

**(12) STANDARD PATENT**  
**(19) AUSTRALIAN PATENT OFFICE**

(11) Application No. **AU 2008240028 B2**

(54) Title  
**Delta 9 elongases and their use in making polyunsaturated fatty acids**

(51) International Patent Classification(s)  
**C12N 9/10** (2006.01) **C12N 15/82** (2006.01)  
**C12N 15/54** (2006.01) **C12P 7/64** (2006.01)

(21) Application No: **2008240028** (22) Date of Filing: **2008.04.16**

(87) WIPO No: **WO08/128240**

(30) Priority Data

(31) Number	(32) Date	(33) Country
<b>60/911,925</b>	<b>2007.04.16</b>	<b>US</b>

(43) Publication Date: **2008.10.23**

(44) Accepted Journal Date: **2014.03.06**

(71) Applicant(s)  
**E. I. du Pont de Nemours and Company**

(72) Inventor(s)  
**Zhu, Quinn Qun;Damude, Howard G.**

(74) Agent / Attorney  
**Houlihan2, Level 1 70 Doncaster Road, BALWYN NORTH, VIC, 3104**

(56) Related Art  
**WO 2002/077213 A2**  
**US 2005/0287652 A1**  
**WO 2005/083093 A2**

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 October 2008 (23.10.2008)

PCT

(10) International Publication Number  
**WO 2008/128240 A1**

(51) International Patent Classification:

*C12N 9/10* (2006.01) *C12P 7/64* (2006.01)  
*C12N 15/54* (2006.01) *C12N 15/82* (2006.01)

(21) International Application Number:

PCT/US2008/060393

(22) International Filing Date: 16 April 2008 (16.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/911,925 16 April 2007 (16.04.2007) US

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

(72) Inventors; and

(75) Inventors/Applicants (for US only): **DAMUDE, Howard, G.** [CA/US]; 4 Kenwick Road, Hockessin, Delaware 19707 (US). **ZHU, Quinn, Qun** [US/US]; 544 Revere Road, West Chester, Pennsylvania 19382 (US).

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

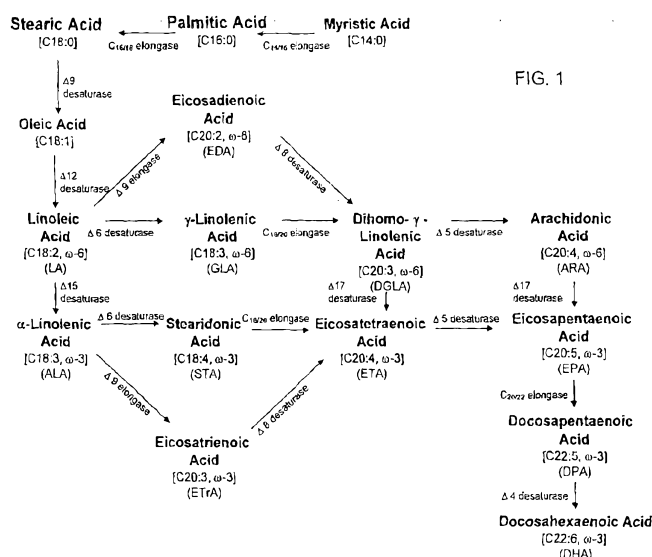
(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, 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.

(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, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description
- with sequence listing part of description published separately in electronic form and available upon request from the International Bureau

(54) Title: DELTA 9 ELONGASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS



(57) Abstract: Isolated nucleic acid fragments and recombinant constructs comprising such fragments encoding delta-9 elongases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) and using these delta-9 elongases in plants.

WO 2008/128240 A1

## TITLE

### DELTA-9 ELONGASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

This application claims the benefit of U.S. Provisional Application  
5 No. 60/911925, filed April 16, 2007, the entire content of which is hereby  
incorporated by reference.

## FIELD OF THE INVENTION

This invention is in the field of biotechnology, in particular, this pertains to  
polynucleotide sequences encoding delta-9 elongases and the use of these  
10 elongases 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  
15 obtained either in the diet or derived by further elongation and desaturation of  
linoleic acid (LA; 18:2  $\omega$ -6) or  $\alpha$ -linolenic acid (ALA; 18:3  $\omega$ -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  
20 several biologically active eicosanoids of importance in mammals (e.g.,  
prostacyclins, eicosanoids, leukotrienes, prostaglandins). Additionally, a high intake  
of long-chain  $\omega$ -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  
25 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  
30 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  
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  $\omega$ -6), eicosapentaenoic acid (EPA; 20:5  $\omega$ -3) and docosahexaenoic acid (DHA; 22:6  $\omega$ -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  $\omega$ -6) and/or eicosatrienoic acid (ETra; 20:3  $\omega$ -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  $\gamma$ -linoleic acid (GLA; 18:3  $\omega$ -6) and/or stearidonic acid (STA; 18:4  $\omega$ -3)) (Figure 6). A delta-6 elongase is also known as a C<sub>18/20</sub> 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<sub>20/22</sub> 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 effort to identify and characterize these enzymes. Initial efforts 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)) discloses 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 C<sub>20</sub> delta-8 desaturase.

5 Also, Applicants' Assignee's co-pending application having U.S. patent application No. 11/737772 (filed April 20, 2007; Attorney Docket No. BB-1566 ) discloses amino acid and nucleic acid sequences for a delta-8 desaturase enzyme from *Pavlova lutheri* (CCMP459). U.S. Patent Application No. 11/876115 (filed October 22, 2007; Attorney Docket No. BB-1574) discloses amino acid and nucleic acid

10 sequences for a delta-8 desaturase enzyme from *Tetruetreptia pomquetensis* CCMP1491, *Eutreptiella* sp. CCMP389 and *Eutreptiella cf. gymnastica* CCMP1594.

Based on the utility of expressing delta-8 desaturases in conjunction with delta-9 elongases, there has also been effort to identify and characterize delta-9 elongases from various sources. Most delta-9 elongase enzymes identified so far

15 have the ability to convert both LA to EDA and ALA to ETrA (wherein DGLA and ETA are subsequently synthesized from EDA and ETrA, respectively, following reaction with a  $\Delta 8$  desaturase; ARA and EPA are subsequently synthesized from DGLA and ETA, respectively, following reaction with a  $\Delta 5$  desaturase; and, DHA synthesis requires subsequent expression of an additional C<sub>20/22</sub> elongase and a  $\Delta 4$

20 desaturase). 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. Application No. 11/601,563 (filed November, 16, 2006, which published May 24, 2007; Attorney Docket No. BB-1562), discloses a delta-9 elongase from

25 *Eulgena gracilis*. Applicants' Assignee's co-pending application having U.S. Application No. 11/601,564 filed November 16, 2006 (Attorney Docket No. CL-3600), discloses a delta-9 elongase from *Eutreptiella* sp. CCMP389.

Applicants' Assignee has a number of patent applications concerning the production of PUFAs in oleaginous yeasts (i.e., *Yarrowia lipolytica*), including: PCT

30 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  
5 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-9 elongase activity, wherein the polypeptide has at least 80% amino acid identity,  
10 based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14;
- (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  
15 nucleotide sequence as set forth in SEQ ID NO:11 or SEQ ID NO:12;
- (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:11 or SEQ ID NO:12;  
or  
20 (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  
25 linked to at least one regulatory sequence.

In a third embodiment, the invention concerns a plant cell comprising in its genome the recombinant DNA construct of the invention.

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

- 5 (a) transforming a plant cell with the recombinant construct of the invention; and
- (b) selecting those transformed plant 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:

- 10 (a) transforming an oilseed plant cell with a first recombinant DNA construct comprising an isolated polynucleotide encoding at least one delta-9 elongase polypeptide, operably linked to at least one regulatory sequence and at least one additional recombinant DNA construct comprising an isolated
- 15 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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub>
- 20 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
- 25 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.

30 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-9 elongase 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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub> 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 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 a representative omega-3 and omega-6 fatty acid pathway providing for the conversion of myristic acid through various intermediates to DHA.

FIG. 2 is a map of plasmid pY115 (SEQ ID NO:19).



FIG. 3 is a map of plasmid pY159 (SEQ ID NO:23).

FIG. 4A is a map of plasmid pY173 (SEQ ID NO:24).

FIG. 4B is a map of plasmid pY174 (SEQ ID NO:25).

FIG. 5 are the fatty acid profiles for *Yarrowia lipolytica* expressing pY173-  
5 pY174 (see Example 4).

FIG. 6 is a map of pKR1140 (SEQ ID NO:30).

FIG. 7 is a map of pKR1151 (SEQ ID NO:39).

FIG. 8 shows a chromatogram of the lipid profile of an *Euglena anabaena* cell  
extract as described in the Examples.

10 FIGs. 9A and 9B shows a comparison of the nucleotide sequences of EaD9E  
(same as EaD9Elo) (SEQ ID NO:11) and EaD9ES (SEQ ID NO:40).

FIG. 10 is map of plasmid pEaD9ES (SEQ ID NO:41).

The sequence descriptions summarize the Sequences Listing attached  
hereto. The Sequence Listing contains one letter codes for nucleotide sequence  
15 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).

FIG. 11 shows five events having the highest average EDA content (average  
of the 5 embryos analyzed) from approximately 30 events transformed with  
20 pKR1140 (SEQ ID NO:30; called Experiment MSE2129). Fatty acids are identified  
as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA and ERA.  
Fatty acid compositions are expressed as a weight percent (wt. %) of total fatty  
acids. Elongation activity is expressed as % delta-9 elongation of C18 fatty acids  
(delta-9 %Elong), calculated according to the following formula:  $([\text{product}]/[\text{substrate}$   
25  $+\text{product}]) \times 100$ . More specifically, the combined percent elongation for LA and ALA  
is determined as:  $([\text{EDA} + \text{ERA}]/[\text{LA} + \text{ALA} + \text{EDA} + \text{ERA}]) \times 100$ . This elongation is  
also referred to as the overall % elongation. The individual omega-6 delta-9  
elongation (LA %Elong) was calculated as:  $([\text{EDA}]/[\text{LA} + \text{EDA}]) \times 100$ . Similarly, the  
individual omega-3 delta-9 elongation (ALA %Elong) was calculated as:  
30  $([\text{ERA}]/[\text{ALA} + \text{ERA}]) \times 100$ . The ratio of delta-9 elongation for omega-6 versus  
omega-3 substrates (Ratio [LA/ALA] %Elong) was obtained by dividing the LA %  
delta-9 elongation by the ALA % delta-9 elongation.

FIG. 12 shows five events having the highest average DGLA content (average of the 5 embryos analyzed) from approximately 30 events transformed with pKR1151 (SEQ ID NO:39; called MSE2131). Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA, ERA, DGLA and ETA. Fatty acid compositions are expressed as a weight percent (wt. %) of total fatty acids. Elongation activity is expressed as % delta-9 elongation of C18 fatty acids (C18 % delta-9 elong), calculated according to the following formula: 
$$\left(\frac{[\text{product}]}{[\text{substrate} + \text{product}]}\right) * 100$$
. More specifically, the combined percent elongation for LA and ALA is determined as: 
$$\left(\frac{[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]}{[\text{LA} + \text{ALA} + \text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]}\right) * 100$$
. The combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as: 
$$\left(\frac{[\text{DGLA} + \text{ETA}]}{[\text{DGLA} + \text{ETA} + \text{EDA} + \text{ERA}]}\right) * 100$$
. This is also referred to as the overall % desaturation.

FIG. 13. shows a schematic depiction of pKR1191.

FIG. 14 shows the lipid profiles of T2 bulk seed for the 18 transformed events transformed with pKR1191. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, , 20:0 (eicosanoic acid), 20:1 (eicosenoic acid), EDA and ERA. Fatty acid compositions are expressed as a weight percent (wt. %) of total fatty acids. The combined percent elongation for LA and ALA is shown as "delta-9 %Elong", determined as: 
$$\left(\frac{[\text{EDA} + \text{ERA}]}{[\text{LA} + \text{ALA}]}\right) * 100$$
. This is also referred to as the overall % elongation.

SEQ ID NO:1 is the nucleotide sequence of the *Euglena anabaena* delta-9 elongase cDNA (EaD9Elo1 cDNA).

SEQ ID NO:2 is the nucleotide sequence of the *Euglena anabaena* delta-9 elongase cDNA (EaD9Elo2 cDNA).

SEQ ID NO:3 is the nucleotide sequence of the *Euglena gracilis* delta-9 elongase coding sequence. (EgD9e).

SEQ ID NO:4 is the nucleotide sequence of the *Euglena gracilis* elongase sense oligonucleotide oEugEL1-1.

SEQ ID NO:5 is the nucleotide sequence of the *Euglena gracilis* elongase sense oligonucleotide oEugEL1-2.

SEQ ID NO:6 is the nucleotide sequence of plasmid pKR906.

SEQ ID NO:7 is the nucleotide sequence of the M13F universal primer.

SEQ ID NO:8 is the nucleotide sequence of the M13-28Rev primer.

SEQ ID NO:9 is the nucleotide sequence of plasmid pLF121-1.

SEQ ID NO:10 is the nucleotide sequence of plasmid pLF121-2.

5 SEQ ID NO:11 is the nucleotide sequence of the *Euglena anabaena* delta-9 elongase coding sequence (EaD8Des1 CDS).

SEQ ID NO:12 is the nucleotide sequence of the *Euglena anabaena* delta-9 elongase coding sequence (EaD8Des2 CDS).

SEQ ID NO:13 is the amino acid sequence of the *Euglena anabaena* delta-9 elongase (EaD9Elo1).

10 SEQ ID NO:14 is the amino acid sequence of the *Euglena anabaena* delta-9 elongase (EaD9Elo2).

SEQ ID NO:15 is the amino acid sequence of the *Isochrysis galbana* delta-9 elongase (IgD9e).

15 SEQ ID NO:16 is the amino acid sequence of the *Euglena gracilis* delta-9 elongase (EgD9e).

SEQ ID NO:17 is the nucleotide sequence of plasmid pDMW263.

SEQ ID NO:18 is the nucleotide sequence of plasmid pDMW237.

SEQ ID NO:19 is the nucleotide sequence of plasmid pY115.

SEQ ID NO:20 is the nucleotide sequence of primer oYFBA1.

20 SEQ ID NO:21 is the nucleotide sequence of primer oYFBA1-6.

SEQ ID NO:22 is the nucleotide sequence of plasmid pY158.

SEQ ID NO:23 is the nucleotide sequence of plasmid pY159.

SEQ ID NO:24 is the nucleotide sequence of plasmid pY173.

SEQ ID NO:25 is the nucleotide sequence of plasmid pY174.

25 SEQ ID NO:26 is the nucleotide sequence of primer oEAd9el1-1.

SEQ ID NO:27 is the nucleotide sequence of primer oEAd9el1-2.

