz trypsin inhibitor	soybean	Jofuku et al., <i>Plant Cell</i> 1:1079-1093 (1989)
Annexin	soybean	WO 2004/071467
glycinin Gy1	soybean	WO 2004/071467
albumin 2S	soybean	U.S. Patent No. 6,177,613
legumin A1	pea	Rerie et al., <i>Mol. Gen. Genet.</i> 225:148-157 (1991)
β -conglycinin β -subunit	soybean	WO 2004/071467
BD30 (also called P34)	soybean	WO 2004/071467
legumin A2	pea	Rerie et al., <i>Mol. Gen. Genet.</i> 225:148-157 (1991)

TABLE 13
Transcription Terminators

Transcription Terminator	Organism	Reference
phaseolin 3'	bean	WO 2004/071467
kunitz trypsin inhibitor 3'	soybean	WO 2004/071467
BD30 (also called P34) 3'	soybean	WO 2004/071467
legumin A2 3'	pea	WO 2004/071467
albumin 2S 3'	soybean	WO 2004/071467

TABLE 14
PUFA Biosynthetic Pathway Genes

Gene	Organism	Reference
delta-6 desaturase	<i>Saprolegnia diclina</i>	WO 2002/081668

delta-6 desaturase	<i>Mortierella alpina</i>	U.S. Patent No. 5,968,809
elongase	<i>Mortierella alpina</i>	WO 2000/12720 U.S. Patent No. 6,403,349
delta-5 desaturase	<i>Mortierella alpina</i>	U.S. Patent No. 6,075,183
delta-5 desaturase	<i>Saprolegnia diclina</i>	WO 2002/081668
delta-15 desaturase	<i>Fusarium moniliforme</i>	WO 2005/047479
delta-17 desaturase	<i>Saprolegnia diclina</i>	WO 2002/081668
elongase	<i>Thraustochytrium aureum</i>	WO 2002/08401 U.S. Patent No. 6,677,145
elongase	<i>Pavlova</i> sp.	Pereira et al., <i>Biochem. J.</i> 384:357-366 (2004)
delta-4 desaturase	<i>Schizochytrium aggregatum</i>	WO 2002/090493
delta-9 elongase	<i>Isochrysis galbana</i>	WO 2002/077213
delta-9 elongase	<i>Euglena gracilis</i>	U.S. Provisional Application No. 60/739,989
delta-8 desaturase	<i>Euglena gracilis</i>	WO 2000/34439 U.S. Patent No. 6,825,017 WO 2004/057001 WO 2006/012325
delta-8 desaturase	<i>Acanthamoeba castellanii</i>	Sayanova et al., <i>FEBS Lett.</i> 580:1946-1952 (2006)
delta-8 desaturase	<i>Pavlova salina</i>	WO 2005/103253
delta-8 desaturase	<i>Pavlova lutheri</i>	U.S. Provisional Application No. 60/795,810

EXAMPLE 15

Chlorsulfuron Selection (ALS) and Plant Regeneration

Chlorsulfuron (ALS) Selection:

Following bombardment, the tissue is divided between 2 flasks with fresh SB196 media and cultured as described in Example 9. Six to seven days post-bombardment, the SB196 is exchanged with fresh SB196 containing selection agent of 100 ng/mL chlorsulfuron (chlorsulfuron stock is 1 mg/mL in 0.01 N ammonium hydroxide). The selection media is refreshed weekly. Four to six weeks post selection, green, transformed tissue may be observed growing from untransformed,

necrotic embryogenic clusters. Isolated, green tissue is removed and inoculated into multiwell plates containing SB196 to generate new, clonally propagated, transformed embryogenic suspension cultures.

Regeneration Of Soybean Somatic Embryos Into Plants:

In order to obtain whole plants from embryogenic suspension cultures, the tissue must be regenerated. Embryos are matured as described in Example 9. After subculturing on medium SB103 for 3 weeks, individual embryos can be removed from the clusters and screened for alterations in their fatty acid compositions as described in Example 10. It should be noted that any detectable phenotype, resulting from the expression of the genes of interest, could be screened at this stage. This would include, but not be limited to, alterations in fatty acid profile, protein profile and content, carbohydrate content, growth rate, viability, or the ability to develop normally into a soybean plant.

Matured individual embryos are desiccated by placing them into an empty, small petri dish (35 x 10 mm) for approximately 4 to 7 days. The plates are sealed with fiber tape (creating a small humidity chamber). Desiccated embryos are planted into SB71-4 medium where they are left to germinate under the same culture conditions described above. Germinated plantlets are removed from germination medium and rinsed thoroughly with water and then are planted in Redi-Earth in 24-cell pack tray, covered with clear plastic dome. After 2 weeks the dome is removed and plants are hardened off for a further week. If plantlets looked hardy they are transplanted to 10" pot of Redi-Earth with up to 3 plantlets per pot. After 10 to 16 weeks, mature seeds are harvested, are chipped and are analyzed for fatty acids as described in Example 10 above.

EXAMPLE 16

Construction of Soybean Expression Vector pKR973 For Co-Expression of the *Pavlova lutheri* Delta-8 Desaturase (PavD8) With the *Euglena gracilis* Delta-9 Elongase (EgD9e) and the *Mortierella alpina* Delta-5 Desaturase (MaD5)

Euglena gracilis delta-9 elongase (EgD9e):

Plasmid pKR906 (SEQ ID NO:79, Example 12) was digested with *NotI* and the fragment containing the *Euglena gracilis* delta-9 elongase was cloned into

plasmid pKR132 (SEQ ID NO:113; which is described in PCT Publication No. WO 2004/071467) to produce pKR953 (SEQ ID NO:114).

Mortierella alpina delta-5 desaturase (MaD5):

Vector pKR287 (SEQ ID NO:115; which is described in PCT Publication No. WO 2004/071467, published August 26, 2004; the contents of which are hereby incorporated by reference), contains the *Mortierella alpina* delta-5 desaturase (MaD5; SEQ ID NO:116, which is described in U.S. Patent No. 6,075,183 and PCT Publication Nos. WO 2004/071467 and WO 2005/047479, the contents of which are hereby incorporated by reference), flanked by the soybean glycinin Gy1 promoter and the pea leguminA2 3' termination region (Gy1/MaD5/legA2 cassette). Vector pKR287 was digested with *Sbf*I/*Bs*WI and the fragment containing the Gy1/MaD5/legA2 cassette was cloned into the *Sbf*I/*Bs*WI fragment of pKR277 (SEQ ID NO:117; which is described in PCT Publication No. WO 2004/071467, the contents of which are hereby incorporated by reference) to produce pK952 (SEQ ID NO:118).

Vector pKR457 (SEQ ID NO:119), which was previously described in PCT Publication No. WO 2005/047479 (the contents of which are hereby incorporated by reference), contains a *Not*I site flanked by the Kunitz soybean Trypsin Inhibitor (KTI) promoter (Jofuku et al., *Plant Cell* 1:1079-1093 (1989)) and the KTI 3' termination region, the isolation of which is described in U.S. Patent No. 6,372,965, followed by the soy albumin transcription terminator, which was previously described in PCT Publication No. WO 2004/071467 (KTI/*Not*I/KTI3'Salb3' cassette). Through a number of sub-cloning steps, sequences containing *Asp*718 restriction sites were added to the 5' and 3' ends of the KTI/*Not*I/KTI3'Salb3' cassette to produce SEQ ID NO:120.

Pavlova lutheri delta-8 desaturase (PavD8):

Pavlova lutheri (CCMP459) was obtained from the Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME) and grown in 250 mL flasks containing 50 mL of F/2-Si medium (made using F/2 Family Medium Kit-KIT20F2 and Filtered Seawater-SEA2 from CCMP) at 26 °C with shaking at 150 rpm. Cultures were transferred to new medium on a weekly basis using 1:4 (old culture:new medium) dilution.

Cultures from 28 flasks (1400 mL) were combined, cells were pelleted by centrifugation at 1,800 x g for 10 min, washed once with water and re-centrifuged.

Total RNA was extracted from the resulting pellet using the RNA STAT-60™ reagent (TEL-TEST, Inc., Friendswood, TX) and following the manufacturer's protocol provided. In this way, 2.6 mg of total RNA (2.6 mg/mL) was obtained from the pellet. The mRNA was isolated from 1.25 mg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol provided. In this way, 112 µg of mRNA was obtained.

cDNA was synthesized from 224 ng of mRNA using the SuperScript™ First-Strand Synthesis System for RT-PCR Kit (Invitrogen™ Life Technologies, Carlsbad, CA) with the provided oligo(dT) primer according to the manufacturer's protocol. After RNase H treatment as per the protocol, the *Pavlova lutheri* delta-8 desaturase (PavD8; SEQ ID NO:121; which is described in U.S. Provisional Application No. 60/795,810 (filed April 28, 2006) and US patent application No: 11/737,772 (filed April 20, 2007) the contents of which are hereby incorporated by reference) was amplified from the resulting cDNA with oligonucleotide primers PvDES5'Not-1 (SEQ ID NO:122) and PvDES3'Not-1 (SEQ ID NO:123) using the conditions described below.

cDNA (2 µL) from the reaction described above was combined with 50 pmol of PvDES5'Not-1 (SEQ ID NO:122), 50 pmol of PvDES3'Not-1 (SEQ ID NO:123), 1 µL of PCR nucleotide mix (10 mM, Promega, Madison, WI), 5 µL of 10X PCR buffer (Invitrogen Corporation), 1.5 µL of MgCl₂ (50 mM, Invitrogen Corporation), 0.5 µL of Taq polymerase (Invitrogen Corporation) and water to 50 µL. The reaction conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 45 sec, 55 °C for 45 sec and 72 °C for 1 min. The PCR was finished at 72 °C for 7 min and then held at 4 °C. The PCR reaction was analyzed by agarose gel electrophoresis on 5 µL and a DNA band with molecular weight around 1.3 kb was observed. The remaining 45 µL of product was separated by agarose gel electrophoresis and the DNA purified using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Orange, CA) following the manufacturer's protocol.

The PavD8, flanked by *NotI* sites, was cloned into the *NotI* site of the modified *Kti/NotI/Kti3'Salb3'* cassette (SEQ ID NO:120), and then the DNA fragment was digested with *Asp718* and cloned into the *SbfI* site of pKR952 (SEQ ID NO:118) to produce pKR970 (SEQ ID NO:124).

Plasmid pKR953 (SEQ ID NO:114) was digested with *Pst*I and the fragment containing the *Euglena gracilis* delta-9 elongase was cloned into the *Sbf*I site of pKR970 (SEQ ID NO:124) to produce pKR973 (SEQ ID NO:125, FIG. 11). In this way, the *Pavlova lutheri* delta-8 desaturase could be co-expressed with the *Mortierella alpina* delta-5 desaturase and the *Euglena gracilis* delta-9 elongase behind strong, seed-specific promoters.

EXAMPLE 17

Construction of Soybean Expression Vector pKR1084 For Co-Expression of the *Euglena gracilis* Delta-9 Elongase (EgD9e) with the *Mortierella alpina* Delta-5 Desaturase (MaD5)

The *Not*I fragment of pKS129 (SEQ ID NO:126; which is described in PCT Publication No. WO 04/071467), containing the MaD5 (SEQ ID NO:116; Example 16) was cloned into the *Not*I site of pKR457 (SEQ ID NO:119; Example 16), to give pKR606 (SEQ ID NO:127).

Vector pKR606 (SEQ ID NO:127) was digested with *Bsi*WI and after filling to blunt the ends, the fragment containing the Gy1/MaD5/legA2 cassette was cloned into the filled *Ngo*MI site of pKR277 (SEQ ID NO:117; Example 16) to produce pKR804 (SEQ ID NO:128).

Plasmid pKR953 (SEQ ID NO:114; Example 16) was digested with *Pst*I and the fragment containing the EgD9e was cloned into the *Sbf*I site of pKR804 (SEQ ID NO:128) to give pKR1084 (SEQ ID NO:129; FIG. 12).

In this way, the *Mortierella alpina* delta-5 desaturase (MaD5) was expressed with the *Euglena gracilis* delta-9 elongase (EgD9e) behind strong, seed-specific promoters.

EXAMPLE 18

Construction of Soybean Expression Vector pKR1123 For Co-Expression of the *Eutreptiella cf. gymnastica* CCMP1594 delta-8 desaturase (E1594D8) With the Delta-9 Elongase From *Euglena gracilis* (EgD9e)

The present Example describes construction of a soybean vector for co-expression of E1594D8 with EgD9e and expression of these genes in somatic embryos.

An intermediate plasmid pKR908 (SEQ ID NO:130) allows the cloning of DNA fragments into an NcoI/XbaI site and thus add a flanking NotI site 5' to the NcoI site.

The NcoI/XbaI fragment of pFBAIn-1594D8 (SEQ ID NO:96; Example 7), containing E1594D8 and where a NotI site is already present just 5' to the XbaI site, was cloned into the NcoI/XbaI sites of pKR908 (SEQ ID NO:130) to produce pKR1118 (SEQ ID NO:131) and where E1594D8 is now flanked by NotI sites at the 5' and 3' ends.

E1594D8 was released from pKR1118 (SEQ ID NO:131) by digestion with NotI and cloned into the NotI site of pKR123r (SEQ ID NO:66; Example 8) to produce pKR1120 (SEQ ID NO:132).

Plasmid pKR1120 (SEQ ID NO:132) was digested with SbfI and the fragment containing E1594D8 was cloned into the SbfI site of pKR912 (SEQ ID NO:81; Example 12), to give pKR1123 (SEQ ID NO:133). In this way, the *Eutreptiella cf gymnastica* CCMP1594 delta-8 desaturase is co-expressed with the *Euglena gracilis* delta-9 elongase behind strong, seed-specific promoters. A schematic depiction of pKR1123 is shown in FIG. 13.

Plasmid pKR1123 was transformed into soybean embryogenic suspension cultures as described in Example 9 and embryos co-expressing E1594D8 and EgD9e were analyzed as described in Example 10.

Embryo fatty acid profiles for each event (6 embryos each) containing pKR1123 were obtained and the lipid profiles of somatic soybean embryos expressing E1594D8 and EgD9e for the top 5 events are shown in FIG. 14. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 14 are expressed as a weight percent (wt. %) of total fatty acids. The activity of E1594D8 is expressed as percent desaturation (% desat), calculated according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$.

More specifically, the combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as: $([\text{DGLA} + \text{ETA}]/[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]) \times 100$. This is also referred to as the overall % desaturation. The individual omega-6 delta-8 desaturation ("EDA % delta-8 desat.") was calculated as: $([\text{DGLA}]/[\text{DGLA} + \text{EDA}]) \times 100$. Similarly, the individual omega-3 delta-8 desaturation

("ERA % delta-8 desat.") was calculated as: $([ETA]/[ETA + ERA]) \times 100$. The ratio of delta-8 desaturation for omega-6 versus omega-3 substrates ("ratio [EDA/ERA] % desat.") was obtained by dividing the EDA % delta-8 desaturation by the ERA % delta-8 desaturation.

In summary of FIG. 14, E1594D8 worked in soybean to convert both EDA and ERA to DGLA and ETA, respectively. The line with the highest average DGLA content (i.e., 2108-6-6) had embryos with an average DGLA content of 13.6% and an average ETA content of 3.9%. The highest DGLA and ETA content for an individual embryo from this line was 17.7% and 4.7%, respectively. The highest average overall % desaturation was 66.4% (2108-5-2) with the highest overall % desaturation for an individual embryo being 71.3%. When broken down into % desaturation for the omega-6 and omega-3 substrates, the highest average % desaturation was 61.6% and 82.0% for EDA and ERA, respectively. The highest % desaturation for an individual embryo from this event was 62.5% and 82.2% for EDA and ERA, respectively. In this example, E1594D8 had a preference for ERA over EDA, with the average desaturation ratio ranging from 0.6-0.8. No GLA was found to accumulate in the embryos.

EXAMPLE 19

Construction of Soybean Expression Vector pKR1122 For Co-Expression of the *Eutreptiella* sp. CCMP389 delta-8 desaturase (E389D8) With the Delta-9 Elongase From *Euglena gracilis* (EgD9e)

The present Example describes construction of a soybean vector for co-expression of E389D8 with EgD9e and expression of these genes in somatic embryos.

The NcoI/XbaI fragment of pFBAIn-389D8 (SEQ ID NO:95; Example 7), containing E389D8 and where a NotI site is already present just 5' to the XbaI site, was cloned into the NcoI/XbaI sites of pKR908 (SEQ ID NO:130) to produce pKR1117 (SEQ ID NO:134) and where E389D8 is now flanked by NotI sites at the 5' and 3' ends.

