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(54) Title: LIPID COMPRISING LONG CHAIN POLYUNSATURATED FATTY ACIDS

(57) Abstract: The present invention relates to extracted plant lipid comprising docosahexaenoic acid (DHA) wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%. The present invention also relates to plant and/or microbial lipid comprising docosapentaenoic acid (DPA) wherein the level of DPA in the total fatty acid content of the extracted lipid is between 7% and 35%, and processes for producing the extracted lipid.



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LIPID COMPRISING LONG CHAIN POLYUNSATURATED FATTY ACIDS

FIELD OF THE INVENTION

The present invention relates to lipid comprising docosahexaenoic acid and/or
5 docosapentaenoic acid, obtained from plant cells or microbial cells, and processes for
producing and using the lipid.

BACKGROUND OF THE INVENTION

Omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) are now widely
10 recognized as important compounds for human and animal health. These fatty acids
may be obtained from dietary sources or by conversion of linoleic (LA, 18:2 ω 6) or α -
linolenic (ALA, 18:3 ω 3) fatty acids, both of which are regarded as essential fatty acids
in the human diet. While humans and many other vertebrate animals are able to
convert LA or ALA, obtained from plant sources to C22 they carry out this conversion
15 at a very low rate. Moreover, most modern societies have imbalanced diets in which at
least 90% of polyunsaturated fatty acids (PUFA) are of the ω 6 fatty acids, instead of
the 4:1 ratio or less for ω 6: ω 3 fatty acids that is regarded as ideal (Trautwein, 2001).
The immediate dietary source of LC-PUFAs such as eicosapentaenoic acid (EPA,
20:5 ω 3) and docosahexaenoic acid (DHA, 22:6 ω 3) for humans is mostly from fish or
20 fish oil. Health professionals have therefore recommended the regular inclusion of fish
containing significant levels of LC-PUFA into the human diet. Increasingly, fish-
derived LC-PUFA oils are being incorporated into food products and in infant formula,
for example. However, due to a decline in global and national fisheries, alternative
sources of these beneficial health-enhancing oils are needed.

25 Flowering plants, in contrast to animals, lack the capacity to synthesise
polyunsaturated fatty acids with chain lengths longer than 18 carbons. In particular,
crop and horticultural plants along with other angiosperms do not have the enzymes
needed to synthesize the longer chain ω 3 fatty acids such as EPA, docosapentaenoic
acid (DPA, 22:5 ω 3) and DHA that are derived from ALA. An important goal in plant
30 biotechnology is therefore the engineering of crop plants which produce substantial
quantities of LC-PUFA, thus providing an alternative source of these compounds.

LC-PUFA Biosynthesis Pathways

Biosynthesis of LC-PUFAs in organisms such as microalgae, mosses and fungi
35 usually occurs as a series of oxygen-dependent desaturation and elongation reactions
(Figure 1). The most common pathway that produces EPA in these organisms includes

a $\Delta 6$ -desaturation, $\Delta 6$ -elongation and $\Delta 5$ -desaturation (termed the $\Delta 6$ -desaturation pathway) whilst a less common pathway uses a $\Delta 9$ -elongation, $\Delta 8$ -desaturation and $\Delta 5$ -desaturation (termed the $\Delta 9$ -desaturation pathway). These consecutive desaturation and elongation reactions can begin with either the $\omega 6$ fatty acid substrate LA, shown schematically as the upper left part of Figure 1 ($\omega 6$) or the $\omega 3$ substrate ALA through to EPA, shown as the lower right part of Figure 1 ($\omega 3$). If the initial $\Delta 6$ -desaturation is performed on the $\omega 6$ substrate LA, the LC-PUFA product of the series of three enzymes will be the $\omega 6$ fatty acid ARA. LC-PUFA synthesising organisms may convert $\omega 6$ fatty acids to $\omega 3$ fatty acids using an $\omega 3$ -desaturase, shown as the $\Delta 17$ -desaturase step in Figure 1 for conversion of arachidonic acid (ARA, 20:4 $\omega 6$) to EPA. Some members of the $\omega 3$ -desaturase family can act on a variety of substrates ranging from LA to ARA. Plant $\omega 3$ -desaturases often specifically catalyse the $\Delta 15$ -desaturation of LA to ALA, while fungal and yeast $\omega 3$ -desaturases may be specific for the $\Delta 17$ -desaturation of ARA to EPA (Pereira et al., 2004a; Zank et al., 2005). Some reports suggest that non-specific $\omega 3$ -desaturases may exist which can convert a wide variety of $\omega 6$ substrates to their corresponding $\omega 3$ products (Zhang et al., 2008).

The conversion of EPA to DHA in these organisms occurs by a $\Delta 5$ -elongation of EPA to produce DPA, followed by a $\Delta 4$ -desaturation to produce DHA (Figure 1). In contrast, mammals use the so-called "Sprecher" pathway which converts DPA to DHA by three separate reactions that are independent of a $\Delta 4$ -desaturase (Sprecher et al., 1995).

The front-end desaturases generally found in plants, mosses, microalgae, and lower animals such as *Caenorhabditis elegans* predominantly accept fatty acid substrates esterified to the *sn*-2 position of a phosphatidylcholine (PC) substrate. These desaturases are therefore known as acyl-PC, lipid-linked, front-end desaturases (Domergue et al., 2003). In contrast, higher animal front-end desaturases generally accept acyl-CoA substrates where the fatty acid substrate is linked to CoA rather than PC (Domergue et al., 2005). Some microalgal desaturases and one plant desaturase are known to use fatty acid substrates esterified to CoA (Table 2).

Each PUFA elongation reaction consists of four steps catalysed by a multi-component protein complex: first, a condensation reaction results in the addition of a 2C unit from malonyl-CoA to the fatty acid, resulting in the formation of a β -ketoacyl intermediate. This is then reduced by NADPH, followed by a dehydration to yield an enoyl intermediate. This intermediate is finally reduced a second time to produce the elongated fatty acid. It is generally thought that the condensation step of these four reactions is substrate specific whilst the other steps are not. In practice, this means that

native plant elongation machinery is capable of elongating PUFA providing that the condensation enzyme (typically called an 'elongase') specific to the PUFA is introduced, although the efficiency of the native plant elongation machinery in elongating the non-native PUFA substrates may be low. In 2007 the identification and
 5 characterisation of the yeast elongation cycle dehydratase was published (Denic and Weissman, 2007).

PUFA desaturation in plants, mosses and microalgae naturally occurs to fatty acid substrates predominantly in the acyl-PC pool whilst elongation occurs to substrates in the acyl-CoA pool. Transfer of fatty acids from acyl-PC molecules to a CoA carrier
 10 is performed by phospholipases (PLAs) whilst the transfer of acyl-CoA fatty acids to a PC carrier is performed by lysophosphatidyl-choline acyltransferases (LPCATs) (Figure 9) (Singh et al., 2005).

Engineered production of LC-PUFA

15 Most LC-PUFA metabolic engineering has been performed using the aerobic $\Delta 6$ -desaturation/elongation pathway. The biosynthesis of γ -linolenic acid (GLA, 18:3 ω 6) in tobacco was first reported in 1996 using a $\Delta 6$ -desaturase from the cyanobacterium *Synechocystis* (Reddy and Thomas, 1996). More recently, GLA has been produced in crop plants such as safflower (73% GLA in seedoil, WO
 20 2006/127789) and soybean (28% GLA; Sato et al., 2004). The production of LC-PUFA such as EPA and DHA involves more complicated engineering due to the increased number of desaturation and elongation steps involved. EPA production in a land plant was first reported by Qi et al. (2004) who introduced genes encoding a $\Delta 9$ -elongase from *Isochrysis galbana*, a $\Delta 8$ -desaturase from *Euglena gracilis* and a $\Delta 5$ -
 25 desaturase from *Mortierella alpina* into *Arabidopsis* yielding up to 3% EPA. This work was followed by Abbadi et al. (2004) who reported the production of up to 0.8% EPA in flax seed using genes encoding a $\Delta 6$ -desaturase and $\Delta 6$ -elongase from *Physcomitrella patens* and a $\Delta 5$ -desaturase from *Phaeodactylum tricornutum*.

The first report of DHA production was in WO 04/017467 where the production
 30 of 3% DHA in soybean embryos is described, but not seed, by introducing genes encoding the *Saprolegnia diclina* $\Delta 6$ -desaturase, *Mortierella alpina* $\Delta 6$ -desaturase, *Mortierella alpina* $\Delta 5$ -desaturase, *Saprolegnia diclina* $\Delta 4$ -desaturase, *Saprolegnia diclina* $\Delta 17$ -desaturase, *Mortierella alpina* $\Delta 6$ -elongase and *Pavlova lutheri* $\Delta 5$ -elongase. The maximal EPA level in embryos also producing DHA was 19.6%,
 35 indicating that the efficiency of conversion of EPA to DHA was poor (WO 2004/071467). This finding was similar to that published by Robert et al. (2005),

where the flux from EPA to DHA was low, with the production of 3% EPA and 0.5% DHA in *Arabidopsis* using the *Danio rerio* $\Delta 5/6$ -desaturase, the *Caenorhabditis elegans* $\Delta 6$ -elongase, and the *Pavlova salina* $\Delta 5$ -elongase and $\Delta 4$ -desaturase. Also in 2005, Wu et al. published the production of 25% ARA, 15% EPA, and 1.5% DHA in *Brassica juncea* using the *Pythium irregulare* $\Delta 6$ -desaturase, a *Thraustochytrid* $\Delta 5$ -desaturase, the *Physcomitrella patens* $\Delta 6$ -elongase, the *Calendula officianalis* $\Delta 12$ -desaturase, a *Thraustochytrid* $\Delta 5$ -elongase, the *Phytophthora infestans* $\Delta 17$ -desaturase, the *Oncorhynchus mykiss* LC-PUFA elongase, a *Thraustochytrid* $\Delta 4$ -desaturase and a *Thraustochytrid* LPCAT (Wu et al., 2005). Summaries of efforts to produce oil-seed crops which synthesize $\omega 3$ LC-PUFAs is provided in Venegas-Caleron et al. (2010) and Ruiz-Lopez et al. (2012). As indicated by Ruiz-Lopez et al. (2012), results obtained to date for the production of DHA in transgenic plants has been no where near the levels seen in fish oils. More recently, Petrie et al (2012) reported the production of about 15% DHA in *Arabidopsis thaliana* seeds, and WO2013/185184 reported the production of certain seedoils having between 7% and 20% DHA. However, there are no reports of production of plant oils having more than 20% DHA.

There are no reports of the production of DPA in recombinant cells to significant levels without concomitant production of DHA. Indeed, the present inventors are unaware of any published suggestion or motivation to produce DPA in recombinant cells without production of DHA.

There therefore remains a need for more efficient production of LC-PUFA in recombinant cells, in particular of DHA or DPA in seeds of oilseed plants.

SUMMARY OF THE INVENTION

The present inventors have identified methods and plants for producing lipid with high levels of DHA and/or DPA. As described in WO2013/185184, the present inventors have previously disclosed extracted plant lipid, and plants and plant parts for producing such lipid, comprising DHA in the total fatty acid content of the extracted lipid of between 7% and 20%. An upper limited of 20% was defined because at the time it was considered a maximal amount of DHA which could be produced in plants. However, as described herein, the inventors were surprised to find that levels of DHA in the total fatty acid content greater than 20% can be obtained. The inventors also found plant lipid, and plant parts and plants for producing lipid comprising DPA in the total fatty acid content of the extracted lipid of between 7% and 35%, particularly in the absence of DHA.

Accordingly, in a first aspect the present invention provides extracted plant lipid, comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA) and docosahexaenoic acid (DHA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and eicosatetraenoic acid (ETA), wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%, preferably between 30% and 35%.

In another aspect, the present invention provides extracted plant lipid, comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA) and docosahexaenoic acid (DHA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and eicosatetraenoic acid (ETA), wherein the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid, if present, is less than 1%, and wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%, preferably between 30% and 35%.

In another aspect, the invention provides extracted lipid, preferably extracted plant lipid or extracted microbial lipid, comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA) and docosapentaenoic acid (DPA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein the level of DPA in the total fatty acid content of the extracted lipid is between about 7% and 35%. In embodiments of this aspect, the level of DPA in the total fatty acid content of the extracted lipid is about 7%, about 8%, about 9%, about 10%, about 12%, about 15%, about 18%, about 20%, about 22%, about 24%, about 26%, about 28%, about 30%, between about 7% and about 28%, between about 7% and about 25%, between about 10% and 35%, between about 10% and about 30%, between about 10% and about 25%, between about 10% and about 22%, between about 14% and 35%, between about 16% and 35%, between about 16% and about 30%, between about 16% and about 25%, or between about 16% and about 22%.

In an embodiment of the above aspect, DHA is present at a level of less than 0.5% of the total fatty acid content of the extracted lipid and more preferably is absent from the total fatty acid content of the lipid.

In another aspect, the invention provides extracted lipid, preferably extracted
 5 plant lipid or extracted microbial lipid, comprising fatty acids in an esterified form, the fatty acids comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG. In an embodiment, the extracted lipid is further characterised by one or more or all of (i) it comprises fatty
 10 acids comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA) and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), (ii) at least about 40%, at least about 45%, at least about 48%, between 35% and about 60%, or between 35% and about 50%, of the DPA and/or DHA
 15 esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG, and (iii) the level of DPA and/DHA in the total fatty acid content of the extracted lipid is between about 1% and 35%, or between about 7% and 35% or between about 20.1% and 35%. In embodiments of this aspect, the level of DPA and/or DHA in the total fatty acid content of the extracted lipid is about 7%, about 8%, about 9%, about
 20 10%, about 12%, about 15%, about 18%, about 20%, about 22%, about 24%, about 26%, about 28%, about 30%, between about 7% and about 28%, between about 7% and about 25%, between about 10% and 35%, between about 10% and about 30%, between about 10% and about 25%, between about 10% and about 22%, between about 14% and 35%, between about 16% and 35%, between about 16% and about 30%, between
 25 about 16% and about 25%, or between about 16% and about 22%. In preferred embodiments, the extracted lipid is characterised by (i) and (ii), (i) and (iii) or (ii) and (iii), more preferably all of (i), (ii) and (iii). Preferably, the extracted lipid is further characterised by a level of palmitic acid in the total fatty acid content of the extracted lipid which is between about 2% and 16%, and a level of myristic acid (C14:0) in the
 30 total fatty acid content of the extracted lipid, if present, is less than 1%.

Embodiments of each of the four above aspects are described in further detail below. As the skilled person would understand, any embodiments described which are broader than the corresponding feature in an above aspect do not apply to that aspect.