SEQ ID NO:28 is the nucleotide sequence of plasmid pKR1137.

SEQ ID NO:29 is the nucleotide sequence of plasmid pKR72.

SEQ ID NO:30 is the nucleotide sequence of plasmid pKR1140.

30 SEQ ID NO:31 is the nucleotide sequence of *Tetruetreptia pomquetensis* CCMP1491 delta-8 desaturase coding sequence (TpomD8) (which is described in U.S. Patent Application No. 11/876115 (filed October 22, 2007; Attorney Docket No. BB-1574).

SEQ ID NO:32 is the nucleotide sequence of the SMART IV oligonucleotide.

SEQ ID NO:33 is the nucleotide sequence of the Adaptor Primer from Invitrogen 3'-RACE kit.

SEQ ID NO:34 is the nucleotide sequence of primer TpomNot-5.

5 SEQ ID NO:35 is the nucleotide sequence of primer TpomNot-3.

SEQ ID NO:36 is the nucleotide sequence of plasmid pLF114-10.

SEQ ID NO:37 is the nucleotide sequence of plasmid pKR457.

SEQ ID NO:38 is the nucleotide sequence of plasmid pKR1145.

SEQ ID NO:39 is the nucleotide sequence of plasmid pKR1151.

10 SEQ ID NO:40 is the nucleotide sequence of the codon-optimized *Euglena anabaena* delta-9 elongase gene (EaD9ES).

SEQ ID NO:41 is the nucleotide sequence of plasmid pEaD9ES.

SEQ ID NO:42 is the nucleotide sequence of plasmid pKR1191.

#### DETAILED DESCRIPTION OF THE INVENTION

15 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  
20 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-9 elongase enzymes and nucleic acid for encoding the same isolated from *Euglena anabaena*. These are useful for, *inter alia*, for the manipulation of biochemical pathways for the production of PUFAs.

25 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  
30 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 C<sub>12</sub> to C<sub>22</sub> (although both longer and shorter chain-length acids are known). The predominant chain lengths are between C<sub>16</sub> and C<sub>22</sub>. 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 $\omega$ -6
gamma-linolenic	GLA	<i>cis</i> -6,9,12-octadecatrienoic	18:3 $\omega$ -6
eicosadienoic	EDA	<i>cis</i> -11,14-eicosadienoic	20:2 $\omega$ -6
dihomo-gamma-linolenic	DGLA	<i>cis</i> -8,11,14-eicosatrienoic	20:3 $\omega$ -6
sciadonic	SCI	<i>cis</i> -5,11,14-eicosatrienoic	20:3b $\omega$ -6
arachidonic	ARA	<i>cis</i> -5,8,11,14-eicosatetraenoic	20:4 $\omega$ -6
alpha-linolenic	ALA	<i>cis</i> -9,12,15-octadecatrienoic	18:3 $\omega$ -3
stearidonic	STA	<i>cis</i> -6,9,12,15-octadecatetraenoic	18:4 $\omega$ -3

eicosatrienoic	ETrA or ERA	<i>cis</i> -11,14,17-eicosatrienoic	20:3 $\omega$ -3
eicosa-tetraenoic	ETA	<i>cis</i> -8,11,14,17-eicosatetraenoic	20:4 $\omega$ -3
juniperonic	JUP	<i>cis</i> -5,11,14,17-eicosatrienoic	20:4b $\omega$ -3
eicosa-pentaenoic	EPA	<i>cis</i> -5,8,11,14,17-eicosapentaenoic	20:5 $\omega$ -3
docosa-pentaenoic	DPA	<i>cis</i> -7,10,13,16,19-docosapentaenoic	22:5 $\omega$ -3
docosa-hexaenoic	DHA	<i>cis</i> -4,7,10,13,16,19-docosahexaenoic	22:6 $\omega$ -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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and/or a C<sub>20/22</sub> elongase.

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. 1, 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, phosphatidyletanolamine (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 elongase) 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 elongase by transforming a suitable host with the gene for the fatty acid elongase and determining its effect on the fatty acid profile of the host.

For the purposes herein, the terms “EaD9Elo1” or “EaD9E” refers to a delta-9 elongase enzyme (SEQ ID NO:13) isolated from *Euglena anabaena*, encoded by SEQ ID NO:11 herein. The term “EaD9Elo2” refers to a delta-9 elongase enzyme (SEQ ID NO:14) isolated from *Euglena anabaena*, encoded by SEQ ID NO:12 herein. Likewise, the term “EaD9ES” refers to a delta-9 elongase codon-optimized for expression in *Yarrowia lipolytica*.

For the purposes herein, the term “IgD9e” refers to a delta-9 elongase (SEQ ID NO:15) (NCBI Accession No. AAL37626 [GI 17226123], locus AAL37626, CDS AF390174; GenBank Accession No. AF390174) isolated from *Isochrysis galbana*. In contrast, the term “IgD9eS” refers to a synthetic (codon-optimized) delta-9 elongase derived from the DNA sequence of the *Isochrysis galbana* delta-9 elongase 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:16) isolated from *Euglena gracilis*, encoded by SEQ ID NO:3. EgD9e is described in U.S. Application No. 11/601,563 (filed November, 16, 2006, which published May 24, 2007; Attorney Docket No. BB-1562).

Similarly, the term “EgD8” refers to a delta-8 desaturase enzyme 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 (CO<sub>2</sub>) and a  $\beta$ -ketoacyl-CoA (where the acyl moiety has been elongated by two carbon atoms). Subsequent reactions include reduction to  $\beta$ -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  $\beta$ -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<sub>14/16</sub> elongase will utilize a C<sub>14</sub> substrate (e.g., myristic acid), a C<sub>16/18</sub> elongase will utilize a C<sub>16</sub> substrate (e.g., palmitate), a C<sub>18/20</sub> elongase will utilize a C<sub>18</sub> substrate (e.g., GLA, STA) and a C<sub>20/22</sub> elongase will utilize a C<sub>20</sub> 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<sub>16/18</sub> elongase, C<sub>18/20</sub> elongase and/or C<sub>20/22</sub> elongase and may have alternate, but not preferred, specificities for delta-5 and delta-6 fatty acids such as EPA and/or GLA, respectively.

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 deosycytidylate, “G” for guanylate or deoxyguanylate, “U” for uridylate, “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  
5 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)  
10 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  
15 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  
20 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  
25 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  
30 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.,  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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*.  
10 "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  
15 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  
20 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.  
25 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  
30 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  
5 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  
10 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*, 2<sup>nd</sup> Ed., Plenum, 1980). A class of plants identified as oleaginous are commonly referred to  
15 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  
20 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  
25 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*.

The term “Euglenophyceae” refers to a group of unicellular colorless or photosynthetic flagellates (“euglenoids”) found living in freshwater, marine, soil, and  
30 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: *Euglena*, *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. 1).

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

#### Biosynthesis of Omega Fatty Acids

The metabolic process wherein oleic acid is converted to 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. 1 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  
5 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  
10 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<sub>20/22</sub> 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  
15 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<sub>18/20</sub> elongase (also known as delta-6 elongase, the terms can be used interchangeably) (i.e., the “delta-6 desaturase/delta-6  
20 elongase pathway”). More specifically, LA and ALA may be converted to GLA and STA, respectively, by a delta-6 desaturase; then, a C<sub>18/20</sub> 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  
25 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  
30 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<sub>18/20</sub> elongases, delta-5 desaturases, delta-17 desaturases, delta-15 desaturases, delta-9 desaturases, delta-12 desaturases, C<sub>14/16</sub> elongases, C<sub>16/18</sub> elongases, delta-9 elongases, delta-8 desaturases, delta-4 desaturases and C<sub>20/22</sub> 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-9 Elongases

In the present invention, nucleotide sequences encoding delta-9 elongases have been isolated from *Euglena anabaena* (designated herein as "EaD9Elo1" and "EaD9Elo2").

Thus, the present invention concerns an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having delta-9 elongase 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:13 [EaD9Elo1] or SEQ ID NO:14 [EaD9Elo2];

5 (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:11 [EaD9Elo1] or SEQ ID NO:12 [EaD9Elo2]; or,

10 (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-9 elongase 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:11 or SEQ ID NO:12.

In alternate embodiments, the instant EaD9Elo1 and EaD9Elo2 sequences can be codon-optimized for expression in a particular host organism (see SEQ ID NO:40). 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., elongase activity can be synthesized in whole or in part using the codons preferred in the host species.

EaD9Elo1 and/or EaD9Elo2 could be codon-optimized for expression in *Yarrowia lipolytica*, as taught in PCT Publication No. WO 04/101757 and U.S. Patent No. 7,125,672. In one embodiment, it may be desirable to modify a portion of the codons encoding EaD9Elo1 and/or EaD9Elo2 (as set forth in SEQ ID NOs:11

and 13, 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-9 elongase proteins suitable for optimal  
5 expression in alternate hosts, based on the wildtype EaD9Elo1 and/or EaD9Elo2 sequences. Accordingly, the instant invention relates to any codon-optimized delta-9 elongase protein that is derived from the wildtype EaD9Elo1 (i.e., encoded by SEQ ID NO:11) or the wildtype EaD9Elo2 (i.e., encoded by SEQ ID NO:12).

#### Identification and Isolation of Homologs

10 Any of the instant elongase sequences (i.e., EaD9Elo1 or EaD9Elo2) or portions thereof may be used to search for delta-9 elongase 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  
15 modifications.

Alternatively, any of the instant elongase sequences or portions thereof may also be employed as hybridization reagents for the identification of delta-9 elongase 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  
20 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  
25 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.

30 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-9 elongase 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),  
5 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-9  
10 elongases 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  
15 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  
20 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  
25 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  
30 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-9 elongase nucleic acid fragments described herein (or any homologs identified thereof) may be used for creation of new and improved fatty acid elongases. 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 elongase genes. Alternatively, improved fatty acids may be synthesized by domain swapping, wherein a functional domain from any of the delta-9 elongase nucleic acid fragments described herein are exchanged with a functional domain in an alternate elongase 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-9 elongases described herein (i.e., EaD9Elo1, EaD9Elo2 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., LA and/or ALA) to the elongase enzymes described herein (e.g., EaD9Elo1 or EaD9Elo2), such that the substrate is converted to the desired fatty acid product (i.e., EDA and/or ETrA).

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

- (a) a recombinant construct encoding a delta-9 elongase polypeptide selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:14;
- 10 and,
- (b) a source of LA;

wherein the plant host cell is grown under conditions such that the delta-9 elongase is expressed and the LA is converted to EDA, and wherein the EDA is optionally recovered.

15 In alternate embodiments of the present invention, the delta-9 elongase may be used for the use of the enzyme for the conversion of ALA to ETrA. Accordingly the invention provides a method for the production of ETrA, wherein the plant host cell comprises:

- (a) a recombinant construct encoding a delta-9 elongase polypeptide selected from the group consisting of SEQ ID NO:13 and SEQ ID NO:14;
- 20 and,
- (b) a source of ALA;

wherein the plant host cell is grown under conditions such that the delta-9 elongase is expressed and the ALA is converted to ETrA, and wherein the ETrA is optionally  
25 recovered.

Alternatively, each delta-9 elongase 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 (see FIG. 1; see also PCT Publication No. WO 2004/101757). Indirect  
30 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-9 elongases described herein (i.e., EaD9Elo1, EaD9Elo2, 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<sub>18/20</sub> elongases, delta-17 desaturases, delta-8 desaturases, delta-15 desaturases, delta-9 desaturases, delta-12 desaturases, C<sub>14/16</sub> elongases, C<sub>16/18</sub> elongases, delta-9 elongases, delta-5 desaturases, delta-4 desaturases, C<sub>20/22</sub> 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-9 elongases of the present invention will minimally be expressed in conjunction with a delta-8 desaturases (e.g., a delta-8 desaturase or a codon-optimized delta-8 desaturase). 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).

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 herein. Addition of a delta-8 desaturase to a delta-6 desaturase/delta-6 elongase pathway would provided a means to convert the "by-product fatty acids" EDA and ETrA back into the "intermediate fatty acids" (as defined previously) 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-9 elongase 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-9 elongase 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-9 elongase 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*; 2<sup>nd</sup> 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.

5       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.

10       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.

15       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.

20       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-9 elongase polynucleotides of the instant invention and regenerating a plant from the transformed plant cell.

25       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

30

*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.

5           Examples of PUFAs having at least twenty carbon atoms and four or more carbon-carbon double bonds include, but are not limited to, omega-3 fatty acids such as EPA, DPA and DHA and the omega-6 fatty acid ARA. Seeds obtained from such plants are also within the scope of this invention as well as oil obtained from such seeds.

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

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding a delta-9 elongase polypeptide, operably linked to at least one regulatory sequence; and,

15           (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  
20 desaturase, a C<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub> elongase.

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.

25           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:

30           (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:
  - 5 (i) an isolated polynucleotide encoding a delta-9 elongase 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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub> elongase;
- 10 (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) or the delta-9 elongase isolated or derived from *Euglena gracilis*.

- 25 In other preferred embodiments, the at least one additional recombinant DNA construct encodes a polypeptide having delta-8 desaturase activity. For example, 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.* 30 580:1946-1952 (2006)) describes the isolation and characterization of a cDNA from the free living soil amoeba *Acanthamoeba castellanii* that, when expressed in *Arabidopsis*, encodes a C<sub>20</sub> delta-8 desaturase. Also, Applicants' Assignee's co-pending application having U.S. patent application No. 11/737772 (filed April 20,

2007; Attorney Docket No. BB-1566) discloses amino acid and nucleic acid sequences for a delta-8 desaturase enzyme from *Pavlova lutheri* (CCMP459). U.S. Patent Application No. 11/876115 (filed October 22, 2007; Attorney Docket No. BB-1574) discloses amino acid and nucleic acid sequences for a delta-8 desaturase  
5 enzyme from *Tetruetrepia pomquetensis* CCMP1491, *Eutrepitiella* sp. CCMP389 and *Eutrepitiella cf\_gymnastica* CCMP1594.

#### Microbial Expression Systems, Cassettes and Vectors, and Transformation

The delta-9 elongase genes and gene products described herein (i.e., EaD9Elo1, EaD9Elo2, or other mutant enzymes, codon-optimized enzymes or  
10 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  
15 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  
20 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.  
25 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  
30 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-9 elongase 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  
5 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*).

10 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  
15 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  
20 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  
25 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  
30 (particularly *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Yarrowia* or *Kluyveromyces*). The 3'-regions of mammalian genes encoding  $\gamma$ -interferon and  $\alpha$ -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-9 elongase 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.

5           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  
10   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)].