E389D8 was released from pKR1117 (SEQ ID NO:134) by digestion with NotI and cloned into the NotI site of pKR123r (SEQ ID NO:66; Example 8) to produce pKR1119 (SEQ ID NO:135).

Plasmid pKR1119 (SEQ ID NO:135) was digested with *SbfI* and the fragment containing E389D8 was cloned into the *SbfI* site of pKR912 (SEQ ID NO:81; Example 12), to give pKR1122 (SEQ ID NO:136). In this way, the *Eutreptiella* sp. CCMP389 delta-8 desaturase is co-expressed with the *Euglena gracilis* delta-9 elongase behind strong, seed-specific promoters. A schematic depiction of pKR1122 is shown in FIG. 15.

Plasmid pKR1122 was transformed into soybean embryogenic suspension cultures as described in Example 9 and embryos co-expressing E389D8 and EgD9e were analyzed as described in Example 10.

Embryo fatty acid profiles for each event (6 embryos each) containing pKR1122 were obtained and the lipid profiles of somatic soybean embryos expressing E389D8 and EgD9e for the top 5 events are shown in FIG. 16. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 16 are expressed as a weight percent (wt. %) of total fatty acids. The activity of E389D8 is expressed as percent desaturation (% desat), calculated according to the following formula: $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$.

More specifically, the combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as: $([\text{DGLA} + \text{ETA}]/[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]) \times 100$. This is also referred to as the overall % desaturation. The individual omega-6 delta-8 desaturation ("EDA % delta-8 desat.") was calculated as: $([\text{DGLA}]/[\text{DGLA} + \text{EDA}]) \times 100$. Similarly, the individual omega-3 delta-8 desaturation ("ERA % delta-8 desat.") was calculated as: $([\text{ETA}]/[\text{ETA} + \text{ERA}]) \times 100$. The ratio of delta-8 desaturation for omega-6 versus omega-3 substrates ("ratio [EDA/ERA] % desat.") was obtained by dividing the EDA % delta-8 desaturation by the ERA % delta-8 desaturation.

In summary of FIG. 16, E389D8 worked in soybean to convert both EDA and ERA to DGLA and ETA, respectively. The line with the highest average DGLA content (i.e., 2107-4-14) had embryos with an average DGLA content of 16.1% and an average ETA content of 5.2%. The highest DGLA and ETA content for an individual embryo from this line was 16.1% and 6.0%, respectively. The highest average overall % desaturation was 68.5% (2107-4-14) with the highest overall % desaturation for an individual embryo being 68.6%. When broken down into %

desaturation for the omega-6 and omega-3 substrates, the highest average % desaturation was 64.0% and 81.7% for EDA and ERA, respectively. The highest % desaturation for an individual embryo from this event was 68.6% and 83.4% for EDA and ERA, respectively. In this example, E389D8 had a preference for ERA over EDA, with the average desaturation ratio ranging from 0.6-0.8. No GLA was found to accumulate in the embryos.

EXAMPLE 20

Construction of Arabidopsis Binary Expression Vector pKR1022R for Co-Expression of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) With the Delta-9 Elongase From *Euglena gracilis* (EgD9e)

The Gy1/Pavelo/legA2 cassette was released from plasmid pKR336 (described in PCT Publication Nos. WO 04/071467; the contents of which are hereby incorporated by reference) by digestion with PstI/BamHI and cloned into the PstI/BamHI site of pKR268 (described in PCT Publication Nos. WO 04/071467) to produce pKR393 (SEQ ID NO:137).

The Pavelo gene was released from pKR393 (SEQ ID NO:137) by digestion with NotI and the vector was re-ligated to form pKR407 (SEQ ID NO:138).

Vector pLF114-10 (SEQ ID NO:63; Example 5) was digested with NotI and the fragment containing TpomD8 was cloned into the NotI site of pKR407 (SEQ ID NO:138) to produce pKR1018 (SEQ ID NO:139).

The PstI fragment of pKR1018 (SEQ ID NO:139), containing the TpomD8 was cloned into the SbfI fragment of pKR911 (previously described in WO2007/061845 published on May 31, 2007 the contents of which are hereby incorporated by reference) to produce pKR1020R (SEQ ID NO:140).

The AscI fragment of pKR1020R (SEQ ID NO:140), containing EgD9e and TpomD8 was cloned into the AscI site of pKR92 (which was previously described in WO2007/061845 published on May 31, 2007 to give pKR1022R (SEQ ID NO:141).

A schematic depiction of pKR1022R is shown in FIG. 17. In this way, EgD9e was expressed in Arabidopsis under control of the soybean beta-conglycinin promoter and TpomD8 was expressed under control of the soybean glycinin Gy1 promoter.

The soybean beta-conglycinin promoter and Gy1 promoter function as a strong, seed-specific promoters in *Arabidopsis*.

EXAMPLE 21

Transformation of *Arabidopsis*

Transformed *Arabidopsis* plants were created by whole plant *Agrobacterium* transformation. Binary vector pKR1022R (SEQ ID NO:141) was transformed into *Agrobacterium tumefaciens* NTL4 (Luo et al., *Molecular Plant-Microbe Interactions* 14(1):98-103 (2001)) by electroporation. Briefly, 1 µg plasmid DNA was mixed with 100 µL of electro-competent cells on ice. The cell suspension was transferred to a 100 µL electro oration curette (1 mm gap width) and electro orated using a BIORAD electro orator set to 1 kV, 400Ω and 25 µF. Cells were transferred to 1 mL LB medium and incubated for 2 h at 30 °C. Cells were plated onto LB medium containing 50 µg/mL kanamycin. Plates were incubated at 30 °C for 60 h. Recombinant agrobacterium cultures (500 mL LB, 50 µg/mL kanamycin) were inoculated from single colonies of transformed *Agrobacterium* cells and grown at 30 °C for 60 h.

Cells were harvested by centrifugation (5000 x g, 10 min) and resuspended in 1 L of 5 % (W/V) sucrose containing 0.05 % (V/V) Silwet L-77 (OSI Specialties, Inc). *Arabidopsis* plants were grown in soil at a density of 10 plants per 100 cm² pot in metromix 360 soil mixture for 4 weeks (22 °C, 16 h light/8 h dark, 100 µE m⁻²s⁻¹). At early bolting, *Arabidopsis* plants were dipped into the *Agrobacterium* suspension. Two days later, the same plants were dipped again with the same *Agrobacterium* strain in sucrose/Silwet. Plants were grown for three to four weeks under standard plant growth conditions described above and plant material was harvested and dried for one week at ambient temperatures in paper bags. Seeds were harvested using a 0.425 mm mesh brass sieve.

Cleaned *Arabidopsis* seeds (2 grams, corresponding to about 100,000 seeds) were sterilized by washes in 45 mL of 80% ethanol, 0.01% triton X-100, followed by 45 mL of 30% (V/V) household bleach in water, 0.01% triton X-100 and finally by repeated rinsing in sterile water. Aliquots of 20,000 seeds were transferred to square plates (20 x 20 cm) containing 150 mL of sterile plant growth

medium comprised of 0.5 x MS salts, 1.0% (W/V) sucrose, 0.05 MES/KOH (pH 5.8), 200 µg/mL timentin, and 50 µg/mL kanamycin solidified with 10 g/L agar.

Homogeneous dispersion of the seed on the medium was facilitated by mixing the aqueous seed suspension with an equal volume of melted plant growth medium.

Plates were incubated under standard growth conditions for fourteen days.

Kanamycin-resistant seedlings were transferred to soil and grown to maturity as described above. T2 seed was obtained from these individual transformants.

EXAMPLE 22

Functional Analysis of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) Co-expressed With the Delta-9 Elongase From *Euglena gracilis* (EgD9e) in Arabidopsis Seed Transformed with Arabidopsis Expression Vector pKR1022R

Wild-type *Arabidopsis thaliana* (Columbia ecotype) were transformed with pKR1022R (SEQ ID NO:141) as described in Example 21 and segregating T2 seed was obtained from a number of individual events for each. Bulk T2 seed lipid profiles for each event were obtained by transesterification with TMSH as described in Example 10 with the following modifications. For each event, a small scoopful of seeds (approximately 25-50 seed each scoopful) was crushed in 50 µL of TMSH in a 1.5 mL eppendorf tube. After shaking in TMSH for 15 min., 400 µL of heptane was added and the tubes were vortexed well, shaken for an additional 15 min and centrifuged at 13,000 x g for 1 min. After shaking, the heptane layer was removed into glass GC vials and the fatty acid methyl esters were analyzed as described in Example 10.

Bulk T2 seed fatty acid profiles were obtained for 22 events where wild-type *Arabidopsis* was transformed with pKR1022R (SEQ ID NO:141). The lipid profiles of T2 bulk seed for the 22 wild-type-transformed events is shown in FIG. 18. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, 20:0 (arachidic acid), 20:1 (eicosenoic acid), EDA, DGLA, ERA and ETA; and, fatty acid compositions listed in FIG. 18 are expressed as a weight percent (wt. %) of total fatty acids.

EXAMPLE 23

Functional analysis of the *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) co-expressed with the *Saprolegnia diclina* Delta-17 Desaturase (SdD17), the *Euglena gracilis* Delta-9 Elongase (EgD9e), the *Pavlova lutheri* delta-8 desaturase (PavD8) and the *Mortierella alpina* Delta-5 Desaturase (MaD5) in Soybean Embryos and Seed Transformed with Soybean Expression Vectors pKR1005 and pKR973

The present Example describes the expression of an EPA biosynthetic pathway using a delta-9 elongase (EgD9e), a delta-5 desaturase (MaD5) and a delta-17 desaturase (SdD17) co-expressed with two delta-8 desaturases (TpomD8 & PavD8).

Soybean embryogenic suspension culture (cv. Jack) was transformed with the *Ascl* fragments of pKR1005 (SEQ ID NO:90; FIG. 4) and pKR973 (SEQ ID NO:125; FIG. 11), as described in in Example 9. Embryos were matured as described in Example 14 and a subset of soybean embryos generated from each event (ten embryos per event) were harvested, picked into glass GC vials, fatty acid methyl esters (FAMES) were prepared by transesterification and analyzed by GC as described in Example 10. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.).

In this way, 373 events transformed with pKR1005 (SEQ ID NO:90; FIG. 4) and pKR973 (SEQ ID NO:125; FIG. 11) (experiment called Heal 17) were analyzed. From the 373 events analyzed, 319 were identified that produced delta-8 desaturation products (i.e. DGLA, ARA, ETA, EPA, DPA, DHA) in at least one embryo out of ten analyzed at a relative abundance greater than 1.0% of the total fatty acids. Of these, 140 were identified that produced delta-8 desaturation products at a relative abundance greater than 10.0% of the total fatty acids, 61 were identified that produced delta-8 desaturation products at a relative abundance greater than 20.0% of the total fatty acids and 20 were identified that produced delta-8 desaturation products at a relative abundance greater than 30.0% of the total fatty acids, in at least one embryo out of ten analyzed.

The average fatty acid profiles (average of 10 embryos per event) for the ten events having the highest amounts of delta-8 desaturation products are shown in FIG. 19. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic

acid), LA, GLA, ALA, EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and, fatty acid compositions listed in FIG. 19 are expressed as a weight percent (wt. %) of total fatty acids. For FIG. 19, fatty acids listed as "others" include: 18:2 (5,9), STA, 20:0, 20:1(11), 20:2 (7,11) or 20:2 (8,11), and DHA. Each of these fatty acids is present at a relative abundance of less than 1.6% of the total fatty acids. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula: $[(DGLA + ETA)/(DGLA + ETA + EDA + ERA)] \times 100$.

In summary of FIG. 19, TpomD8 and PavD8 functioned in soybean to convert both EDA and ERA to DGLA and ETA, respectively. Additionally, the activity of the delta-5 desaturase also functioned to convert the DGLA and ETA produced to ARA and EPA, respectively. In events such as AFS 4881-6-5 & 4881-4-5, delta-5 desaturase is somewhat limiting and DGLA and ETA are high while in others (e.g. AFS 4829-6-5 & AFS 4885-1-2), delta-5 desaturase activity is strong and the delta-8 desaturated products are further converted to ARA and EPA, respectively. Further, the presence of the delta-17 desaturase also functioned to convert DGLA and ARA to ETA and EPA, respectively. In events such as AFS 4880-8-8, the delta-17 desaturase is somewhat limiting while in others (e.g. AFS 4881-6-5 & AFS 4829-6-5), delta-17 desaturase activity is strong with DGLA and ARA being efficiently converted to ETA and EPA, respectively. The individual embryo with the highest total delta-8 desaturated products came from event AFS 4881-6-5, with as high as 43% of total fatty acids. The average concentration of delta-8 desaturated products from the top ten events was 27.7% of the total fatty acids.

The fatty acid profiles for ten individual T1 seeds from 2 plants from event AFS 4882-4-6 (plant #4882-4-6-1 & #4882-4-6-2) having some of the highest amounts of total delta-8 desaturation products are shown in FIG. 20. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (5,9), LA, GLA, ALA, 20:1 (11), EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and, fatty acid compositions listed in FIG. 20 are expressed as a weight percent (wt. %) of total fatty acids. For FIG. 20, fatty acids listed as "others" include: STA, 20:0, 20:2 (7,11) or 20:2 (8,11), and DHA. Each of these fatty acids is present at a

relative abundance of less than 1.0% of the total fatty acids. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula: $([DGLA + ETA]/[DGLA + ETA + EDA + ERA]) \times 100$.

In summary of FIG. 20, TpomD8 and PavD8 worked in soybean seed to convert both EDA and ERA to DGLA and ETA, respectively. Fatty acid compositions in T1 seed are similar to those in embryos. The T1 seed is segregating as expected with some wild-type present.

EXAMPLE 24

Functional analysis of the *Tetruetrepia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) co-expressed with the *Saprolegnia diclina* Delta-17 Desaturase (SdD17), the *Euglena gracilis* Delta-9 Elongase (EgD9e) and the *Mortierella alpina* Delta-5 Desaturase (MaD5) in Soybean Embryos and Seed Transformed with Soybean Expression Vectors pKR1005 and pKR1084

The present Example describes the expression of an EPA biosynthetic pathway using a delta-9 elongase (EgD9e), a delta-5 desaturase (MaD5) and a delta-17 desaturase (SdD17) co-expressed with one delta-8 desaturases (TpomD8).

Soybean embryogenic suspension culture (cv. Jack) was transformed with the *AscI* fragments of pKR1005 (SEQ ID NO:90; FIG. 4) and pKR1084 (SEQ ID NO:129; FIG. 12), as described in in Example 9. Embryos were matured as described in Example 14 and a subset of soybean embryos generated from each event (ten embryos per event) were harvested, picked into glass GC vials, fatty acid methyl esters (FAMES) were prepared by transesterification and analyzed by GC as described in Example 10. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.).

In this way, 182 events transformed with pKR1005 (SEQ ID NO:90; FIG. 4) and pKR1084 (SEQ ID NO:129; FIG. 11) (experiment called Heal21) were analyzed. From the 182 events analyzed, 172 were identified that produced delta-8 desaturation products (i.e. DGLA, ARA, ETA, EPA, DPA, DHA) in at least one embryo out of ten analyzed at a relative abundance greater than 1.0% of the total fatty acids. Of these, 103 were identified that produced delta-8 desaturation

products at a relative abundance greater than 10.0% of the total fatty acids, 59 were identified that produced delta-8 desaturation products at a relative abundance greater than 20.0% of the total fatty acids and 9 were identified that produced delta-8 desaturation products at a relative abundance greater than 30.0% of the total fatty acids, in at least one embryo out of ten analyzed.

The average fatty acid profiles (average of 10 embryos per event) for the ten events having the highest amounts of delta-8 desaturation products are shown in FIG. 21. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, GLA, ALA, EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and, fatty acid compositions listed in FIG. 21 are expressed as a weight percent (wt. %) of total fatty acids. For FIG. 21, fatty acids listed as "others" include: 18:2 (5,9), STA, 20:0, 20:1(11), 20:2 (7,11) or 20:2 (8,11), and DHA. Each of these fatty acids is present at a relative abundance of less than 2.0% of the total fatty acids. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula: $[(DGLA + ETA)/(DGLA + ETA + EDA + ERA)] \times 100$.