In an embodiment, the extracted lipid has one or more of the following features

- i) the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 18%, between about 2% and 16%, between about 2% and 15%, or between about 3% and about 10%,
- 5 ii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid is less than 6%, less than 3%, less than 2%, less than 1%, or about 0.1%,
- 10 iii) the level of oleic acid in the total fatty acid content of the extracted lipid is between about 1% and about 30%, between about 3% and about 30%, between about 6% and about 30%, between 1% and about 20%, between about 30% and about 60%, about 45% to about 60%, about 30%, or between about 15% and about 30%,
- 15 iv) the level of linoleic acid (LA) in the total fatty acid content of the extracted lipid is between about 4% and about 35%, between about 4% and about 20%, between about 4% and about 17%, or between about 5% and about 10%,
- 20 v) the level of α -linolenic acid (ALA) in the total fatty acid content of the extracted lipid is between about 4% and about 40%, between about 7% and about 40%, between about 10% and about 35%, between about 20% and about 35%, between about 4% and 16%, or between about 2% and 16%,
- 25 vi) the level of γ -linolenic acid (GLA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, between 0.05% and about 7%, between 0.05% and about 4%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 30 vii) the level of stearidonic acid (SDA) in the total fatty acid content of the extracted lipid is less than about 10%, less than about 8%, less than about 7%, less than about 6%, less than about 4%, less than about 3%, between about 0.05% and about 7%, between about 0.05% and about 6%, between about 0.05% and about 4%, between about 0.05% and about 3%, between about 0.05% and about 10%, or between 0.05% and about 2%,
- 35 viii) the level of eicosatetraenoic acid (ETA) in the total fatty acid content of the extracted lipid is less than about 6%, less than about 5%, less than about 4%, less than about 1%, less than about 0.5%, between 0.05% and

- about 6%, between 0.05% and about 5%, between 0.05% and about 4%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 5 ix) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 2%, less than about 1%, between 0.05% and 4%, between 0.05% and 3%, or between 0.05% and about 2%, or between 0.05% and about 1%,
- 10 x) the level of eicosapentaenoic acid (EPA) in the total fatty acid content of the extracted lipid is between 4% and 15%, less than 4%, less than about 3%, less than about 2%, between 0.05% and 10%, between 0.05% and 5%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 15 xi) if the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 35%, the level of docosapentaenoic acid (DPA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 3%, less than about 2%, between 0.05% and 8%, between 0.05% and 5%, between 0.05% and about 3%, between 5% and 15%, between 5% and 10%, or between 0.05% and about 2%,
- 20 xii) the level of DHA in the total fatty acid content of the extracted lipid is about 22%, about 24%, about 26%, about 28%, about 31%, between 20.1% and 29%, between 20.1% and 28%, between 20.1% and about 27%, between 20.1% and about 26%, between 20.1% and about 25%, between 20.1% and about 24%, between 21% and 35%, between 21% and 30%, between 21% and 28%, between 21% and about 26%, or between 21% and about 24%,
- 25 xiii) the lipid comprises ω 6-docosapentaenoic acid ($22:5^{\Delta 4,7,10,13,16}$) in its fatty acid content,
- xiv) the lipid comprises less than 0.1% of ω 6-docosapentaenoic acid ($22:5^{\Delta 4,7,10,13,16}$) in its fatty acid content,
- xv) the lipid comprises less than 0.1% of one or more or all of SDA, EPA and ETA in its fatty acid content,
- 30 xvi) the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 25%, between about 4% and about 20%, between about 6% and about 20%, or between about 6% and about 12%,
- 35 xvii) the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 40%, between about 4% and about 35%, between about 8% and about 25%,

- between 8% and about 22%, between about 15% and about 40% or between about 15% and about 35%,
- 5 xviii) the level of total polyunsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 20% and about 75%, between 30% and 75%, between about 50% and about 75%, about 60%, about 65%, about 70%, about 75%, or between about 60% and about 75%,
- 10 xix) the level of total $\omega 6$ fatty acids in the total fatty acid content of the extracted lipid is between about 35% and about 50%, between about 20% and about 35%, between about 6% and 20%, less than 20%, less than about 16%, less than about 10%, between about 1% and about 16%, between about 2% and about 10%, or between about 4% and about 10%,
- 15 xx) the level of new $\omega 6$ fatty acids in the total fatty acid content of the extracted lipid is less than about 10%, less than about 8%, less than about 6%, less than 4%, between about 1% and about 20%, between about 1% and about 10%, between 0.5% and about 8%, or between 0.5% and 4%,
- 20 xxi) the level of total $\omega 3$ fatty acids in the total fatty acid content of the extracted lipid is between 36% and about 65%, between 36% and about 70%, between 40% and about 60%, between about 30% and about 60%, between about 35% and about 60%, between 40% and about 65%, between about 30% and about 65%, between about 35% and about 65%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, about 65% or about 70%,
- 25 xxii) the level of new $\omega 3$ fatty acids in the total fatty acid content of the extracted lipid is between 21% and about 45%, between 21% and about 35%, between about 23% and about 35%, between about 25% and about 35%, between about 27% and about 35%, about 23%, about 25%, about 27%, about 30%, about 35%, about 40% or about 45%,
- 30 xxiii) the ratio of total $\omega 6$ fatty acids: total $\omega 3$ fatty acids in the fatty acid content of the extracted lipid is between about 1.0 and about 3.0, between about 0.1 and about 1, between about 0.1 and about 0.5, less than about 0.50, less than about 0.40, less than about 0.30, less than about 0.20, less than about 0.15, about 1.0, about 0.1, about 0.10 to about 0.4, or about 0.2,
- 35 xxiv) the ratio of new $\omega 6$ fatty acids: new $\omega 3$ fatty acids in the fatty acid content of the extracted lipid is between about 1.0 and about 3.0,

- between about 0.02 and about 0.1, between about 0.1 and about 1, between about 0.1 and about 0.5, less than about 0.50, less than about 0.40, less than about 0.30, less than about 0.20, less than about 0.15, about 0.02, about 0.05, about 0.1, about 0.2 or about 1.0,
- 5 xxv) the fatty acid composition of the lipid is based on an efficiency of conversion of oleic acid to LA by $\Delta 12$ -desaturase of at least about 60%, at least about 70%, at least about 80%, between about 60% and about 98%, between about 70% and about 95%, or between about 75% and about 90%,
- 10 xxvi) the fatty acid composition of the lipid is based on an efficiency of conversion of ALA to SDA by $\Delta 6$ -desaturase of at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 30% and about 70%, between about 35% and about 60%, or between about 50% and about 70%,
- 15 xxvii) the fatty acid composition of the lipid is based on an efficiency of conversion of SDA to ETA acid by $\Delta 6$ -elongase of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 95%, between about 70% and about 88%, or between about 75% and about 85%,
- 20 xxviii) the fatty acid composition of the lipid is based on an efficiency of conversion of ETA to EPA by $\Delta 5$ -desaturase of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 99%, between about 70% and about 99%, or between about 75% and about 98%,
- 25 xxix) the fatty acid composition of the lipid is based on an efficiency of conversion of EPA to DPA by $\Delta 5$ -elongase of at least about 80%, at least about 85%, at least about 90%, between about 50% and about 99%, between about 85% and about 99%, between about 50% and about 95%, or between about 85% and about 95%,
- 30 xxx) if the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%, the fatty acid composition of the lipid is based on an efficiency of conversion of DPA to DHA by $\Delta 4$ -desaturase of at least about 80%, at least about 90%, at least about 93%, between about 50% and about 95%, between about 80% and about 95%, or between about 85% and about 95%,
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- xxxii) the fatty acid composition of the lipid is based on an efficiency of conversion of oleic acid to DHA and/or DPA of at least about 10%, at least about 15%, at least about 20%, at least about 25%, about 20%, about 25%, about 30%, between about 10% and about 50%, between about 10% and about 30%, between about 10% and about 25% or between about 20% and about 30%,
- xxxiii) the fatty acid composition of the lipid is based on an efficiency of conversion of LA to DHA and/or DPA of at least about 15%, at least about 20%, at least about 22%, at least about 25%, at least about 30%, at least about 40%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, between about 15% and about 50%, between about 20% and about 40%, or between about 20% and about 30%,
- xxxiiii) the fatty acid composition of the lipid is based on an efficiency of conversion of ALA to DHA and/or DPA of at least about 17%, at least about 22%, at least about 24%, at least about 30%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, between about 22% and about 70%, between about 17% and about 55%, between about 22% and about 40%, or between about 24% and about 40%,
- xxxiv) the total fatty acid in the extracted lipid has less than 1.5% C20:1, less than 1% C20:1 or about 1% C20:1,
- xxxv) the triacylglycerol (TAG) content of the lipid is at least about 70%, at least about 80%, at least about 90%, at least 95%, between about 70% and about 99%, or between about 90% and about 99%,
- xxxvi) the lipid comprises diacylglycerol (DAG), which DAG preferably comprises DHA and/or DPA,
- xxxvii) the lipid comprises less than about 10%, less than about 5%, less than about 1%, or between about 0.001% and about 5%, free (non-esterified) fatty acids and/or phospholipid, or is essentially free thereof,
- xxxviii) at least 70%, at least 72% or at least 80%, of the DHA and/or DPA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG,
- xxxix) the most abundant DHA-containing TAG species in the lipid is DHA/18:3/18:3 (TAG 58:12), the lipid comprises tri-DHA TAG (TAG 66:18), and
- xl) the level of DPA in the total fatty acid content of the extracted lipid is about 7%, about 8%, about 9%, about 10%, about 12%, about 15%,

about 18%, about 20%, about 22%, about 24%, about 26%, about 28%, about 31%, between about 7% and about 31%, between about 7% and about 28%, between about 10% and about 35%, between about 10% and about 30%, between about 10% and about 25%, between about 10% and about 22%, between about 14% and about 35%, between about 16% and about 35%, between about 16% and about 30%, between about 16% and about 25%, or between about 16% and about 22%, optionally wherein the level of DHA is less than 0.5% of the total fatty acid content of the extracted lipid.

- 10 In another embodiment, the extracted lipid has one or more of the following features
- i) the level of palmitic acid in the total fatty acid content of the extracted plant lipid is between 2% and 15%,
 - ii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted plant lipid is about 0.1%,
 - 15 iii) the level of oleic acid in the total fatty acid content of the extracted plant lipid is between 1% and 30%,
 - iv) the level of linoleic acid (LA) in the total fatty acid content of the extracted plant lipid is between 4% and 20%,
 - 20 v) the level of α -linolenic acid (ALA) in the total fatty acid content of the extracted plant lipid is between 4% and 40%,
 - vi) the level of γ -linolenic acid (GLA) in the total fatty acid content of the extracted plant lipid is between 0.05% and 7%,
 - vii) the level of stearidonic acid (SDA) in the total fatty acid content of the extracted plant lipid is between 0.05% and 10%,
 - 25 viii) the level of eicosatetraenoic acid (ETA) in the total fatty acid content of the extracted plant lipid is less than 6%,
 - ix) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted plant lipid is less than 4%,
 - 30 x) the extracted plant lipid comprises less than 0.1% of ω 6-docosapentaenoic acid (22:5 ^{Δ 4,7,10,13,16}) in its fatty acid content,
 - xi) the level of new ω 6 fatty acids in the total fatty acid content of the extracted plant lipid is less than 10%,
 - xii) the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted plant lipid is between 1.0 and 3.0, or between 0.1 and 1,
 - 35

- xiii) the ratio of new $\omega 6$ fatty acids: new $\omega 3$ fatty acids in the fatty acid content of the extracted plant lipid is between 1.0 and 3.0, between 0.02 and 0.1, or between 0.1 and 1,
- xiv) the fatty acid composition of the extracted plant lipid is based on an efficiency of conversion of oleic acid to DHA of at least 10%,
- xv) the fatty acid composition of the extracted plant lipid is based on an efficiency of conversion of LA to DHA of at least 15%,
- xvi) the fatty acid composition of the extracted plant lipid is based on an efficiency of conversion of ALA to DHA of at least 17%,
- xvii) the total fatty acid in the extracted plant lipid has less than 1.5% C20:1, and
- xviii) the triacylglycerol (TAG) content of the extracted plant lipid is at least 70%, and may be characterised by one or more of the following features
- xix) the extracted plant lipid comprises diacylglycerol (DAG) which comprises DHA,
- xx) the extracted plant lipid comprises less than 10% free (non-esterified) fatty acids and/or phospholipid, or is essentially free thereof,
- xxi) at least 70% of the DHA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG,
- xxii) the most abundant DHA-containing TAG species in the extracted plant lipid is DHA/18:3/18:3 (TAG 58:12), and
- xxiii) the extracted plant lipid comprises tri-DHA TAG (TAG 66:18).

In an embodiment, the level of eicosapentaenoic acid (EPA) in the total fatty acid content of the extracted plant lipid is between 0.05% and 10%.

In another embodiment, where DHA is present between 20.1% and 35%, the level of docosapentaenoic acid (DPA) in the total fatty acid content of the extracted plant lipid is less than about 4%.

In a further embodiment, the level of DHA in the total fatty acid content of the extracted plant lipid is between 20.1% and 30%.

In another embodiment, the extracted lipid is in the form of an oil, wherein at least about 90%, least about 95%, at least about 98%, or between about 95% and about 98%, by weight of the oil is the lipid.

In a preferred embodiment of the first two aspects above, the lipid or oil, preferably a seedoil, has the following features: in the total fatty acid content of the lipid or oil, the level of DHA is between about 20.1% and 30% or between 20.1% and 35%, the level of palmitic acid is between about 2% and about 16%, the level of myristic acid is less than about 6%, the level of oleic acid is between about 1% and

about 30%, the level of LA is between about 4% and about 35%, ALA is present, the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 25%, the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and about 3.0, and the triacylglycerol (TAG) content of the lipid is at least about 70%, and optionally the lipid is essentially free of cholesterol and/or the lipid comprises tri-DHA TAG (TAG 66:18). More preferably, the lipid or oil, preferably a seedoil, additionally has one or more or all of the following features: at least 70% of the DHA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG), ALA is present at a level of between 4% and 40% of the total fatty acid content, GLA is present and/or the level of GLA is less than 4% of the total fatty acid content, the level of SDA is between 0.05% and about 10%, the level of ETA is less than about 4%, the level of EPA is between 0.05% and about 10%, the level of DPA is between 0.05% and about 8%, the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 35%, the level of total polyunsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 20% and about 75%, the ratio of new ω 6 fatty acids: new ω 3 fatty acids in the fatty acid content of the extracted lipid is between about 0.03 and about 3.0, preferably less than about 0.50, the fatty acid composition of the lipid is based on: an efficiency of conversion of oleic acid to LA by Δ 12-desaturase of at least about 60%, an efficiency of conversion of SDA to ETA acid by Δ 6-elongase of at least about 60%, an efficiency of conversion of EPA to DPA by Δ 5-elongase of between about 50% and about 95%, an efficiency of conversion of DPA to DHA by Δ 4-desaturase of between about 50% and about 95%, an efficiency of conversion of oleic acid to DHA of at least about 10%. Most preferably, at least 81% of the DHA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG).

In a preferred embodiment of the third aspect above, the lipid or oil, preferably a seedoil, has the following features: in the total fatty acid content of the lipid or oil, the level of DPA is between about 7% and 30% or between about 7% and 35%, the level of palmitic acid is between about 2% and about 16%, the level of myristic acid is less than 1%, the level of oleic acid is between about 1% and about 30%, the level of LA is between about 4% and about 35%, ALA is present, the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 25%, the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and about 3.0, and the triacylglycerol (TAG) content of the lipid is at least about 70%, and optionally the lipid is essentially free of cholesterol and/or the lipid comprises tri-DPA TAG (TAG 66:15). More preferably, the lipid or oil,

preferably a seedoil, additionally has one or more or all of the following features: at least 70% of the DPA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG), ALA is present at a level of between 4% and 40% of the total fatty acid content, GLA is present and/or the level of GLA is less than 4% of the total fatty acid content, the level of SDA is between 0.05% and about 10%, the level of ETA is less than about 4%, the level of EPA is between 0.05% and about 10%, the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 35%, the level of total polyunsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 20% and about 75%, the ratio of new ω 6 fatty acids: new ω 3 fatty acids in the fatty acid content of the extracted lipid is between about 0.03 and about 3.0, preferably less than about 0.50, the fatty acid composition of the lipid is based on: an efficiency of conversion of oleic acid to LA by Δ 12-desaturase of at least about 60%, an efficiency of conversion of SDA to ETA acid by Δ 6-elongase of at least about 60%, an efficiency of conversion of EPA to DPA by Δ 5-elongase of between about 50% and about 95%, an efficiency of conversion of oleic acid to DPA of at least about 10%. Most preferably, at least 81% of the DPA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG).

In another preferred embodiment of the fourth aspect above, the lipid or oil, preferably a seedoil, comprising DHA and/or DPA has the following features: in the total fatty acid content of the lipid or oil, the level of palmitic acid is between about 2% and about 16%, the level of myristic acid is less than 1%, the level of oleic acid is between about 1% and about 30%, the level of LA is between about 4% and about 35%, ALA is present, the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 25%, the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and about 3.0, the triacylglycerol (TAG) content of the lipid is at least about 70%, and optionally the lipid comprises tri-DHA TAG (TAG 66:18) and/or tri-DPA TAG (TAG 66:15), wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG. More preferably, the lipid or oil, preferably a seedoil, additionally has one or more or all of the following features: ALA is present at a level of between 4% and 40% of the total fatty acid content, GLA is present and/or the level of GLA is less than 4% of the total fatty acid content, the level of SDA is between 0.05% and about 10%, the level of ETA is less than about 4%, the level of EPA is between 0.05% and about 10%, the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 35%, the level of total polyunsaturated fatty acids in the

total fatty acid content of the extracted lipid is between about 20% and about 75%, the ratio of new $\omega 6$ fatty acids: new $\omega 3$ fatty acids in the fatty acid content of the extracted lipid is between about 0.03 and about 3.0, preferably less than about 0.50, the fatty acid composition of the lipid is based on: an efficiency of conversion of oleic acid to LA by $\Delta 12$ -desaturase of at least about 60%, an efficiency of conversion of SDA to ETA acid by $\Delta 6$ -elongase of at least about 60%, an efficiency of conversion of EPA to DPA by $\Delta 5$ -elongase of between about 50% and about 95%, an efficiency of conversion of DPA to DHA by $\Delta 4$ -desaturase (if present) of between about 50% and about 95%, an efficiency of conversion of oleic acid to DPA of at least about 10%.