15           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  
20   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  
25   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  
30   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. Thus, the term "transformed" and "recombinant" are used interchangeably 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. WO 2004/101757 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<sup>-</sup> 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<sup>-</sup> 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<sup>-</sup> 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<sup>+</sup> phenotype. Subsequent integration produces a new Ura3<sup>-</sup> 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-9 elongase (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  
5 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  
10 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*  
15 *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  
20 (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;  $\gamma$ -decalactone;  $\gamma$ -dodecalatone; and pyruvic acid. Specific teachings applicable for engineering ARA, EPA and DHA production  
25 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  
30 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-9 elongase

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

5 Thraustochytriales microorganisms are disclosed in U.S. 7,001,772.

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  
10 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  
15 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,  
20 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  
25 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-  
30 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-9 elongase 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 should 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 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  
5 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.,  $\text{Mn}^{+2}$ ,  $\text{Co}^{+2}$ ,  $\text{Zn}^{+2}$ ,  $\text{Mg}^{+2}$ ) 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)).

10 Preferred growth media 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  
15 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  
20 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.

#### 25 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  
30 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  
5 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  
10 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  
15 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  
20 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.

25 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  
30 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.

5 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<sup>®</sup>, Ensure<sup>®</sup>, Jevity<sup>®</sup>, and Alimentum<sup>®</sup> from Ross Products Division,  
10 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  
15 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  
20 of Enfamil LIPIL<sup>™</sup> (Mead Johnson & Company) and Similac Advance<sup>™</sup> (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.

25 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.

30 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  
5 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  
10 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  
15 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  
20 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  
25 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  
30 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  
5 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  
10 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.

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,  
15 "M" means molar, "mmol" means millimole(s), "μmole" means 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).

#### General Methods:

##### 20 Transformation and Cultivation of *Yarrowia lipolytica*:

*Yarrowia lipolytica* strains with ATCC Accession Nos. #20362, #76982 and #90812 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).

25 Transformation of *Yarrowia lipolytica* was performed according to the method of Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.* 48(2):232-235 (1997)), unless otherwise noted. Briefly, *Yarrowia* was streaked onto a YPD plate and grown at 30 °C for approximately 18 h. Several large loopfuls of cells were scraped from the plate and resuspended in 1 mL of transformation buffer, comprising: 2.25 mL of  
30 50% PEG, average MW 3350; 0.125 mL of 2 M lithium 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 μL 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 uracil were added as appropriate to a final concentration of 0.01% (thereby producing "MMU" selection media, 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 µL of 1%) was added to the sample, and then the sample was vortexed and rocked for 20 min. After adding 3 drops of 1 M NaCl and 400 µL hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.

### EXAMPLE 1

#### Synthesis of a cDNA Library From

#### *Euglena anabaena* UTEX 373

The present Example describes the synthesis of a cDNA library from *Euglena*  
5 *anabaena* UTEX 373. This work included the generation of RNA, synthesis of cDNA,  
and generation of a cDNA library.

#### Growth of *Euglena anabaena* UTEX 373 and preparation of RNA

*Euglena anabaena* UTEX 373 was obtained from Dr. Richard Triemer's lab at  
Michigan State University (East Lansing, MI). Approximately 2 mL of culture was  
10 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 incubation, 0.5 mL of  
hexane was added and the vials were further incubated for 15 min at room  
15 temperature with shaking. Fatty acid methyl esters (5 µL 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., Cat. No. 24152). The oven temperature was programmed to hold at 170 °C for  
1.0 min, increase to 240 °C at 5 °C /min and then hold for an additional 1.0 min.  
20 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. Cat. No. U-99-A) and the resulting chromatogram is shown in FIG. 8. The  
presence of EDA, ERA, EPA and DHA in the fatty acid profile, with the absence of  
GLA and STA, suggested that *Euglena anabaena* uses the delta-9 elongase/delta-8  
25 desaturase pathway for LC-PUFA biosynthesis and would be a good source for LC-  
PUFA biosynthetic genes such as, but not limited to, delta-9 elongases.

The remaining 5 mL of an actively growing culture was transferred into 25 mL  
of AF-6 Medium (Watanabe & Hiroki, NIES-Collection List of Strains, 5<sup>th</sup> ed.,  
National Institute for Environmental Studies, Tsukuba, 127 pp (2004)) in a 125 mL  
30 glass flask. *Euglena anabaena* cultures were grown at 22 °C with a 16 h light, 8 h  
dark cycle for 2 weeks with very gentle agitation.

After 2 weeks, the culture (25 mL) was transferred to 100 mL of AF-6 medium  
in a 500 mL glass bottle and the culture was grown for 1 month as described above.

After this time, two 50 mL aliquots were transferred into two separate 500 mL glass bottles containing 250 mL of AF-6 medium and the cultures were grown for two months as described above (giving a total of ~600 mL of culture). After this, the cultures were pelleted by centrifugation at 1,800 x g for 10 min, washed once with water and re-centrifuged. Total RNA was extracted from one of the resulting pellets 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, 340 µg of total RNA (680 µg/mL) was obtained from the pellet. The remaining pellet was frozen in liquid nitrogen and stored at -80 °C. The mRNA was isolated from all 340 µg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol provided. In this way, 9.0 µg of mRNA was obtained.

#### Preparation of *Euglena anabaena* cDNA and generation of cDNA library eug1c

A cDNA library was generated using the Cloneminer™ cDNA Library Construction Kit (Cat. 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 5.12 µg of mRNA (described above) using the Biotin-*attB2*-Oligo(dT) primer. After synthesis of the first and second strand, the *attB1* adapter was added, ligated and the cDNA was size fractionated using column chromatography. DNA from fractions were concentrated, recombined into pDONR™222 and transformed into *E. coli* ElectroMAX™ DH10B™ T1 Phage-Resistant cells (Invitrogen Corporation). The *Euglena anabaena* library was named eug1c.

The cDNA library eug1c was plated onto LBKan plates (approx. 100,000 colonies), the colonies were scraped off and DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. In this way, a plasmid DNA sub-library from eug1c was obtained.

### EXAMPLE 2

#### Isolation of the Full-length Delta-9 Elongases

from *Euglena anabaena* UTEX 373

The present Example describes the identification of cDNAs (SEQ ID NOs:1 and 2) encoding delta-9 elongases from *Euglena anabaena* UTEX 373. This work

included the generation of a probe derived from the *Euglena gracilis* delta-9 elongase (EgD9e; SEQ ID NO:3) and the hybridization of the probe to the cDNA library eug1c in order to identify delta-9 elongase homologs from *Euglena anabaena* UTEX 373.

5 *Euglena gracilis* delta-9 elongase (EgD9e):

A clone from the *Euglena* cDNA library (eeg1c), called eeg1c.pk001.n5f, containing the *Euglena gracilis* delta-9 elongase (EgD9e; SEQ ID NO:3; which is described in U.S. Application No. 11/601,563 (filed November, 16, 2006, which published May 24, 2007; Attorney Docket No. BB-1562) the contents of which are  
10 hereby incorporated by reference) was used as template to amplify EgD9e with oligonucleotide primers oEugEL1-1 (SEQ ID NO:4) and oEugEL1-2 (SEQ ID NO:5) using the VentR<sup>®</sup> DNA Polymerase (Cat. No. M0254S, New England Biolabs Inc., Beverly, MA) following the manufacturer's protocol. The resulting DNA fragment was cloned into the pCR-Blunt<sup>®</sup> cloning vector using the Zero Blunt<sup>®</sup> PCR Cloning  
15 Kit (Invitrogen Corporation), following the manufacturer's protocol, to produce pKR906 (SEQ ID NO:6).

Colony Lifts:

Approximately 17,000 clones of cDNA library eug1c were plated onto three large square (24 cm x 24 cm) petri plates (Corning, Corning, NY) each containing  
20 LB + 50 µg/mL kanamycin agar media. Cells were grown overnight at 37 °C and plates were then cooled to room temperature.

Biodyne B 0.45 µm membrane (Cat. No. 60207, Pall Corporation, Pensacola, FL) was trimmed to approximately 22 cm x 22 cm and the membrane was carefully  
25 layed on top of the agar to avoid air bubbles. After incubation for 2 min at room temperature, the membrane was marked for orientation, lifted off with tweezers and placed colony-side up on filter paper soaked with 0.5 M sodium hydroxide and 1.5 M sodium chloride. After denaturation for 4 min, the sodium hydroxide was  
neutralized by placing the membrane on filter paper soaked with 0.5 M Tris-HCL (pH 7.5) and 1.5 M sodium chloride for 4 min. This step was repeated and the  
30 membrane was rinsed briefly in 2X SSC buffer (20X SSC is 3M sodium chloride, 0.3 M sodium citrate; pH 7.0) and air dried on filter paper.

Hybridization:

Membranes were pre-hybridized at 65 °C in 200 mL hybridization solution for 2 h. Hybridization solution contained 6X SSPE (20X SSPE is 3 M sodium chloride, 0.2 M sodium phosphate, 20 mM EDTA; pH 7.4), 5X Denhardt's reagent (100X  
5 Denhardt's reagent is 2%(w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) acetylated bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 100 µg/mL sheared salmon sperm DNA and 5% dextran sulfate.

A DNA probe was made using an agarose gel purified NcoI/NotI DNA fragment, containing the *Euglena gracilis* delta-9 elongase gene, from pKR906  
10 (SEQ ID NO:6) labeled with P<sup>32</sup> dCTP using the RadPrime DNA Labeling System (Cat. No. 18428-011, Invitrogen, Carlsbad, CA) following the manufacture's instructions. Unincorporated P<sup>32</sup> dCTP was separated using a NICK column (Cat. No. 17-0855-02, Amersham Biosciences, Piscataway, NJ) following the  
15 manufacturer's instructions. The probe was denatured for 5 min at 100 °C, placed on ice for 3 min and half was added to the hybridization solution.

The membrane was hybridized with the probe overnight at 65 °C with gentle shaking and then washed the following day twice with 2X SSC containing 0.5% SDS (5 min each) and twice with 0.2X SSC containing 0.1% SDS (15 min each). After washing, hyperfilm (Cat. No. RPN30K, Amersham Biosciences, Piscataway,  
20 NJ) was exposed to the membrane overnight at -80 °C.

Based on alignment of plates with the exposed hyperfilm, positive colonies were picked using the blunt end of a Pasteur pipette into 1 mL of water and vortexed. Several dilutions were made and plated onto small round Petri dishes (82 mm) containing LB media plus 50 µg/mL kanamycin to obtain around 100 well  
25 isolated colonies on a single plate. Lifts were done as described above except NytranN membrane circles (Cat. No. 10416116, Schleicher & Schuell, Keene, NH) were used and hybridization was carried out in 100 mL using the remaining radiolabeled probe. In this way, positive clones were confirmed.

Individual positive clones were grown at 37 °C in LB + 50 µg/mL kanamycin  
30 liquid media and plasmid was purified using the QIAprep® Spin Miniprep Kit (Qiagen Inc.) following the manufacturer's protocol.

DNA inserts were end-sequenced in 384-well plates, using vector-primed M13F universal primer (SEQ ID NO:7), M13rev-28 primer (SEQ ID NO:8) and the

poly(A) tail-primed WobbleT oligonucleotides, with the ABI BigDye version 3 Prism sequencing kit. For the sequencing reaction, 100-200 ng of template and 6.4 pmol of primer 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 3700 automated sequencers. 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.

Sequences were aligned and compared using Sequencher™ (Version 4.2, Gene Codes Corporation, Ann Arbor, MI) and in this way, the clones could be categorized into one of two distinct groups based on insert sequence (called EaD9Elo1 and EaD9Elo2). Representative clones containing the cDNA for each class of sequence were chosen for further study and sequences for each representative plasmid (pLF121-1 and pLF121-2) are shown in SEQ ID NO:9 and SEQ ID NO:10, respectively. The sequence shown by a string of NNNN's represents a region of the polyA tail which was not sequenced. The coding sequences for EaD9Elo1 and EaD9Elo2 are shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. The corresponding amino acid sequences for EaD9Elo1 and EaD9Elo2 are shown in SEQ ID NO:13 and SEQ ID NO:14, respectively.

### EXAMPLE 3

#### Primary Sequence Analysis of the Delta-9 Elongase Sequences of *Euglena anabaena* UTEX 373 (EaD9Elo1 and EaD9Elo2) and Comparison to a Delta-9 Elongase Sequence of *Euglena gracilis* (EgD9e)

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were compared using 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)) 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).

Compared to a EaD9Elo1 (SEQ ID NO:13), EaD9Elo2 (SEQ ID NO:14) has 1 amino acid substitution (R254Q; based on numbering for EaD9Elo1).

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were evaluated by BLASTP (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) using default parameters with the filter turned off. 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.

Both sequences yielded a pLog value of 38.70 (P value of  $2e-39$ ) versus the *Isochrysis galbana* long chain polyunsaturated fatty acid elongation enzyme (IgD9e; SEQ ID NO:15) (NCBI Accession No. AAL37626(GI 17226123), locus AAL37626, CDS AF390174 ; Qi et al., *FEBS Lett.* 510:159-165 (2002)) when compared to the "nr" database. BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire *Euglena anabaena* delta-9 fatty acid elongase.

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were compared to the *Isochrysis galbana* long chain polyunsaturated fatty acid elongation enzyme (IgD9e; SEQ ID NO:15) and the *Euglena gracilis* delta-9 elongase amino acid sequence (EgD9e; SEQ ID NO:16; which is described in U.S. Application No. 11/601,563 (filed November, 16, 2006, which published May 24, 2007; Attorney Docket No. BB-1562) the contents of which are hereby incorporated by reference) using BlastP, Clustal V and the Jotun Hein methods of sequence comparison. The % identity against the IgD9e and EgD9e using each method is shown in Table 4 and Table 5, respectively.

Sequence percent identity calculations performed by the BlastP and Clustal V method as described above. 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).

TABLE 4

Sequence Comparison of EaD9Elo1 (SEQ ID NO:13) and  
EaD9Elo2 (SEQ ID NO:14) to IgD9e (SEQ ID NO:15)

Desaturase	% Identity to IgD9e by BLASTP	% Identity to IgD9e by the Jotun Hein Method	% Identity to IgD9e by the Clustal V Method
EaD9Elo1 (SEQ ID NO:13)	37%	40.4%	32.9%
EaD9Elo2 (SEQ ID NO:14)	37%	41.2%	32.9%

TABLE 5

Sequence Comparison of EaD9Elo1 (SEQ ID NO:13) and  
EaD9Elo2 (SEQ ID NO:14) to EgD9e (SEQ ID NO:16)

Desaturase	% Identity to EgD9e by BLASTP	% Identity to EgD9e by the Jotun Hein Method	% Identity to EgD9e by the Clustal V Method
EaD9Elo1 (SEQ ID NO:13)	77%	77.2%	77.1%
EaD9Elo2 (SEQ ID NO:14)	77%	77.2%	77.1%

## EXAMPLE 4

Functional Analysis of the *Euglena gracilis* UTEX 373 Delta-9 Elongases  
in *Yarrowia lipolytica*

The present Example describes functional analysis of EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) in *Yarrowia lipolytica*. This work included the following steps: (1) Construction of Gateway®-compatible *Yarrowia* expression vector pY159; (2) transfer of EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) into pY159 to produce pY173 and pY174; and, (3) comparison of lipid profiles within transformant organisms comprising pY173 and pY174.