As similar to that seen for the Heal17 embryos in Example 23, the Tpom delta-8 functioned alone to convert EDA and ERA to DGLA and ETA, respectively. Downstream products also varied depending on the expression of the delta-5 desaturase and delta-17 desaturase activities. But, while the range of delta-8 desaturated products for the Heal17 embryos, expressing 2 delta-8 desaturases, ranged from 25.5-33.7% of total fatty acids, those for the Heal21 embryos expressing only the single TpomD8 ranged from 18.4-22.7. The average delta-8 desaturated products for Heal17 and Heal21 embryos was 27.7% and 20.2%, respectively. With the decrease in overall delta-8 desaturase activity in Heal21 embryos compared to Heal17 embryos, EDA and ERA levels also increased from an average of 3.3% EDA and 1.2% ERA to 5.2% EDA and 2.0% ERA, respectively. An increase in the amounts of the fatty acid by-products, SCI and JUP, in Heal21 embryos compared to Heal17 embryos from 0% SCI and 0.6% JUP to 0.4% SCI and 2.3% JUP, respectively, was also observed.

The fatty acid profiles for individual T1 seeds from 2 plants from event AFS 5003-1-8 (plant #5003-1-8-1 & #5003-1-8-2) having some of the highest amounts of total delta-8 desaturation products are shown in FIG. 22. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), 18:2 (5,9), LA, GLA, ALA, 20:1 (11), EDA, SCI, DGLA, ARA, ERA, JUP, ETA, EPA and DPA; and, fatty acid compositions listed in FIG. 22 are expressed as a weight percent (wt. %) of total fatty acids. For FIG. 22, fatty acids listed as "others" include: STA, 20:0, 20:2 (7,11) or 20:2 (8,11), and DHA. Each of these fatty acids is present at a relative abundance of less than 1.0% of the total fatty acids. The total wt. % of fatty acids containing a delta-8 double bond is expressed as C20 delta-8 desat (DGLA + ARA + ETA + EPA + DPA) and the delta-8 desaturase activity is expressed as percent desaturation (C20 % delta-8 desat), calculated according to the following formula:
$$([DGLA + ETA]/[DGLA + ETA + EDA + ERA]) \times 100.$$

In summary of FIG. 22, TpomD8 worked in soybean seed to convert both EDA and ERA to DGLA and ETA, respectively. Fatty acid compositions in T1 seed are similar to those in embryos. The T1 seed is segregating as expected with some wild-type present.

CLAIMS

What is claimed is:

1. An isolated polynucleotide comprising:
 - (a) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57;
 - (b) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62;
 - (c) a nucleotide sequence encoding a polypeptide having delta-8 desaturase activity, wherein the nucleotide sequence hybridizes under stringent conditions to a nucleotide sequence as set forth in SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62; or
 - (d) a complement of the nucleotide sequence of (a), (b) or (c), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.
2. The polypeptide of Claim 1, wherein the amino acid sequence of the polypeptide has at least 85% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57.
3. The polypeptide of Claim 1, wherein the amino acid sequence of the polypeptide has at least 90% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57.
4. The polypeptide of Claim 1, wherein the amino acid sequence of the polypeptide has at least 95% sequence identity, based on the Clustal V method of alignment, when compared to SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57.
5. The polynucleotide of Claim 1 wherein the nucleotide sequence comprises SEQ ID NO:92, SEQ ID NO:93 or SEQ ID NO:62.
6. The polypeptide of Claim 1, wherein the amino acid sequence of the polypeptide comprises
 - (a) SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57; or

(b) an amino acid sequence that differs from the amino acid sequences in (a) by at least one conservative amino acid substitution.

7. The polypeptide of Claim 1, wherein the amino acid sequence of the polypeptide comprises SEQ ID NO:47, SEQ ID NO:49 or SEQ ID NO:57.

8. A recombinant DNA construct comprising the polynucleotide of Claim 1, 5, 6 or 7 operably linked to at least one regulatory sequence.

9. A cell comprising in its genome the recombinant DNA construct of Claim 8.

10. The cell of Claim 9 wherein said cell is selected from the group consisting of plants and yeast.

11. A transformed *Yarrowia* sp. comprising the recombinant construct of Claim 8.

12. A method for transforming a cell, comprising transforming a cell with the recombinant construct of Claim 8 and selecting those cells transformed with the recombinant construct of Claim 8.

13. A method for producing a transformed plant comprising transforming a plant cell with the polynucleotide of Claim 1, 5, 6 or 7 and regenerating a plant from the transformed plant cell.

14. The method of Claim 13 wherein the plant is a soybean plant.

15. A method for producing yeast comprising transforming a yeast cell with the polynucleotide of Claim 1, 5, 6 or 7 and growing yeast from the transformed yeast cell.

16. A method for producing yeast comprising transforming a yeast cell with the polynucleotide of Claim 1, 5, 6 or 7 and growing yeast from the transformed yeast cell, wherein the yeast is an oleaginous yeast selected from the group of: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

17. A transgenic seed comprising in its genome the recombinant construct of Claim 8.

18. A transgenic seed obtained from the plant made by the method of Claim 13 or 14.

19. A method for making long-chain polyunsaturated fatty acids in a cell comprising:

(a) transforming a cell with the recombinant construct of Claim 8;

(b) selecting those transformed cells that make long-chain polyunsaturated fatty acids.

20. Oil or by-products obtained from the seed of Claim 17.

21. Oil or by-products obtained from the seed of Claim 18.

22. Oil obtained from the yeast made by the method of Claim 15 or 16.

23. A method for making long-chain polyunsaturated fatty acids in a plant cell comprising:

(a) transforming a cell with the recombinant construct of Claim 8; and

(b) selecting those transformed cells that make long-chain polyunsaturated fatty acids.

24. A method for producing at least one polyunsaturated fatty acid in an oilseed plant cell comprising:

(a) transforming an oilseed plant cell with a first recombinant DNA construct comprising an isolated polynucleotide encoding at least one delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence and at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase;

(b) regenerating an oilseed plant from the transformed cell of step (a); and

(c) selecting those seeds obtained from the plants of step (b) having an altered level of polyunsaturated fatty acids when compared to the level in seeds obtained from a nontransformed oilseed plant.

25. The method of claim 24 wherein the oilseed plant is selected from the group consisting of soybean, *Brassica* species, sunflower, maize, cotton, flax, and safflower.

26. An oilseed plant comprising in its genome the recombinant construct of Claim 8.

27. An oilseed plant comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding at least one delta-8 desaturase polypeptide, operably linked to at least one regulatory sequence; and

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of a delta-4 desaturase, a delta-5 desaturase, a delta-6 desaturase, a delta-8 desaturase, a delta-12 desaturase, a delta-15 desaturase, a delta-17 desaturase, a delta-9 desaturase, a delta-9 elongase, a C_{14/16} elongase, a C_{16/18} elongase, a C_{18/20} elongase and a C_{20/22} elongase.

28. The oilseed plant of Claim 26 or 27, wherein the oilseed plant is selected from the group consisting of soybean, *Brassica* species, sunflower, maize, cotton, flax and safflower.

29. A transgenic seed obtained from the oilseed plant of Claim 25.

30. A transgenic seed obtained from the oilseed plant of Claim 26.

31. Oil or by-products obtained from the seed of Claim 29.

32. Oil or by-products obtained from the seed of Claim 30.

33. Oil obtained by the method of Claim 23 or 24.

34. Food or feed which incorporates the oil of Claim 31.

35. Food or feed which incorporates the oil of Claim 32.

36. Food or feed which incorporates the oil of Claim 33.

37. Food or feed comprising the seed of Claim 29.

38. Food or feed comprising the seed of Claim 31.

39. Food or feed comprising an ingredient derived from the processing of the seeds of Claim 29.

40. Food or feed comprising an ingredient derived from the processing of the seed of Claim 30.

41. The oil or by-products of Claim 31 wherein the by-product is lecithin.

42. The oil or by-products of Claim 32 wherein the by-product is lecithin.

43. A method for making long-chain polyunsaturated fatty acids in a cell having a reduced level of by-product fatty acids, said method comprising:

(a) transforming a host cell with at least one recombinant DNA construct comprising an isolated polynucleotide encoding at least two delta-8 desaturases operably linked to at least one regulatory sequence; and

(b) selecting those transformed host cells obtained having a reduced level of by-product fatty acids, when compared to the level of such metabolic by-product fatty acids in a transformed host cell having at least one recombinant DNA construct

comprising an isolated polynucleotide encoding one delta-8 desaturase operably linked to a regulatory sequence.

44. Progeny plants obtained from the plant made by the method of Claim 13 or 14.

45. Progeny plants obtained from the oilseed plant of Claim 29 or 30.

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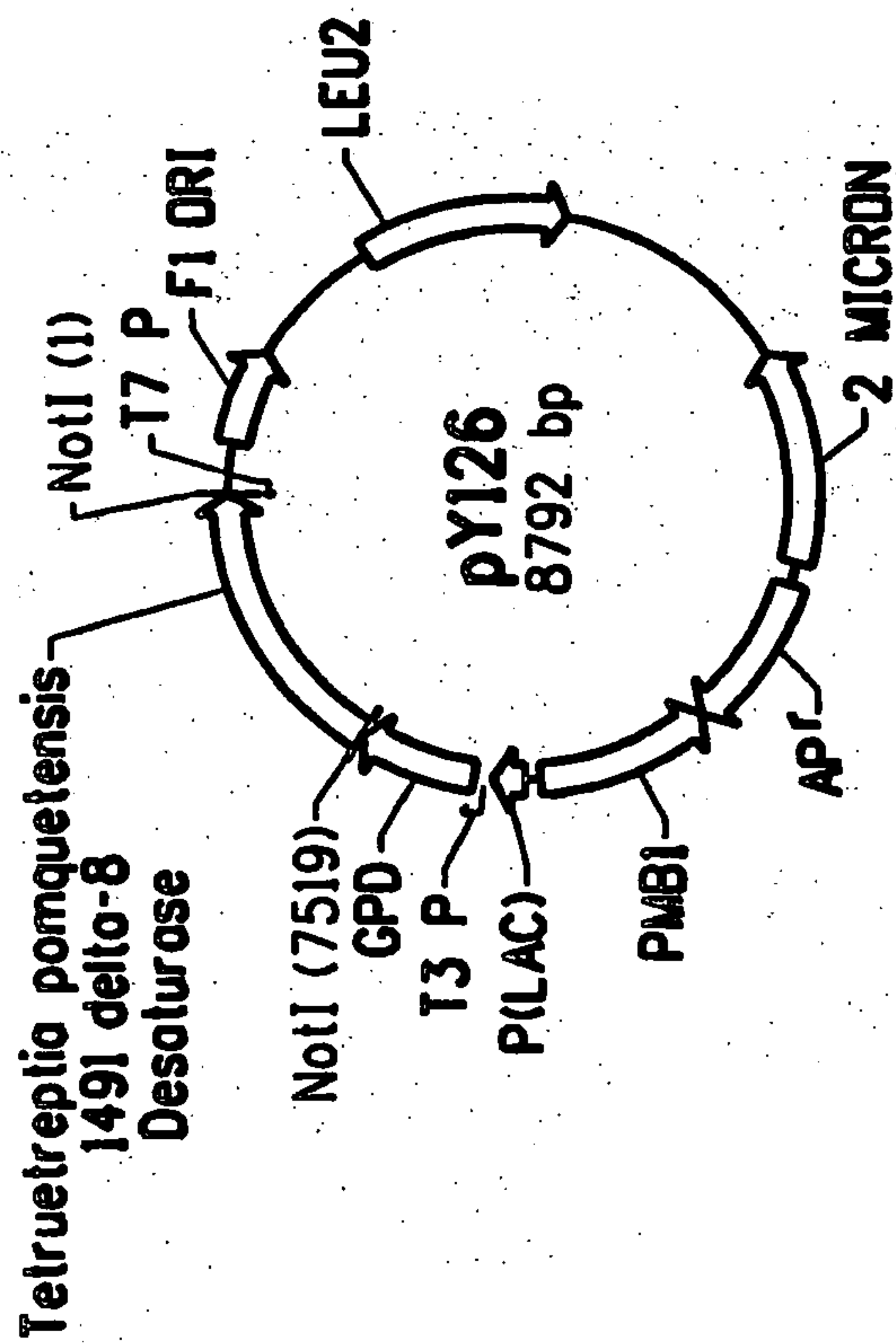