- 10 In the context of the extracted lipid or oil of the invention, the level of DHA and/or DPA in the extracted lipid or oil has not been increased, or is substantially the same as, the level of DHA and/or DPA in the lipid or oil of the plant part or microbe prior to extraction. In other words, no procedure has been performed to increase the level of DHA and/or DPA in the lipid or oil relative to other fatty acids post-extraction.
- 15 As would be apparent, the lipid or oil may subsequently be treated by fractionation or other procedures to alter the fatty acid composition.

In a further preferred embodiment, the lipid or oil, preferably a seedoil, has the following features: in the total fatty acid content of the lipid or oil, the level of DHA is between about 20.1% and 30% or between about 20.1% and 35%, the level of palmitic acid is between about 2% and about 16%, the level of myristic acid is less than about 6% and preferably less than 1%, the level of oleic acid is between about 1% and about 30%, the level of LA is between about 4% and about 35%, ALA is present, GLA is present, the level of SDA is between about 0.05% and about 10%, the level of ETA is less than about 6%, the level of EPA is between about 0.05% and about 10%, the level of DPA is between about 0.05% and about 8%.

In another preferred embodiment, the lipid or oil, preferably a seedoil and more preferably a *Brassica* seedoil such as mustard oil or canola oil, has the following features: in the total fatty acid content of the lipid or oil, the level of DPA is between about 7% and 35%, the level of palmitic acid is between about 2% and about 16%, the level of myristic acid is less than about 6% and preferably less than 1%, the level of oleic acid is between about 1% and about 30%, the level of LA is between about 4% and about 35%, ALA is present, the level of SDA is between about 0.05% and about 10%, the level of ETA is less than about 6%, the level of EPA is between about 0.05% and about 10%. DHA may or may not be present in the lipid or oil. Preferably, DHA, if present, is present at a level of not more than 0.5% of the total fatty acid content of the lipid or oil and more preferably is absent from the total fatty acid content of the

lipid or oil. Optionally, the lipid is essentially free of cholesterol and/or the lipid comprises tri-DPA TAG (TAG 66:15). More preferably, the lipid or oil, preferably a seedoil, additionally has one or more or all of the following features: at least 70% of the DPA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG), ALA is present
 5 at a level of between 4% and 40% of the total fatty acid content, GLA is present and/or the level of GLA is less than 4% of the total fatty acid content, the level of SDA is between 0.05% and about 10%, the level of ETA is less than about 4%, the level of EPA is between 0.05% and about 10%, the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 35%,
 10 the level of total polyunsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 20% and about 75%, the ratio of new ω 6 fatty acids: new ω 3 fatty acids in the fatty acid content of the extracted lipid is between about 0.03 and about 3.0, preferably less than about 0.50, the fatty acid composition of the lipid is based on: an efficiency of conversion of oleic acid to LA by Δ 12-desaturase of at least
 15 about 60%, an efficiency of conversion of SDA to ETA acid by Δ 6-elongase of at least about 60%, an efficiency of conversion of EPA to DPA by Δ 5-elongase of between about 50% and about 95%, an efficiency of conversion of oleic acid to DPA of at least about 10%. In an embodiment, at least 81% of the DPA is esterified at the *sn*-1 or *sn*-3 position of triacylglycerol (TAG). Alternatively, at least 35% of the DPA esterified in
 20 the form of TAG is esterified at the *sn*-2 position of TAG.

In a further embodiment, the extracted lipid of the invention further comprises one or more sterols, preferably plant sterols.

In another embodiment, the extracted lipid is in the form of an oil, and comprises less than about 10 mg of sterols/g of oil, less than about 7 mg of sterols/g of
 25 oil, between about 1.5 mg and about 10 mg of sterols/g of oil, or between about 1.5 mg and about 7 mg of sterols/g of oil.

Examples of sterols which can be in the extracted lipid include, but are not necessarily limited to, one or more or all of campesterol/24-methylcholesterol, Δ 5-stigmasterol, eburicol, β -sitosterol/24-ethylcholesterol, Δ 5-avenasterol/isofucosterol,
 30 Δ 7-stigmasterol/stigmast-7-en-3 β -ol, and Δ 7-avenasterol.

In an embodiment, the plant species is one listed in Table 14, such as canola, and the level of sterols are about the same as that listed in Table 14 for that particular plant species. The plant species may be mustard (*B. juncea*) or *C. sativa* and comprise a level of sterols about that found in wild-type mustard or *C. sativa* extracted oil,
 35 respectively.

In an embodiment, the extracted plant lipid comprises one or more or all of campesterol/24-methylcholesterol, Δ^5 -stigmasterol, eburicol, β -sitosterol/24-ethylcholesterol, Δ^5 -avenasterol/isofucosterol, Δ^7 -stigmasterol/stigmast-7-en-3 β -ol, and Δ^7 -avenasterol, or which has a sterol content essentially the same as wild-type
5 canola oil.

In an embodiment, the extracted lipid has a sterol content essentially the same as wild-type canola oil, mustard oil or *C. sativa* oil.

In an embodiment, the extracted lipid comprises less than about 0.5 mg of cholesterol/g of oil, less than about 0.25 mg of cholesterol/g of oil, between about 0 mg
10 and about 0.5 mg of cholesterol/g of oil, or between about 0 mg and about 0.25 mg of cholesterol/g of oil, or which is essentially free of cholesterol.

In a further embodiment, the lipid is an oil, preferably oil from an oilseed. Examples of such oils include, but are not limited to, *Brassica sp.* oil such as for example canola oil or mustard oil, *Gossypium hirsutum* oil, *Linum usitatissimum* oil,
15 *Helianthus sp.* oil, *Carthamus tinctorius* oil, *Glycine max* oil, *Zea mays* oil, *Arabidopsis thaliana* oil, *Sorghum bicolor* oil, *Sorghum vulgare* oil, *Avena sativa* oil, *Trifolium sp.* oil, *Elaeisis guineensis* oil, *Nicotiana benthamiana* oil, *Hordeum vulgare* oil, *Lupinus angustifolius* oil, *Oryza sativa* oil, *Oryza glaberrima* oil, *Camelina sativa* oil, *Crambe abyssinica* oil, *Miscanthus x giganteus* oil, or *Miscanthus sinensis* oil. More preferably,
20 the oil is a *Brassica sp.* oil, a *Camelina sativa* oil or a *Glycine max* (soybean) oil. In an embodiment the lipid comprises or is *Brassica sp.* oil such as *Brassica napus* oil or *Brassica juncea* oil, *Gossypium hirsutum* oil, *Linum usitatissimum* oil, *Helianthus sp.* oil, *Carthamus tinctorius* oil, *Glycine max* oil, *Zea mays* oil, *Elaeisis guineensis* oil, *Nicotiana benthamiana* oil, , *Lupinus angustifolius* oil,, *Camelina sativa* oil, *Crambe*
25 *abyssinica* oil, *Miscanthus x giganteus* oil, or *Miscanthus sinensis* oil. In a further embodiment, the oil is canola oil, mustard (*B. juncea*) oil, soybean (*Glycine max*) oil, *Camelina sativa* oil or *Arabidopsis thaliana* oil. In an alternate embodiment, the oil is a plant oil other than *A. thaliana* oil and/or other than *C. sativa* oil. In an embodiment, the plant oil is an oil other than *G. max* (soybean) oil. In an embodiment, the oil was
30 obtained from a plant grown under standard conditions, for Example as described in Example 1, or from a plant grown in the field or in a glasshouse under standard conditions.

In another aspect, the present invention provides a process for producing extracted plant lipid, comprising the steps of

35 i) obtaining a plant part comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids

which comprise linoleic acid (LA) and γ -linolenic acid (GLA), ω 3 fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally one or more of eicosapentaenoic acid (EPA) and eicosatetraenoic acid (ETA), wherein the level of DHA in the total fatty acid content of extractable lipid in the plant part is between 20.1% and 30% or between 20.1% and 35%, and

ii) extracting lipid from the plant part,

wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%.

10 In a further aspect, the present invention provides a process for producing extracted plant lipid, comprising the steps of

i) obtaining a plant part comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA) and γ -linolenic acid (GLA), ω 3 fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally one or more of eicosapentaenoic acid (EPA) and eicosatetraenoic acid (ETA), wherein the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid, if present, is less than 1%, and wherein the level of DHA in the total fatty acid content of extractable lipid in the plant part is between 20.1% and 30% or between 20.1% and 35%, and

ii) extracting lipid from the plant part,

wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%.

In a further aspect, the present invention provides a process for producing extracted plant lipid, comprising the steps of

i) obtaining a plant part comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally one or more of eicosapentaenoic acid (EPA) and eicosatetraenoic acid (ETA), wherein the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid, if present, is

less than 1%, and wherein the level of DHA in the total fatty acid content of extractable lipid in the plant part is between 20.1% and 30% or between 20.1% and 35%, and

ii) extracting lipid from the plant part,

wherein the level of DHA in the total fatty acid content of the extracted lipid is between
5 20.1% and 30% or between 20.1% and 35%.

In an embodiment of the three above aspect, the invention provides a process for producing extracted plant lipid, comprising the steps of

i) obtaining a plant part comprising lipid, the lipid comprising fatty acids in an esterified form, wherein the lipid has a fatty acid composition comprising oleic acid,
10 palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA) and docosahexaenoic acid (DHA), and one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and eicosatetraenoic acid (ETA), wherein (i) the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%,
15 preferably between 30% and 35%, (ii) the level of palmitic acid in the total fatty acid content of the extracted lipid is between 2% and 16%, (iii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid is less than 1%, (iv) the level of oleic acid in the total fatty acid content of the extracted lipid is between 1% and 30%, (v) the level of linoleic acid (LA) in the total fatty acid content of the extracted
20 lipid is between 4% and 35%, (vi) the level of α -linolenic acid (ALA) in the total fatty acid content of the extracted lipid is between 4% and 40%, (vii) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted lipid is less than 4%, (viii) the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between 4% and 25%, (ix) the ratio of total ω 6 fatty acids: total ω 3
25 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and 1, (x) the triacylglycerol (TAG) content of the lipid is at least 70%, and (xi) at least 70% of the DHA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG, and

ii) extracting lipid from the plant part,

wherein the level of DHA in the total fatty acid content of the extracted lipid is between
30 about 20.1% and 30% or between 20.1% and 35%, preferably between 30% and 35%. Preferably, at least 81% or at least 90% of the DHA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG.

In a further aspect, the invention provides a process for producing extracted plant lipid or microbial lipid, comprising the steps of

35 i) obtaining a plant part or microbial cells comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, ω 6

fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA) and docosapentaenoic acid (DPA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein the level of DPA in the total fatty acid content of the lipid of the plant part or
 5 microbial cells between about 7% and 35%, and

ii) extracting lipid from the plant part or microbial cells, wherein the level of DPA in the total fatty acid content of the extracted lipid is between about 7% and 35%. In an embodiment, the level of DPA in the total fatty acid content of the extracted lipid is between about 7% and 20%, or between 20.1% and 35%.

10 In an embodiment of the above aspect, the invention provides a process for producing extracted plant lipid or microbial lipid, comprising the steps of

i) obtaining a plant part or microbial cells comprising lipid, the lipid comprising fatty acids in an esterified form, wherein the lipid has a fatty acid composition comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA),
 15 ω 3 fatty acids which comprise α -linolenic acid (ALA) and docosahexaenoic acid (DPA), and one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein (i) the level of DPA in the total fatty acid content of the extracted lipid is between 7% and 30% or between 7% and 35%, preferably between 30% and 35%, (ii) the level of palmitic acid in the total fatty acid content of
 20 the extracted lipid is between 2% and 16%, (iii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid is less than 6%, preferably less than 1%, (iv) the level of oleic acid in the total fatty acid content of the extracted lipid is between 1% and 30%, (v) the level of linoleic acid (LA) in the total fatty acid content of the extracted lipid is between 4% and 35%, (vi) the level of α -linolenic acid (ALA) in the
 25 total fatty acid content of the extracted lipid is between 4% and 40%, (vii) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted lipid is less than 4%, (viii) the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between 4% and 25%, (ix) the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and 1, (x) the
 30 triacylglycerol (TAG) content of the lipid is at least 70%, and (xi) at least 70% of the DPA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG and

ii) extracting lipid from the plant part, wherein the level of DPA in the total fatty acid content of the extracted lipid is between about 7% and 30% or between 7% and 35%, preferably between 30% and 35%.
 35 Preferably, at least 81% or at least 90% of the DPA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG.

In another aspect, the present invention provides a process for producing extracted lipid, comprising the steps of

- i) obtaining cells, preferably a plant part comprising the cells or microbial cells, comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG, and

- ii) extracting lipid from the cells, wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) in the total fatty acid content of the extracted lipid is esterified at the *sn*-2 position of the TAG. In an embodiment, the extracted lipid produced by the process is further characterised by one or more or all of (i) it comprises fatty acids comprising oleic acid, palmitic acid, and fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA) and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), (ii) at least about 40%, at least about 45%, at least about 48%, between 35% and about 60%, or between 35% and about 50%, of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG, and (iii) the level of DPA and/DHA in the total fatty acid content of the extracted lipid is between about 1% and 35%, or between about 7% and 35% or between about 20.1% and 35%. In embodiments of this aspect, the level of DPA and/or DHA in the total fatty acid content of the extracted lipid is about 7%, about 8%, about 9%, about 10%, about 12%, about 15%, about 18%, about 20%, about 22%, about 24%, about 26%, about 28%, about 30%, between about 7% and about 28%, between about 7% and about 25%, between about 10% and 35%, between about 10% and about 30%, between about 10% and about 25%, between about 10% and about 22%, between about 14% and 35%, between about 16% and 35%, between about 16% and about 30%, between about 16% and about 25%, or between about 16% and about 22%. In preferred embodiments, the extracted lipid is characterised by (i) and (ii), (i) and (iii) or (ii) and (iii), more preferably all of (i), (ii) and (iii). Preferably, the extracted lipid is further characterised by a level of palmitic acid in the total fatty acid content of the extracted lipid which is between about 2% and 16%, and a level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid, if present, is less than 1%.

In an embodiment of the above aspect, the invention provides a process for producing extracted lipid, comprising the steps of

- i) obtaining cells, preferably a plant part comprising the cells or microbial cells, comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), and further comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA), and one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein (i) the level of palmitic acid in the total fatty acid content of the extracted lipid is between 2% and 16%, (ii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid is less than 1%, (iii) the level of oleic acid in the total fatty acid content of the extracted lipid is between 1% and 30%, (iv) the level of linoleic acid (LA) in the total fatty acid content of the extracted lipid is between 4% and 35%, (v) the level of α -linolenic acid (ALA) in the total fatty acid content of the extracted lipid is between 4% and 40%, (vi) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted lipid is less than 4%, (vii) the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between 4% and 25%, (viii) the ratio of total ω 6 fatty acids: total ω 3 fatty acids in the fatty acid content of the extracted lipid is between 0.05 and 1, (ix) the triacylglycerol (TAG) content of the lipid is at least 70%, and (x) at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG, and
- ii) extracting lipid from the plant part, wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) in the total fatty acid content of the extracted lipid is esterified at the *sn*-2 position of the TAG.

The step of obtaining the plant part or microbial cells may comprise harvesting plant parts, preferably seed, from plants that produce the plant parts, recovery of the microbial cells from cultures of such cells, or obtaining the plant parts or microbial cells by purchase from a producer or supplier, or by importation. The process may comprise a step of determining the fatty acid composition of the lipid in a sample of the plant parts or microbial cells, or of the extracted lipid.

In a preferred embodiment, the extracted lipid obtained by a process of the invention has, where relevant, one or more of the features defined herein, for example as defined above in relation to the first four aspects.

Embodiments of each of the five above aspects are described in further detail below. As the skilled person would understand, any embodiments described which are broader than the corresponding feature in an above aspect do not apply to that aspect.