Construction of Gateway®-compatible *Yarrowia* expression vector pY159

Plasmid pY5-30 (which was previously described in PCT Publication No. WO 2005/003310 (the contents of which are hereby incorporated by reference), is a



shuttle plasmid that can replicate both in *E. coli* and *Yarrowia lipolytica*. Plasmid pY5-30 contains the following: a *Yarrowia* autonomous replication sequence (ARS18); a ColE1 plasmid origin of replication; an ampicillin-resistance gene (Amp<sup>R</sup>), for selection in *E. coli*; a *Yarrowia* LEU2 gene, for selection in *Yarrowia*;  
 5 and a chimeric TEF::GUS::XPR gene. Plasmid pDMW263 (SEQ ID NO:17) was created from pY5-30, by replacing the TEF promoter with the *Yarrowia lipolytica* FBAINm promoter (PCT Publication No. WO 2005/049805) using techniques well known to one skilled in the art. Briefly, this promoter refers to a modified promoter which is located in the 5' upstream untranslated region in front of the 'ATG'  
 10 translation initiation codon of the fructose-bisphosphate aldolase enzyme (E.C. 4.1.2.13) encoded by the *fab1* gene and that is necessary for expression, plus a portion of 5' coding region that has an intron, wherein FBAINm has a 52 bp deletion between the ATG translation initiation codon and the intron of the FBAIN promoter (thereby including only 22 amino acids of the N-terminus) and a new translation  
 15 consensus motif after the intron. Table 6 summarizes the components of pDMW263 (SEQ ID NO:17).

TABLE 6Components of Plasmid pDMW263 (SEQ ID NO:17)

RE Sites and Nucleotides Within SEQ ID NO:17	Description of Fragment and Chimeric Gene Components
4992-4296	ARS18 sequence (GenBank Accession No. A17608)
<i>Sall/SacII</i> (8505-2014)	FBAINm::GUS::XPR, comprising: <ul style="list-style-type: none"> <li>• FBAINm: FBAINm promoter (WO2005/049805)</li> <li>• GUS: <i>E. coli</i> gene encoding <math>\beta</math>-glucuronidase (Jefferson, R.A. <i>Nature</i>. 14:342:837-838 (1989))</li> <li>• XPR: ~100 bp of the 3' region of the <i>Yarrowia Xpr</i> gene (GenBank Accession No. M17741)</li> </ul>
6303-8505	<i>Yarrowia Leu2</i> gene (GenBank Accession No. AF260230)

20 The *NcoI/SalI* DNA fragment from pDMW263 (SEQ ID NO:17), containing the *Yarrowia lipolytica* FBAINm promoter, was cloned into the *NcoI/SalI* DNA fragment

of pDMW237 (SEQ ID NO:18), previously described in PCT Publication No. WO 2006/012325 (the contents of which are hereby incorporated by reference), containing a synthetic delta-9 elongase gene derived from *Isochrysis galbana* and codon-optimized for expression in *Yarrowia lipolytica* (IgD9eS), to produce pY115 (SEQ ID NO:19; FIG. 2). In FIG. 2, the modified FBAINm promoter is called FBA1 + Intron. It is also FBA1 + Intron in other figures, as well as YAR FBA1 PRO + Intron and these terms are used interchangeably with FBAINm.

The FBAINm promoter was amplified from plasmid pY115 (SEQ ID NO:19), using PCR with oligonucleotide primers oYFBA1 (SEQ ID NO:20) and oYFBA1-6 (SEQ ID NO:21). Primer oYFBA1 (SEQ ID NO:20) was designed to introduce an *Bgl*II site at the 5' end of the promoter and primer oYFBA1-6 (SEQ ID NO:21) was designed to introduce a *Not*I site at the 3' end of the promoter while removing the *Nco*I site and thus, the ATG start codon. The resulting PCR fragment was digested with *Bgl*II and *Not*I and cloned into the *Bgl*II/*Not*I fragment of pY115, containing the vector backbone, to form pY158 (SEQ ID NO:22).

Plasmid pY158 (SEQ ID NO:22) was digested with *Not*I and the resulting DNA ends were filled. After filling to form blunt ends, the DNA fragments were treated with calf intestinal alkaline phosphatase and separated using agarose gel electrophoresis. The 6992 bp fragment containing the *Yarrowia lipolytica* FBAINm promoter was excised from the agarose gel and purified using the QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. The purified 6992 bp fragment was ligated with cassette rfA using the Gateway Vector Conversion System (Cat. No. 11823-029, Invitrogen Corporation) following the manufacturer's protocol to form *Yarrowia lipolytica* Gateway® destination vector pY159 (SEQ ID NO:23; FIG. 3).

#### Construction of *Yarrowia* expression vectors pY173 and pY174

Using the Gateway® LR Clonase™ II enzyme mix (Cat. No. 11791-020, Invitrogen Corporation) and following the manufacturer's protocol, the cDNA inserts from pLF121-1 (SEQ ID NO:9) and pLF121-2 (SEQ ID NO:10) were transferred to pY159 (SEQ ID NO:23) to form pY173 (SEQ ID NO:24, FIG. 4A) and pY174 (SEQ ID NO:25; FIG. 4B), respectively.

Functional analysis of EaD9Elo1 and EaD9Elo2 in *Yarrowia lipolytica*

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.

Strain Y2224 was transformed with pY173 (SEQ ID NO:24, FIG. 4A) and pY174 (SEQ ID NO:25; FIG. 4B) as described in the General Methods.

Single colonies of transformant *Yarrowia lipolytica* containing pY173 and pY174 were grown in 3 mL minimal media lacking uraci at 30 °C for 16 h after which cells were centrifuged at 250 rpm to pellet. 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 1M sodium chloride and 100 µL of heptane were added. After thorough mixing and centrifugation, fatty acid methyl esters (FAMES) were analyzed by GC. FAMES (5 µL injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Cat. No. 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.).

The fatty acid profiles for *Yarrowia lipolytica* expressing pY173 and pY174 are shown in FIG. 5. Percent delta-9 elongation (delta-9 %Elong) was calculated either by dividing the wt. % for EDA by the sum of the wt. % for EDA and LA and multiplying by 100 to express as a %. Average is indicated by Ave. followed by appropriate header.

### EXAMPLE 5

#### Construction of Soybean Expression Vector pKR1140 for Expression of *Euglena anabaena* UTEX 373 Delta-9 Elongase (EaD9Elo1)

The present Example describes construction of a soybean vector for  
5 expression of EaD9Elo1. This work included the following steps: (1) PCR  
amplification of EaD9Elo1 with appropriate restriction sites for cloning from plasmids  
described in Example 2; (2) cloning of the EaD9Elo1 PCR products into cloning  
vector pCR-Blunt® (Invitrogen Corporation) to produce pKR1137; (3) cloning  
EaD9Elo1 into soybean expression vector pKR72 to produce pKR1140.

10 In order to introduce *NotI* and *NcoI* restriction sites at the 5' end of the coding  
sequences and a *NotI* site at the 3' end of the coding sequences, EaD9Elo1 was  
PCR amplified. The coding sequence for EaD9Elo1 (SEQ ID NO:11) was amplified  
from pLF121-1 (SEQ ID NO:9) with oligonucleotide primers oEAd9el1-1 (SEQ ID  
NO:26) and oEAd9el1-2 (SEQ ID NO:27) using the Phusion™ High-Fidelity DNA  
15 Polymerase (Cat. No. F553S, Finnzymes Oy, Finland) following the manufacturer's  
protocol. The resulting DNA fragments were cloned into the pCR-Blunt® cloning  
vector using the Zero Blunt® PCR Cloning Kit (Invitrogen Corporation), following the  
manufacturer's protocol, to produce pKR1137 (SEQ ID NO:28).

A starting plasmid pKR72 (ATCC Accession No. PTA-6019; SEQ ID NO:29,  
20 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  
25 replication (*ori*) for selection and replication in bacteria (e.g., *E. coli*). In addition,  
pKR72 (SEQ ID NO:29) 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 (SEQ ID NO:29) also contains a *NotI* restriction  
30 site, flanked by the promoter for the  $\alpha'$  subunit of  $\beta$ -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.

EaD9Elo1 was released from pKR1137 (SEQ ID NO:28) by digestion with *NotI* and cloned into the *NotI* site of pKR72 (SEQ ID NO:29) to produce pKR1140 (SEQ ID NO:30).

#### EXAMPLE 6

##### Construction of Soybean Expression Vector pKR1151 for Co-Expression of the *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8) with a Delta-9 Elongase Derived from *Euglena anabaena* (EaD9Elo1)

The present Example describes construction of a soybean vector for co-expression of TpomD8 (SEQ ID NO:31; which is described in U.S. Patent Application No. 11/876115 (filed October 22, 2007; Attorney Docket No. BB-1574)) with EaD9Elo1.

##### *Tetruetreptia pomquetensis* CCMP1491 Delta-8 Desaturase (TpomD8):

*Tetruetreptia pomquetensis* CCMP1491 cells (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 was isolated using the trizol reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's protocol. The cell pellet was 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 mixture was centrifuged in an Eppendorf centrifuge for 30 sec at 14,000 rpm to remove debris and glass beads. Supernatant was extracted with 150  $\mu$ L of 24:1 chloroform:isoamy alcohol. The upper aqueous phase was used for RNA isolation.

For RNA isolation, the aqueous phase was mixed with 0.375 mL of isopropyl alcohol and allowed to incubate at room temperature for 5 min. Precipitated RNA was collected by centrifugation at 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, 95  $\mu$ g of total RNA was obtained from *Tetruetreptia pomquetensis* CCMP1491.

Total RNA (0.95  $\mu$ g of total RNA in 1  $\mu$ L) was used as template to synthesize double stranded cDNA. The Creator<sup>TM</sup> SMART<sup>TM</sup> cDNA Library Construction Kit from BD Bioscience Clontech (Palo Alto, CA) was used. Total RNA (1  $\mu$ L) was

mixed with 1  $\mu$ L of SMART IV oligonucleotide (SEQ ID NO:32) 1  $\mu$ L of the Adaptor Primer from Invitrogen 3'-RACE kit (SEQ ID NO:33) and 2  $\mu$ L of water. The mixture was heated to 75 °C for 5 min and then cooled on ice for 5 min. To the mixture was added, 2  $\mu$ L of 5X first strand buffer, 1  $\mu$ L 20 mM DTT, 1  $\mu$ L of dNTP mix (10 mM  
5 each of dATP, dCTP, dGTP and dTTP) and 1  $\mu$ L of PowerScript reverse transcriptase. The sample was incubated at 42 °C for 1 h. The resulting first strand cDNAs were then used as template for amplification.

The *Tetruetreptia pomquetensis* CCMP1491 (TpomD8; SEQ ID NO:31) was amplified from the cDNA with oligonucleotide primers TpomNot-5 (SEQ ID NO:34)  
10 and TpomNot-3 (SEQ ID NO:35) using *Taq* polymerase (Invitrogen Corporation) following the manufacturer's protocol.

*Tetruetreptia pomquetensis* CCMP1491 cDNA (1  $\mu$ L) was combined with 50 pmol of TpomNot-5 (SEQ ID NO:34), 50 pmol of TpomNot-3 (SEQ ID NO:35), 1  $\mu$ L of PCR nucleotide mix (10 mM, Promega, Madison, WI), 5  $\mu$ L of 10X PCR buffer  
15 (Invitrogen Corporation), 1.5  $\mu$ L of MgCl<sub>2</sub> (50 mM, Invitrogen Corporation), 0.5  $\mu$ L of *Taq* polymerase (Invitrogen Corporation) and water to 50  $\mu$ 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  $\mu$ L  
20 and a DNA band with molecular weight around 1.3 kb was observed.

The remaining 45  $\mu$ 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  
25 manufacturer's protocol to produce pLF114-10 (SEQ ID NO:36).

Vector pKR457 (SEQ ID NO:37), which was previously described in PCT Publication No. WO 2005/047479 (the contents of which are hereby incorporated by reference), contains a *NotI* site flanked by the Kunitz soybean Trypsin Inhibitor (KTI) promoter (Jofuku et al., *Plant Cell* 1:1079-1093 (1989)) and the KTI 3' termination  
30 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/*NotI*/Kti3'Salb3' cassette).

The *NotI* fragment of pLF114-10 (SEQ ID NO:36), containing the *TpomD8* gene was cloned into the *NotI* site of pKR457 (SEQ ID NO:37), to produce pKR1145 (SEQ ID NO:38).

5 The *BsWI* fragment from pKR1145 (SEQ ID NO:38), containing the *TpomD8* gene, was cloned into the *BsWI* site of pKR1140 (SEQ ID NO:30) to produce pKR1151 (SEQ ID NO:39; FIG. 7). the *Euglena anabaena* delta-9 elongase (EaD9Elo1) is called EA D9elong in fig. 7.

#### EXAMPLE 7

#### Production and Model System Transformation of Somatic Soybean Embryo Cultures

#### 10 with Soybean Expression Vectors and Plant Regeneration

##### Culture Conditions:

Soybean embryogenic suspension cultures (cv. Jack) are maintained in 35 mL liquid medium SB196 (*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  
15  $\mu\text{E}/\text{m}^2/\text{s}$ . Cultures are 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 are transformed with the soybean expression plasmids by the method of particle gun bombardment (Klein et al.,  
20 *Nature* 327:70 (1987)) using a DuPont Biolistic PDS1000/HE instrument (helium retrofit) for all transformations.

##### Soybean Embryogenic Suspension Culture Initiation:

Soybean cultures are initiated twice each month with 5-7 days between each initiation. Pods with immature seeds from available soybean plants are picked 45-  
25 55 days after planting. Seeds are removed from the pods and placed into a sterilized magenta box. The soybean seeds are 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 are rinsed using 2 1-liter bottles of sterile distilled water and those less than 4 mm are placed  
30 on individual microscope slides. The small end of the seed is cut and the cotyledons pressed out of the seed coat. When cultures are being prepared for production transformation, cotyledons are transferred to plates containing SB1 medium (25-30 cotyledons per plate). Plates are wrapped with fiber tape and are

maintained at 26 °C with cool white fluorescent lights on 16:8 h day/night photoperiod at light intensity of 60-80  $\mu\text{E}/\text{m}^2/\text{s}$  for eight weeks, with a media change after 4 weeks. When cultures are being prepared for model system experiments, cotyledons are transferred to plates containing SB199 medium (25-30 cotyledons per plate) for 2 weeks, and then transferred to SB1 for 2-4 weeks. Light and temperature conditions are the same as described above. After incubation on SB1 medium, secondary embryos are 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 are used for bombardment. Fragments from soybean expression plasmids are obtained by gel isolation of digested plasmids. In each case, 100  $\mu\text{g}$  of plasmid DNA is used in 0.5 mL of the specific enzyme mix described below. Plasmids are digested with *AscI* (100 units) in NEBuffer 4 (20 mM Tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 1 mM dithiothreitol, pH 7.9), 100  $\mu\text{g}/\text{mL}$  BSA, and 5 mM beta-mercaptoethanol at 37 °C for 1.5 hr. The resulting DNA fragments are separated by gel electrophoresis on 1% SeaPlaque GTG agarose (BioWhittaker Molecular Applications) and the DNA fragments containing gene cassettes are cut from the agarose gel. DNA is purified from the agarose using the GELase digesting enzyme following the manufacturer's protocol.