FIG. 1

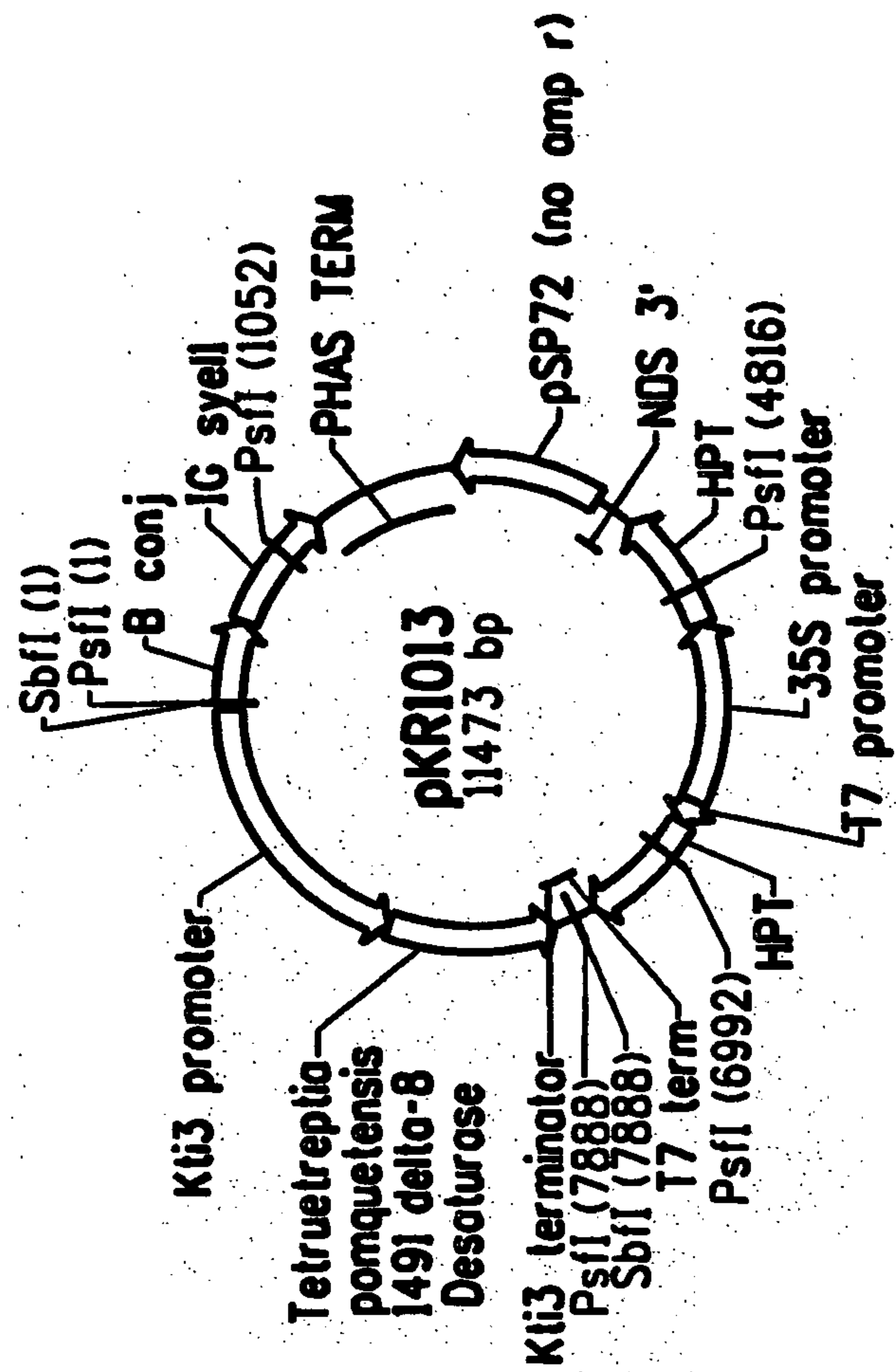


FIG. 2

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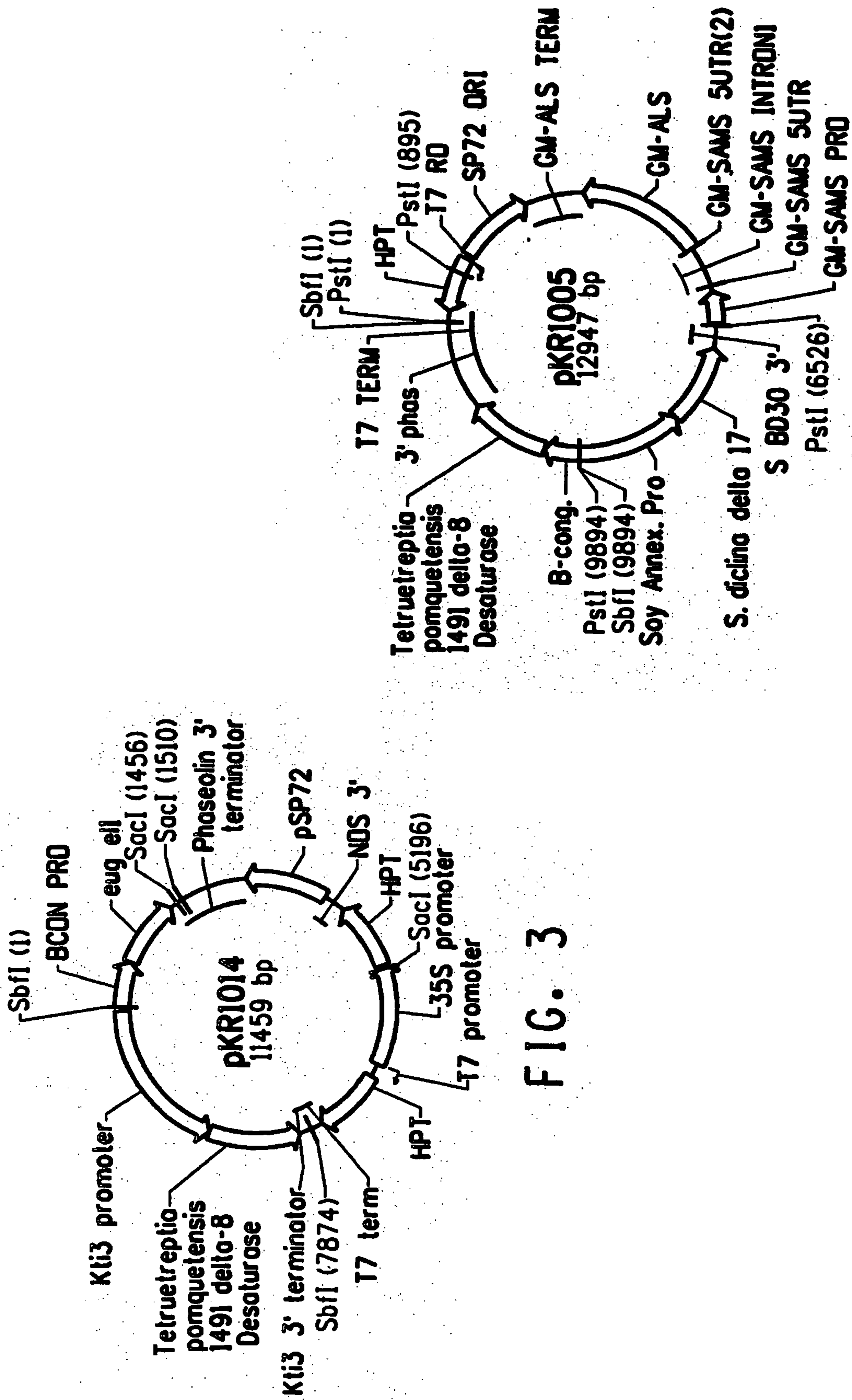


FIG. 4

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Event	Fatty Acid Composition (wt. %)											C20 % delta-8 desat	EDA		ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	GLA	ALA	EDA	DGLA	ERA	ETA	% delta-8					
											desat		% desat			
1974-1-1-1	14.5	3.6	14.9	40.3	0.0	13.4	9.9	1.2	1.7	0.4	11.8	10.7	17.9	0.6		
1974-1-1-2	14.7	4.8	19.3	37.6	0.0	12.5	8.0	1.4	1.2	0.5	17.3	15.2	29.2	0.5		
1974-1-1-3	14.0	3.7	14.5	39.0	0.0	12.9	11.8	1.8	1.9	0.5	14.4	13.1	22.0	0.6		
1974-1-1-4	15.9	2.7	10.5	41.9	0.0	17.0	8.0	1.7	1.5	0.7	20.1	17.8	29.9	0.6		
1974-1-1-5	15.9	4.1	11.3	41.2	0.0	16.4	4.7	5.0	0.5	1.0	53.5	51.4	67.5	0.8		
1974-1-1-6	15.3	3.2	10.5	40.6	0.0	15.8	9.8	2.1	1.8	0.8	19.9	17.8	29.6	0.6		
1974-1-2-1	13.8	4.0	13.8	31.2	0.0	7.1	16.1	10.0	2.1	1.9	39.7	38.3	48.6	0.8		
1974-1-2-2	14.5	2.9	14.3	29.0	0.0	8.5	16.4	9.9	2.3	2.2	39.4	37.7	49.5	0.8		
1974-1-2-3	14.0	4.0	11.4	36.7	0.0	9.8	15.9	4.7	2.4	1.1	24.1	23.0	30.2	0.8		
1974-1-2-4	15.2	3.1	10.9	36.7	0.0	10.3	13.5	6.7	2.0	1.6	34.8	33.1	44.3	0.7		
1974-1-2-5	13.8	3.8	14.0	36.1	0.0	10.0	12.6	6.6	1.7	1.4	36.0	34.4	46.2	0.7		
1974-1-2-6	13.2	2.8	14.6	35.0	0.0	8.8	17.1	4.2	3.1	1.2	21.2	19.7	28.4	0.7		
1974-3-1-1	14.7	6.2	20.1	27.9	0.0	11.6	10.5	5.0	2.1	1.8	35.0	32.3	45.4	0.7		
1974-3-1-2	15.8	5.2	17.4	27.2	0.0	14.1	10.7	5.0	2.7	1.8	34.0	32.1	40.3	0.8		
1974-3-1-3	15.4	5.5	18.3	27.3	0.0	12.2	12.6	4.6	2.6	1.5	28.5	26.6	36.7	0.7		
1974-3-1-4	16.2	6.2	19.7	25.1	0.0	12.8	9.7	5.5	2.7	2.1	38.0	36.2	43.5	0.8		
1974-3-1-5	16.2	4.7	19.1	28.7	0.0	13.5	9.8	4.3	2.2	1.4	32.5	30.6	39.7	0.8		
1974-3-1-6	15.8	5.3	19.9	26.3	0.0	13.0	9.9	5.6	2.4	1.9	37.7	36.1	43.7	0.8		

FIG. 5A

Event	Fatty Acid Composition (wt. %)										C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	GLA	ALA	EDA	DGLA	ERA	ETA				
1974-1-4-1	13.6	3.2	7.0	39.4	0.0	10.2	16.8	6.3	2.3	1.1	27.7	27.2	31.1	0.9
1974-1-4-2	13.2	2.7	10.4	41.2	0.0	8.4	16.1	5.5	1.7	0.9	26.5	25.5	34.5	0.7
1974-1-4-3	13.6	3.0	8.8	36.3	0.0	7.3	17.7	9.6	2.1	1.7	36.3	35.1	44.9	0.8
1974-1-4-4	13.6	2.9	8.2	35.8	0.0	8.4	17.8	9.3	2.4	1.6	35.1	34.2	40.6	0.8
1974-1-4-5	13.3	2.8	9.9	40.3	0.0	8.2	16.5	5.8	2.0	1.2	27.7	26.1	38.4	0.7
1974-1-4-6	13.4	4.1	10.4	41.1	0.0	9.7	13.6	4.9	1.8	1.0	27.9	26.5	36.9	0.7
1974-5-6-1	15.3	3.8	12.5	29.6	0.0	9.0	12.2	13.7	1.1	2.9	55.5	52.9	72.7	0.7
1974-5-6-2	14.7	3.8	12.9	28.0	0.0	8.8	14.8	12.4	1.7	2.8	47.9	45.6	61.6	0.7
1974-5-6-3	15.2	3.9	9.3	27.1	0.0	9.9	14.8	14.6	1.9	3.4	52.0	49.6	64.9	0.8
1974-5-6-4	13.4	3.9	15.0	30.3	0.1	9.4	12.1	12.0	1.2	2.6	52.2	49.7	68.1	0.7
1974-5-6-5	13.7	3.3	11.6	30.5	0.0	9.1	14.0	13.4	1.5	2.9	51.1	48.9	65.3	0.7
1974-5-6-6	15.9	2.2	8.1	30.2	0.1	12.1	15.1	11.4	2.1	2.8	45.3	43.0	57.6	0.7

FIG. 5B

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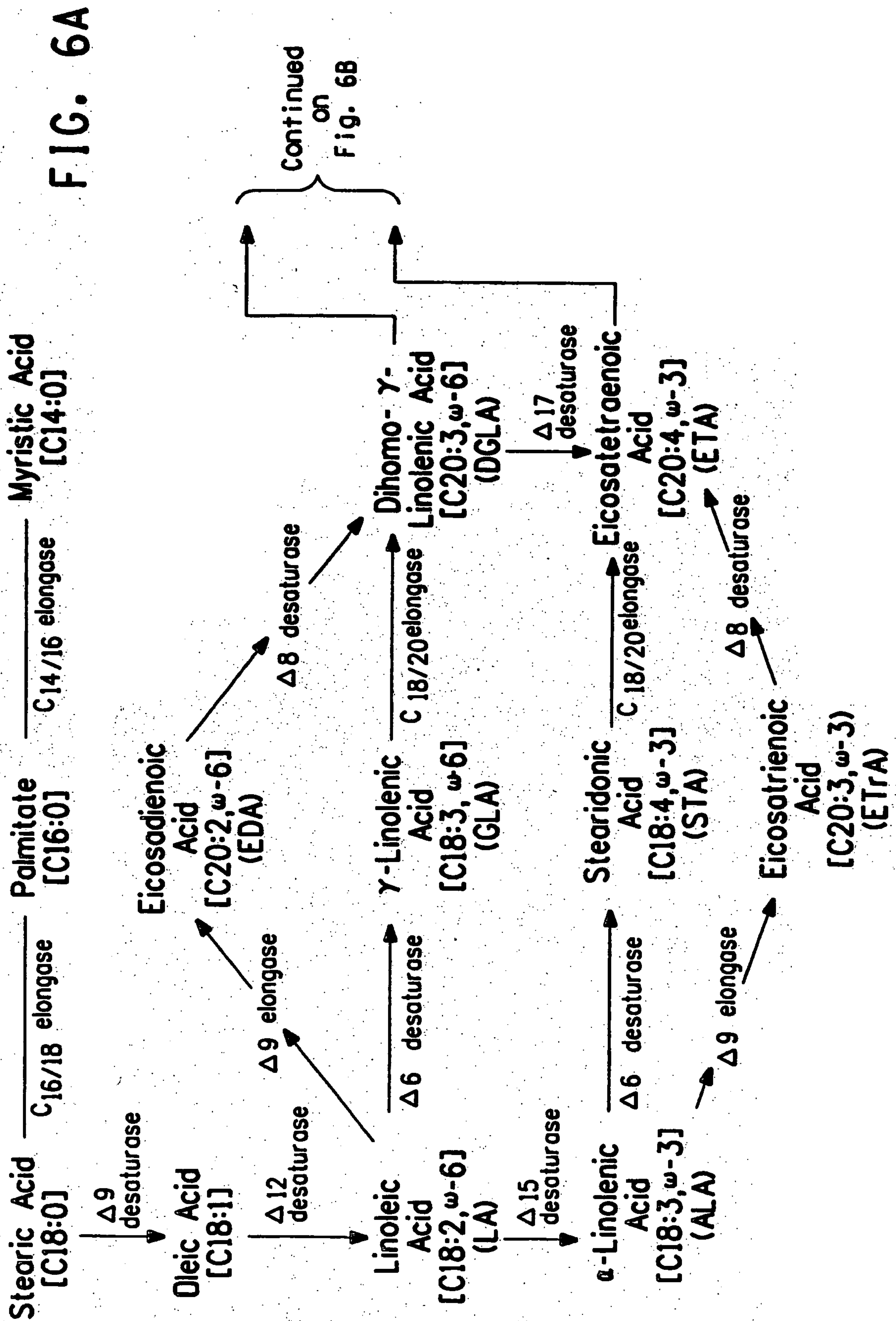
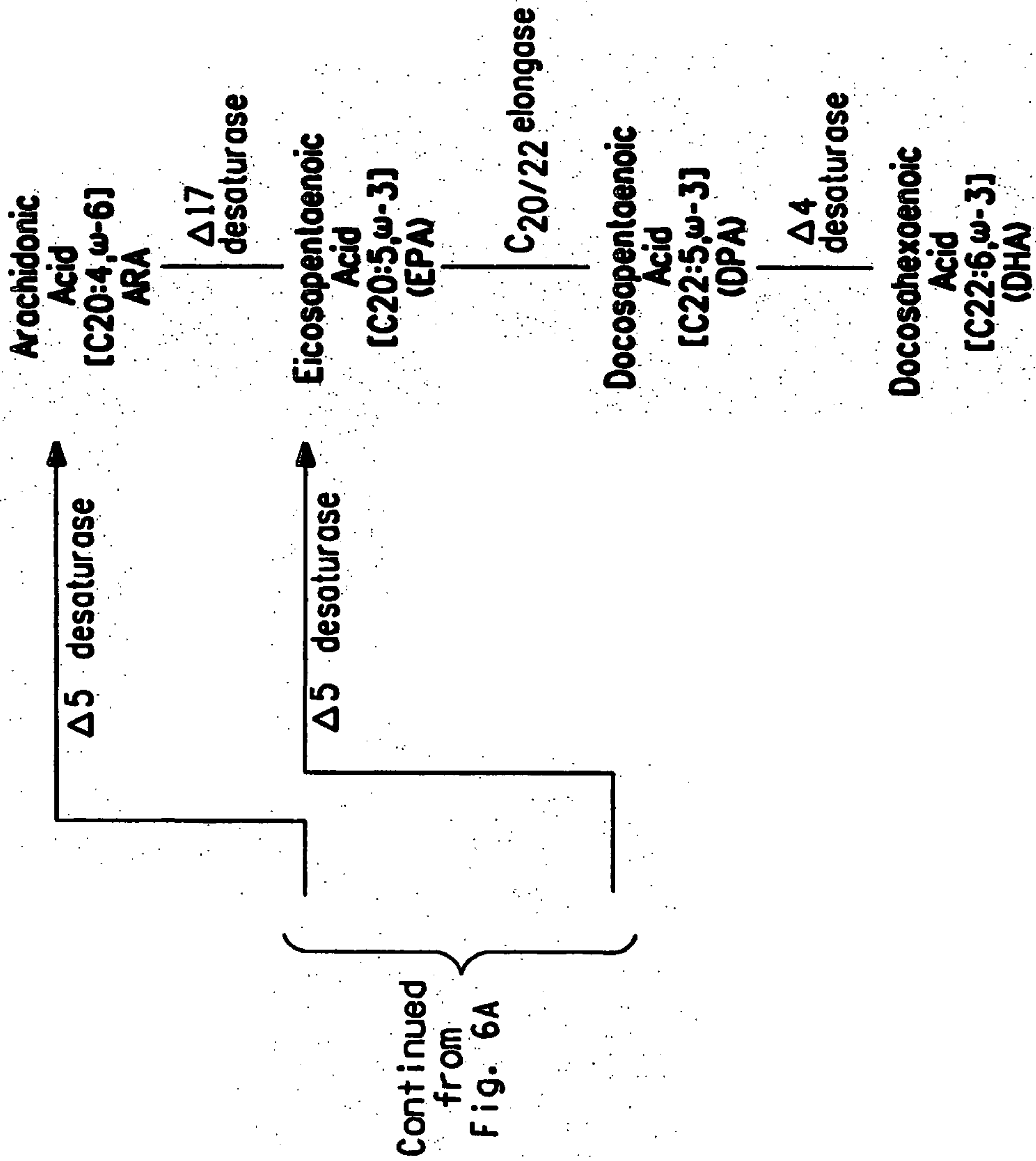