- In an embodiment, the plant part is a seed, preferably an oilseed. Examples of such seeds include, but are not limited to, *Brassica sp.*, *Gossypium hirsutum*, *Linum usitatissimum*, *Helianthus sp.*, *Carthamus tinctorius*, *Glycine max*, *Zea mays*, *Arabidopsis thaliana*, *Sorghum bicolor*, *Sorghum vulgare*, *Avena sativa*, *Trifolium sp.*,
- 5 *Elaeisis guineensis*, *Nicotiana benthamiana*, *Hordeum vulgare*, *Lupinus angustifolius*, *Oryza sativa*, *Oryza glaberrima*, *Camelina sativa*, or *Crambe abyssinica*, preferably a *Brassica sp.* seed, a *C. sativa* seed or a *G. max* (soybean) seed, more preferably a *Brassica napus*, *B. juncea* or *C. sativa* seed. In an embodiment, the plant part is a seed, preferably an oilseed such as *Brassica sp.* such as *Brassica napus* or *Brassica juncea*,
- 10 *Gossypium hirsutum*, *Linum usitatissimum*, *Helianthus sp.*, *Carthamus tinctorius*, *Glycine max*, *Zea mays*, *Elaeisis guineensis*, *Nicotiana benthamiana*, *Lupinus angustifolius*, *Camelina sativa*, or *Crambe abyssinica*, preferably a *Brassica napus*, *B. juncea* or *C. sativa* seed. In an embodiment, the seed is canola seed, mustard seed, soybean seed, *Camelina sativa* seed or *Arabidopsis thaliana* seed. In an alternate
- 15 embodiment, the seed is a seed other than *A. thaliana* seed and/or other than *C. sativa* seed. In an embodiment, the seed is a seed other than soybean seed. In an embodiment, the plant part is *Brassica sp.* seed. In an embodiment, the seed was obtained from a plant grown under standard conditions, for Example as described in Example 1, or from a plant grown in the field or in a glasshouse under standard conditions.
- 20 In another embodiment, the seed comprises at least about 18 mg, at least about 22 mg, at least about 26 mg, between about 18 mg and about 100 mg, between about 22 mg and about 70 mg, about 80 mg, between about 30mg and about 80mg, or between about 24 mg and about 50 mg, of DHA and/or DPA per gram of seed.
- In a further embodiment, the plant part such as a seed comprises exogenous
- 25 polynucleotides encoding one of the following sets of enzymes;
- i) an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
 - ii) a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
 - 30 iii) a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
 - iv) a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
 - v) an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-
- 35 elongase and an Δ 5-elongase,

vi) a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and a $\Delta 5$ -elongase,

vii) a $\Delta 12$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

- 5 viii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,
and wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in a cell of the plant part.

In a further embodiment, the plant part such as a seed or recombinant cells such
10 as microbial cells comprise exogenous polynucleotides encoding one of the following sets of enzymes;

i) an $\omega 3$ -desaturase and/or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and a $\Delta 5$ -elongase,

15 ii) a $\Delta 12$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

iii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

iv) an $\omega 3$ -desaturase and/or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and a $\Delta 5$ -elongase,

20 v) a $\Delta 12$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

vi) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

25 and wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in a cell of the plant part or the cells.

In an embodiment, if the plant part or cells comprise lipid comprising fatty acids in an esterified form, the fatty acids comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), wherein at least 35% of the DPA and/or DHA esterified
30 in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG, the plant part such as a seed or recombinant cells such as microbial cells comprise an exogenous polynucleotide encoding an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), wherein the polynucleotide is operably linked to one or more promoters that are capable of directing expression of the polynucleotide in a cell of the plant part or
35 the cells. In a further embodiment, the cells comprises exogenous polynucleotides encoding one of the following sets of enzymes;

i) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

ii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

iii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

iv) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase and a Δ 5-elongase and optionally a Δ 4-desaturase,

v) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

vi) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

vii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

viii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell. Preferably, the LPAAT can use a C22 polyunsaturated fatty acyl-CoA substrate such as DHA-CoA and/or DPA-CoA.

In an embodiment, the Δ 12-desaturase also has ω 3-desaturase and/or Δ 15-desaturase activity, i.e. the activities are conferred by a single polypeptide. Alternatively, the Δ 12-desaturase does not have ω 3-desaturase activity and does not have Δ 15-desaturase activity i.e. the Δ 12-desaturase is a separate polypeptide to the polypeptide having ω 3-desaturase activity and/or Δ 15-desaturase.

In yet a further embodiment, the plant part such as a seed or recombinant cells such as microbial cells have one or more or all of the following features:

- i) the $\Delta 12$ -desaturase converts oleic acid to linoleic acid in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 60%, at least about 70%, at least about 80%, between about 60% and about 95%, between about 70% and about 90%, or between about 75% and about 85%,
- 5 ii) the $\omega 3$ -desaturase converts $\omega 6$ fatty acids to $\omega 3$ fatty acids in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 65%, at least about 75%, at least about 85%, between about 65% and about 95%, between about 75% and about 91%, or between about 80% and about 91%,
- 10 iii) the $\Delta 6$ -desaturase converts ALA to SDA in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 30% and about 70%, between about 35% and about 60%, or between about 50% and about 70%,
- 15 iv) the $\Delta 6$ -desaturase converts linoleic acid to γ -linolenic acid in one or more cells of the plant part or in the recombinant cells with an efficiency of less than about 5%, less than about 2.5%, less than about 1%, between about 0.1% and about 5%, between about 0.5% and about 2.5%, or between about 0.5% and about 1%,
- 20 v) the $\Delta 6$ -elongase converts SDA to ETA in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 95%, between about 70% and about 80%, or between about 75% and about 80%,
- 25 vi) the $\Delta 5$ -desaturase converts ETA to EPA in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, between about 60% and about 95%, between about 70% and about 95%, or between about 75% and about 95%,
- vii) the $\Delta 5$ -elongase converts EPA to DPA in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 80%, at least about 85%, at least about 90%, between about 50% and about 90%, or between about 85% and about 95%,
- 30 viii) the $\Delta 4$ -desaturase converts DPA to DHA in one or more cells of the plant part or in the recombinant cells with an efficiency of at least about 80%, at least about 90%, at least about 93%, between about 50% and about 95%, between about 80% and about 95%, or between about 85% and about 95%,
- 35 ix) the efficiency of conversion of oleic acid to DHA or DPA in one or more cells of the plant part or in the recombinant cells is at least about 10%, at least about 15%, at least about 20%, at least about 25%, about 20%, about 25%, about 30%,

between about 10% and about 50%, between about 10% and about 30%, between about 10% and about 25%, or between about 20% and about 30%,

x) the efficiency of conversion of LA to DHA or DPA in one or more cells of the plant part or in the recombinant cells is at least about 15%, at least about 20%, at least about 22%, at least about 25%, at least about 30%, about 25%, about 30%, about 35%, between about 15% and about 50%, between about 20% and about 40%, or between about 20% and about 30%,

xi) the efficiency of conversion of ALA to DHA or DPA in one or more cells of the plant part or in the recombinant cells is at least about 17%, at least about 22%, at least about 24%, at least about 30%, about 30%, about 35%, about 40%, between about 17% and about 55%, between about 22% and about 35%, or between about 24% and about 35%,

xii) one or more cells of the plant part or the recombinant cells comprise at least about 25%, at least about 30%, between about 25% and about 40%, or between about 27.5% and about 37.5%, more ω 3 fatty acids than corresponding cells lacking the exogenous polynucleotides,

xiii) the Δ 6-desaturase preferentially desaturates α -linolenic acid (ALA) relative to linoleic acid (LA),

xiv) the Δ 6-elongase also has Δ 9-elongase activity,

xv) the Δ 12-desaturase also has Δ 15-desaturase activity,

xvi) the Δ 6-desaturase also has Δ 8-desaturase activity,

xvii) the Δ 8-desaturase also has Δ 6-desaturase activity or does not have Δ 6-desaturase activity,

xviii) the Δ 15-desaturase also has ω 3-desaturase activity on GLA,

xix) the ω 3-desaturase also has Δ 15-desaturase activity on LA,

xx) the ω 3-desaturase desaturates both LA and/or GLA,

xxi) the ω 3-desaturase preferentially desaturates GLA relative to LA,

xxii) one or more or all of the desaturases, preferably the Δ 6-desaturase and/or the Δ 5-desaturase, have greater activity on an acyl-CoA substrate than a corresponding acyl-PC substrate,

xxiii) the Δ 6-desaturase has greater Δ 6-desaturase activity on ALA than LA as fatty acid substrate,

xxiv) the Δ 6-desaturase has greater Δ 6-desaturase activity on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxv) the $\Delta 6$ -desaturase has at least about a 2-fold greater $\Delta 6$ -desaturase activity, at least 3-fold greater activity, at least 4-fold greater activity, or at least 5-fold greater activity, on ALA as a substrate compared to LA,

xxvi) the $\Delta 6$ -desaturase has greater activity on ALA-CoA as fatty acid substrate
5 than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxvii) the $\Delta 6$ -desaturase has at least about a 5-fold greater $\Delta 6$ -desaturase activity or at least 10-fold greater activity, on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxviii) the desaturase is a front-end desaturase, and

10 xxix) the $\Delta 6$ -desaturase has no detectable $\Delta 5$ -desaturase activity on ETA.

In yet a further embodiment, the plant part such as a seed or the recombinant cell such as microbial cells has one or more or all of the following features

i) the $\Delta 12$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:4, a biologically active fragment thereof, or an amino acid sequence which
15 is at least 50% identical to SEQ ID NO:4,

ii) the $\omega 3$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:6, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:6,

iii) the $\Delta 6$ -desaturase comprises amino acids having a sequence as provided in
20 SEQ ID NO:9, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:9,

iv) the $\Delta 6$ -elongase comprises amino acids having a sequence as provided in SEQ ID NO:16, a biologically active fragment thereof such as SEQ ID NO:17, or an amino acid sequence which is at least 50% identical to SEQ ID NO:16 and/or SEQ ID
25 NO:17,

v) the $\Delta 5$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:20, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:20,

vi) the $\Delta 5$ -elongase comprises amino acids having a sequence as provided in
30 SEQ ID NO:25, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:25, and

vii) the $\Delta 4$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:28, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:28.

35 In an embodiment, the plant part such as a seed or the recombinant cells such as microbial cells further comprise(s) an exogenous polynucleotide encoding a

diacylglycerol acyltransferase (DGAT), monoacylglycerol acyltransferase (MGAT), glycerol-3-phosphate acyltransferase (GPAT), 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT) preferably an LPAAT which can use a C22 polyunsaturated fatty acyl-CoA substrate such as DHA-CoA and/or DPA-CoA, acyl-
 5 CoA:lysophosphatidylcholine acyltransferase (LPCAT), phospholipase A₂ (PLA₂), phospholipase C (PLC), phospholipase D (PLD), CDP-choline diacylglycerol choline phosphotransferase (CPT), phosphatidylcholine diacylglycerol acyltransferase (PDAT), phosphatidylcholine:diacylglycerol choline phosphotransferase (PDCT), acyl-CoA synthase (ACS), or a combination of two or more thereof.

- 10 In another embodiment, the plant part such as a seed or the recombinant cells such as microbial cells further comprise(s) an introduced mutation or an exogenous polynucleotide which down regulates the production and/or activity of an endogenous enzyme in a cell of the plant part selected from FAEL, DGAT, MGAT, GPAT, LPAAT, LPCAT, PLA₂, PLC, PLD, CPT, PDAT, a thioesterase such as FATB, or a
 15 Δ 12-desaturase, or a combination of two or more thereof.

In a further embodiment, at least one, or all, of the promoters are seed specific promoters. In an embodiment, at least one, or all, of the promoters have been obtained from an oil biosynthesis or accumulation gene such as a gene encoding oleosin, or from a seed storage protein genes such as a gene encoding conlinin.

- 20 In another embodiment, the promoter(s) directing expression of the exogenous polynucleotides encoding the Δ 4-desaturase and the Δ 5-elongase initiate expression of the polynucleotides in developing seed of the plant or the recombinant cells such as the microbial cells before, or reach peak expression before, the promoter(s) directing expression of the exogenous polynucleotides encoding the Δ 12-desaturase and the ω 3-
 25 desaturase.

- In a further embodiment, the exogenous polynucleotides are covalently linked in a DNA molecule, preferably a T-DNA molecule, integrated into the genome of cells of the plant part or the recombinant cells such as the microbial cells and preferably where the number of such DNA molecules integrated into the genome of the cells of the plant
 30 part or the recombinant cells is not more than one, two or three, or is two or three.

In yet another embodiment, the plant part comprises at least two different, exogenous polynucleotides each encoding a Δ 6-desaturase which have the same or different amino acid sequences.

- In a further embodiment, the total oil content of the plant part comprising the
 35 exogenous polynucleotides is at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 50% and about 80%, or between about 80%

and about 100% of the total oil content of a corresponding plant part lacking the exogenous polynucleotides. In a further embodiment, the seed comprising the exogenous polynucleotides has a seed weight at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 50% and about 80%, or between
5 about 80% and about 100% of the weight of a corresponding seed lacking the exogenous polynucleotides.

In another embodiment, the lipid is in the form of an oil, preferably a seedoil from an oilseed, and wherein at least about 90%, or about least 95%, at least about 98%, or between about 95% and about 98%, by weight of the lipid is triacylglycerols.

10 In a further embodiment, the process further comprises treating the lipid to increase the level of DHA and/or DPA as a percentage of the total fatty acid content. For example, the treatment comprises transesterification. For example, the lipid such as canola oil may be treated to convert the fatty acids in the oil to alkyl esters such as methyl or ethyl esters, which may then be fractionated to enrich the lipid or oil for the
15 DHA and/or DPA. In embodiments, the fatty acid composition of the lipid after such treatment comprises at least 40%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% DHA and/or DPA. The ratio of DHA:DPA in the lipid after treatment is preferably greater than 2:1, or alternatively less than 0.5:1. Alternatively, the level of DHA in the total fatty acid content of the lipid after treatment is less than 2.0% or less
20 than 0.5%, preferably is absent from the lipid.

Also provided is lipid, or oil comprising the lipid, produced using a process of the invention.

In another aspect, the present invention provides a process for producing methyl or ethyl esters of polyunsaturated fatty acids, the process comprising reacting
25 triacylglycerols in extracted plant lipid, or during the process of extraction, with methanol or ethanol, respectively, wherein the extracted plant lipid comprises fatty acids esterified in the form of TAG, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), and docosahexaenoic acid (DHA), and optionally one or more of
30 stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and eicosatetraenoic acid (ETA), wherein the level of DHA in the total fatty acid content of the extracted lipid is between about 20.1% and 30%, or between 20.1% and 35%, preferably between 30% and 35%, thereby producing the methyl or ethyl esters of polyunsaturated fatty acids.

35 In another aspect, the present invention provides a process for producing methyl or ethyl esters of polyunsaturated fatty acids, the process comprising reacting

triacylglycerols in extracted plant lipid, or during the process of extraction, with methanol or ethanol, respectively, wherein the extracted plant lipid comprises fatty acids esterified in the form of TAG, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), and docosapentaenoic acid (DPA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein the level of DPA in the total fatty acid content of the extracted lipid is between about 7% and 35%, preferably between 20.1% and 30% or between 20.1% and 35%, thereby producing the methyl or ethyl esters of polyunsaturated fatty acids.

10 In another aspect, the present invention provides a process for producing methyl or ethyl esters of docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), the process comprising reacting triacylglycerols (TAG) in extracted plant lipid, or during the process of extraction, with methanol or ethanol, respectively, wherein the extracted plant lipid comprises fatty acids in an esterified form, the fatty acids
15 comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), wherein at least 35% of the DPA and/or DHA esterified in the form of TAG is esterified at the *sn*-2 position of the TAG, thereby producing the methyl or ethyl esters of polyunsaturated fatty acids.

In a preferred embodiment, the lipid which is used in the process of the above
20 three aspects has one or more of the features defined herein in the context of the extracted lipid or oil of the invention.

In another aspect, the present invention provides an oilseed plant or part thereof such as a seed comprising

- a) lipid in its seed, the lipid comprising fatty acids in an esterified form, and
- 25 b) exogenous polynucleotides encoding one of the following sets of enzymes;
 - i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or
 - ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

30 wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, wherein the fatty acids comprise oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA) and γ -linolenic acid (GLA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA),
35 docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level

of DHA in the total fatty acid content of the lipid of the seed is between 20.1% and 30%, or between 20.1% and 35%, preferably between 30% and 35%.

In another aspect, the present invention provides an oilseed plant or part thereof such as a seed comprising

- 5 a) lipid in its seed, the lipid comprising fatty acids in an esterified form, and
- b) exogenous polynucleotides encoding one of the following sets of enzymes;
 - i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or
 - 10 ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, wherein the fatty acids comprise oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level of DHA in the total fatty acid content of the lipid of the seed is between 20.1% and 30%, or between 20.1% and 35%, preferably between 30% and 35%.

- 20 In another aspect, the present invention provides an oilseed plant or part thereof such as a seed comprising

- a) lipid in its seed, the lipid comprising fatty acids in an esterified form, and
- b) exogenous polynucleotides encoding one of the following sets of enzymes;
 - i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or
 - 25 ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

- wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, wherein the fatty acids comprise oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA) and γ -linolenic acid (GLA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level
- 30 of DHA in the total fatty acid content of the lipid of the seed is between 20.1% and 30%, or between 20.1% and 35%, and wherein the level of palmitic acid in the total
- 35

fatty acid content of the lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the lipid, if present, is less than 1%.