A 50  $\mu\text{L}$  aliquot of sterile distilled water containing 3 mg of gold particles (3 mg gold) is added to 30  $\mu\text{L}$  of a 10 ng/ $\mu\text{L}$  DNA solution (either intact plasmid or DNA fragment prepared as described herein), 25  $\mu\text{L}$  5M  $\text{CaCl}_2$  and 20  $\mu\text{L}$  of 0.1 M spermidine. The mixture is shaken 3 min on level 3 of a vortex shaker and spun for 10 sec in a bench microfuge. The supernatant is removed, followed by a wash with 400  $\mu\text{L}$  100% ethanol and another brief centrifugation. The 400  $\mu\text{L}$  ethanol is removed and the pellet is resuspended in 40  $\mu\text{L}$  of 100% ethanol. Five  $\mu\text{L}$  of DNA suspension is dispensed to each flying disk of the Biolistic PDS1000/HE instrument disk. Each 5  $\mu\text{L}$  aliquot contains approximately 0.375 mg gold per bombardment (e.g., per disk).

For model system transformations, the protocol is identical except for a few minor changes (ie, 1 mg of gold particles is added to 5  $\mu\text{L}$  of a 1  $\mu\text{g}/\mu\text{L}$  DNA



solution, 50  $\mu$ L of a 2.5M  $\text{CaCl}_2$  is used and the pellet is ultimately resuspended in 85  $\mu$ L of 100% ethanol thus providing 0.058 mg of gold particles per bombardment).

Tissue Preparation and Bombardment with DNA:

Approximately 150-200 mg of seven day old embryogenic suspension  
5 cultures is placed in an empty, sterile 60 x 15 mm petri dish and the dish is covered with plastic mesh. The chamber is evacuated to a vacuum of 27-28 inches of mercury, and tissue is bombarded one or two shots per plate with membrane rupture pressure set at 1100 PSI. Tissue is placed approximately 3.5 inches from the retaining /stopping screen. Model system transformation conditions are identical  
10 except 100-150 mg of embryogenic tissue is used, rupture pressure is set at 650 PSI and tissue is place approximately 2.5 inches from the retaining screen.

Selection of Transformed Embryos:

Transformed embryos are selected either using hygromycin (when the hygromycin B phosphotransferase (HPT) gene is used as the selectable marker) or  
15 chlorsulfuron (when the acetolactate synthase (ALS) gene is used as the selectable marker).

Following bombardment, the tissue is placed into fresh SB196 media and cultured as described above. Six to eight days post-bombardment, the SB196 is exchanged with fresh SB196 containing either 30 mg/L hygromycin or 100 ng/mL  
20 chlorsulfuron, depending on the selectable marker used. The selection media is refreshed weekly. Four to six weeks post-selection, green, transformed tissue is observed growing from untransformed, necrotic embryogenic clusters.

Embryo Maturation:

For production transformations, isolated, green tissue is removed and  
25 inoculated into multiwell plates to generate new, clonally propagated, transformed embryogenic suspension cultures. Transformed embryogenic clusters are cultured for four-six weeks in multiwell plates 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  
30 time embryo clusters are removed to a solid agar media, SB166, for one-two weeks and then subcultured to SB103 medium for 3-4 weeks to mature embryos. After maturation on plates in SB103, individual embryos are removed from the clusters,

dried and screened for alterations in their fatty acid compositions as described previously.

For model system transformations, embryos are matured in soybean histodifferentiation and maturation liquid medium (SHaM liquid media; Schmidt et al., *Cell Biology and Morphogenesis* 24:393 (2005)) using a modified procedure. Briefly, after 4 weeks of selection in SB196 as described above, embryo clusters are removed to 35 mL of SB228 (SHaM liquid media) in a 250 mL Erlenmeyer flask. Tissue is maintained in SHaM liquid media 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  $\mu\text{E}/\text{m}^2/\text{s}$  for 2 weeks as embryos mature. Embryos grown for 2 weeks in SHaM liquid media are equivalent in size and fatty acid content to embryos cultured on SB166/SB103 for 5-8 weeks.

After maturation in SHaM liquid media, individual embryos are removed from the clusters, dried and screened for alterations in their fatty acid compositions as described previously.

#### Media Recipes:

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

	MS FeEDTA - 100x Stock 1	10 mL
20	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 (10mg/L final concentration)	1.0 mL
25	KNO <sub>3</sub>	2.83 gm
	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.463 gm
	asparagine	1.0 gm
	sucrose (1%)	10 gm
	pH 5.8	

30

FN Lite Stock Solutions

	Stock Number		1000 mL	500 mL
	1	MS Fe EDTA 100x Stock		
		Na <sub>2</sub> EDTA*	3.724 g	1.862 g
5		FeSO <sub>4</sub> - 7H <sub>2</sub> O	2.784 g	1.392 g
		*Add first, dissolve in dark bottle while stirring		
	2	MS Sulfate 100x stock		
		MgSO <sub>4</sub> - 7H <sub>2</sub> O	37.0 g	18.5 g
10		MnSO <sub>4</sub> - H <sub>2</sub> O	1.69 g	0.845 g
		ZnSO <sub>4</sub> - 7H <sub>2</sub> O	0.86 g	0.43 g
		CuSO <sub>4</sub> - 5H <sub>2</sub> O	0.0025 g	0.00125 g
	3	FN Lite Halides 100x Stock		
15		CaCl <sub>2</sub> - 2H <sub>2</sub> O	30.0 g	15.0 g
		KI	0.083 g	0.0715 g
		CoCl <sub>2</sub> - 6H <sub>2</sub> O	0.0025 g	0.00125 g
	4	FN Lite P, B, Mo 100x Stock		
20		KH <sub>2</sub> PO <sub>4</sub>	18.5 g	9.25 g
		H <sub>3</sub> BO <sub>3</sub>	0.62 g	0.31 g
		Na <sub>2</sub> MoO <sub>4</sub> - 2H <sub>2</sub> O	0.025 g	0.0125 g

SB1 Solid Medium (per liter)

25	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
30	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  
30g Sucrose  
5 4 ml 2,4-D (40 mg/L final concentration)  
pH 7.0  
2 gm Gelrite

SB 166 Solid Medium (per liter)

- 1 package MS salts (Gibco/ BRL – Cat. No. 11117-066)  
10 1 mL B5 vitamins 1000X stock  
60 g maltose  
750 mg MgCl<sub>2</sub> hexahydrate  
5 g activated charcoal  
pH 5.7  
15 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  
20 750 mg MgCl<sub>2</sub> 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)  
25 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)

- 30 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)

5	DDI H <sub>2</sub> 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
10	B5 Vitamins 1000x	1 mL
	L-Methionine	0.149 g
	Sucrose	30 g
	Sorbitol	30 g

Adjust volume to 900 mL

15 pH 5.8

Autoclave

Add to cooled media ( $\leq 30$  °C):

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

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

20 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 <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (ammonium sulfate)	4.63 g
25	KNO <sub>3</sub> (potassium nitrate)	28.3 g
	MgSO <sub>4</sub> *7H <sub>2</sub> O (magnesium sulfate heptahydrate)	3.7 g
	KH <sub>2</sub> PO <sub>4</sub> (potassium phosphate, monobasic)	1.85 g

Bring to volume

Autoclave

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

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

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

5 Bring to volume

Autoclave

FeEDTA 100X- Stock #3 (per liter)

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

10 \*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)

15 CaCl<sub>2</sub>\*2H<sub>2</sub>O (calcium chloride dihydrate) 44 g

Bring to Volume

Autoclave

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

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

Bring to Volume

Store frozen

25 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.

30 Bring to Volume

Filter Sterilize

Store frozen\*

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

### 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 above. After subculturing on medium SB103 for 3 weeks, individual embryos can be removed  
5 from the clusters and screened for alterations in their fatty acid compositions as described in Example 7. 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  
10 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  
15 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 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  
20 16 weeks, mature seeds are harvested, chipped and analyzed for fatty acids.

### EXAMPLE 8

#### Fatty Acid Analysis of Transgenic Somatic Soybean Embryos

Mature somatic soybean embryos are a good model for zygotic embryos. While in the globular embryo state in liquid culture, somatic soybean embryos  
25 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  
30 the prominent seed proteins,  $\alpha'$ -subunit of  $\beta$ -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  $\alpha'$ -subunit of  $\beta$ -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.

A subset of soybean embryos for each event generated from either production transformation or model system transformation (as described in Example 6) are harvested in the following way. Embryos (5-10 embryos) from each event are picked into glass GC vials and fatty acid methyl esters are prepared by transesterification. For transesterification, 50  $\mu$ L of trimethylsulfonium hydroxide (TMSH) and 0.5 mL of hexane is added to the embryos in glass vials and incubated for 30 min at room temperature while shaking. Fatty acid methyl esters (5  $\mu$ L injected from hexane layer) are separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Cat. No. 24152, Supelco Inc.). The oven temperature is 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 is supplied by a Whatman hydrogen generator. Retention times are compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.). Events having good phenotype can be re-analyzed by GC using identical conditions except the oven temperature is held at 150 °C for 1 min and then increased to 240 °C at 5 °C.

#### EXAMPLE 9

##### Construction of Alternate Soybean Expression Vectors For Expression of *Euglena anabaena* UTEX 373 Delta-9 Elongase (EaD9Elo1)

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 EaD9Elo1. Similarly, it may be desirable to express other PUFA genes (such as those described below in Table 9), for co-expression with any of the delta-9 elongases 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

5 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 7) and a transcription terminator (such as those listed in, but not limited to, Table 8) 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 9 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 7

Seed-specific Promoters

Promoter	Organism	Promoter Reference
$\beta$ -conglycinin $\alpha'$ -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)
$\beta$ -conglycinin $\beta$ -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 8

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

5

TABLE 9

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-5 desaturase	<i>Peridinium</i> sp.	U.S. Patent Application No. 11/748637
delta-5 desaturase	<i>Euglena gracilis</i>	U.S. Patent Application No. 11/748629
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 U.S. Patent No. 7,045,683
delta-4 desaturase	<i>Isochrysis galbana</i>	WO 2002/090493 U.S. Patent No. 7,045,683
delta-4 desaturase	<i>Thraustochytrium aureum</i>	WO 2002/090493 U.S. Patent No. 7,045,683
delta-4 desaturase	<i>Euglena gracilis</i>	U.S. Patent Application No. 10/552,127
delta-9 elongase	<i>Isochrysis galbana</i>	WO 2002/077213
delta-9 elongase	<i>Euglena gracilis</i>	U.S. Patent Application No. 11/601,563
delta-9 elongase	<i>Eutreptiella</i> sp. CCMP389	U.S. Patent Application No. 11/601,564
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. Patent Application No. 11/737772
delta-8 desaturase	<i>Tetruetreptia pomquetensis</i> CCMP1491	U.S. Patent Application No. 11/876115
delta-8 desaturase	<i>Eutreptiella</i> sp. CCMP389	U.S. Patent Application No. 11/876115
delta-8 desaturase	<i>Eutreptiella cf. gymnastica</i> CCMP1594	U.S. Patent Application No. 11/876115

### EXAMPLE 10

#### Synthesis of a Codon-Optimized Delta-9 Elongase Gene for *Yarrowia lipolytica* (EaD9ES)

The codon usage of the delta-9 elongase gene (EaD9Elo1; SEQ ID NO:11) of *Euglena anabaena* was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in PCT Publication No. WO 2004/101753. Specifically, a codon-optimized delta-9 elongase gene (designated "EaD9ES", SEQ ID NO:40) was designed based on the coding sequence of EaD9E (SEQ ID NO:11), 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, 106 bp of the 774 bp coding region were modified (13.7%) and 98 codons were optimized (38.0%). The GC content (52.1%) was about the same between the wild type gene (i.e., EaD9Elo1) and the synthetic gene (i.e., EaD9ES). A *Nco*I site and *Not*I sites were incorporated around the translation initiation codon and after the stop codon of EaD9ES (SEQ ID NO:40), respectively. FIGs. 9A and 9B shows a comparison of the nucleotide sequences of EaD9E (same as EaD9Elo1) (SEQ ID NO:11) and EaD9ES (SEQ ID NO:40). The codon optimized EaD9ES gene did not change any amino acid sequence of EaD9Elo1 (SEQ ID NO:13). The designed EaD9ES gene was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pEaD9ES (SEQ ID NO:41; FIG. 10).

Based on the teachings herein concerning vector construction and suitable promoter and terminators for use in *Yarrowia lipolytica*, one of skill in the art will be able to construct additional plasmids suitable for expression of EaD9ES (SEQ ID NO:40).

### EXAMPLE 11

#### Functional Analyses Of *Euglena anabaena* Delta-9 Elongase In Soy

The present example describes the transformation and expression in soybean somatic embryos of either pKR1140 (SEQ ID NO:30; Example 5), comprising EaD9Elo1 or pKR1151 (SEQ ID NO:39; Example 6), comprising EaD9Elo1 and TpomD8.

Soybean embryogenic suspension culture (cv. Jack) was transformed with each of the vectors above and embryos were matured in soybean histodifferentiation and maturation liquid medium (SHaM liquid media; Schmidt et al., *Cell Biology and Morphogenesis*, 24:393 (2005)) as described in Example 7 and previously described in PCT Publication No. WO 2007/136877, published November 29, 2007 (the contents of which are hereby incorporated by reference).

After maturation in SHaM liquid media a subset of transformed soybean embryos (i.e., 5-6 embryos per event) were harvested and analyzed as described herein.

10 In this way, approximately 30 events transformed with either pKR1140 (SEQ ID NO:30; called Experiment MSE2129) or pKR1151 (SEQ ID NO:39; called MSE2131) were analyzed and the five events having the highest average EDA or DGLA content (average of the 5 embryos analyzed) are shown in FIG.s 11 or 12, respectively.