FIG. 6B



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	10	20	30	40	50	60	
1	MSPKRQAL-PITIDGATYDVSAWNHHPGGADIENYRNRDATDAFVMHMQEAVAKLKR						SEQ ID NO57
1	MSPKRREAL-PITIDGTTYDVSAWNHHPGGADIMENYRNRDATDVFMVHSHDALNKLKR						SEQ ID NO47
1	MSPKRDAL-PLTIDGTTYDVSAWNHHPGGAGQIENYRNRDATDVFMVHMQEALNKLKR						SEQ ID NO49
1	MKSQRQALSPLQLMQTYDV---VNFHPGGAEIENYQGRDATDAFVMHMQEAFDKLKR						SEQ ID NO98
1	MKSQRQAL-PLTIDGTTYDVSAWNHHPGGAEIENYQGRDATDAFVMHMQEAFDKLKR						SEQ ID NO112
	70	80	90	100	110	120	
60	MPVMEPSSPDTPVAPKPKRDEPQEDFRKLRREEPIKGMFETSEFLWYFYKTSSTTVGLMVL						SEQ ID NO57
60	MPVMEPTSP---RSPKTPNDEVAEDFRKLRKDMIAKGMFNASPLFYVYKSAATTVAGALA						SEQ ID NO47
60	MPVMEPSSP---LTPKSPSDDISXDFRKLRSMSVEKGMFNASPLFYVYKSLTTVALGAVG						SEQ ID NO49
58	MPKINPSFE---LPPQAAVNEAQEDFRKLREELIATGMFDASPLWYSYKISTTGLGLVGL						SEQ ID NO98
60	MPKINPSSE---LPPQAAVNEAQEDFRKLREELIATGMFDASPLWYSYKISTTGLGLVGL						SEQ ID NO112
	130	140	150	160	170	180	
120	ILMTVYTNWYFTAALVLGVCYQQQLGWLSDYCHHQVFTNRKINDAFGLFFGNVMQGYST						SEQ ID NO57
117	ILMVMLQWYIYPAIILLGLCYQQQLGWLSDYCHHQVFTNRKINDAFGLFFGNVMQGYST						SEQ ID NO47
117	VLMVMYQWYYSAMFLLGLCYQQQLGWLSDYCHHQVFTNRKINDAFGLFFGNVMQGYST						SEQ ID NO49
115	YFLMVQYQMYFIFGAVLLGMHYQQMGWLSDYCHHQVFTNRKINDAFGLFFGNVMQGYST						SEQ ID NO98
117	YFLMVQYQMYFIFGAVLLGMHYQQMGWLSDYCHHQVFTNRKINDAFGLFFGNVMQGYST						SEQ ID NO112
	190	200	210	220	230	240	
180	WKDRHNGHHAATNVVGHDPDIDNLPILAWSPEDEVKRAATPSTRNLIKYQQYFIPTIASL						SEQ ID NO57
177	WKDRHNGHHAATNVQGHDPDIDNLPVLAWSPEDEVKRAATPSTRNLIKYQQYFIPTIASL						SEQ ID NO47
177	WKDRHNGHHAATNVQGHDPDIDNLPVLAWSPEDEVKRAATPSTRNLIKYQQYFIPTIASL						SEQ ID NO49
175	CWKDRHNAHSAATNVQGHDPDIDNLPVLAWSPEDEVKRAATPSTRNLIKYQQYFIPTIASL						SEQ ID NO98

FIG. 7A

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	250	260	270	280	290	300	
240	RFIWC	LSIGGVMSYKSEERNLYYKRRYTK	EALPWLKATF-YCSAMP	SFATGLGC			SEQ ID NO57
237	RFIWC	FQSILAVMAYKTDARNIYYQRQYAKE	AVGLALHWILKGVFMFCY-MPGILTGLAF				SEQ ID NO47
237	RFIWC	FQSILGVMSYKTDSDXNLYYKRQYRRE	AGLALHWILKSVFLFCY-MPSFLTGLAF				SEQ ID NO49
235	RFIWC	FQCVLTVRSLK-DRDNQFYRSQYKKE	AGLALHWTLLKALFHLFF-MPSILTSLLV				SEQ ID NO98
237	RFIWC	FQSVLTVRSLK-DRDNQFYRSQYKKE	AGLALHWTLLKTLFHLFF-MPSILTSLV				SEQ ID NO112
	310	320	330	340	350	360	
299	FLISE	LGGFGIAIVVFLNHYPLDKVEETV	WDEHGFSASQIHETLN	IKPGLLTDWVFGGL			SEQ ID NO57
296	FLISE	CLGGFGIAIVVFLNHYPLEKVEESV	WDHSHGFCAGQIHTTMNIQRGV	IVDWF			SEQ ID NO47
296	FLISE	CLGGFGIAIVVFLNHYPLDKVEESV	WDHSHGFCAGQIHTTMNIQRGL	ITDWF			SEQ ID NO49
293	FFVSE	LVGGFGIAIVVFMNHYPLEKIGDP	VWDHGHGFSVGOIHETMNI	RRGIITDWF			SEQ ID NO98
295	FFVSE	LVGGFGIAIVVFMNHYPLEKIGDS	VWDHGHGFSVGOIHETMNI	RRGIITDWF			SEQ ID NO112
	370	380	390	400	410	420	
359	NYQIE	HHLWPNMPPRHNLTAA	SLEVQKLC	AKHNLPYRAPAI	IPGVQKLV	SFLGEIAQLA--	SEQ ID NO57
356	NYQIE	HHLWPTLPRHHLKA	ASFEVEKICQKHKL	PPYRAPPM	SDGVAQLL	GFLGKIAKLA--	SEQ ID NO47
356	NYQIE	HHLWPNLPRHHLKA	VSFEVEKLCQKHNL	PPYRAPPM	HTGVAQLL	GFLGKIAQLA--	SEQ ID NO49
353	NYQIE	HHLWPTLPRHNLTA	VSQVEQLCQKHNL	PPYRNPLP	HEGLVILL	RYLAVFARMAEK	SEQ ID NO98
355	NYQIE	HHLWPTLPRHNLTA	VSQVEQLCQKHNL	PPYRNPLP	HEGLVILL	RYLAVFARMAEK	SEQ ID NO112
417	---	AVPE					SEQ ID NO57
414	---	AVPV					SEQ ID NO47
414	---	AVPV					SEQ ID NO49
413	Q	PAGKAL					SEQ ID NO98
415	Q	P					SEQ ID NO112

FIG. 7B

FIG. 7B

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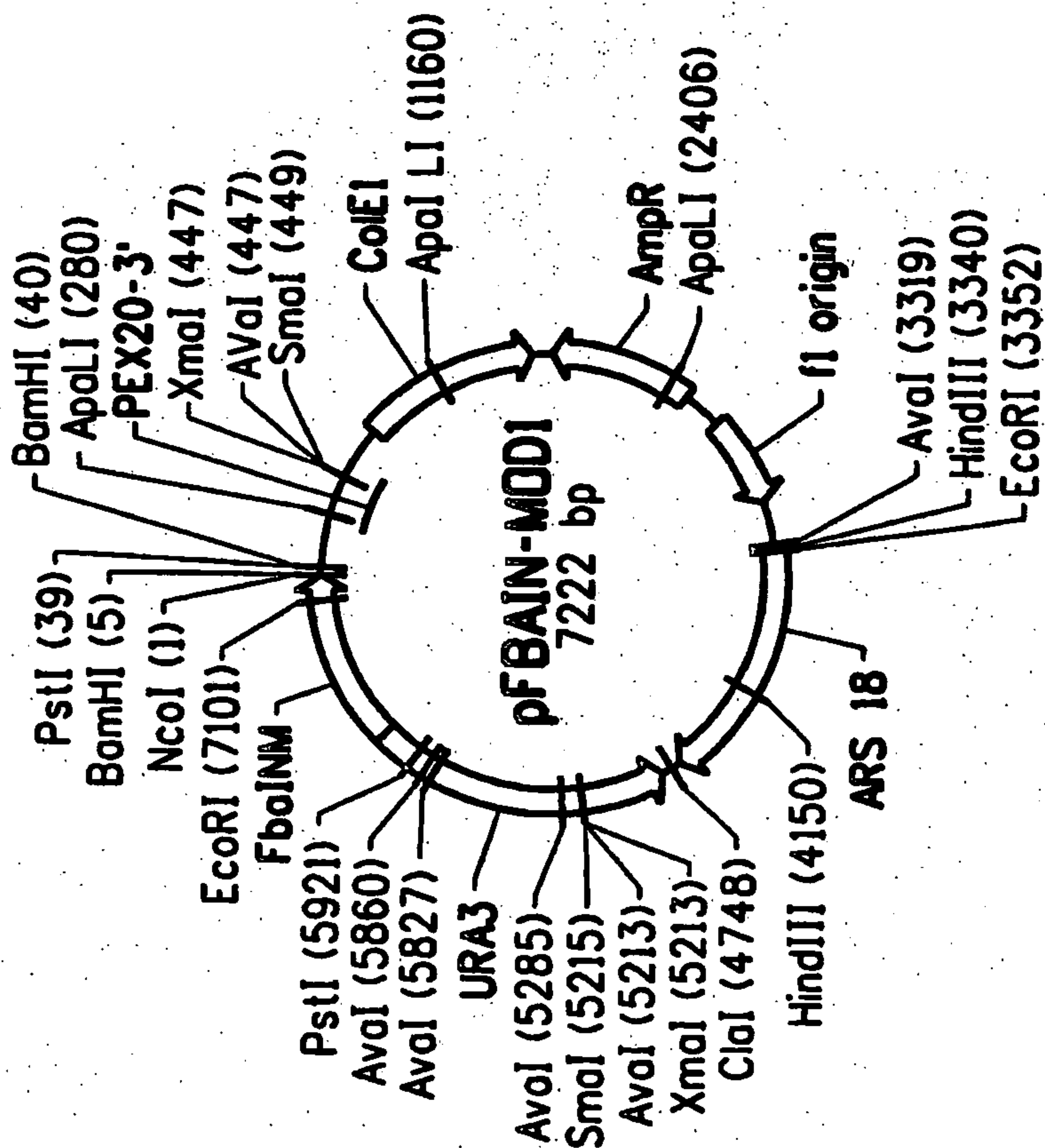


FIG. 8

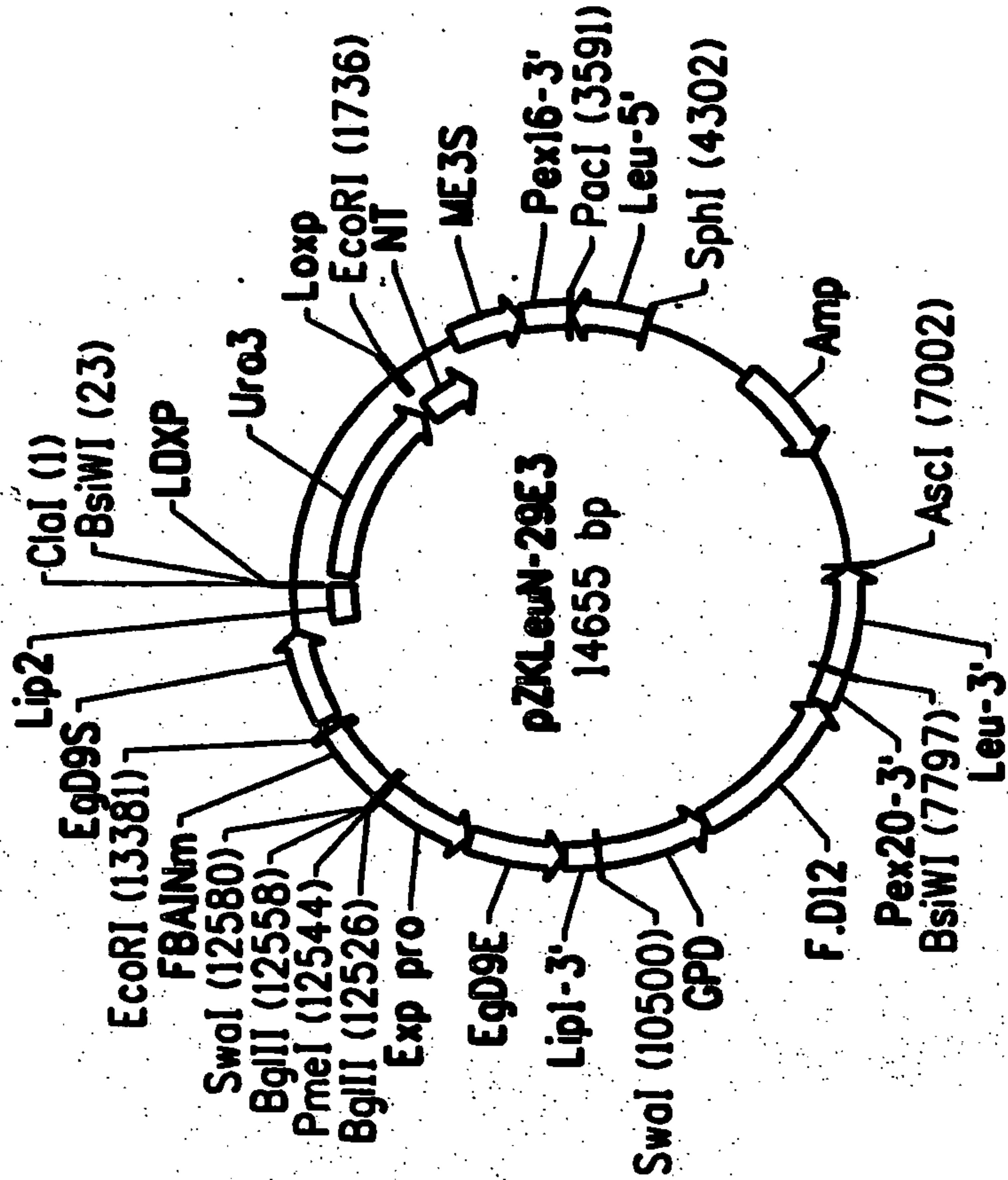


FIG. 9

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Event	Fatty acid composition (wt.%)												C20		EDA		ERA		Ratio	
	16:0	18:0	18:1	LA	GLA	ALA	EDA	DGLA	ERA	ETA			% delta-8	desat	% delta-8	desat	% delta-8	desat	(EDA/ERA)	% desat
'2024-2-11-1	20.3	6.6	16.3	29.6	0.0	12.3	4.9	6.7	1.0	2.4			60.7		57.7		71.1		0.8	0.8
'2024-2-11-2	17.1	4.8	21.7	28.7	0.0	12.1	4.8	7.4	0.7	2.7			64.7		60.5		79.9		0.8	0.8
'2024-2-11-3	18.3	5.1	17.7	30.7	0.0	13.6	4.5	6.7	0.9	2.5			63.3		60.1		74.1		0.8	0.8
'2024-2-11-4	18.9	5.0	13.2	32.5	0.0	14.7	5.0	7.2	0.9	2.7			62.6		59.0		75.1		0.8	0.8
'2024-2-11-5	21.1	5.9	17.5	29.1	0.1	11.5	5.4	6.2	1.0	2.3			57.2		53.5		70.5		0.8	0.8
'2024-2-11-6	19.5	6.2	17.7	29.7	0.0	11.9	5.7	6.5	0.8	2.1			57.0		53.3		72.5		0.7	0.7
'2024-3-5-1	16.9	4.9	17.2	23.2	0.0	7.5	11.8	13.2	1.7	3.8			55.7		52.8		69.4		0.8	0.8
'2024-3-5-2	17.1	5.3	24.1	23.2	0.0	9.3	8.1	9.0	1.2	2.7			55.6		52.6		68.9		0.8	0.8
'2024-3-5-3	15.3	6.1	34.5	16.6	0.0	6.0	7.8	9.7	1.0	3.0			59.1		55.7		73.9		0.8	0.8
'2024-3-5-4	17.4	7.2	24.5	20.8	0.0	6.5	9.0	10.7	1.3	2.5			55.9		54.1		65.2		0.8	0.8
'2024-3-5-5	17.6	6.6	22.5	22.1	0.0	8.8	8.3	9.8	1.5	2.8			56.1		54.0		64.8		0.8	0.8
'2024-3-5-6	17.0	6.7	23.2	19.8	0.0	6.1	10.7	11.9	1.4	3.2			55.5		52.8		68.6		0.8	0.8
'2024-3-9-1	16.9	5.1	24.7	16.5	0.0	6.2	11.2	14.1	1.5	3.7			58.3		55.7		70.8		0.8	0.8
'2024-3-9-2	16.2	5.2	19.9	20.0	0.0	6.1	11.5	16.0	1.3	3.9			60.8		58.1		74.8		0.8	0.8
'2024-3-9-3	16.4	5.6	25.3	17.1	0.0	5.7	10.4	14.3	1.4	3.8			60.5		57.9		73.1		0.8	0.8
'2024-3-9-4	16.3	5.5	28.7	15.9	0.0	6.4	9.2	13.1	1.3	3.7			61.4		58.7		73.2		0.8	0.8
'2024-3-9-5	15.8	6.4	27.6	14.2	0.0	5.5	10.3	15.2	1.3	3.7			62.1		59.6		74.6		0.8	0.8
'2024-3-9-6	16.3	5.0	22.2	17.9	0.0	5.6	11.8	15.9	1.4	3.8			59.9		57.4		73.1		0.8	0.8