In another aspect, the present invention provides an oilseed plant or part thereof
5 such as a seed comprising lipid in its seed, or a microbial cell, comprising

a) lipid comprising fatty acids in an esterified form, and

b) exogenous polynucleotides encoding one of the following sets of enzymes;

i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

10 ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

iii) a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or

15 iv) a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, or one or more promoters that are capable of directing expression of said polynucleotides in the microbial cell, wherein the fatty acids
20 comprise oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA) and optionally γ -linolenic acid (GLA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), and docosapentaenoic acid (DPA), and optionally docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level of DPA in the total fatty acid content of the lipid of
25 the seed or microbial cell is between 7% and 35%. In a preferred embodiment of this aspect, DHA is present at a level of less than 0.5% of the total fatty acid content of the lipid of the seed and of the extracted lipid and more preferably is absent from the total fatty acid content of the lipids.

In another aspect, the present invention provides a cell, preferably a cell in or
30 from a plant such as an oilseed plant or part thereof such as a seed, or an oilseed plant or part thereof, or a microbial cell, comprising

a) fatty acids in an esterified form, the fatty acids comprising docosapentaenoic acid (DPA) and/or docosahexaenoic acid (DHA), wherein at least 35% of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2
35 position of the TAG, and

b) exogenous polynucleotides encoding one of the following sets of enzymes;

i) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

5 ii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

iii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, an Δ 5-elongase and optionally a Δ 4-desaturase,

10 iv) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase and an Δ 5-elongase and optionally a Δ 4-desaturase,

v) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, an Δ 5-elongase and
15 optionally a Δ 4-desaturase,

vi) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

vii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-
20 desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, an Δ 5-elongase and optionally a Δ 4-desaturase,

viii) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, an Δ 5-elongase and optionally a Δ 4-desaturase,

25 wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell. Preferably, the LPAAT can use a C22 polyunsaturated fatty acyl-CoA substrate such as DHA-CoA and/or DPA-CoA and the level of DPA and/or DHA in the total fatty acid content of the extracted lipid is between about 1% and 35%, or between about 7% and 35% or
30 between about 20.1% and 35%. In embodiments, at least about 40%, at least about 45%, at least about 48%, between 35% and about 60%, or between 35% and about 50%, of the DPA and/or DHA esterified in the form of triacylglycerol (TAG) is esterified at the *sn*-2 position of the TAG.

In embodiments of each of the above five aspects, the Δ 15-desaturase is a fungal
35 Δ 15-desaturase and the ω 3-desaturase is a fungal ω 3-desaturase.

In a preferred embodiment, the oilseed plant, microbial cell or cell of the invention has, where relevant, one or more of the features defined herein, for example as defined above in relation to extracted plant lipid, extracted microbial lipid or a process for the production thereof.

- 5 Examples of oilseed plants include, but are not limited to, *Brassica sp.*, *Gossypium hirsutum*, *Linum usitatissimum*, *Helianthus sp.*, *Carthamus tinctorius*, *Glycine max*, *Zea mays*, *Arabidopsis thaliana*, *Sorghum bicolor*, *Sorghum vulgare*, *Avena sativa*, *Trifolium sp.*, *Elaeisis guineensis*, *Nicotiana benthamiana*, *Hordeum vulgare*, *Lupinus angustifolius*, *Oryza sativa*, *Oryza glaberrima*, *Camelina sativa*, or
10 *Crambe abyssinica*. In an embodiment, the plant is a *Brassica sp.* plant, a *C. sativa* plant or a *G. max* (soybean) plant. In an embodiment, the oilseed plant is a canola, *B. juncea*, *Glycine max*, *Camelina sativa* or *Arabidopsis thaliana* plant. In an alternate embodiment, the oilseed plant is other than *A. thaliana* and/or other than *C. sativa*. In an embodiment, the oilseed plant is a plant other than *G. max* (soybean). In an
15 embodiment, the oilseed plant is in the field, or was grown in the field, or was grown in a glasshouse under standard conditions, for example as described in Example 1.

- In an embodiment, one or more of the desaturases used in a process of the invention or present in a cell, or plant or part thereof of the invention, is capable of using an acyl-CoA substrate. In a preferred embodiment, one or more of the $\Delta 6$ -
20 desaturase, $\Delta 5$ -desaturase, $\Delta 4$ -desaturase and $\Delta 8$ -desaturase, if present, is capable of using an acyl-CoA substrate, preferably each of the i) $\Delta 6$ -desaturase, $\Delta 5$ -desaturase and $\Delta 4$ -desaturase or ii) $\Delta 5$ -desaturase, $\Delta 4$ -desaturase and $\Delta 8$ -desaturase is capable of using an acyl-CoA substrate. In an embodiment, a $\Delta 12$ -desaturase and/or an $\omega 3$ -desaturase is capable of using an acyl-CoA substrate. The acyl-CoA substrate is preferably an ALA-
25 CoA for $\Delta 6$ -desaturase, ETA-CoA for $\Delta 5$ -desaturase, DPA-CoA for $\Delta 4$ -desaturase, and ETrA-CoA for $\Delta 8$ -desaturase, oleoyl-CoA for the $\Delta 12$ -desaturase, or one or more of LA-CoA, GLA-CoA, and ARA-CoA for $\omega 3$ -desaturase.

- In an embodiment, mature, harvested seed of the plant has a DHA and/or DPA content of at least about 28mg per gram seed, preferably at least about 32mg per gram
30 seed, at least about 36mg per gram seed, at least about 40mg per gram seed, more preferably at least about 44mg per gram seed or at least about 48mg per gram seed, about 80 mg per gram seed, or between about 30mg and about 80mg per gram seed.

- In a further aspect, the present invention provides a *Brassica napus*, *B. juncea* or *Camelina sativa* plant which is capable of producing seed comprising DHA and/or
35 DPA, wherein mature, harvested seed of the plant has a DHA and/or DPA content of at least about 28mg per gram seed, preferably at least about 32mg per gram seed, at least

about 36mg per gram seed, at least about 40mg per gram seed, more preferably at least about 44mg per gram seed or at least about 48mg per gram seed, about 80 mg per gram seed, or between about 30mg and about 80mg per gram seed.

In another aspect, the present invention provides a plant cell of a plant of the invention comprising the exogenous polynucleotides defined herein.

Also provided is a plant part, preferably a seed, or recombinant cells such as microbial cells which has one or more of the following features

- i) is from a plant of the invention,
- ii) comprises lipid as defined herein, or
- iii) can be used in a process of the invention.

In yet another aspect, the present invention provides mature, harvested *Brassica napus*, *B. juncea* or *Camelina sativa* seed comprising DHA and/or DPA and a moisture content of between about 4% and about 15% by weight, preferably between about 6% and about 8% by weight or between about 4% and about 8% by weight, more preferably between about 4% and about 6% by weight, wherein the DHA and/or DPA content of the seed is at least about 28mg per gram seed, preferably at least about 32mg per gram seed, at least about 36mg per gram seed, at least about 40mg per gram seed, more preferably at least about 44mg per gram seed or at least about 48mg per gram seed, about 80 mg per gram seed, or between about 30mg and about 80mg per gram seed.

In an embodiment, the cell of the invention, the oilseed plant of the invention, the *Brassica napus*, *B. juncea* or *Camelina sativa* plant of the invention, the plant part of the invention, or the seed of the invention, can be used to produce extracted lipid comprising one or more or all of the features defined herein.

In yet a further aspect, the present invention provides a method of producing a plant or cell which can be used to produce extracted lipid of the invention, the method comprising

- a) assaying the level of DHA and/or DPA in lipid produced by one or more plant parts such as seeds or recombinant cells such as microbial cells from a plurality of plants or recombinant cells such as microbial cells, each plant or recombinant cell such as a microbial cell comprising one or more exogenous polynucleotides encoding one of the following sets of enzymes;

- i) an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- ii) a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,

iii) a $\Delta 12$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

iv) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

5 v) an $\omega 3$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

vi) a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and a $\Delta 5$ -elongase,

vii) a $\Delta 12$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

viii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

ix) an $\omega 3$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and a $\Delta 5$ -elongase,

15 x) a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and a $\Delta 5$ -elongase,

xi) a $\Delta 12$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,

xiii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or

xiv) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase.

wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in a cell of a plant part or

25 recombinant cell, and

b) identifying a plant or recombinant cell, from the plurality of plants or recombinant cells, which can be used to produce extracted plant lipid or cell lipid of the invention in one or more of its parts, and

c) optionally, producing progeny plants or recombinant cells from the identified plant or recombinant cell, or seed therefrom.

In an embodiment, the plant or recombinant cell further comprises an exogenous polynucleotide encoding an LPAAT as defined herein.

Preferably, the progeny plant is at least a second or third generation removed from the identified plant, and is preferably homozygous for the one or more polynucleotides. More preferably, the one or more polynucleotides are present in the progeny plant at only a single insertion locus. That is, the invention provides such a

method which can be used as a screening method to identify a plant or seed therefrom from a plurality of transformed candidate plants or seeds, wherein the identified plant or its progeny plant produces lipid of the invention, preferably in its seed. Such a plant or progeny plant or its seed is selected if it produces lipid of the invention, in particular
5 having the specified DHA level and/or DPA level, or is not selected if it does not produce lipid of the invention.

In an embodiment, the exogenous polynucleotide(s) present in a cell such as a microbial cell, or plant or part thereof as defined herein, become stably integrated into the genome of the cell, plant or the plant part such as seed. Preferably, the exogenous
10 polynucleotide(s) become stably integrated into the genome of the cell, plant or plant part such as seed at a single locus in the genome, and is preferably homozygous for the insertion. More preferably, the plant, plant part or seed is further characterised in that it is lacking exogenous polynucleotides other than one or more T-DNA molecules. That is, no exogenous vector sequences are integrated into the genome other than the T-
15 DNA sequences.

In an embodiment, before step a) the method includes introducing the one or more exogenous polynucleotides into one or more cells of the plant.

Also provided is a plant produced using a method of the invention, and seeds of such plants.

20 In an embodiment, the plant of the invention is both male and female fertile, preferably has levels of both male and female fertility that are at least 70% relative to, or preferably are about the same as, a corresponding wild-type plant. In an embodiment, the pollen produced by the plant of the invention or the plant produced from the seed of the invention is 90-100% viable as determined by staining with a
25 viability stain. For example, the pollen viability may be assessed as described in Example 1.

In another aspect, the present invention provides a method of producing seed, the method comprising,

a) growing a plant of the invention, or a plant which produces a part of the
30 invention, preferably in a field as part of a population of at least 1000 or 2000 or 3000 such plants or in an area of at least 1 hectare or 2 hectares or 3 hectares planted at a standard planting density, alternatively in a glasshouse under standard conditions,

b) harvesting seed from the plant or plants, and

c) optionally, extracting lipid from the seed, preferably to produce oil with a
35 total DHA yield and/or DPA yield of at least 60kg or 70kg or 80kg DHA and/or DPA /hectare.

In an embodiment, the plant, plant cell, plant part or seed, or recombinant cell, of the invention has one or more of the following features

- i) its oil is as defined herein, or
- ii) the plant part or seed or recombinant cell is capable of being used in a process of the invention.

For example, the seed can be used to produce a plant of the invention. The plant may be grown in the field or in a glasshouse under standard conditions, for example as described in Example 1.

In a further aspect, the present invention provides lipid, or oil, produced by, or obtained from, using the process of the invention, the cell of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *G. max* or *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, or the plant, plant cell, plant part or seed of the invention. Preferably, the lipid or oil is purified to remove contaminants such as nucleic acid (DNA and/or RNA), protein and/or carbohydrate, or pigments such as chlorophyll. The lipid or oil may also be purified to enrich the proportion of TAG, for example by removal of free fatty acids (FFA) or phospholipid.

In an embodiment, the lipid or oil is obtained by extraction of oil from an oilseed. Examples of oil from oilseeds include, but are not limited to, canola oil (*Brassica napus*, *Brassica rapa* ssp.), mustard oil (*Brassica juncea*), other Brassica oil, sunflower oil (*Helianthus annuus*), linseed oil (*Linum usitatissimum*), soybean oil (*Glycine max*), safflower oil (*Carthamus tinctorius*), corn oil (*Zea mays*), tobacco oil (*Nicotiana tabacum*), peanut oil (*Arachis hypogaea*), palm oil, cottonseed oil (*Gossypium hirsutum*), coconut oil (*Cocos nucifera*), avocado oil (*Persea americana*), olive oil (*Olea europaea*), cashew oil (*Anacardium occidentale*), macadamia oil (*Macadamia intergrifolia*), almond oil (*Prunus amygdalus*) or *Arabidopsis* seed oil (*Arabidopsis thaliana*).

In an embodiment, a cell (recombinant cell) of, or used in, the invention is a microbial cell such as a cell suitable for fermentation, preferably an oleaginous microbial cell which is capable of accumulating triacylglycerols to a level of at least 25% on a weight basis. Preferred fermentation processes are anaerobic fermentation processes, as are well known in the art. Suitable fermenting cells, typically microorganisms are able to ferment, i.e., convert, sugars, such as glucose or maltose, directly or indirectly into the desired fatty acids. Examples of fermenting microorganisms include fungal organisms, such as yeast. As used herein, "yeast" includes *Saccharomyces* spp., *Saccharomyces cerevisiae*, *Saccharomyces*

carlbergensis, *Candida* spp., *Kluyveromyces* spp., *Pichia* spp., *Hansenula* spp., *Trichoderma* spp., *Lipomyces starkey*, and preferably *Yarrowia lipolytica*.

In a further aspect, the present invention provides fatty acid produced by, or obtained from, using the process of the invention, the cell of the invention, the oilseed
5 plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *G. max* or *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, or the plant, plant cell, plant part or seed of the invention. Preferably the fatty acid is DHA and/or DPA. The fatty acid may be in a mixture of fatty acids having a fatty acid composition as described herein, or may be enriched so that the fatty acid comprises at
10 least 40% or at least 90% of the fatty acid content of the mixture. In an embodiment, the fatty acid is non-esterified. Alternatively, the fatty acid is esterified such as, for example, to a methyl, ethyl, propyl or butyl group.

Also provided is seedmeal obtained from seed of the invention or obtained from a plant of the invention. Preferred seedmeal includes, but not necessarily limited to,
15 *Brassica* sp., *Brassica napus*, *B. juncea*, *Camelina sativa* or *Glycine max* seedmeal. In an embodiment, the seedmeal comprises an exogenous polynucleotide(s) and/or genetic constructs as defined herein. In a preferred embodiment, the seedmeal retains some of the lipid or oil produced in the seed from which the seedmeal is obtained, but at a low level (for example, less than 2% by weight) after extraction of most of the lipid
20 or oil. The seedmeal may be used as an animal feed or as an ingredient in food production.

In another aspect, the present invention provides a composition comprising one or more of the lipid or oil of the invention, the fatty acid of the invention, the cell according of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica*
25 *napus*, *B. juncea*, *Glycine max* or *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, or the seedmeal of the invention. In embodiments, the composition comprises a carrier suitable for pharmaceutical, food or agricultural use, a seed treatment compound, a fertiliser, another food or feed ingredient, or added protein or vitamins.

Also provided is feedstuffs, cosmetics or chemicals comprising one or more of
30 the lipid or oil of the invention, the fatty acid of the invention, the cell according of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *Glycine max* or *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, the seedmeal of the invention, or the composition
35 of the invention.

In another aspect, the present invention provides a method of producing a feedstuff, the method comprising mixing one or more of the lipid or oil of the invention, the fatty acid of the invention, the cell according of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *Glycine max* or
5 *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, the seedmeal of the invention, or the composition of the invention, with at least one other food ingredient. The method may comprise steps of blending, cooking, baking, extruding, emulsifying or otherwise formulating the feedstuff, or packaging the feedstuff, or of analysing the amount of lipid or oil in the feedstuff.

10 In another aspect, the present invention provides a method of treating or preventing a condition which would benefit from a PUFA, preferably DHA and/or DPA, the method comprising administering to a subject one or more of the lipid or oil of the invention, the fatty acid of the invention, the cell according of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *Glycine max*
15 or *Camelina sativa* plant of the invention, the plant part of the invention, the seed of the invention, the seedmeal of the invention, the composition of the invention, or the feedstuff of the invention. In a preferred embodiment, the PUFA is administered in the form of a pharmaceutical composition comprising an ethyl ester of the PUFA. The subject may be a human or an animal other than a human.