15 In FIG. 11, fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA and ERA. Fatty acid compositions are expressed as a weight percent (wt. %) of total fatty acids. In FIG. 11, elongation activity is expressed as % delta-9 elongation of C18 fatty acids (delta-9 %Elong), calculated according to the following formula:  $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$ . More specifically, the combined percent elongation for LA and ALA is determined as:  $([\text{EDA} + \text{ERA}]/[\text{LA} + \text{ALA} + \text{EDA} + \text{ERA}]) \times 100$ . This elongation is also referred to as the overall % elongation. The individual omega-6 delta-9 elongation (LA %Elong) was calculated as:  $([\text{EDA}]/[\text{LA} + \text{EDA}]) \times 100$ . Similarly, the individual omega-3 delta-9 elongation (ALA %Elong) was calculated as:  $([\text{ERA}]/[\text{ALA} + \text{ERA}]) \times 100$ . The ratio of delta-9 elongation for omega-6 versus omega-3 substrates (Ratio [LA/ALA] %Elong) was obtained by dividing the LA % delta-9 elongation by the ALA % delta-9 elongation.

20 In FIG. 12, fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA, ERA, DGLA and ETA. Fatty acid compositions are expressed as a weight percent (wt. %) of total fatty acids. In FIG.12, elongation activity is expressed as % delta-9 elongation of C18 fatty acids (C18 % delta-9 elong), calculated according to the following formula:  $([\text{product}]/[\text{substrate} + \text{product}]) \times 100$ . More specifically, the combined percent elongation for LA and ALA is

determined as:  $([DGLA + ETA + EDA + ERA]/[LA + ALA + DGLA + ETA + EDA + ERA]) \times 100$ . In FIG. 12, the combined percent desaturation for EDA and ERA is shown as "C20 % delta-8 desat", determined as:  $([DGLA + ETA]/[DGLA + ETA + EDA + ERA]) \times 100$ . This is also referred to as the overall % desaturation.

5 In summary of FIG. 11, the *Euglena anabeana* delta-9 elongase functioned in soybean to convert both LA and ALA to EDA and ERA, respectively. The line with the highest average EDA content (i.e., 2129-2-6) had embryos with an average EDA content of 26.7% and an average ERA content of 4.4%. The highest EDA and ERA content for an individual embryo from this line was 30.5% and 4.3%, respectively.

10 The highest average overall % delta-9 elongation (i.e. 2129-2-2) was 47.9% with the highest overall % delta-9 elongation for an individual embryo being 53.3%. When broken down into % delta-9 elongation for the omega-6 and omega-3 substrates, the highest average % delta-9 elongation (i.e. 2129-2-2) was 47.3% and 49.9% for LA and ALA, respectively. The highest % delta-9 elongation for an individual

15 embryo in this event was 52.2% and 56.8% for LA and ALA, respectively. In this example, the *Euglena anabaena* delta-9 elongase had no preference for ALA over LA, with the average desaturation ratio ranging from 0.9 to 1.1.

In summary of FIG. 12, the *Euglena anabeana* delta-9 elongase functioned in soybean, along with the TpomD8, to convert both LA and ALA to DGLA and ETA, respectively.

20 The line with the highest average DGLA content (i.e., 2131-2-24) had embryos with an average DGLA content of 23.8% and an average ERA content of 7.2%. The highest DGLA and ETA content for an individual embryo from this line was 26.8% and 8.0%, respectively. The highest average overall % delta-9 elongation for this event was 63.2% with the highest overall % delta-9 elongation for

25 an individual embryo being 65.7%.

### EXAMPLE 12

#### Functional Analysis of Arabidopsis Seed Transformed with pKR1191 for Expression of *Euglena anabaena* delta-9 elongase in Arabidopsis

The present example describes the synthesis of Arabidopsis expression plasmid pKR1191, comprising EaD9Elo1, and its transformation and

30 expression in Arabidopsis seed.

### Construction of pKR1191

The *Ascl* fragment of pKR1140 (SEQ ID NO:30; Example 5), containing the EaD9Elo1, was cloned into the *Ascl* site of pKR92 (which was previously described in WO2007/061845 published on May 31, 2007 to produce pKR1191 (SEQ ID NO:42). A schematic depiction of pKR1191 is shown in FIG. 13. In FIG. 13, EaD9Elo1 is called EA D9elong but they are identical. In this way, EaD9Elo1 was expressed in *Arabidopsis* under control of the soybean beta-conglycinin promoter. The soybean beta-conglycinin promoter functions as a strong, seed-specific promoter in *Arabidopsis*. Functional analysis of EaD9Elo1 in *Arabidopsis* Seed

A *fad3/fae1* double mutant (Smith et al., *Planta* 217:507-516 (2003)) of *Arabidopsis* produces seed where the ALA and 20:1 fatty acid content is less than 2.0%. The *fad3/fae1* double mutant *Arabidopsis* plants were transformed with pKR1191 (SEQ ID NO:42), and plants were grown, maintained and seed was harvested as previously described in WO 2007/061845 (the contents of which are hereby incorporated by reference).

Segregating T2 seed was obtained from 18 individual events for each and bulk T2 seed lipid profiles for each event were obtained by transesterification with TMSH as described in herein with the following modifications. For each event, a small scoopful of seeds (approximately 25-50 seed each scoopful) was crushed in 50  $\mu$ L of TMSH in a 1.5 mL eppendorf tube. After shaking in TMSH for 15 min., 400  $\mu$ L 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 herein.

The lipid profiles of T2 bulk seed for the 18 transformed events is shown in FIG. 14. Fatty acids are identified as 16:0 (palmitate), 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, , 20:0 (eicosanoic acid), 20:1 (eicosenoic acid), EDA and ERA; and, fatty acid compositions listed in FIG. 14 are expressed as a weight percent (wt. %) of total fatty acids. In FIG. 14, the combined percent elongation for LA and ALA is shown as "delta-9 %Elong", determined as:  $([EDA + ERA]/[LA + ALA]) \times 100$ . This is also referred to as the overall % elongation.

In summary of FIG. 14, the event with the highest EDA content (i.e. ff1191-16) in bulk T2 seed analysis contained 32.9% EDA and 1.6% ERA. In this event, The

2008240028 19 Aug 2009

delta-9 %Elong was 50.9%, calculated as described above. Because bulk analysis of T2 seed (still segregating for the phenotype and thus having some wild-type seed) was performed, it is likely that individual seed within an event that are homozygous for the EaD9Elo1 gene will have higher EDA and ERA contents and thus higher overall % elongation.

Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.

Further, any prior art reference or statement provided in the specification is not to be taken as an admission that such art constitutes, or is to be understood as constituting, part of the common general knowledge in Australia.



The Claims defining the invention are as follows:

1. An isolated polynucleotide comprising:
  - (a) a nucleotide sequence encoding a polypeptide having delta-9 elongase activity, wherein the polypeptide has at least 90% amino acid identity, based on the Clustal V method of alignment, when compared to the amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14;
  - (b) a nucleotide sequence encoding a polypeptide having delta-9 elongase activity, wherein the nucleotide sequence has at least 90% sequence identity, based on the BLASTN method of alignment, when compared to the nucleotide sequence as set forth in SEQ ID NO:11 or SEQ ID NO:12; 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.
2. The polynucleotide of Claim 1 wherein the nucleotide sequence comprises SEQ ID NO:11 or SEQ ID NO:12.
3. The polynucleotide of Claim 1 wherein the amino acid sequence of the polypeptide comprises SEQ ID NO:13 or SEQ ID NO:14.
4. A recombinant DNA construct comprising the polynucleotide of Claim 1 operably linked to at least one regulatory sequence.
5. A plant cell comprising in its genome the recombinant DNA construct of Claim 4.
6. A method for transforming a plant cell, comprising transforming a plant cell with the recombinant construct of Claim 4 and selecting those plant cells transformed with the recombinant construct of Claim 4.
7. A method for producing a transformed plant comprising transforming a plant cell with the polynucleotide of Claim 1 and regenerating a plant from the transformed plant cell.
8. A transgenic seed comprising in its genome the recombinant construct of Claim 4.
9. A method for making long-chain polyunsaturated fatty acids in a plant cell comprising:
  - (a) transforming a plant cell with the recombinant construct of Claim 4; and
  - (b) selecting those transformed plant cells that make long-chain polyunsaturated fatty acids.
10. 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 as defined in Claim 1, 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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub> 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.
- 11. The method of claim 10 wherein the oilseed plant is selected from the group consisting of soybean, *Brassica species*, sunflower, maize, cotton, flax, and safflower.
- 12. An oilseed plant comprising:
  - (a) a first recombinant DNA construct comprising an isolated polynucleotide as defined in Claim 1, 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<sub>14/16</sub> elongase, a C<sub>16/18</sub> elongase, a C<sub>18/20</sub> elongase and a C<sub>20/22</sub> elongase.
- 13. The oilseed plant of Claim 12 wherein the oilseed plant is selected from the group consisting of soybean, *Brassica species*, sunflower, maize, cotton, flax, and safflower.
- 14. A transgenic seed obtained from the plant made by the method of Claim 7 or the oilseed plant of Claim 12, wherein said seed comprises in its genome the recombinant construct of Claim 4.
- 15. Food or feed comprising the seed of Claim 14.
- 16. Progeny plants obtained from the plant made by the method of claim 7 or from the oilseed plant of Claim 12.