FIG. 10A

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Event	Fatty acid composition (wt.%)											C20		EDA		ERA		Ratio
	16:0	18:0	18:1	LA	GLA	ALA	EDA	DGLA	ERA	ETA	% delta-8	desat	% delta-8	desat	% delta-8	desat	Ratio	
'2024-3-11-1	17.9	6.5	24.3	22.9	0.0	7.9	8.1	9.1	1.0	2.3		55.5	53.1		68.2		0.8	
'2024-3-11-2	16.0	6.2	29.6	20.5	0.0	6.4	9.4	8.3	1.2	2.3		49.9	46.8		65.3		0.7	
'2024-3-11-3	18.1	7.8	24.5	18.9	0.0	5.4	9.7	11.9	1.1	2.9		57.9	55.2		72.3		0.8	
'2024-3-11-4	16.3	6.5	27.0	21.7	0.0	6.7	8.2	9.8	1.1	2.5		56.7	54.3		68.5		0.8	
'2024-3-11-5	16.0	6.4	28.5	19.8	0.0	6.3	8.3	10.8	1.1	2.8		59.3	56.6		72.3		0.8	
'2024-3-11-6	17.2	7.2	29.1	19.3	0.0	5.4	8.3	9.9	1.1	2.5		56.8	54.2		69.5		0.8	
'2024-3-15-1	16.9	4.3	15.7	28.9	0.3	11.0	6.9	11.5	1.1	3.4		65.0	62.4		75.9		0.8	
'2024-3-15-2	15.8	4.2	19.5	27.4	0.3	7.9	7.5	13.5	0.7	3.1		66.8	64.2		80.9		0.8	
'2024-3-15-3	19.3	7.0	19.3	22.8	0.0	9.1	11.4	6.5	2.9	1.7		36.5	36.3		37.2		1.0	
'2024-3-15-4	16.9	3.7	9.6	30.2	0.2	13.2	7.4	13.4	2.0	3.2		63.7	64.4		60.8		1.1	
'2024-3-15-5	17.0	4.5	12.9	29.8	0.1	13.6	6.9	10.6	1.8	2.8		60.6	60.5		60.9		1.0	
'2024-3-15-6	17.7	5.5	20.4	25.2	0.3	10.7	5.4	10.7	1.0	3.2		68.7	66.7		76.5		0.9	

FIG. 10B

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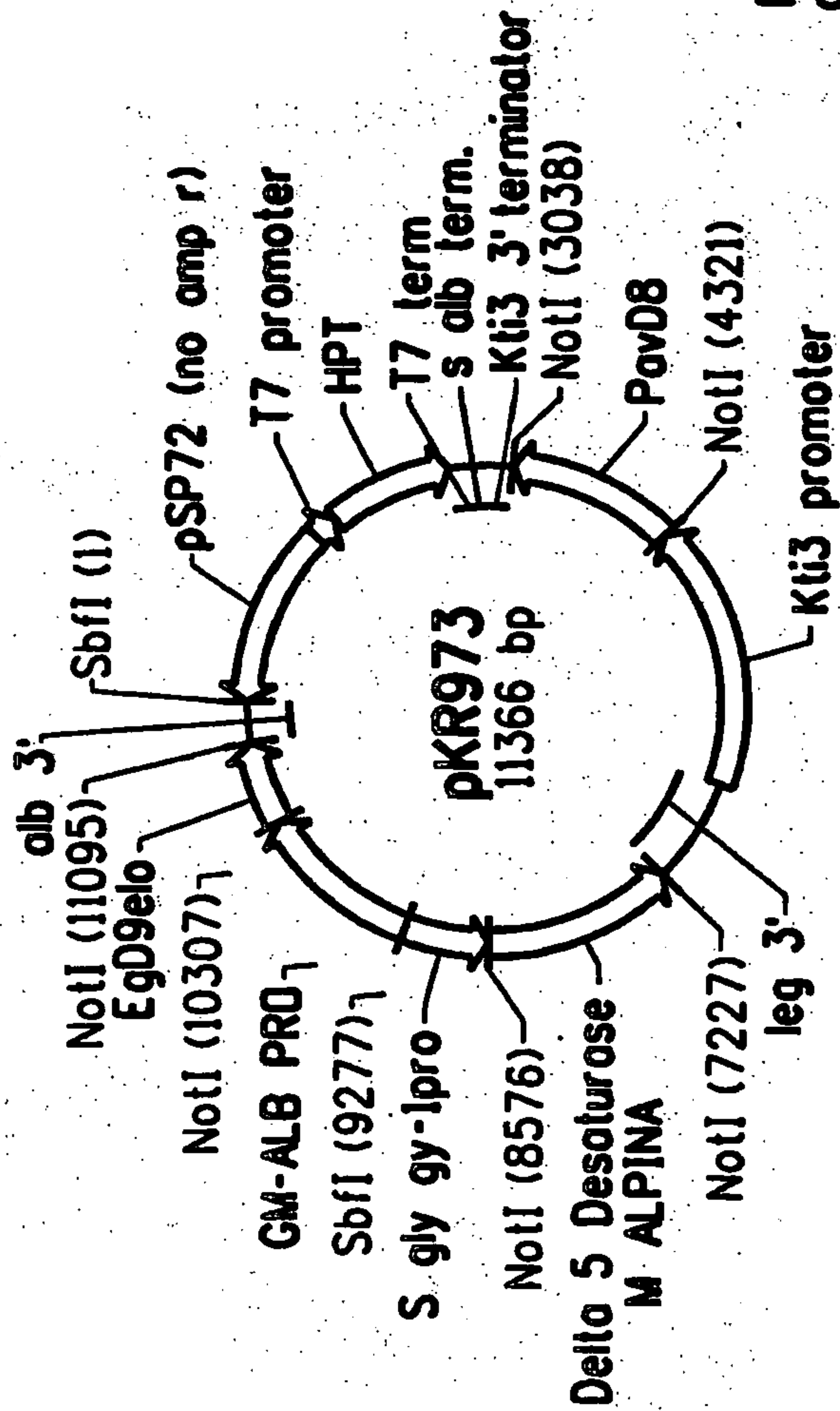


FIG. 11

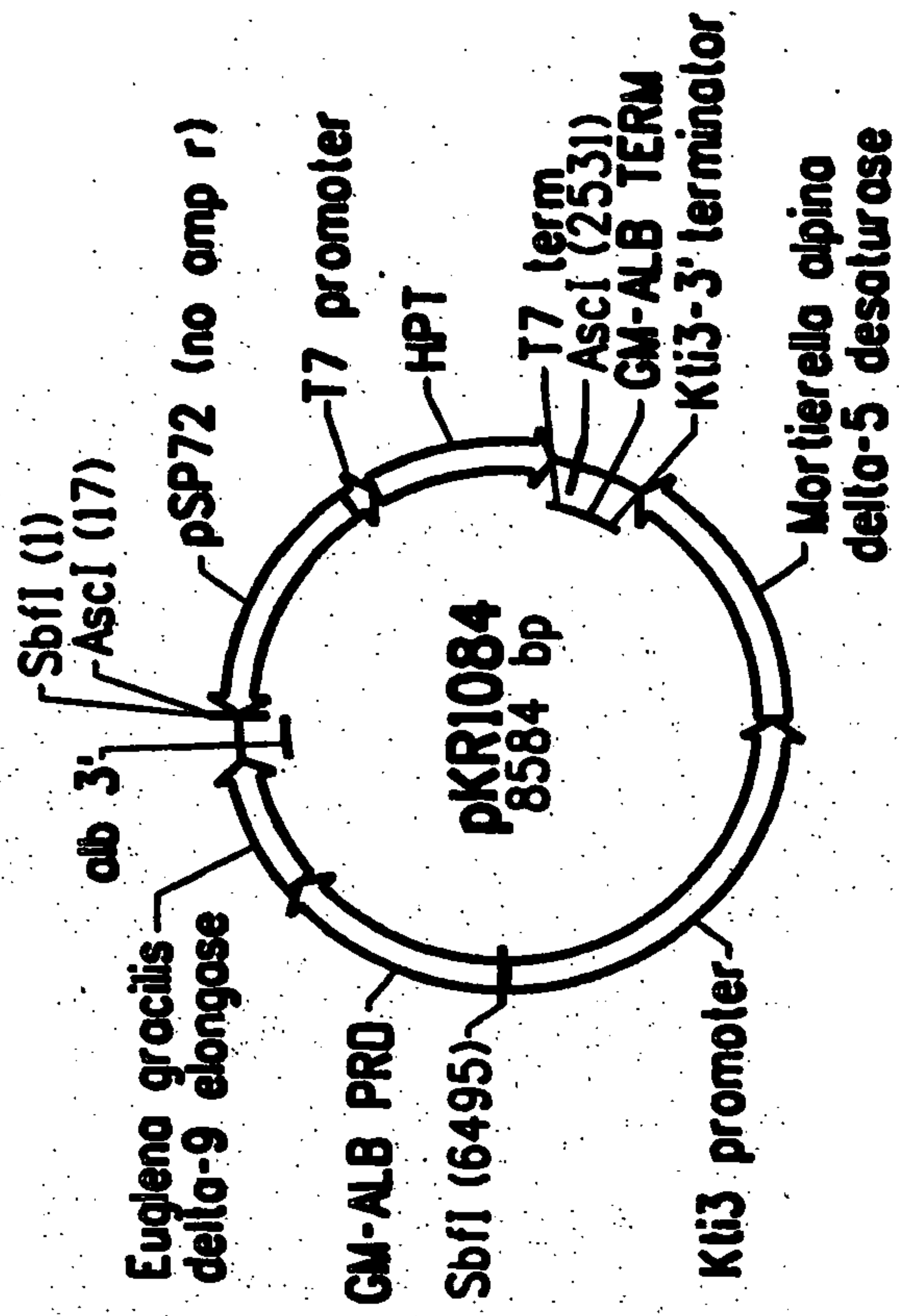


FIG. 12

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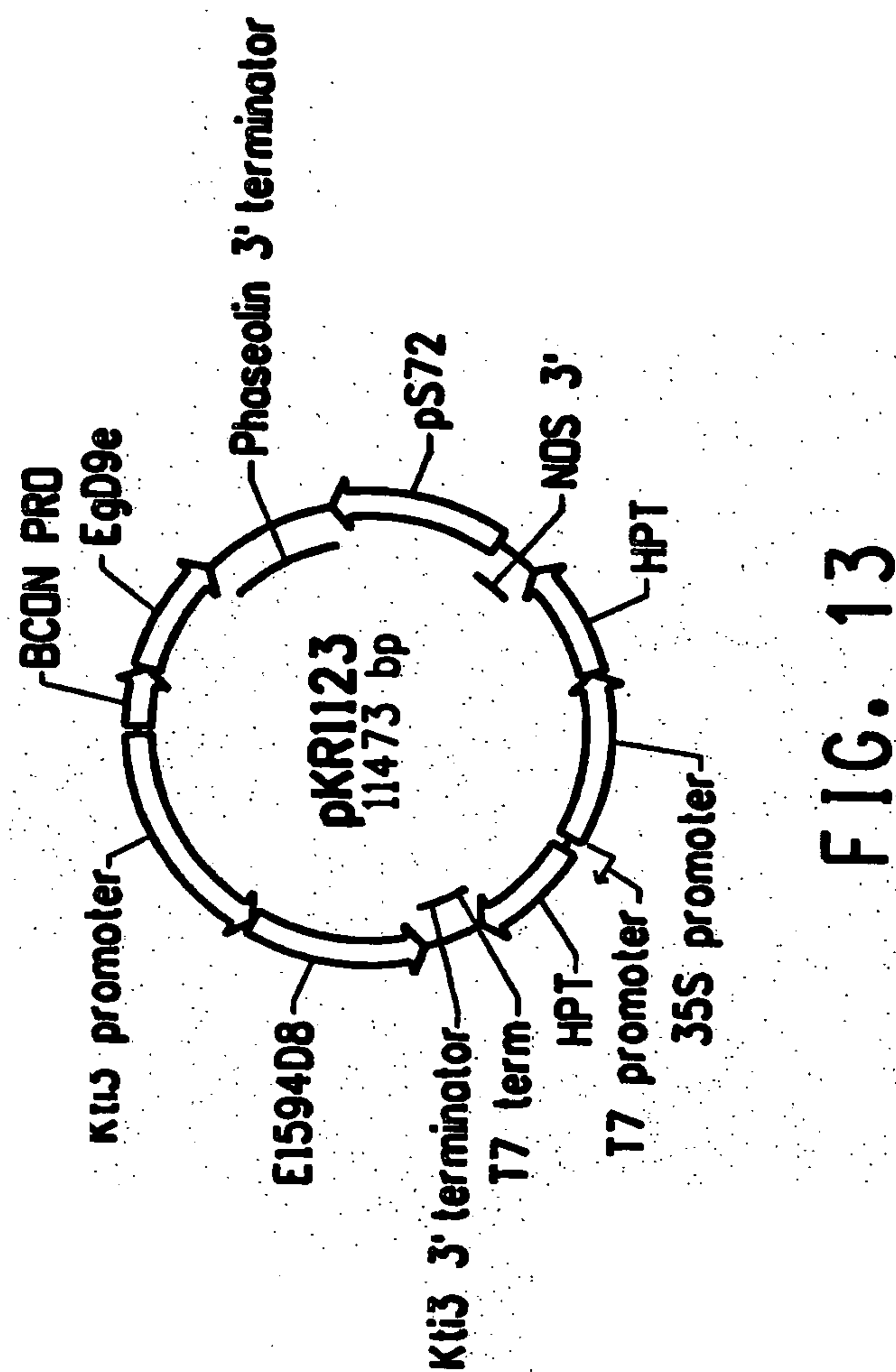


FIG. 13

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Event	Fatty acid composition (wt.%)									C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	ALA	EDA	DGLA	ERA	ETA				
2108-2-2-1	16.1	3.9	15.9	25.3	10.7	8.6	13.3	1.0	5.3	66.0	60.8	84.4	0.7
2108-2-2-2	16.1	2.8	12.0	27.9	14.1	6.9	12.8	1.1	6.3	70.4	64.9	85.0	0.8
2108-2-2-3	16.6	4.3	13.6	27.4	12.9	6.3	12.5	0.9	5.4	71.3	66.4	85.9	0.8
2108-2-2-4	16.5	4.2	14.9	25.8	12.0	8.4	11.7	1.1	5.3	64.0	58.3	82.2	0.7
2108-2-2-5	14.9	3.1	13.5	29.1	10.9	11.3	11.1	1.4	4.7	55.5	49.5	77.3	0.6
2108-5-2-1	17.7	4.0	16.2	31.0	11.8	6.0	9.1	0.8	3.6	65.2	60.4	81.9	0.7
2108-5-2-2	15.9	3.0	12.1	33.2	12.8	6.6	11.0	0.9	4.4	67.0	62.5	82.2	0.8
2108-5-2-3	17.2	3.4	13.9	30.8	12.4	6.5	10.2	1.0	4.4	66.0	60.9	81.3	0.7
2108-5-2-4	16.0	3.2	12.5	33.1	12.6	6.8	10.6	1.0	4.2	65.7	60.9	81.4	0.7
2108-5-2-5	15.3	2.5	11.6	33.0	14.6	6.4	11.1	0.9	4.5	68.1	63.4	83.1	0.8
2108-5-5-1	13.9	3.0	13.1	31.1	9.7	12.5	11.1	1.4	4.3	52.6	47.0	75.6	0.6
2108-5-5-2	16.3	3.4	12.1	30.5	12.8	10.1	9.6	1.4	3.8	53.8	48.5	73.8	0.7
2108-5-5-3	14.9	3.5	13.2	29.9	9.9	11.8	11.3	1.4	4.3	54.1	48.8	75.7	0.6
2108-5-5-4	15.0	3.7	15.0	28.3	8.9	12.4	11.2	1.4	4.1	52.6	47.4	74.8	0.6
2108-5-5-5	15.7	2.8	11.0	32.2	14.2	10.2	8.5	1.7	3.6	50.3	45.3	67.8	0.7

FIG. 14A

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Event	Fatty acid composition (wt.%)										C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	ALA	EDA	DGLA	ERA	ETA					
2108-5-9-1	20.3	5.8	14.9	23.1	9.7	9.6	10.6	1.7	4.2	56.8	52.5	71.3	0.7	
2108-5-9-2	22.4	7.3	13.7	21.5	10.9	9.3	9.7	1.3	3.8	56.0	51.1	74.8	0.7	
2108-5-9-3	24.1	7.5	18.7	18.9	7.3	9.4	9.2	1.4	3.6	54.3	49.6	72.1	0.7	
2108-5-9-4	20.6	4.3	11.6	23.8	11.2	11.0	10.9	2.0	4.5	54.3	50.0	68.9	0.7	
2108-5-9-5	20.6	6.0	20.9	20.8	8.5	9.7	8.5	1.6	3.5	51.4	46.8	68.1	0.7	
2108-6-6-1	21.5	5.4	9.6	22.0	7.0	10.8	17.7	1.3	4.7	64.9	62.0	78.7	0.8	
2108-6-6-2	25.4	7.9	17.2	19.0	7.4	8.1	10.5	1.3	3.4	59.7	56.4	72.7	0.8	
2108-6-6-3	23.9	8.4	18.4	17.1	5.5	9.0	13.1	1.1	3.5	62.3	59.3	76.6	0.8	
2108-6-6-4	21.2	7.9	19.1	19.5	5.9	9.0	12.5	1.1	3.7	61.5	58.0	77.3	0.7	
2108-6-6-5	20.1	5.0	10.8	23.7	10.0	10.3	14.3	1.5	4.3	61.1	58.0	74.1	0.8	