20 Examples of conditions which would benefit from a PUFA include, but are not limited to, elevated serum triglyceride levels, elevated serum cholesterol levels such as elevated LDL cholesterol levels, cardiac arrhythmia's, angioplasty, inflammation, asthma, psoriasis, osteoporosis, kidney stones, AIDS, multiple sclerosis, rheumatoid arthritis, Crohn's disease, schizophrenia, cancer, foetal alcohol syndrome, attention
25 deficient hyperactivity disorder, cystic fibrosis, phenylketonuria, unipolar depression, aggressive hostility, adrenoleukodystrophy, coronary heart disease, hypertension, diabetes, obesity, Alzheimer's disease, chronic obstructive pulmonary disease, ulcerative colitis, restenosis after angioplasty, eczema, high blood pressure, platelet aggregation, gastrointestinal bleeding, endometriosis, premenstrual syndrome, myalgic
30 encephalomyelitis, chronic fatigue after viral infections or an ocular disease.

Also provided is the use of one or more of the lipid or oil of the invention, the fatty acid of the invention, the cell according of the invention, the oilseed plant of the invention, the *Brassica* sp., *Brassica napus*, *B. juncea*, *Glycine max* or *Camelina sativa*
35 plant of the invention, the plant part of the invention, the seed of the invention, the seedmeal of the invention, the composition of the invention, or the feedstuff of the

invention for the manufacture of a medicament for treating or preventing a condition which would benefit from a PUFA preferably DHA and/or DPA.

The production of the medicament may comprise mixing the oil of the invention with a pharmaceutically acceptable carrier, for treatment of a condition as described
5 herein. The method may comprise firstly purifying the oil and/or transesterification, and/or fractionation of the oil to increase the level of DHA and/or DPA. In a particular embodiment, the method comprises treating the lipid or oil such as canola oil to convert the fatty acids in the oil to alkyl esters such as methyl or ethyl esters. Further treatment such as fractionation or distillation may be applied to enrich the lipid or oil for the
10 DHA and/or DPA. In a preferred embodiment, the medicament comprises ethyl esters of DHA and/or DPA. In an even more preferred embodiment, the level of ethyl esters of DHA and/or DPA in the medicament is between 30% and 50%, or at least 80% or at least about 85% or at least 90% or at least about 95%. The medicament may further comprise ethyl esters of EPA, such as between 30% and 50%, or at least 90%, of the
15 total fatty acid content in the medicament. Such medicaments are suitable for administration to human or animal subjects for treatment of medical conditions as described herein.

In another aspect, the present invention provides a method of trading seed, comprising obtaining seed of the invention, and trading the obtained seed for pecuniary
20 gain.

In an embodiment, obtaining the seed comprises cultivating plants of the invention and/or harvesting the seed from the plants.

In another embodiment, obtaining the seed further comprises placing the seed in a container and/or storing the seed.

25 In a further embodiment, obtaining the seed further comprises transporting the seed to a different location.

In yet another embodiment, the method further comprises transporting the seed to a different location after the seed is traded.

In a further embodiment, the trading is conducted using electronic means such
30 as a computer.

In yet a further aspect, the present invention provides a process of producing bins of seed comprising:

- a) swathing, windrowing and/or reaping above-ground parts of plants comprising seed of the invention,
- 35 b) threshing and/or winnowing the parts of the plants to separate the seed from the remainder of the plant parts, and

c) sifting and/or sorting the seed separated in step b), and loading the sifted and/or sorted seed into bins, thereby producing bins of seed.

In an embodiment, where relevant, the lipid or oil, preferably seedoil, of, or useful for, the invention has fatty levels about those provided in a Table in the Examples section, such as seed CT136-27-18-2 or CT136-27-18-19 of Table 10, or the seedoil of Tables 12, 20, 22, 23 or 24.

Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1. Aerobic DHA biosynthesis pathways.

Figure 2. Map of the T-DNA insertion region between the left and right borders of pJP3416-GA7. RB denotes right border; LB, left border; TER, transcription terminator/ polyadenylation region; PRO, promoter; Coding regions are indicated above the arrows, promoters and terminators below the arrows. Micpu-Δ6D, *Micromonas pusilla* Δ6-desaturase; Pyrco-Δ6E, *Pyramimonas cordata* Δ6-elongase; Pavsa-Δ5D, *Pavlova salina* Δ5-desaturase; Picpa-ω3D, *Pichia pastoris* ω3-desaturase; Pavsa-Δ4D, *P. salina* Δ4-desaturase; Lack1-Δ12D, *Lachancea kluyveri* Δ12-desaturase; Pyrco-Δ5E, *Pyramimonas cordata* Δ5-elongase. NOS denotes the *Agrobacterium tumefaciens* nopaline synthase transcription terminator/polyadenylation region; FP1, *Brassica napus* truncated napin promoter; FAE1, *Arabidopsis thaliana* FAE1 promoter; Lectin, *Glycine max* lectin transcription terminator/polyadenylation region; Cn11 and Cn12 denotes the *Linum usitatissimum* conlinin1 or conlinin2 promoter or terminator. MAR denotes the Rb7 matrix attachment region from *Nicotiana tabacum*.

Figure 3. Map of the T-DNA insertion region between the left and right borders of pJP3404. Labels are as in Figure 2.

- 5 **Figure 4.** Oil content (w/w) vs. DHA content, as a percentage of total fatty acid content of lipid from transgenic *Arabidopsis thaliana* seeds.

Figure 5. Positional distribution analysis by NMR on A) Tuna oil and, B) transgenic DHA *Arabidopsis* seed oil. The peaks labelled 'DHA-alpha' represent the amount of
10 DHA present at the *sn*-1 and *sn*-3 positions of TAG (with no positional preference this would equal 66% of total DHA) whilst the peaks labelled 'DHA-beta' represent the amount of DHA present at the *sn*-2 position of TAG (with no preference this would equal 33% of DHA).

- 15 **Figure 6.** LC-MS analysis of major DHA-containing triacylglycerol species in transgenic *A. thaliana* developing (grey) and mature (black) seeds. The number following the DHA denotes the total number of carbon atoms and total number of double bonds in the other two fatty acids. Therefore DHA/34:1 can also be designated TAG 56:7, etc.

20

Figure 7. (A) Basic phytosterol structure with ring and side chain numbering. (B) Chemical structures of some of the phytosterols.

Figure 8. Phylogenetic tree of known LPAATs.

25

Figure 9. The various acyl exchange enzymes which transfer fatty acids between PC, CoA pools, and TAG pools. Adapted from Singh et al. (2005).

- Figure 10.** DHA levels in the total fatty acid content of seedoil obtained from
30 individual T2 seeds from *B. napus* seeds transformed with the T-DNA from the GA7-modB construct. Each dot shows the DHA level in an individual seed, with each column of dots representing T2 seeds from an individual T1 plant.

KEY TO THE SEQUENCE LISTING

- 35 SEQ ID NO:1 – pJP3416-GA7 nucleotide sequence.
SEQ ID NO:2 – pGA7- mod_B nucleotide sequence.

- SEQ ID NO:3 - Codon-optimized open reading frame for expression of *Lachancea kluyveri* $\Delta 12$ desaturase in plants.
- SEQ ID NO:4 - *Lachancea kluyveri* $\Delta 12$ -desaturase.
- SEQ ID NO:5 - Codon-optimized open reading frame for expression of *Pichia pastoris* $\omega 3$ desaturase in plants.
- 5 SEQ ID NO:6 - *Pichia pastoris* $\omega 3$ desaturase.
- SEQ ID NO:7 - Open reading frame encoding *Micromonas pusilla* $\Delta 6$ -desaturase.
- SEQ ID NO:8 - Codon-optimized open reading frame for expression of *Micromonas pusilla* $\Delta 6$ -desaturase in plants.
- 10 SEQ ID NO:9 - *Micromonas pusilla* $\Delta 6$ -desaturase.
- SEQ ID NO:10 - Open reading frame encoding *Ostreococcus lucimarinus* $\Delta 6$ -desaturase.
- SEQ ID NO:11 - Codon-optimized open reading frame for expression of *Ostreococcus lucimarinus* $\Delta 6$ -desaturase in plants.
- 15 SEQ ID NO:12 - *Ostreococcus lucimarinus* $\Delta 6$ -desaturase.
- SEQ ID NO:13 - *Ostreococcus tauri* $\Delta 6$ -desaturase.
- SEQ ID NO:14 - Open reading frame encoding *Pyramimonas cordata* $\Delta 6$ -elongase.
- SEQ ID NO:15 - Codon-optimized open reading frame for expression of *Pyramimonas cordata* $\Delta 6$ -elongase in plants (truncated at 3' end and encoding functional elongase).
- 20 SEQ ID NO:16 - *Pyramimonas cordata* $\Delta 6$ -elongase.
- SEQ ID NO:17 - Truncated *Pyramimonas cordata* $\Delta 6$ -elongase.
- SEQ ID NO:18 - Open reading frame encoding *Pavlova salina* $\Delta 5$ -desaturase.
- SEQ ID NO:19 - Codon-optimized open reading frame for expression of *Pavlova salina* $\Delta 5$ -desaturase in plants.
- 25 SEQ ID NO:20 - *Pavlova salina* $\Delta 5$ -desaturase.
- SEQ ID NO:21 - Open reading frame encoding *Pyramimonas cordata* $\Delta 5$ -desaturase.
- SEQ ID NO:22 - *Pyramimonas cordata* $\Delta 5$ -desaturase.
- SEQ ID NO:23 - Open reading frame encoding *Pyramimonas cordata* $\Delta 5$ -elongase.
- SEQ ID NO:24 - Codon-optimized open reading frame for expression of *Pyramimonas*
- 30 *cordata* $\Delta 5$ -elongase in plants.
- SEQ ID NO:25 - *Pyramimonas cordata* $\Delta 5$ -elongase.
- SEQ ID NO:26 - Open reading frame encoding *Pavlova salina* $\Delta 4$ -desaturase.
- SEQ ID NO:27 - Codon-optimized open reading frame for expression of *Pavlova salina* $\Delta 4$ -desaturase in plants.
- 35 SEQ ID NO:28 - *Pavlova salina* $\Delta 4$ -desaturase.
- SEQ ID NO:29 - *Isochrysis galbana* $\Delta 9$ -elongase.

- SEQ ID NO:30 - Codon-optimized open reading frame for expression of *Emiliania huxleyi* $\Delta 9$ -elongase in plants.
- SEQ ID NO:31 - *Emiliania huxleyi* CCMP1516 $\Delta 9$ -elongase.
- SEQ ID NO:32 - Open reading frame encoding *Pavlova pinguis* $\Delta 9$ -elongase.
- 5 SEQ ID NO:33 - *Pavlova pinguis* $\Delta 9$ -elongase.
- SEQ ID NO:34 - Open reading frame encoding *Pavlova salina* $\Delta 9$ -elongase.
- SEQ ID NO:35 - *Pavlova salina* $\Delta 9$ -elongase.
- SEQ ID NO:36 - Open reading frame encoding *Pavlova salina* $\Delta 8$ -desaturase.
- SEQ ID NO:37 - *Pavlova salina* $\Delta 8$ -desaturase.
- 10 SEQ ID NO:38 - V2 viral suppressor.
- SEQ ID NO:39 - Open reading frame encoding V2 viral suppressor.
- SEQ ID NO: 40 - *Arabidopsis thaliana* LPAAT2.
- SEQ ID NO: 41 - *Limnanthes alba* LPAAT.
- SEQ ID NO: 42 - *Saccharomyces cerevisiae* LPAAT.
- 15 SEQ ID NO: 43 - *Micromonas pusilla* LPAAT.
- SEQ ID NO: 44 - *Mortierella alpina* LPAAT.
- SEQ ID NO: 45 - *Brassica napus* LPAAT.
- SEQ ID NO: 46 - *Brassica napus* LPAAT.
- SEQ ID NO: 47 - *Phytophthora infestans* $\omega 3$ desaturase.
- 20 SEQ ID NO: 48 - *Thalassiosira pseudonana* $\omega 3$ desaturase.
- SEQ ID NO: 49 - *Pythium irregulare* $\omega 3$ desaturase.
- SEQ ID NO's: 50 to 58 - Oligonucleotide primers/probes.

DETAILED DESCRIPTION OF THE INVENTION

25 General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in cell culture, molecular genetics, fatty acid synthesis, transgenic plants, recombinant cells, protein chemistry, and biochemistry).

- 30 Unless otherwise indicated, the protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual,
- 35 Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M.

Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors), Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), and J.E. Coligan et al. (editors), Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

As used herein, the term "about" unless stated to the contrary, refers to +/- 10%, more preferably +/- 5%, more preferably +/- 1% of the designated value.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Selected Definitions

As used herein, the terms "extracted plant lipid" and "isolated plant lipid" refer to a lipid composition which has been extracted from, for example by crushing, a plant or part thereof such as seed. The extracted lipid can be a relatively crude composition obtained by, for example, crushing a plant seed, or a more purified composition where most, if not all, of one or more or each of the water, nucleic acids, proteins and carbohydrates derived from the plant material have been removed. Examples of purification methods are described below. In an embodiment, the extracted or isolated plant lipid comprises at least about 60%, at least about 70%, at least about 80%, at least about 90%, or at least about 95% (w/w) lipid by weight of the composition. The lipid may be solid or liquid at room temperature, when liquid it is considered to be an oil. In an embodiment, extracted lipid of the invention has not been blended with another lipid such as DHA and/or DPA produced by another source (for example, DHA from fish oil). In an embodiment, following extraction the ratio of one or more or all of, oleic acid to DHA and/or DPA, palmitic acid to DHA and/or DPA, linoleic acid to DHA and/or DPA, and total $\omega 6$ fatty acids: total $\omega 3$ fatty acids, has not been significantly altered (for example, no greater than a 10% or 5% alteration) when compared to the ratio in the intact seed or cell. In an another embodiment, the extracted plant lipid has not been exposed to a procedure, such as hydrogenation or fractionation, which may

alter the ratio of one or more or all of, oleic acid to DHA and/or DPA, palmitic acid to DHA and/or DPA, linoleic acid to DHA and/or DPA, and total $\omega 6$ fatty acids: total $\omega 3$ fatty acids, when compared to the ratio in the intact seed or cell. When the extracted plant lipid of the invention is comprised in an oil, the oil may further comprise non-
5 fatty acid molecules such as sterols.

As used herein, the terms "extracted plant oil" and "isolated plant oil" refer to a substance or composition comprising extracted plant lipid or isolated plant lipid and which is a liquid at room temperature. The oil is obtained from a plant or part thereof such as seed. The extracted or isolated oil can be a relatively crude composition
10 obtained by, for example, crushing a plant seed, or a more purified composition where most, if not all, of one or more or each of the water, nucleic acids, proteins and carbohydrates derived from the plant material have been removed. The composition may comprise other components which may be lipid or non-lipid. In an embodiment, the oil composition comprises at least about 60%, at least about 70%, at least about
15 80%, at least about 90%, or at least about 95% (w/w) extracted plant lipid. In an embodiment, extracted oil of the invention has not been blended with another oil such as DHA and/or DPA produced by another source (for example, DHA from fish oil). In an embodiment, following extraction, the ratio of one or more or all of, oleic acid to DHA and/or DPA, palmitic acid to DHA and/or DPA, linoleic acid to DHA and/or
20 DPA, and total $\omega 6$ fatty acids: total $\omega 3$ fatty acids, has not been significantly altered (for example, no greater than a 10% or 5% alteration) when compared to the ratio in the intact seed or cell. In an another embodiment, the extracted plant oil has not been exposed to a procedure, such as hydrogenation or fractionation, which may alter the ratio of one or more or all of, oleic acid to DHA and/or DPA, palmitic acid to DHA
25 and/or DPA, linoleic acid to DHA and/or DPA, and total $\omega 6$ fatty acids: total $\omega 3$ fatty acids, when compared to the ratio in the intact seed or cell. Extracted plant oil of the invention may comprise non-fatty acid molecules such as sterols.

As used herein, terms such as "extracted microbial lipid" or "extracted microbial oil" have analogous meanings as the corresponding terms "extracted plant lipid" and
30 "extracted plant oil" respectively, with the main difference being the source of the lipid or oil.

As used herein, an "oil" is a composition comprising predominantly lipid and which is a liquid at room temperature. For instance, oil of the invention preferably comprises at least 75%, at least 80%, at least 85% or at least 90% lipid by weight.
35 Typically, a purified oil comprises at least 90% triacylglycerols (TAG) by weight of the

lipid in the oil. Minor components of an oil such as diacylglycerols (DAG), free fatty acids (FFA), phospholipid and sterols may be present as described herein.