1/15

FIG. 1

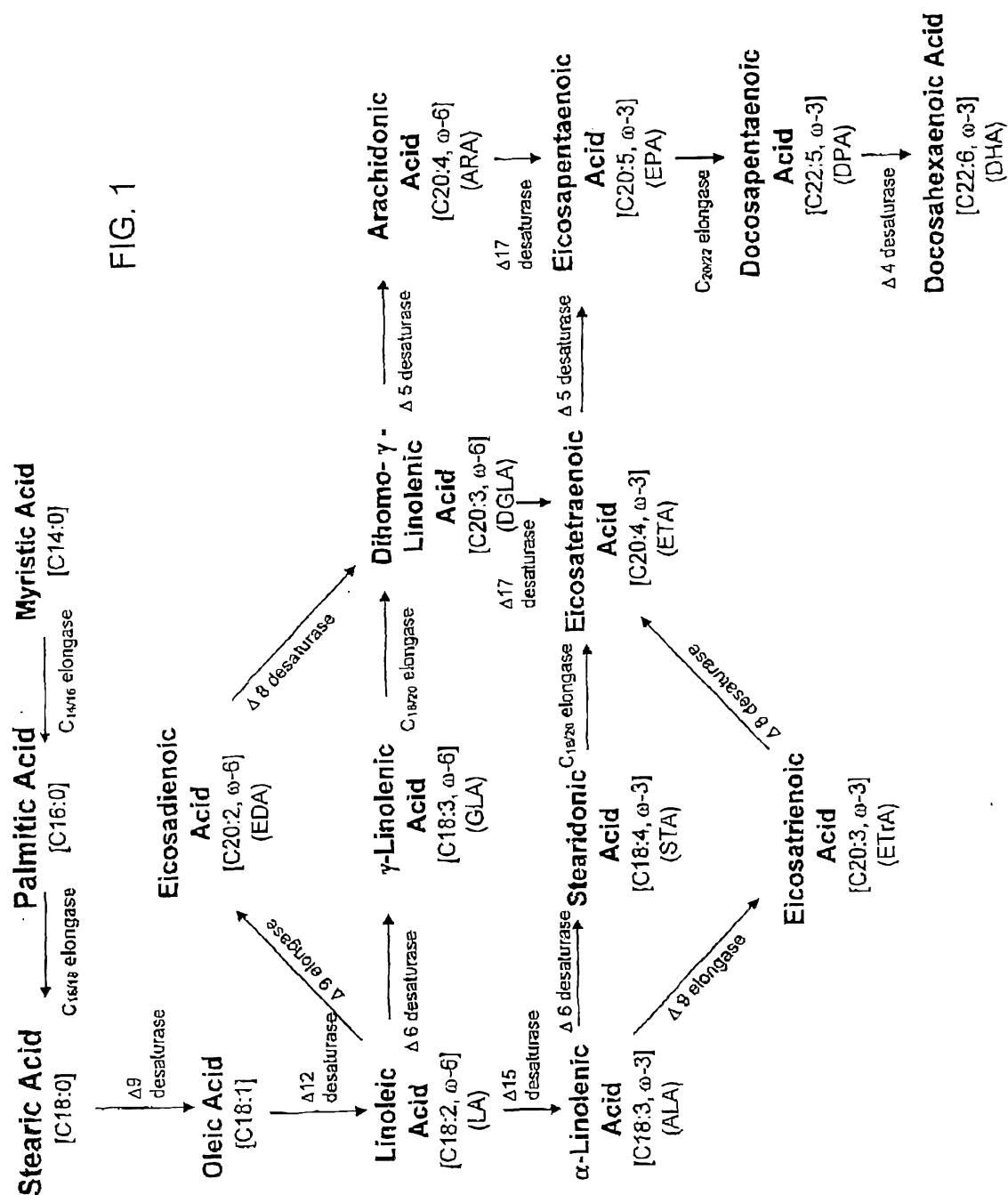


FIG. 2

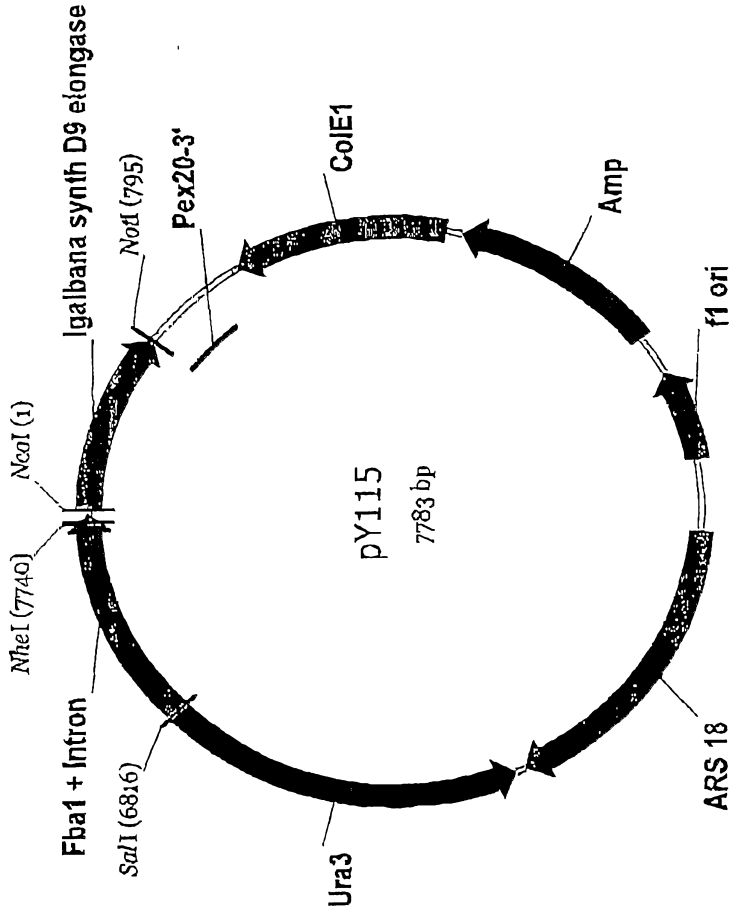


FIG. 3

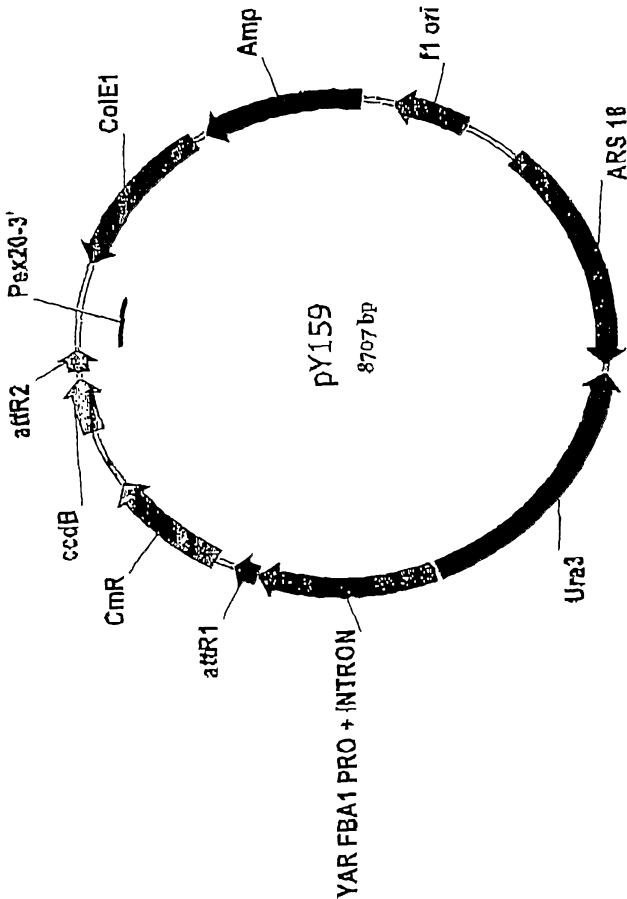


FIG. 4

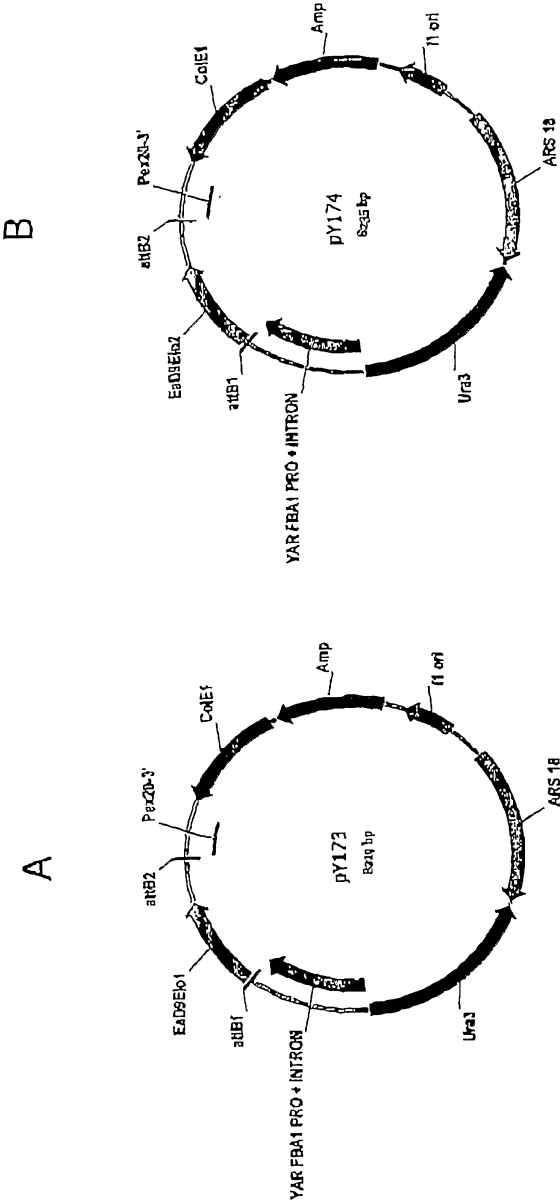




FIG. 6

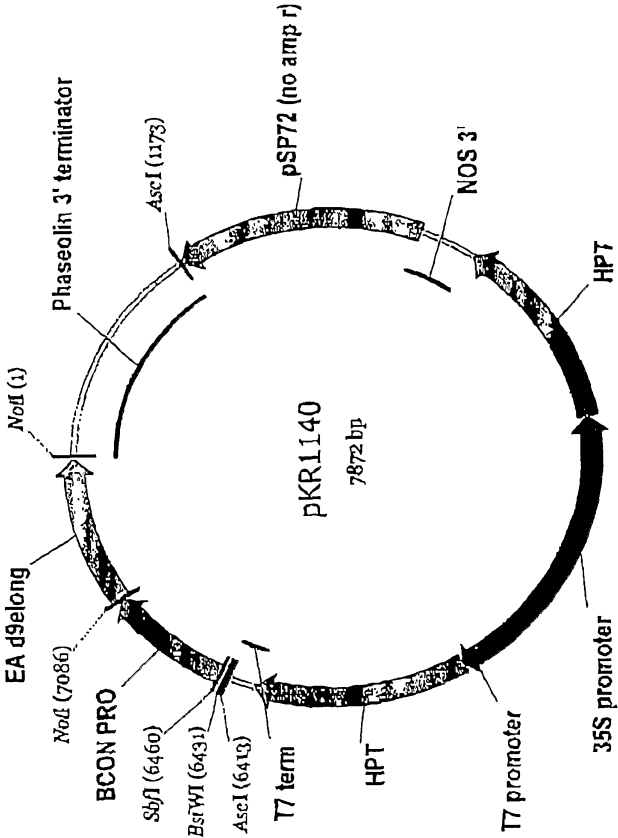
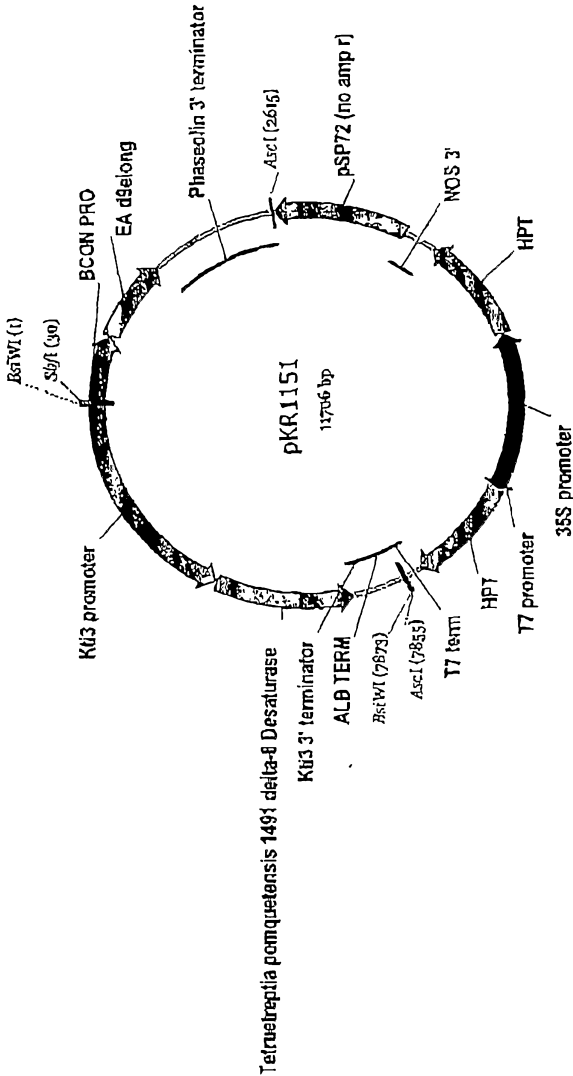




FIG. 7



8/15

FIG. 8

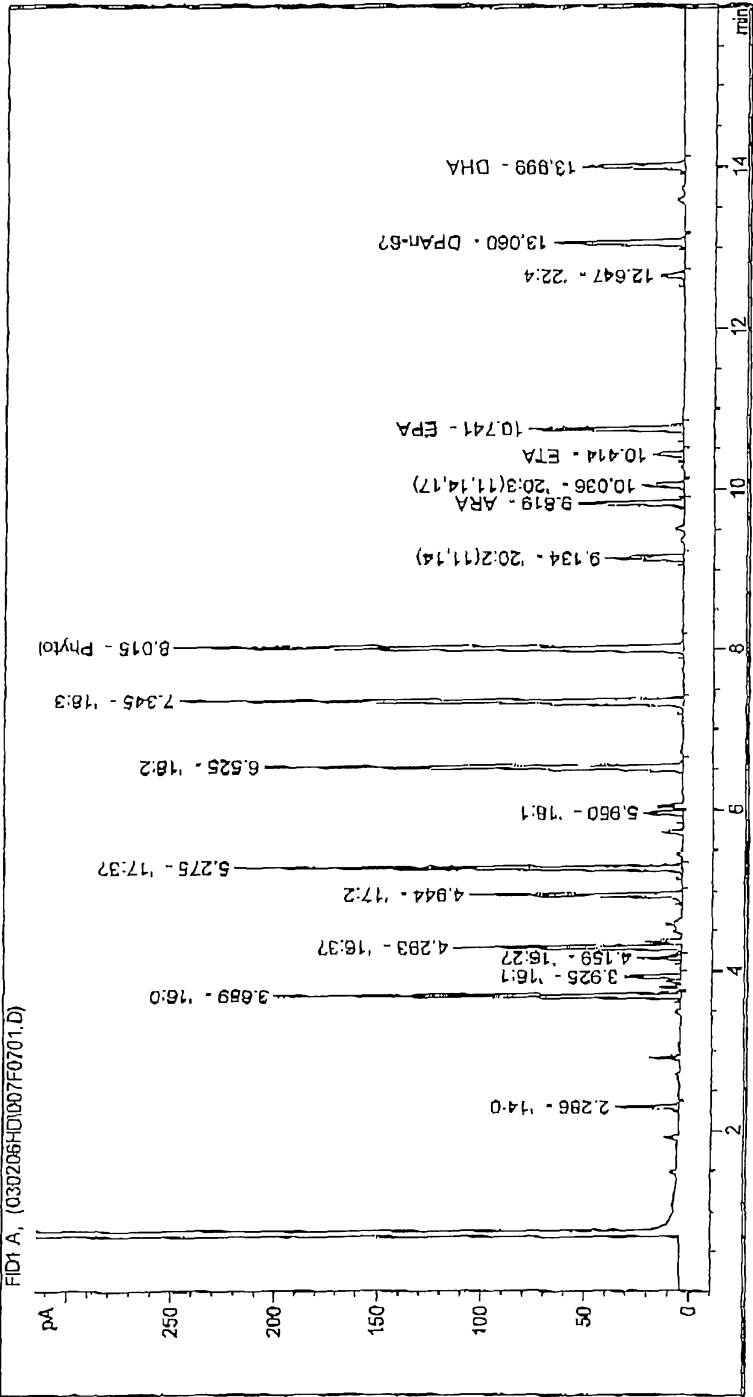


FIG. 9A

1	A T G G A A G C A G C C A A A A A T T G G T T T C C A T C G T C C A A G A G G A G G C T C C C A A	EaD9E
1	A T E A G G C T G C C A A G G A G C T G G T C F C C A T C G T C C A G A G A A C I T C C C A A	EaD9ES
51	G T G G A C T A T G C C C A G C T T T G G C A G G A T G C C A G C A G C T G T G A G G T C C T T T	EaD9E
51	G T G G A C T A C G C C C A G C T C T E G C A G A C G C C T C C T C T T G C G A G G T T C T G T	EaD9ES
101	A C C T C T C G G T G G C A T C G T G C C G A T C A B G T T C A T G C T G C S C C C A C T G G A C	EaD9E
101	A C C T C T C G G T C C T T C S T E S C A T C A A G T T C A T G C T T C G A C C T C T G G A C	EaD9ES
151	C T G A A G C C C A G C C A C C T T E A A G A A G C T G T T C A C A G C A T A C A A C T T C C T	EaD9E
151	C T C A A G C C A C A A G C C A C C C I C A A A A A G C T G T T C A C C G C A T A C A A C T T C T	EaD9ES
201	C A T G T C E A T C T A T P C C T T T E G C C C C T T C C T G G C C A T G G C C T A T G C C C A T	EaD9E
201	C A T G T C C A T C T A C T C G T T C G G C T C C T T C C T G G C G A T G G C C T A C G C T C T C T	EaD9ES
251	C A G T A A C T G G C A P C C T C T C G G C C A C T G T G A G A C G E C G I T C A A C A A C G A T	EaD9E
251	C T G T C A C T G E T A T T C T T C C G G C G A T T G T G A G A C T E C C T T C A A C A A T G A C	EaD9ES
301	G T G T T C A G G A T C A C A A C T C A G C T G T T C T A C C T C A G C A A G T T C G T A G A G T A	EaD9E
301	G T G T T C C E A T C A C C A C T C A G C T G T T C T A C C T C A G C A A G T T C G T C G A G T A	EaD9ES
351	C A T C G A C T C C T C T A C C T T C C C C T T A T G G A C A A G C C A C T G T C G T T C C T C	EaD9E
351	C A T C G A C T C C T C T A C C T T C C C C I C A T G G A C A A G C C T T G T C G T T T C T G C	EaD9ES
401	A G T T C T T C A T C A T T T G E S G G C C C C A T T G A C A T G T G G C T A T T C T A C A A A	EaD9E
401	A G T T C T T C A C C A T C C G A A S C T C C C A T C S A C A T G T G G C T G T T C T A C A A G	EaD9ES
451	T A C C G C A A C G A A G S A G T C T G G A T C T T T G T C C T G T T G A A T G G C T T C A T T C A	EaD9E
451	I A T C G A A A C C E A A G C C G T C T G E A T C T T T G T T C T G C T C A A C G C T T C A T T C A	EaD9ES
501	C T G G A T C A T G T A C G G T A C T A T T G G A C G C G G C T C A A G C T G A A C T T C C	EaD9E
501	C T G G A T C A T G T A C G G T A C T A T T G G A C G C G A C C C A T C A A G C T G A A C T T C C	EaD9ES
551	C C A T C C C A A G A A C C C A T T A C C C T C C A T T G C A A T T A T T C C A A T G T C	EaD9E
551	C T A T G C C C A A G A A C C C A T T A C C C T C C A T T A T T C C A A T T C A A C G T C	EaD9ES