FIG. 14B

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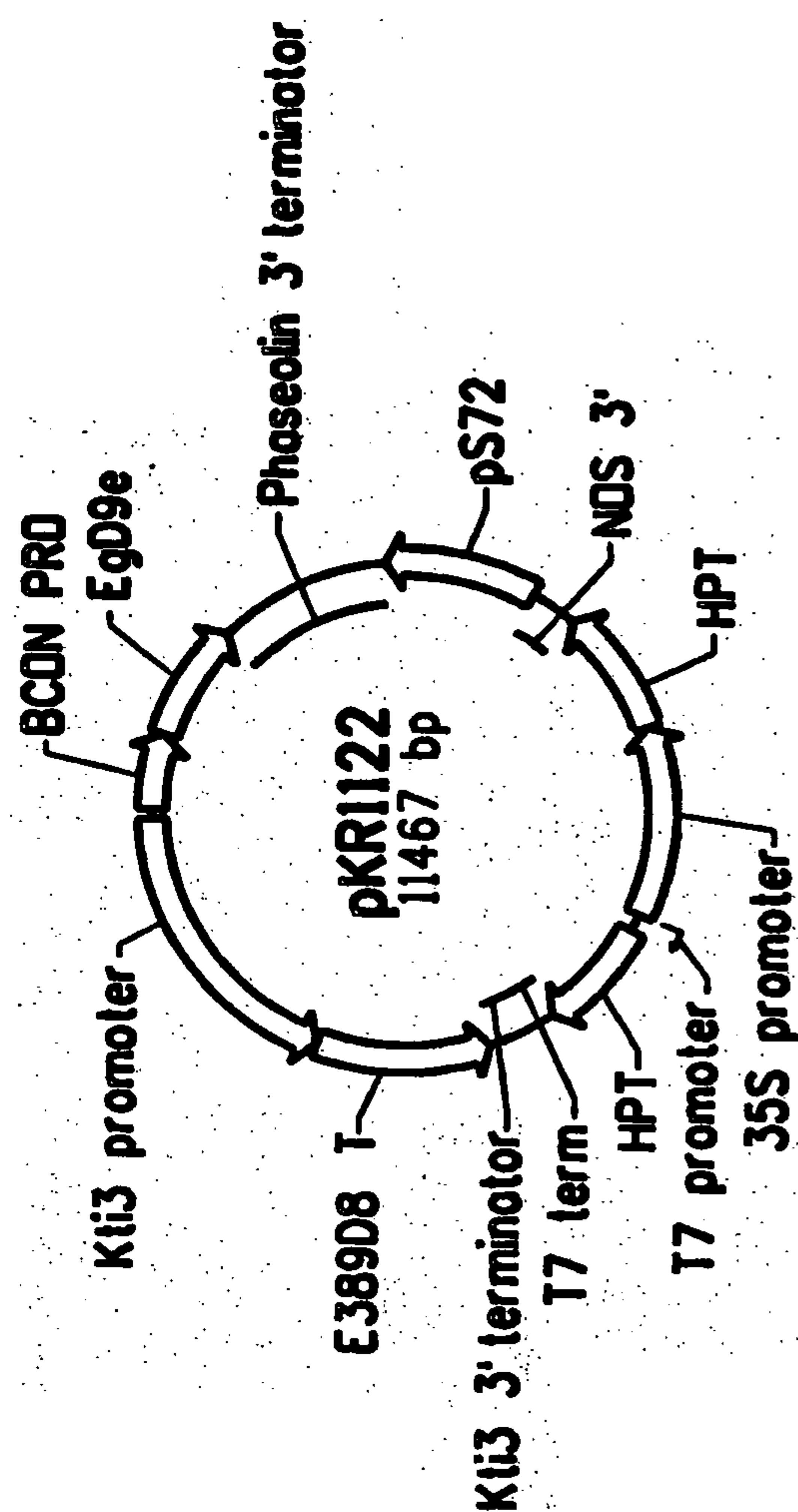


FIG. 15

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Event	Fatty acid composition (wt.%)										C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	ALA	EDA	DGLA	ERA	ETA					
2107-3-5-1	17.6	4.3	12.1	30.3	15.6	8.1	7.0	1.5	3.5	52.3	46.4	70.4	0.7	
2107-3-5-2	16.4	4.6	14.9	27.4	12.1	10.1	9.4	1.3	3.8	53.5	48.1	74.1	0.6	
2107-3-5-3	16.7	4.3	12.4	29.6	13.9	9.0	9.1	1.3	3.5	55.1	50.4	72.9	0.7	
2107-3-5-4	16.8	3.8	13.0	28.9	12.1	9.3	11.0	1.1	4.1	59.2	54.3	78.1	0.7	
2107-3-5-5	16.6	4.3	12.8	27.1	12.8	9.0	11.7	1.3	4.6	61.4	56.5	78.5	0.7	
2107-3-11-1	15.7	4.6	14.1	27.2	12.7	8.0	11.7	1.2	4.9	64.6	59.5	81.0	0.7	
2107-3-11-2	17.5	5.1	13.9	26.1	13.0	7.3	11.3	1.0	4.9	66.0	60.8	82.4	0.7	
2107-3-11-3	16.5	4.9	14.9	25.1	11.4	8.5	12.3	1.2	5.1	64.0	59.1	80.2	0.7	
2107-3-11-4	16.6	4.3	12.2	29.2	16.4	6.7	9.2	1.1	4.2	63.2	57.9	78.6	0.7	
2107-3-11-5	16.7	4.6	12.9	27.0	13.8	7.3	11.3	1.2	5.1	65.7	60.7	80.6	0.8	
2107-4-11-1	18.1	5.5	11.2	29.0	14.0	8.4	7.9	1.7	4.2	54.6	48.6	71.1	0.7	
2107-4-11-2	17.9	5.8	16.4	23.8	11.2	7.3	11.8	1.1	4.7	66.2	61.6	81.3	0.8	
2107-4-11-3	16.8	6.2	15.3	26.0	10.8	12.6	5.6	3.2	3.5	36.6	30.8	52.2	0.6	
2107-4-11-4	17.5	5.4	14.7	24.3	12.4	7.6	12.1	1.1	5.0	66.2	61.6	81.2	0.8	
2107-4-11-5	18.0	5.2	11.7	27.3	13.2	7.7	11.1	1.3	4.7	63.9	59.2	78.9	0.7	

FIG. 16A

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Event	Fatty acid composition (wt.%)									C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA) % desat
	16:0	18:0	18:1	LA	ALA	EDA	DGLA	ERA	ETA				
2107-4-14-1	19.3	5.0	11.0	22.9	11.0	7.4	16.1	1.2	6.0	72.1	68.6	83.4	0.8
2107-4-14-2	18.6	5.1	10.3	27.7	16.6	6.0	10.0	1.1	4.6	67.5	62.6	81.1	0.8
2107-4-14-3	19.6	4.7	11.6	21.5	11.2	7.6	15.2	1.4	7.1	71.2	66.6	83.3	0.8
2107-4-14-4	18.8	6.5	11.7	32.1	16.1	4.0	6.7	0.9	3.1	66.9	62.5	78.7	0.8
2107-4-14-5	17.7	5.7	10.6	26.3	12.0	8.6	12.6	1.2	5.3	64.8	59.6	81.9	0.7
2107-5-3-1	17.0	4.4	12.3	28.2	10.0	10.8	11.8	1.3	4.3	57.1	52.2	76.9	0.7
2107-5-3-2	16.6	4.9	13.0	27.7	9.0	10.9	12.9	1.1	3.9	58.3	54.2	77.8	0.7
2107-5-3-3	17.4	3.7	13.4	28.4	10.0	10.6	11.4	1.3	3.8	56.0	51.7	74.3	0.7
2107-5-3-4	16.7	4.4	14.6	25.1	9.4	10.8	13.0	1.4	4.6	59.1	54.7	76.6	0.7
2107-5-3-5	19.0	3.8	12.1	37.7	18.6	3.0	4.1	0.3	1.3	62.3	58.2	79.7	0.7

FIG. 16B

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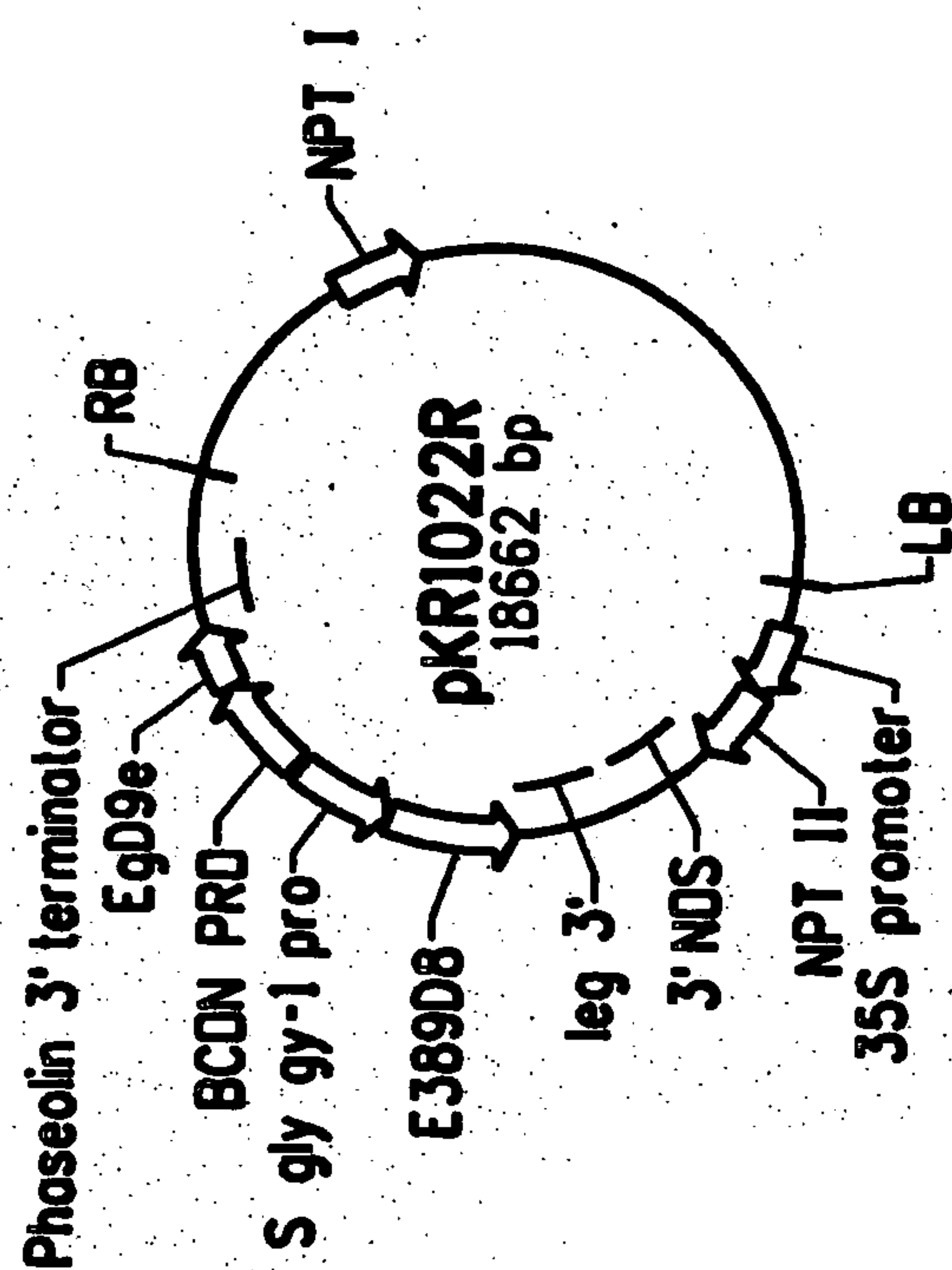


FIG. 17

Event	Fatty acid composition (wt.%)										C20 % delta-8 desat	EDA % delta-8 desat	ERA % delta-8 desat	Ratio (EDA/ERA)	
	20:1														
	16:0	18:0	18:1	LA	ALA	20:0	(11)	EDA	DGLA	ERA					ETA
pKR1022R-1	7.4	2.8	16.5	26.2	10.1	1.4	15.5	12.3	2.3	5.0	0.4	13.6	15.8	7.9	2.0
pKR1022R-2	7.0	2.7	18.3	26.0	11.8	1.5	17.3	7.8	3.9	3.0	0.8	30.4	33.3	21.4	1.8
pKR1022R-3	9.1	3.0	16.6	20.1	7.1	1.3	13.3	13.0	8.7	5.5	2.2	37.1	40.3	28.0	1.4
pKR1022R-4	7.7	3.0	16.0	27.7	12.2	1.6	16.5	8.5	2.5	3.9	0.5	19.2	22.3	11.2	2.0
pKR1022R-5	8.3	2.8	15.5	28.9	12.0	1.4	16.0	8.2	2.9	3.4	0.6	22.7	25.9	14.0	1.9
pKR1022R-6	8.5	3.0	17.8	32.4	16.2	1.8	17.9	2.0	0.1	0.4	0.0	4.3	5.2	0.0	
pKR1022R-7	7.8	2.6	17.6	31.8	16.6	1.7	19.6	1.9	0.0	0.4	0.0	0.0	0.0	0.0	
pKR1022R-8	7.9	3.1	16.4	27.4	12.3	1.5	16.2	7.6	3.5	3.4	0.8	27.9	31.4	18.5	1.7
pKR1022R-9	7.7	2.7	18.6	31.4	16.4	1.7	19.2	1.9	0.1	0.4	0.0	3.2	3.9	0.0	
pKR1022R-10	8.3	2.8	15.9	25.7	11.2	1.4	15.7	8.3	5.8	3.5	1.4	38.0	41.2	28.6	1.4
pKR1022R-11	8.1	3.0	15.4	27.9	13.1	1.6	16.3	8.2	2.2	3.8	0.4	17.7	20.7	10.4	2.0
pKR1022R-12	7.7	2.8	16.1	28.0	11.9	1.5	16.9	8.4	2.6	3.6	0.5	20.5	23.6	12.3	1.9
pKR1022R-13	8.2	3.0	14.9	27.7	12.9	1.6	16.2	7.9	3.2	3.7	0.7	25.1	28.6	16.2	1.8
pKR1022R-14	8.5	3.0	14.9	27.8	12.6	1.5	15.0	8.4	3.7	4.0	0.9	26.9	30.5	18.0	1.7
pKR1022R-15	8.1	2.9	15.5	28.0	13.6	1.6	16.4	7.1	2.8	3.3	0.6	25.0	28.4	16.4	1.7
pKR1022R-16	8.4	3.1	16.5	23.4	10.0	1.5	15.5	9.5	6.6	3.8	1.5	38.0	41.0	28.8	1.4
pKR1022R-17	8.7	2.7	17.1	27.3	14.6	1.7	17.3	5.3	2.3	2.3	0.6	27.0	29.8	19.7	1.5
pKR1022R-18	8.2	2.9	16.8	25.9	10.9	1.5	16.6	8.1	4.8	3.2	1.1	34.2	37.2	25.2	1.5
pKR1022R-19	8.6	3.0	15.3	28.2	12.9	1.5	15.4	7.7	3.1	3.5	0.7	25.4	28.8	16.8	1.7
pKR1022R-20	7.8	3.0	16.2	28.2	12.3	1.7	16.5	8.3	2.1	3.5	0.4	17.7	20.6	10.0	2.1
pKR1022R-21	8.1	3.0	15.6	26.8	9.9	1.5	15.7	9.7	4.8	3.9	1.0	29.6	32.9	19.8	1.7
pKR1022R-22	8.0	3.1	16.0	26.2	10.2	1.5	16.6	9.3	4.5	3.8	0.9	29.3	32.5	20.0	1.6