As used herein, the term "fatty acid" refers to a carboxylic acid (or organic acid), often with a long aliphatic tail, either saturated or unsaturated. Typically fatty acids have a carbon-carbon bonded chain of at least 8 carbon atoms in length, more preferably at least 12 carbons in length. Preferred fatty acids of the invention have carbon chains of 18-22 carbon atoms (C18, C20, C22 fatty acids), more preferably 20-22 carbon atoms (C20, C22) and most preferably 22 carbon atoms (C22). Most naturally occurring fatty acids have an even number of carbon atoms because their biosynthesis involves acetate which has two carbon atoms. The fatty acids may be in a free state (non-esterified) or in an esterified form such as part of a triglyceride, diacylglyceride, monoacylglyceride, acyl-CoA (thio-ester) bound or other bound form. The fatty acid may be esterified as a phospholipid such as a phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol forms. In an embodiment, the fatty acid is esterified to a methyl or ethyl group, such as, for example, a methyl or ethyl ester of a C20 or C22 PUFA. Preferred fatty acids are the methyl or ethyl esters of EPA, DPA or DHA, or the mixtures EPA and DHA, or EPA, DPA and DHA, or EPA and DPA.

"Saturated fatty acids" do not contain any double bonds or other functional groups along the chain. The term "saturated" refers to hydrogen, in that all carbons (apart from the carboxylic acid [-COOH] group) contain as many hydrogens as possible. In other words, the omega (ω) end contains 3 hydrogens (-CH₃-) and each carbon within the chain contains 2 hydrogens (-CH₂-).

"Unsaturated fatty acids" are of similar form to saturated fatty acids, except that one or more alkene functional groups exist along the chain, with each alkene substituting a singly-bonded "-CH₂-CH₂-" part of the chain with a doubly-bonded "-CH=CH-" portion (that is, a carbon double bonded to another carbon). The two next carbon atoms in the chain that are bound to either side of the double bond can occur in a *cis* or *trans* configuration, preferably in the *cis* configuration. In an embodiment, the lipid or oil or the invention has a fatty acid composition which comprises less than 1% fatty acids having a carbon-carbon double bond in the *trans* configuration (*trans* fatty acids).

As used herein, the term "monounsaturated fatty acid" refers to a fatty acid which comprises at least 12 carbon atoms in its carbon chain and only one alkene group (carbon-carbon double bond) in the chain. As used herein, the terms "polyunsaturated

fatty acid" or "PUFA" refer to a fatty acid which comprises at least 12 carbon atoms in its carbon chain and at least two alkene groups (carbon-carbon double bonds).

As used herein, the terms "long-chain polyunsaturated fatty acid" and "LC-PUFA" refer to a fatty acid which comprises at least 20 carbon atoms in its carbon chain and at least two carbon-carbon double bonds, and hence include VLC-PUFAs. As used herein, the terms "very long-chain polyunsaturated fatty acid" and "VLC-PUFA" refer to a fatty acid which comprises at least 22 carbon atoms in its carbon chain and at least three carbon-carbon double bonds. Ordinarily, the number of carbon atoms in the carbon chain of the fatty acids refers to an unbranched carbon chain. If the carbon chain is branched, the number of carbon atoms excludes those in sidegroups. In one embodiment, the long-chain polyunsaturated fatty acid is an $\omega 3$ fatty acid, that is, having a desaturation (carbon-carbon double bond) in the third carbon-carbon bond from the methyl end of the fatty acid. In another embodiment, the long-chain polyunsaturated fatty acid is an $\omega 6$ fatty acid, that is, having a desaturation (carbon-carbon double bond) in the sixth carbon-carbon bond from the methyl end of the fatty acid. In a further embodiment, the long-chain polyunsaturated fatty acid is selected from the group consisting of; arachidonic acid (ARA, 20:4 Δ 5,8,11,14; $\omega 6$), eicosatetraenoic acid (ETA, 20:4 Δ 8,11,14,17, $\omega 3$), eicosapentaenoic acid (EPA, 20:5 Δ 5,8,11,14,17; $\omega 3$), docosapentaenoic acid (DPA, 22:5 Δ 7,10,13,16,19, $\omega 3$), or docosahexaenoic acid (DHA, 22:6 Δ 4,7,10,13,16,19, $\omega 3$). The LC-PUFA may also be dihomo- γ -linoleic acid (DGLA) or eicosatrienoic acid (ETra, 20:3 Δ 11,14,17, $\omega 3$). It would readily be apparent that the LC-PUFA that is produced according to the invention may be a mixture of any or all of the above and may include other LC-PUFA or derivatives of any of these LC-PUFA. In a preferred embodiment, the $\omega 3$ fatty acids are at least DHA and/or DPA, preferably, DPA and DHA, or EPA, DPA and DHA, or EPA and DPA. As extracted from the plant, DHA is present in the lipid or oil at a level of 20.1-30% or between 20.1% and 35%, preferably between 30% to 35% of the total fatty acid composition. For example, DHA is present at a level of between 30.1% and 35% of the total fatty acid composition. In an embodiment, the level of DHA is greater than the level of DPA, more preferably greater than the level of each of EPA and DPA, most preferably greater than the combined level of EPA and DPA. In an alternative embodiment, DPA is present at a level of between about 7% and 30% or 35% and DHA is either absent or, if present, is present at a level of less than 2.0%, preferably less than 1.0%, more preferably less than 0.5% of the total fatty acid composition and most preferably absent or undetectable. This may be accomplished by the absence of a $\Delta 4$ -desaturase activity in the cell. In an embodiment, the level of DPA is greater than the

level of EPA, more preferably greater than the level of each of EPA and DHA, most preferably greater than the combined level of EPA and DHA. In this embodiment, DHA may be absent or, if present, is present at a level of less than 0.5% of the total fatty acid composition.

5 Furthermore, as used herein the terms "long-chain polyunsaturated fatty acid" (LC-PUFA) and "very long-chain polyunsaturated fatty acid" (VLC-PUFA) refer to the fatty acid being in a free state (non-esterified) or in an esterified form such as part of a triglyceride (triacylglycerol), diacylglyceride, monoacylglyceride, acyl-CoA bound or other bound form. In the triglyceride, the LC-PUFA or VLC-PUFA such as DHA or
10 DPA may be esterified at the *sn*-1/3 or *sn*-2 positions, or the triglyceride may comprise two or three acyl groups selected from LC-PUFA and VLC-PUFA acyl groups. For example, the triglyceride may comprise DHA or DPA at both of the *sn*-1 and *sn*-3 positions. The fatty acid may be esterified as a phospholipid such as a phosphatidylcholine (PC), phosphatidylethanolamine, phosphatidylserine,
15 phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol forms. Thus, the LC-PUFA may be present as a mixture of forms in the lipid of a cell or a purified oil or lipid extracted from cells, tissues or organisms. In preferred embodiments, the invention provides oil comprising at least 75% or at least 85% triacylglycerols, with the remainder present as other forms of lipid such as those mentioned, with at least said
20 triacylglycerols comprising the LC-PUFA. The oil may subsequently be further purified or treated, for example by hydrolysis with a strong base to release the free fatty acids, or by transesterification, distillation or the like.

As used herein, "total ω 6 fatty acids" or "total ω 6 fatty acid content" or the like refers to the sum of all the ω 6 fatty acids, esterified and non-esterified, in the extracted
25 lipid, oil, recombinant cell, plant part or seed, as the context determines, expressed as a percentage of the total fatty acid content. These ω 6 fatty acids include (if present) LA, GLA, DGLA, ARA, EDA and ω 6-DPA, and exclude any ω 3 fatty acids and monounsaturated fatty acids. The ω 6 fatty acids present in the plants, seeds, lipid or oils of the invention are all included in the class of polyunsaturated fatty acids (PUFA).

30 As used herein, "new ω 6 fatty acids" or "new ω 6 fatty acid content" or the like refers to the sum of all the ω 6 fatty acids excluding LA, esterified and non-esterified, in the extracted lipid, oil, recombinant cell, plant part or seed, as the context determines, expressed as a percentage of the total fatty acid content. These new ω 6 fatty acids are the fatty acids that are produced in the cells, plants, plant parts and seeds of the
35 invention by the expression of the genetic constructs (exogenous polynucleotides) introduced into the cells, and include (if present) GLA, DGLA, ARA, EDA and ω 6-

DPA, but exclude LA and any $\omega 3$ fatty acids and monounsaturated fatty acids. Exemplary total $\omega 6$ fatty acid contents and new $\omega 6$ fatty acid contents are determined by conversion of fatty acids in a sample to FAME and analysis by GC, as described in Example 1.

5 As used herein, "total $\omega 3$ fatty acids" or "total $\omega 3$ fatty acid content" or the like refers to the sum of all the $\omega 3$ fatty acids, esterified and non-esterified, in the extracted lipid, oil, recombinant cell, plant part or seed, as the context determines, expressed as a percentage of the total fatty acid content. These $\omega 3$ fatty acids include (if present) ALA, SDA, ETrA, ETA, EPA, DPA and DHA, and exclude any $\omega 6$ fatty acids and
10 monounsaturated fatty acids. The $\omega 3$ fatty acids present in the plants, seeds, lipid or oils of the invention are all included in the class of polyunsaturated fatty acids (PUFA).

As used herein, "new $\omega 3$ fatty acids" or "new $\omega 3$ fatty acid content" or the like refers to the sum of all the $\omega 3$ fatty acids excluding ALA, esterified and non-esterified, in the extracted lipid, oil, recombinant cell, plant part or seed, as the context
15 determines, expressed as a percentage of the total fatty acid content. These new $\omega 3$ fatty acids are the $\omega 3$ fatty acids that are produced in the cells, plants, plant parts and seeds of the invention by the expression of the genetic constructs (exogenous polynucleotides) introduced into the cells, and include (if present) SDA, ETrA, ETA, EPA, DPA and DHA, but exclude ALA and any $\omega 6$ fatty acids and monounsaturated
20 fatty acids. Exemplary total $\omega 3$ fatty acid contents and new $\omega 3$ fatty acid contents are determined by conversion of fatty acids in a sample to FAME and analysis by GC, as described in Example 1.

As the skilled person would appreciate, the term "obtaining a plant part" as a step in the process of the invention can include obtaining one or more plant parts for
25 use in the process. Obtaining the plant part includes harvesting the plant part from a plant such as with a mechanical harvester, or purchasing the plant part, or receiving the plant part from a supplier. In another example, obtaining a plant part may be acquiring the plant from someone else who has harvested the plant part.

The desaturase, elongase and acyl transferase proteins and genes encoding them
30 that may be used in the invention are any of those known in the art or homologues or derivatives thereof. Examples of such genes and encoded protein sizes are listed in Table 1. The desaturase enzymes that have been shown to participate in LC-PUFA biosynthesis all belong to the group of so-called "front-end" desaturases. Preferred proteins, or combinations of proteins, are those encoded by the genetic constructs
35 provided herein as SEQ ID NOs: 1 and 2.

Table 1. Cloned genes involved in LC-PUFA biosynthesis

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
Δ4-desaturase	Protist	<i>Euglena gracilis</i>	AY278558	541	Meyer et al., 2003
	Algae	<i>Pavlova lutheri</i>	AY332747	445	Tanon et al., 2003
		<i>Isochrysis galbana</i>	AAV33631	433	Pereira et al., 2004b
		<i>Pavlova salina</i>	AAV15136	447	Zhou et al., 2007
	Thraustochytrid	<i>Thraustochytrium aureum</i>	AAN75707 AAN75708 AAN75709 AAN75710	515	N/A
Δ5-desaturase		<i>Thraustochytrium</i> sp. ATCC21685	AAM09688	519	Qiu et al. 2001
	Mammals	<i>Homo sapiens</i>	AF199596	444	Cho et al., 1999b Leonard et al., 2000b
	Nematode	<i>Caenorhabditis elegans</i>	AF11440, NM_069350	447	Michaelson et al., 1998b; Watts and Browne, 1999b
	Fungi	<i>Mortierella alpina</i>	AF067654	446	Michaelson et al., 1998a; Knutzon et al., 1998
		<i>Pythium irregulare</i>	AF419297	456	Hong et al., 2002a
		<i>Dictyostelium discoideum</i>	AB022097	467	Saito et al., 2000
		<i>Saprolegnia diclina</i>		470	WO20081668
	Diatom	<i>Phaeodactylum tricornutum</i>	AY082392	469	Domergue et al., 2002
	Algae	<i>Thraustochytrium</i> sp	AF489588	439	Qiu et al., 2001
		<i>Thraustochytrium aureum</i>		439	WO20081668
		<i>Isochrysis galbana</i>		442	WO20081668
	Moss	<i>Marchantia polymorpha</i>	AY583465	484	Kajikawa et al., 2004

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
$\Delta 6$ -desaturase	Mammals	<i>Homo sapiens</i>	NM_013402	444	Cho et al., 1999a; Leonard et al., 2000
		<i>Mus musculus</i>	NM_019699	444	Cho et al., 1999a
	Nematode	<i>Caenorhabditis elegans</i>	Z70271	443	Napier et al., 1998
	Plants	<i>Borago officinalis</i>	U79010	448	Sayanova et al., 1997
		<i>Echium</i>	AY055117 AY055118		Garcia-Maroto et al., 2002
		<i>Primula vialii</i>	AY234127	453	Sayanova et al., 2003
		<i>Anemone leveillei</i>	AF336525	446	Whitney et al., 2003
	Mosses	<i>Ceratodon purpureus</i>	AJ250735	520	Sperling et al., 2000
		<i>Marchantia polymorpha</i>	AY583463	481	Kajikawa et al., 2004
		<i>Physcomitrella patens</i>	CAA11033	525	Girke et al., 1998
	Fungi	<i>Mortierella alpina</i>	AF110510 AB020032	457	Huang et al., 1999; Sakuradani et al., 1999
		<i>Pythium irregulare</i>	AF419296	459	Hong et al., 2002a
		<i>Mucor circinelloides</i>	AB052086	467	NCBI*
		<i>Rhizopus sp.</i>	AY320288	458	Zhang et al., 2004
		<i>Saprolegnia diclina</i>		453	WO02081668
	Diatom	<i>Phaeodactylum tricornutum</i>	AY082393	477	Domergue et al., 2002
	Bacteria	<i>Synechocystis</i>	L11421	359	Reddy et al., 1993
	Algae	<i>Thraustochytrium aureum</i>		456	WO02081668
	Fish	<i>Danio rerio</i>	AF309556	444	Hastings et al., 2001
Bifunctional $\Delta 5/\Delta 6$ -desaturase					
C20 $\Delta 8$ -desaturase	Algae	<i>Euglena gracilis</i>	AF139720	419	Wallis and Browse, 1999
	Plants	<i>Borago officinalis</i>	AAG43277	446	Sperling et al., 2001

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
$\Delta 6$ -elongase	Nematode	<i>Caenorhabditis elegans</i>	NM_069288	288	Beaudoin et al., 2000
	Mosses	<i>Physcomitrella patens</i>	AF428243	290	Zank et al., 2002
		<i>Marchantia polymorpha</i>	AY583464	290	Kajikawa et al., 2004
	Fungi	<i>Mortierella alpina</i>	AF206662	318	Parker-Barnes et al., 2000
	Algae	<i>Pavlova lutheri</i> **		501	WO 03078639
		<i>Thraustochytrium</i>	AX951565	271	WO 03093482
		<i>Thraustochytrium</i> sp**	AX214454	271	WO 0159128
PUFA-elongase	Mammals	<i>Homo sapiens</i>	AF231981	299	Leonard et al., 2000b; Leonard et al., 2002
		<i>Rattus norvegicus</i>	AB071985	299	Inagaki et al., 2002
		<i>Rattus norvegicus</i> **	AB071986	267	Inagaki et al., 2002
		<i>Mus musculus</i>	AF170907	279	Tvdik et al., 2000
		<i>Mus musculus</i>	AF170908	292	Tvdik et al., 2000
	Fish	<i>Danio rerio</i>	AF532782	291 (282)	Agaba et al., 2004
		<i>Danio rerio</i> **	NM_199532	266	Lo et al., 2003
	Worm	<i>Caenorhabditis elegans</i>	Z68749	309	Abbott et al., 1998 Beaudoin et al., 2000
	Algae	<i>Thraustochytrium aureum</i> **	AX464802	272	WO 0208401-A2
		<i>Pavlova lutheri</i> **		320	WO 03078639
$\Delta 9$ -elongase	Algae	<i>Ischrysis galbana</i>	AF390174	263	Qi et al., 2002
		<i>Euglena gracilis</i>		258	WO 08/128241
$\Delta 5$ -elongase	Algae	<i>Ostreococcus tauri</i>	AAV67798	300	Meyer et al., 2004
		<i>Pyramimonas cordata</i>		268	WO 2010/057246
		<i>Pavlova</i> sp. CCMP459	AAV33630	277	Pereira et al., 2004b
		<i>Pavlova salina</i>	AAV15135	302	Robert et al., 2009
	Diatom	<i>Thalassiosira pseudonana</i>	AAV67800	358	Meyer et al., 2004
	Fish	<i>Oncorhynchus mykiss</i>	CAM55862	295	WO 06/008099
	Moss	<i>Marchantia polymorpha</i>	BAE71129	348	Kajikawa et al., 2006

* <http://www.ncbi.nlm.nih.gov/> ** Function not proven/not demonstrated

As used herein, the term "front-end desaturase" refers to a member of a class of enzymes that introduce a double bond between the carboxyl group and a pre-existing unsaturated part of the acyl chain of lipids, which are characterized structurally by the presence of an N-terminal cytochrome b5 domain, along with a typical fatty acid
5 desaturase domain that includes three highly conserved histidine boxes (Napier et al., 1997).