FIG. 9B

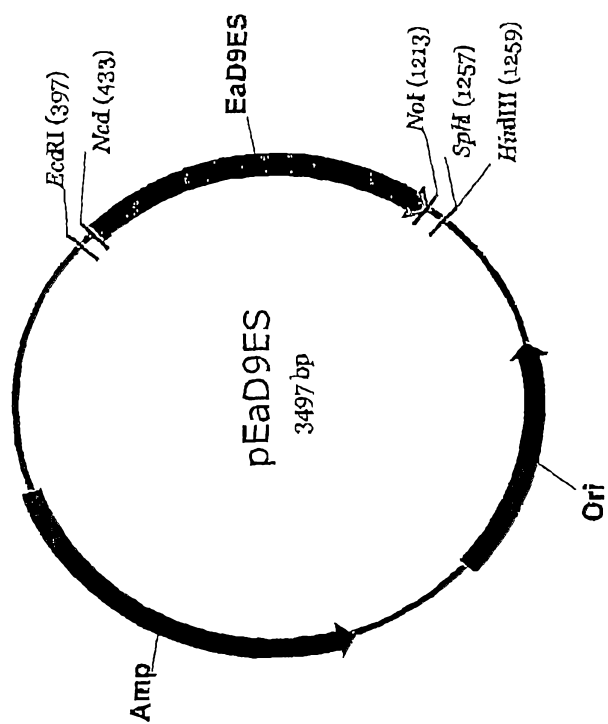
601	G G G T T C T A C A T C C E T C T G G A A E T A C C G C A A T G T G C C A T G C T A C C G C A G G A	EaD9E
601	G E A T T C I A C A T C C E T C T G G A A G T A C C G A A A C G T G C C C T G C T A C C G G C A G G A	EaD9ES
651	T G G G A T E C G C A T G G T T T G C C T G G A T C T T C A A C T A C T G G I A T G T C C G G G A C G G	EaD9E
651	C G G T A T G C G A A T G T T T E C C T G G A T C T T C A A C T A C T G G T A T G T C G G C A C G G	EaD9ES
701	T C T T G C T G C T G T C C T C A A C T T T A C G T G C A G A C C T A C A T C C G A A G C C G	EaD9E
701	T G C T E C T T C T G T C C C T C A A C T T C T A C G T C A G A C C T A C A T T C G G A H G C C T	EaD9ES
751	A G G A A G A A C C G A A G G G A A G A A G G A G	EaD9E
751	C G A A A G A A C C C G A G G C A A A A A G G A G	EaD9ES

10/15

EaD9E (same as EaD9E101) (SEQ ID NO:11)  
EaD9ES (SEQ ID NO:40)

11/15

FIG. 10



12/15

FIG. 11

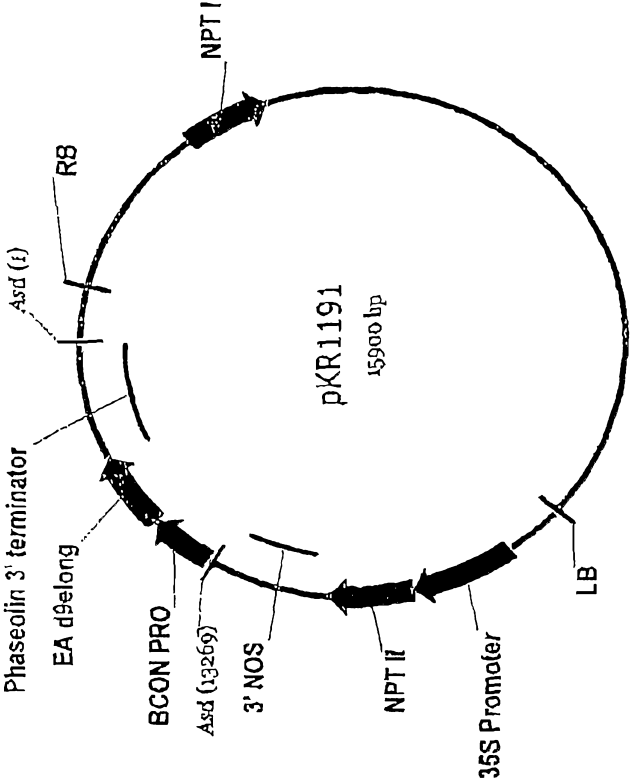
Event	Fatty acid composition (wt.%)							delta-9 %Elong	LA %Elong	ALA %Elong	Ratio (LA/ALA) %Elong
	16:0	18:0	18:1	LA	ALA	EDA	ERA				
2129-2-2-1	15.4	5.8	11.0	32.1	8.2	21.8	5.9	40.6	40.3	41.8	1.0
2129-2-2-2	14.9	3.8	15.5	28.3	6.2	24.8	6.5	47.6	46.7	51.2	0.9
2129-2-2-3	15.1	4.8	13.9	26.6	7.2	25.5	7.3	49.4	49.1	50.4	1.0
2129-2-2-4	17.3	8.0	9.6	25.1	8.5	23.3	8.2	48.4	48.2	49.2	1.0
2129-2-2-5	15.2	4.1	10.5	25.2	7.5	27.6	9.9	53.3	52.2	56.8	0.9
Avg.	15.6	5.2	12.1	27.4	7.5	24.6	7.6	47.9	47.3	49.9	0.9
2129-2-5-1	12.8	4.5	10.9	31.9	5.8	28.6	5.5	47.5	47.3	48.8	1.0
2129-2-5-2	18.5	3.7	3.6	34.0	13.7	20.6	6.0	35.8	37.7	30.4	1.2
2129-2-5-3	13.3	4.3	8.2	35.8	6.0	27.5	4.9	43.8	43.5	45.2	1.0
2129-2-5-4	13.4	5.2	9.5	38.0	6.5	22.9	4.4	38.0	37.6	40.0	0.9
2129-2-5-5	12.8	5.3	11.5	31.9	6.3	27.6	4.8	45.7	46.4	42.1	1.1
Avg.	14.2	4.6	8.8	34.3	7.7	25.4	5.1	42.1	42.5	41.3	1.0
2129-2-6-1	13.4	3.4	10.3	33.8	6.2	28.1	4.9	45.2	45.4	44.0	1.0
2129-2-6-2	13.3	4.2	12.8	31.1	3.8	30.5	4.3	50.0	49.5	53.3	0.9
2129-2-6-3	14.8	4.7	12.7	30.7	5.4	27.1	4.7	46.8	46.9	46.8	1.0
2129-2-6-4	14.4	4.0	11.3	34.8	6.5	25.3	3.6	41.1	42.1	35.3	1.2
2129-2-6-5	18.1	4.7	12.5	29.7	7.9	22.4	4.7	41.9	43.0	37.5	1.1
Avg.	14.8	4.2	11.9	32.0	6.0	28.7	4.4	45.0	45.4	43.4	1.1
2129-6-1-1	13.4	2.9	14.9	27.4	10.8	22.9	7.8	44.5	45.4	41.8	1.1
2129-6-1-2	12.3	3.3	19.1	25.1	7.3	28.0	6.9	50.4	50.9	48.5	1.1
2129-6-1-3	12.3	3.2	18.8	26.8	7.3	26.9	6.7	48.6	50.1	47.7	1.1
2129-6-1-4	14.0	3.3	13.2	33.0	13.2	17.5	5.9	33.5	34.6	30.7	1.1
2129-6-1-5	12.6	3.0	18.8	27.5	8.9	23.2	7.0	44.7	45.7	41.5	1.1
Avg.	12.9	3.1	18.1	28.0	9.7	23.3	6.8	44.5	45.3	42.0	1.1
2129-6-3-1	13.1	3.8	16.7	25.7	9.2	24.0	7.5	47.5	48.3	45.0	1.1
2129-6-3-2	13.3	3.6	13.9	27.1	10.0	24.0	8.1	46.3	47.0	44.5	1.1
2129-6-3-3	13.4	3.9	17.3	27.4	10.8	20.4	7.0	41.9	42.7	39.6	1.1
2129-6-3-4	13.3	3.2	16.4	29.3	9.8	21.7	6.2	41.7	42.6	38.8	1.1
2129-6-3-5	14.1	3.6	16.9	29.4	9.7	20.9	5.3	40.2	41.6	36.3	1.2
Avg.	13.5	3.6	16.2	27.8	9.9	22.2	6.8	43.5	44.4	40.6	1.1

13/15

FIG. 12

Event	Fatty acid composition (wt.%)									C18	C20
	16:0	18:0	18:1	LA	ALA	EDA	DGLA	ERA	ETA	% delta-9 elong	% delta-8 desat
2131-2-9-1	15.4	4.0	19.9	27.7	6.8	8.5	13.4	0.8	3.0	42.7	83.7
2131-2-9-2	17.0	3.6	10.5	29.9	10.9	7.7	15.1	1.4	3.5	40.4	87.1
2131-2-9-3	16.8	3.7	9.4	26.2	9.2	8.7	19.5	1.4	4.5	49.0	70.5
2131-2-9-4	16.2	3.7	14.7	29.2	8.8	8.0	14.6	0.9	3.3	41.4	88.9
2131-2-9-5	16.9	3.8	12.5	27.9	9.5	8.8	14.8	1.3	3.6	43.4	84.4
Avg.	16.5	3.8	13.4	28.2	9.0	8.3	15.5	1.2	3.6	43.4	86.5
2131-2-15-1	16.0	3.9	13.3	29.1	7.4	8.8	16.0	1.0	4.1	44.9	87.5
2131-2-15-2	15.4	3.4	10.3	27.3	5.8	11.6	20.6	1.4	4.1	53.4	85.5
2131-2-15-3	16.9	3.8	13.7	28.6	8.2	8.9	14.7	1.0	3.6	43.3	84.9
2131-2-15-4	17.3	3.2	6.6	22.2	10.3	6.5	24.1	1.7	7.3	54.9	79.3
2131-2-15-5	14.6	3.7	11.0	26.0	6.0	8.9	22.3	1.6	5.2	54.2	72.4
Avg.	16.0	3.6	11.0	28.6	7.5	8.9	19.5	1.3	4.8	50.2	69.9
2131-2-22-1	16.2	4.7	6.5	17.5	4.8	15.1	24.7	3.0	6.2	68.7	63.0
2131-2-22-2	17.3	4.9	7.0	24.2	9.7	12.5	16.4	2.6	4.4	51.5	58.0
2131-2-22-3	17.3	5.1	9.5	20.7	6.5	14.6	18.7	2.4	4.3	59.5	57.5
2131-2-22-4	18.7	5.2	7.4	18.5	5.4	12.3	23.9	2.0	5.6	64.8	67.4
2131-2-22-5	18.4	5.0	8.8	18.0	5.6	11.1	24.3	2.0	5.8	64.8	68.6
Avg.	17.6	5.0	7.8	19.8	6.4	13.1	21.6	2.4	5.3	61.9	63.1
2131-2-24-1	17.0	4.0	5.3	19.3	8.1	11.6	21.6	3.7	8.0	62.1	65.9
2131-2-24-2	17.4	4.1	6.4	19.8	5.8	9.0	26.8	2.0	7.6	63.9	75.8
2131-2-24-3	16.0	4.2	6.3	23.0	6.6	16.8	17.5	3.8	4.8	59.2	52.0
2131-2-24-4	18.0	5.9	8.4	17.2	5.6	7.9	26.4	1.7	7.8	65.7	78.0
2131-2-24-5	18.1	4.8	7.3	18.0	5.9	8.1	26.5	2.1	8.0	65.2	77.1
Avg.	17.3	4.6	6.7	19.5	6.4	10.7	23.8	2.6	7.2	63.2	69.8
2131-6-14-1	17.5	4.1	17.3	21.0	7.8	8.1	17.4	1.1	5.0	52.4	70.9
2131-6-14-2	17.5	4.7	16.4	26.4	8.5	8.3	13.1	1.2	3.1	42.4	63.2
2131-6-14-3	17.2	3.2	12.3	22.4	9.1	10.2	18.1	1.7	5.4	52.9	66.5
2131-6-14-4	18.1	3.6	10.9	24.6	9.1	7.7	19.6	1.0	4.8	49.7	73.6
2131-6-14-5	16.6	3.8	13.6	26.3	8.9	8.1	17.0	1.0	4.4	46.5	70.1
Avg.	17.4	3.9	14.1	24.1	8.7	8.5	17.1	1.2	4.6	48.8	68.8

FIG. 13





15/15

FIG. 14

Event	Fatty acid composition (wt.%)										delta-9 %Elong
	16:0	18:0	18:1	LA	ALA	20:0	20:1 (11)	EDA	ERA		
#1191-1	7.8	2.6	22.8	41.8	1.6	0.9	1.8	19.1	1.3	32.0	
#1191-2	7.8	3.2	27.2	44.0	0.5	0.7	1.3	14.5	0.6	25.4	
#1191-3	8.2	2.8	19.8	35.4	0.6	0.7	2.1	27.9	1.1	44.0	
#1191-4	7.6	2.8	21.1	41.6	1.3	0.8	2.2	21.3	0.9	34.1	
#1191-5	8.5	2.9	18.5	41.2	1.1	0.9	1.9	23.6	1.0	36.8	
#1191-6	7.8	3.1	17.8	37.0	0.8	0.8	2.0	29.2	1.2	44.5	
#1191-7	8.5	2.6	19.9	34.4	0.6	0.7	2.4	29.4	1.2	46.7	
#1191-8	8.1	2.4	18.4	36.7	0.5	0.5	2.0	30.3	1.2	45.9	
#1191-9	7.6	2.6	21.8	41.9	0.6	0.7	1.8	22.0	0.8	35.0	
#1191-10	7.9	2.5	20.3	36.2	0.4	0.6	2.1	28.6	1.0	44.7	
#1191-11	8.2	2.8	19.3	37.6	0.5	0.8	2.2	27.2	1.1	42.6	
#1191-12	8.6	3.4	21.9	40.4	0.6	0.8	1.3	21.9	1.0	35.8	
#1191-13	8.6	3.0	30.6	55.0	1.1	0.8	0.4	0.2	0.0	0.4	
#1191-14	8.9	3.0	17.2	39.3	1.2	0.9	1.3	26.5	1.2	40.6	
#1191-15	15.9	0.0	20.5	41.4	0.0	0.0	0.0	22.3	0.0	35.0	
#1191-16	9.0	2.9	18.0	32.6	0.6	0.6	1.7	32.9	1.6	50.9	
#1191-17	8.7	3.2	17.2	37.9	1.0	1.0	1.5	27.8	1.3	42.8	
#1191-18	8.0	2.8	21.2	41.1	0.5	0.7	1.5	23.1	0.9	36.6	