FIG. 18

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Event	Fatty acid composition (wt.%)																C20		C20	
	16:0	18:0	18:1	LA	GLA	ALA	EDA	SCI	DGLA	ARA	ERA	JUP	ETA	EPA	DPA	Other	delta-8	desat	% delta-8	desat
AFS 4881-6-5	15.2	1.8	12.3	20.9	3.0	5.9	2.8	0.0	5.8	0.1	2.0	0.3	18.3	9.5	0.1	2.2	33.7		87.5	
AFS 4885-1-2	14.9	3.0	14.5	20.8	1.3	4.6	8.3	0.0	8.8	0.7	1.6	0.7	6.5	12.8	0.1	1.3	28.9		74.4	
AFS 4829-6-5	13.3	4.2	13.3	23.9	0.9	6.3	5.8	0.0	6.9	0.8	1.6	0.9	4.1	16.6	0.1	1.2	28.6		79.5	
AFS 4880-1-8	17.7	4.1	12.6	24.8	1.0	5.3	3.7	0.0	11.6	0.5	1.4	0.3	5.3	10.1	0.3	1.1	27.9		84.6	
AFS 4880-8-8	16.4	3.6	13.2	26.2	1.4	8.2	1.3	0.0	11.4	6.6	0.6	0.5	2.0	7.2	0.1	1.3	27.3		93.5	
AFS 4882-5-5	15.9	4.1	16.7	22.6	1.6	5.2	3.7	0.0	9.0	0.4	1.0	0.7	4.4	12.5	0.3	1.8	26.6		84.9	
AFS 4828-2-22	11.0	2.1	13.1	33.3	1.2	9.9	0.9	0.0	5.1	0.8	0.7	0.4	5.0	15.1	0.2	1.4	26.2		94.3	
AFS 4881-4-5	15.4	3.1	18.0	22.7	1.7	7.4	3.0	0.0	10.5	0.1	1.2	0.0	12.6	3.0	0.0	1.5	26.1		86.2	
AFS 4829-3-2	14.6	2.3	10.7	30.9	1.0	10.3	1.2	0.0	6.1	0.7	0.9	1.0	5.0	13.8	0.3	1.3	25.8		92.5	
AFS 4882-4-6	15.8	3.9	15.2	25.3	0.9	6.7	2.7	0.0	9.1	0.4	1.3	1.2	4.4	11.3	0.3	1.8	25.5		86.6	
Average	15.0	3.2	14.0	25.1	1.4	7.0	3.3	0.0	8.4	1.1	1.2	0.6	6.8	11.2	0.2	1.5	27.7		86.4	

FIG. 19

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Event	Fatty acid composition (wt.%)																			C20	
	18:2 (5,9)				LA	GLA	ALA	20:1 (11)	EDA	SCI	DGLA	ARA	ERA	JUP	ETA	EPA	DPA	Other	delta-8 desat	C20 % delta-8 desat	
	16:0	18:0	18:1	18:2																	
4882-4-6-1-1	11.7	3.7	11.8	0.0	56.7	0.0	15.5	0.1	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.2	63.1	
4882-4-6-1-2	13.8	2.9	35.6	7.1	13.6	1.7	2.5	1.0	1.5	0.4	5.2	0.1	0.7	0.6	2.1	10.3	0.2	1.2	18.0	88.9	
4882-4-6-1-3	11.7	2.6	36.0	11.1	12.0	1.9	2.4	1.2	1.0	0.6	5.1	0.1	0.7	0.5	2.0	10.1	0.2	1.3	17.5	91.2	
4882-4-6-1-4	24.3	7.4	19.3	6.4	15.4	1.0	6.4	0.7	3.2	0.0	3.8	0.7	1.1	0.5	2.4	6.2	0.0	1.1	13.1	75.2	
4882-4-6-1-5	12.5	3.1	36.6	8.2	13.3	1.6	2.6	1.2	1.7	0.4	5.9	0.3	0.7	0.4	2.0	8.3	0.2	1.1	16.9	87.7	
4882-4-6-1-6	13.8	2.8	35.4	18.2	5.2	2.2	2.1	1.1	0.8	1.0	2.8	0.3	0.4	0.3	1.9	9.9	0.8	2.0	15.7	92.9	
4882-4-6-1-7	15.1	2.8	18.1	4.5	18.1	2.6	2.9	0.4	1.8	0.3	5.8	0.1	0.9	0.8	2.7	21.6	0.6	1.2	30.9	91.9	
4882-4-6-1-8	13.2	2.9	32.8	8.2	12.3	2.2	2.2	1.0	1.1	0.5	6.0	0.2	0.6	0.4	2.4	12.7	0.4	1.3	21.7	93.0	
4882-4-6-1-9	11.1	3.6	12.6	0.0	56.2	0.0	16.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	44.2	
4882-4-6-1-10	12.0	4.4	13.5	0.0	54.4	0.0	15.1	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.1	60.3	
4882-4-6-2-1	11.9	4.0	15.5	0.0	53.3	0.0	14.8	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	57.4	
4882-4-6-2-2	10.7	3.8	49.2	13.7	7.1	0.8	1.6	2.3	0.9	0.5	2.5	0.0	0.7	0.4	1.1	3.7	0.0	1.5	7.3	81.9	
4882-4-6-2-3	12.0	3.4	40.9	9.0	10.7	1.7	1.7	1.4	1.2	0.5	5.0	0.1	0.5	0.3	2.2	8.6	0.1	1.2	16.1	90.4	
4882-4-6-2-4	12.7	3.1	32.5	11.6	9.3	2.7	2.1	1.3	0.8	0.7	4.4	0.3	0.5	0.4	2.1	14.2	0.5	1.6	21.4	94.2	
4882-4-6-2-5	15.8	2.3	22.9	9.1	12.4	3.8	2.4	0.7	0.8	0.4	6.0	0.4	0.5	0.4	2.5	18.0	0.6	1.4	27.5	95.3	
4882-4-6-2-6	10.0	5.2	58.5	9.3	5.7	0.3	1.1	3.1	0.8	0.3	1.6	0.0	0.5	0.4	0.7	1.8	0.0	1.1	4.2	76.5	
4882-4-6-2-7	10.3	3.9	55.2	10.6	6.4	0.5	1.1	2.7	0.9	0.6	2.4	0.0	0.6	0.3	0.9	2.8	0.0	1.3	6.1	80.1	
4882-4-6-2-8	11.0	3.9	50.3	10.1	7.3	0.6	1.0	2.3	1.8	0.6	3.8	0.0	0.6	0.3	1.3	4.3	0.0	1.3	9.4	79.6	
4882-4-6-2-9	12.2	5.7	57.6	8.8	3.1	0.0	1.0	2.8	0.5	0.6	2.4	0.0	0.0	0.0	1.6	2.7	0.0	1.6	6.7	92.8	
4882-4-6-2-10	12.2	3.3	18.6	0.0	52.9	0.0	12.5	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0	

FIG. 20

Event	Fatty acid composition (wt.%)																C20	C20
	16:0	18:0	18:1	LA	GLA	ALA	EDA	SCI	DGLA	ARA	ERA	JUP	ETA	EPA	DPA	Other	delta-8 desat	% delta-8 desat
AFS 5002-1-4	14.8	2.9	17.7	22.1	1.0	10.1	4.7	0.6	8.0	10.3	0.9	0.8	0.9	3.3	0.2	1.7	22.7	80.2
AFS 5001-7-1	15.2	2.0	25.5	17.9	1.9	9.3	1.7	0.0	2.2	0.0	1.5	0.9	11.5	7.9	0.1	2.4	21.8	87.3
AFS 5006-1-20	17.3	3.0	22.6	17.9	0.8	6.7	4.2	0.0	3.2	0.2	1.7	3.6	2.9	14.3	0.4	1.3	21.0	78.0
AFS 5001-6-25	14.3	3.4	23.1	19.2	0.6	7.4	4.1	0.0	2.1	0.0	3.1	3.0	7.7	10.2	0.2	1.6	20.2	73.6
AFS 5001-3-4	13.5	2.7	17.8	27.9	0.6	8.0	6.1	0.1	6.6	1.9	1.3	1.0	2.6	8.6	0.2	1.2	19.7	72.8
AFS 5001-7-7	14.6	2.9	16.0	26.0	0.6	9.2	5.4	0.1	2.6	0.3	2.7	1.4	3.5	13.2	0.2	1.4	19.7	70.8
AFS 5003-6-12	17.9	2.4	11.8	23.3	0.8	8.8	5.8	0.1	2.8	0.1	3.3	4.7	5.3	10.8	0.1	1.7	19.2	67.7
AFS 5003-1-8	14.8	3.2	18.4	24.3	0.4	6.4	6.2	0.1	2.6	0.2	2.5	3.2	3.6	12.0	0.4	1.6	18.8	68.2
AFS 5003-5-7	13.1	2.9	12.9	24.9	0.3	7.2	9.5	2.8	5.2	9.5	1.6	2.6	0.7	4.7	0.2	1.9	20.2	64.4
AFS 5002-7-2	14.2	2.9	18.7	27.5	1.6	8.0	4.3	0.2	6.9	2.6	1.1	1.8	1.4	7.4	0.3	1.4	18.4	77.5
Average	15.0	2.8	18.5	23.1	0.9	8.1	5.2	0.4	4.2	2.5	2.0	2.3	4.0	9.2	0.2	1.6	20.2	74.1

FIG. 21

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Event	Fatty acid composition (wt.%)																			C20 delta-8 desat	C20 % delta-8 desat
	16:0	18:0	18:1	18:2 (5,9)	LA	GLA	ALA	20:1 (11)	EDA	SCI	DGLA	ARA	ERA	JUP	ETA	EPA	DPA	Other			
5003-1-8-1-1	11.1	2.8	21.9	0.0	50.4	0.0	13.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.3	0.3	100.0	
5003-1-8-1-2	12.7	2.9	12.7	0.0	49.1	0.0	21.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.3	100.0	
5003-1-8-1-3	16.8	3.3	50.2	2.3	7.1	0.7	6.3	0.4	0.4	0.3	1.0	0.0	0.5	0.1	3.0	4.8	0.2	2.7	9.0	91.3	
5003-1-8-1-4	14.0	3.1	41.4	1.6	9.2	0.5	13.3	0.6	1.3	0.0	1.3	0.1	0.8	0.3	2.8	6.4	0.1	3.2	10.7	83.8	
5003-1-8-1-5	14.3	4.1	36.3	2.4	9.8	1.3	10.5	0.5	1.3	0.2	2.5	0.0	0.6	0.4	4.0	9.4	0.3	2.1	16.2	89.2	
5003-1-8-1-6	15.4	4.5	49.8	2.4	6.3	1.2	2.9	0.5	0.8	0.0	1.6	0.0	0.5	0.4	4.0	7.1	0.3	2.5	13.1	91.2	
5003-1-8-1-7	12.3	2.6	21.3	0.0	49.3	0.0	13.9	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.2	0.2	100.0	
5003-1-8-1-8	12.4	4.6	54.6	2.7	8.0	0.7	1.4	1.0	2.8	0.0	2.0	0.0	0.9	0.4	2.0	4.6	0.1	1.8	8.8	70.0	
5003-1-8-1-9	11.4	6.4	45.8	2.1	13.0	0.7	1.7	0.6	5.5	0.0	1.9	0.1	1.3	0.9	1.5	5.9	0.2	1.0	9.5	58.2	
5003-1-8-1-10	13.3	4.6	53.2	2.8	7.7	0.6	1.4	1.1	3.1	0.0	1.3	0.0	1.2	0.7	2.3	5.1	0.2	1.5	8.8	67.4	
5003-1-8-2-1	16.0	3.6	42.1	2.5	8.0	1.7	6.8	0.2	0.9	0.2	1.7	0.0	0.6	0.3	3.3	8.5	0.2	3.5	13.7	90.2	
5003-1-8-2-2	12.2	3.1	21.1	0.0	47.7	0.0	15.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.3	0.1	100.0	
5003-1-8-2-3	9.1	3.3	28.8	0.0	47.0	0.0	10.9	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.3	0.4	98.5	
5003-1-8-2-4	13.8	3.5	14.6	0.0	53.4	0.0	14.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.3	0.3	100.0	
5003-1-8-2-5	11.0	3.5	50.7	2.2	11.2	0.4	1.6	1.5	7.4	0.3	1.1	0.2	1.7	1.9	1.0	3.2	0.1	1.1	5.5	37.6	
5003-1-8-2-6	10.5	3.5	22.2	0.0	37.0	0.0	25.1	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.2	1.1	0.4	84.1	
5003-1-8-2-7	13.7	3.5	40.9	2.0	9.2	0.8	13.5	0.4	1.4	0.2	1.2	0.1	0.8	0.4	1.9	6.9	0.1	3.1	10.2	82.3	
5003-1-8-2-8	12.5	2.9	15.7	0.0	47.4	0.0	20.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.2	0.5	0.4	100.0	

FIG. 22

Tetruetreptia pomquetensis
1491 delta-8
Desaturase

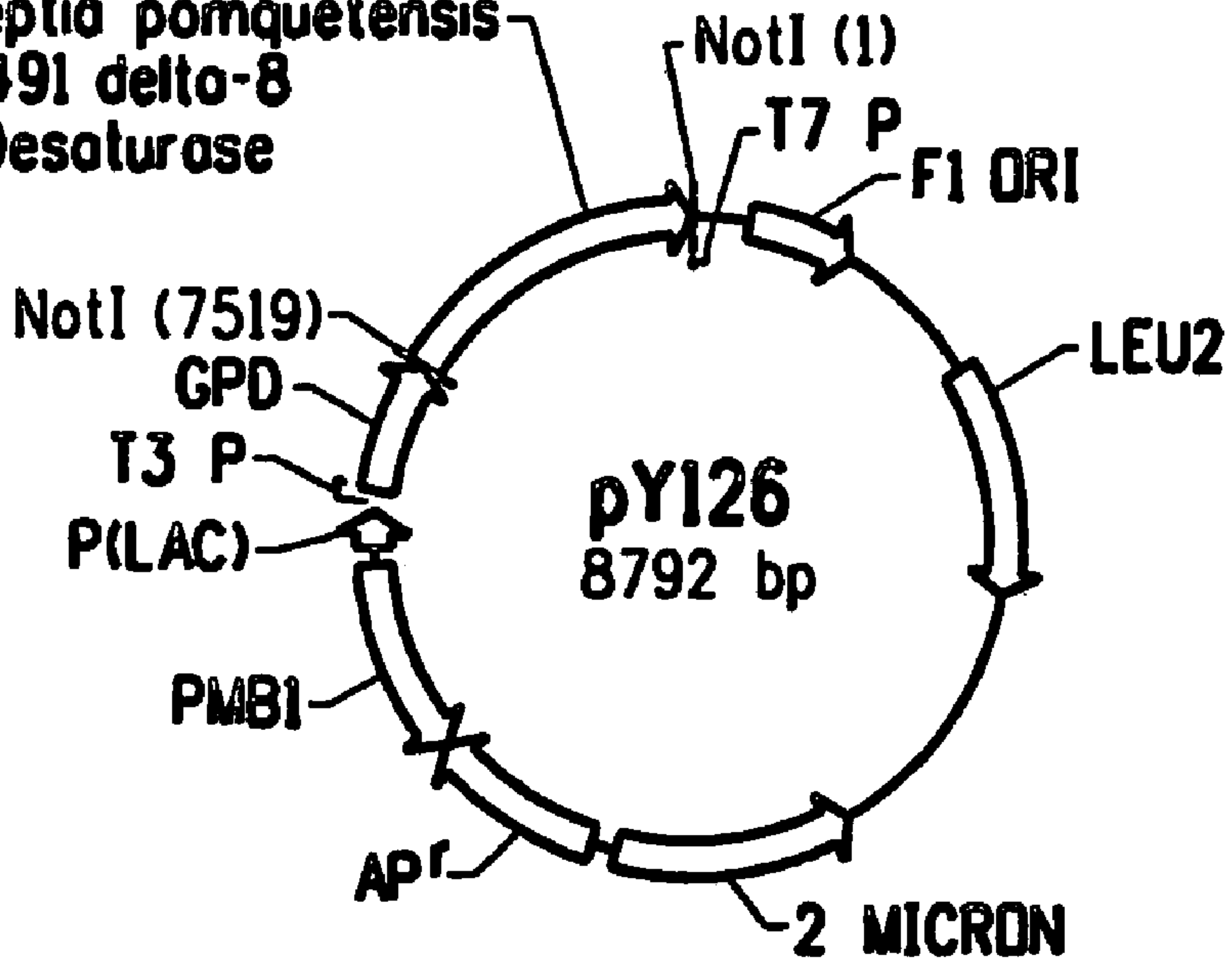


FIG. 1