Activity of any of the elongases or desaturases for use in the invention may be tested by expressing a gene encoding the enzyme in a cell such as, for example, a plant cell or preferably in somatic embryos or transgenic plants, and determining whether the
10 cell, embryo or plant has an increased capacity to produce LC-PUFA compared to a comparable cell, embryo or plant in which the enzyme is not expressed.

In one embodiment one or more of the desaturases and/or elongases for use in the invention can purified from a microalga, i.e. is identical in amino acid sequence to a polypeptide which can be purified from a microalga.

15 Whilst certain enzymes are specifically described herein as "bifunctional", the absence of such a term does not necessarily imply that a particular enzyme does not possess an activity other than that specifically defined.

Desaturases

20 As used herein, the term "desaturase" refers to an enzyme which is capable of introducing a carbon-carbon double bond into the acyl group of a fatty acid substrate which is typically in an esterified form such as, for example, acyl-CoA esters. The acyl group may be esterified to a phospholipid such as phosphatidylcholine (PC), or to acyl carrier protein (ACP), or in a preferred embodiment to CoA. Desaturases generally
25 may be categorized into three groups accordingly. In one embodiment, the desaturase is a front-end desaturase.

As used herein, a " $\Delta 4$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 4th carbon-carbon bond from the carboxyl end of a fatty acid substrate. The " $\Delta 4$ -desaturase" is at
30 least capable of converting DPA to DHA. Preferably, the " $\Delta 4$ -desaturase" is capable of converting DPA-CoA to DHA-CoA, i.e. it is an acyl-CoA desaturase. In an embodiment, the " $\Delta 4$ -desaturase" is capable of converting DPA esterified at the *sn*-2 position of PC to DHA-PC. Preferably the $\Delta 4$ -desaturase has greater activity on DPA-CoA than on DPA-PC. The desaturation step to produce DHA from DPA is catalysed
35 by a $\Delta 4$ -desaturase in organisms other than mammals, and a gene encoding this enzyme has been isolated from the freshwater protist species *Euglena gracilis* and the marine

species *Thraustochytrium* sp. (Qiu et al., 2001; Meyer et al., 2003). In one embodiment, the $\Delta 4$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:28, or a *Thraustochytrium* sp. $\Delta 4$ -desaturase, a biologically active fragment thereof, or an amino acid sequence which is at least 80% identical to SEQ ID NO:28. In an embodiment, a plant, plant part (such as seed) or cell of, or used in, the invention which produces high levels of DPA, such as 5% to 35% of the total extractable fatty acid content is DPA, does not comprise a gene encoding a functional $\Delta 4$ -desaturase.

As used herein, a " $\Delta 5$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 5th carbon-carbon bond from the carboxyl end of a fatty acid substrate. In an embodiment, the fatty acid substrate is ETA and the enzyme produces EPA. Preferably, the " $\Delta 5$ -desaturase" is capable of converting ETA-CoA to EPA-CoA, i.e. it is an acyl-CoA desaturase. In an embodiment, the " $\Delta 5$ -desaturase" is capable of converting ETA esterified at the *sn*-2 position of PC. Preferably the $\Delta 5$ -desaturase has greater activity on ETA-CoA than on ETA-PC. Examples of $\Delta 5$ -desaturases are listed in Ruiz-Lopez et al. (2012) and Petrie et al. (2010a) and in Table 1 herein. In one embodiment, the $\Delta 5$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:20, a biologically active fragment thereof, or an amino acid sequence which is at least 80% identical to SEQ ID NO:20. In another embodiment, the $\Delta 5$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:22, a biologically active fragment thereof, or an amino acid sequence which is at least 53% identical to SEQ ID NO:22. In another embodiment, the $\Delta 5$ -desaturase is from *Thraustochytrium* sp or *Emiliania huxleyi*.

As used herein, a " $\Delta 6$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 6th carbon-carbon bond from the carboxyl end of a fatty acid substrate. In an embodiment, the fatty acid substrate is ALA and the enzyme produces SDA. Preferably, the " $\Delta 6$ -desaturase" is capable of converting ALA-CoA to SDA-CoA, i.e. it is an acyl-CoA desaturase. In an embodiment, the " $\Delta 6$ -desaturase" is capable of converting ALA esterified at the *sn*-2 position of PC. Preferably the $\Delta 6$ -desaturase has greater activity on ALA-CoA than on ALA-PC. The $\Delta 6$ -desaturase may also have activity as a $\Delta 5$ -desaturase, being termed a $\Delta 5/\Delta 6$ bifunctional desaturase, so long as it has greater $\Delta 6$ -desaturase activity on ALA than $\Delta 5$ -desaturase activity on ETA. Examples of $\Delta 6$ -desaturases are listed in Ruiz-Lopez et al. (2012) and Petrie et al. (2010a) and in Table

1 herein. Preferred $\Delta 6$ -desaturases are from *Micromonas pusilla*, *Pythium irregulare* or *Ostreococcus tauri*.

In an embodiment, the $\Delta 6$ -desaturase is further characterised by having at least two, preferably all three and preferably in a plant cell, of the following: i) greater $\Delta 6$ -
 5 desaturase activity on α -linolenic acid (ALA, 18:3 $\Delta 9,12,15$, $\omega 3$) than linoleic acid (LA, 18:2 $\Delta 9,12$, $\omega 6$) as fatty acid substrate; ii) greater $\Delta 6$ -desaturase activity on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate; and iii) $\Delta 8$ -desaturase activity on ETrA. Examples of such $\Delta 6$ -desaturases are provided in Table 2.

10

Table 2. Desaturases demonstrated to have activity on an acyl-CoA substrate

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
$\Delta 6$ -desaturase	Algae	<i>Mantoniella squamata</i>	CAQ30479	449	Hoffmann et al., 2008
		<i>Ostreococcus tauri</i>	AAW70159	456	Domergue et al., 2005
		<i>Micromonas pusilla</i>	EEH58637		Petrie et al., 2010a (SEQ ID NO: 7)
$\Delta 5$ -desaturase	Algae	<i>Mantoniella squamata</i>	CAQ30478	482	Hoffmann et al., 2008
	Plant	<i>Anemone leveillei</i>	N/A		Sayanova et al., 2007
$\omega 3$ -desaturase	Fungi	<i>Pythium aphanidermatum</i>	FW362186.1	359	Xue et al., 2012; WO2008/054565
	Fungi (oomycete)	<i>Phytophthora sojae</i>	FW362214.1	363	Xue et al., 2012; WO2008/054565
	Fungi (oomycete)	<i>Phytophthora ramorum</i>	FW362213.1	361	Xue et al., 2012; WO2008/054565

In an embodiment the $\Delta 6$ -desaturase has greater activity on an $\omega 3$ substrate than the corresponding $\omega 6$ substrate and has activity on ALA to produce octadecatetraenoic
 15 acid (stearidonic acid, SDA, 18:4 $\Delta 6,9,12,15$, $\omega 3$) with an efficiency of at least 30%, more preferably at least 40%, or most preferably at least 50% when expressed from an exogenous polynucleotide in a recombinant cell such as a plant cell, or at least 35% when expressed in a yeast cell. In one embodiment, the $\Delta 6$ -desaturase has greater

activity, for example, at least about a 2-fold greater $\Delta 6$ -desaturase activity, on ALA than LA as fatty acid substrate. In another embodiment, the $\Delta 6$ -desaturase has greater activity, for example, at least about 5 fold greater $\Delta 6$ -desaturase activity or at least 10-fold greater activity, on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate. In a further embodiment, the $\Delta 6$ -desaturase has activity on both fatty acid substrates ALA-CoA and on ALA joined to the *sn*-2 position of PC.

In one embodiment, the $\Delta 6$ -desaturase has no detectable $\Delta 5$ -desaturase activity on ETA. In another embodiment, the $\Delta 6$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:9, SEQ ID NO:12 or SEQ ID NO:13, a biologically active fragment thereof, or an amino acid sequence which is at least 77% identical to SEQ ID NO:9, SEQ ID NO:12 or SEQ ID NO:13. In another embodiment, the $\Delta 6$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:12 or SEQ ID NO:13, a biologically active fragment thereof, or an amino acid sequence which is at least 67% identical to one or both of SEQ ID NO:12 or SEQ ID NO:13. The $\Delta 6$ -desaturase may also have $\Delta 8$ -desaturase activity.

As used herein, a " $\Delta 8$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 8th carbon-carbon bond from the carboxyl end of a fatty acid substrate. The $\Delta 8$ -desaturase is at least capable of converting ETrA to ETA. Preferably, the $\Delta 8$ -desaturase is capable of converting ETrA-CoA to ETA-CoA, i.e. it is an acyl-CoA desaturase. In an embodiment, the $\Delta 8$ -desaturase is capable of converting ETrA esterified at the *sn*-2 position of PC. Preferably the $\Delta 8$ -desaturase has greater activity on ETrA-CoA than on ETrA-PC. The $\Delta 8$ -desaturase may also have activity as a $\Delta 6$ -desaturase, being termed a $\Delta 6/\Delta 8$ bifunctional desaturase, so long as it has greater $\Delta 8$ -desaturase activity on ETrA than $\Delta 6$ -desaturase activity on ALA. Examples of $\Delta 8$ -desaturases are listed in Table 1. In one embodiment, the $\Delta 8$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:37, a biologically active fragment thereof, or an amino acid sequence which is at least 80% identical to SEQ ID NO:37.

As used herein, an " $\omega 3$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 3rd carbon-carbon bond from the methyl end of a fatty acid substrate. A $\omega 3$ -desaturase therefore may convert LA to ALA and GLA to SDA (all C18 fatty acids), or DGLA to ETA and/or ARA to EPA (C20 fatty acids). Some $\omega 3$ -desaturases (group I) have activity only on C18 substrates, such as plant and cyanobacterial $\omega 3$ -desaturases. Such $\omega 3$ -desaturases are also $\Delta 15$ -desaturases. Other $\omega 3$ -desaturases have activity on C20

substrates with no activity (group II) or some activity (group III) on C18 substrates. Such ω 3-desaturases are also Δ 17-desaturases. Preferred ω 3-desaturases are group III type which convert LA to ALA, GLA to SDA, DGLA to ETA and ARA to EPA, such as the *Pichia pastoris* ω 3-desaturase (SEQ ID NO: 6). Examples of ω 3-desaturases include those described by Pereira et al. (2004a) (*Saprolegnia diclina* ω 3-desaturase, group II), Horiguchi et al. (1998), Berberich et al. (1998) and Spychalla et al. (1997) (*C. elegans* ω 3-desaturase, group III). In a preferred embodiment, the ω 3-desaturase is a fungal ω 3-desaturase. As used herein, a "fungal ω 3-desaturase" refers to an ω 3-desaturase which is from a fungal source, including an oomycete source, or a variant thereof whose amino acid sequence is at least 95% identical thereto. Genes encoding numerous ω 3-desaturases have been isolated from fungal sources such as, for example, from *Phytophthora infestans* (Accession No. CAJ30870, WO2005083053; SEQ ID NO: 47), *Saprolegnia diclina* (Accession No. AAR20444, Pereira et al., 2004a & US 7211656), *Pythium irregulare* (WO2008022963, Group II; SEQ ID NO: 49), *Mortierella alpina* (Sakuradani et al., 2005; Accession No. BAD91495; WO2006019192), *Thalassiosira pseudonana* (Armbrust et al., 2004; Accession No. XP_002291057; WO2005012316, SEQ ID NO: 48), *Lachancea kluyveri* (also known as *Saccharomyces kluyveri*; Oura et al., 2004; Accession No. AB118663). Xue et al. (2012) describes ω 3-desaturases from the oomycetes *Pythium aphanidermatum*, *Phytophthora sojae*, and *Phytophthora ramorum* which were able to efficiently convert ω 6 fatty acid substrates to the corresponding ω 3 fatty acids, with a preference for C20 substrates, i.e. they had stronger Δ 17-desaturase activity than Δ 15-desaturase activity. These enzymes lacked Δ 12-desaturase activity, but could use fatty acids in both acyl-CoA and phospholipid fraction as substrates.

In a more preferred embodiment, the fungal ω 3-desaturase is the *Pichia pastoris* (also known as *Komagataella pastoris*) ω 3-desaturase/ Δ 15-desaturase (Zhang et al., 2008; Accession No. EF116884; SEQ ID NO: 6), or a polypeptide which is at least 95% identical thereto.

In an embodiment, the ω 3-desaturase is at least capable of converting one of ARA to EPA, DGLA to ETA, GLA to SDA, both ARA to EPA and DGLA to ETA, both ARA to EPA and GLA to SDA, or all three of these.

In one embodiment, the ω 3-desaturase has Δ 17-desaturase activity on a C20 fatty acid which has at least three carbon-carbon double bonds, preferably ARA. In another embodiment, the ω 3-desaturase has Δ 15-desaturase activity on a C18 fatty acid which has three carbon-carbon double bonds, preferably GLA. Preferably, both activities are present.

As used herein, a " $\Delta 12$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 12th carbon-carbon bond from the carboxyl end of a fatty acid substrate. $\Delta 12$ -desaturases typically convert either oleoyl-phosphatidylcholine or oleoyl-CoA to linoleoyl-phosphatidylcholine (18:1-PC) or linoleoyl-CoA (18:1-CoA), respectively. The subclass using the PC linked substrate are referred to as phospholipid-dependent $\Delta 12$ -desaturases, the latter subclass as acyl-CoA dependent $\Delta 12$ -desaturases. Plant and fungal $\Delta 12$ -desaturases are generally of the former sub-class, whereas animal $\Delta 12$ -desaturases are of the latter subclass, for example the $\Delta 12$ -desaturases encoded by genes cloned from insects by Zhou et al. (2008). Many other $\Delta 12$ -desaturase sequences can be easily identified by searching sequence databases.

As used herein, a " $\Delta 15$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 15th carbon-carbon bond from the carboxyl end of a fatty acid substrate. Numerous genes encoding $\Delta 15$ -desaturases have been cloned from plant and fungal species. For example, US5952544 describes nucleic acids encoding plant $\Delta 15$ -desaturases (FAD3). These enzymes comprise amino acid motifs that were characteristic of plant $\Delta 15$ -desaturases. WO200114538 describes a gene encoding soybean FAD3. Many other $\Delta 15$ -desaturase sequences can be easily identified by searching sequence databases.

As used herein, a " $\Delta 17$ -desaturase" refers to a protein which performs a desaturase reaction that introduces a carbon-carbon double bond at the 17th carbon-carbon bond from the carboxyl end of a fatty acid substrate. A $\Delta 17$ -desaturase is also regarded as an $\omega 3$ -desaturase if it acts on a C20 substrate to introduce a desaturation at the $\omega 3$ bond.

In a preferred embodiment, the $\Delta 12$ -desaturase and/or $\Delta 15$ -desaturase is a fungal $\Delta 12$ -desaturase or fungal $\Delta 15$ -desaturase. As used herein, a "fungal $\Delta 12$ -desaturase" or "a fungal $\Delta 15$ -desaturase" refers to a $\Delta 12$ -desaturase or $\Delta 15$ -desaturase which is from a fungal source, including an oomycete source, or a variant thereof whose amino acid sequence is at least 95% identical thereto. Genes encoding numerous desaturases have been isolated from fungal sources. US 7211656 describes a $\Delta 12$ desaturase from *Saprolegnia diclina*. WO2009016202 describes fungal desaturases from *Helobdella robusta*, *Laccaria bicolor*, *Lottia gigantea*, *Microcoleus chthonoplastes*, *Monosiga brevicollis*, *Mycosphaerella fijiensis*, *Mycosphaerella graminicola*, *Naegleria gruberi*, *Nectria haematococca*, *Nematostella vectensis*, *Phycomyces blakesleeana*, *Trichoderma reesei*, *Physcomitrella patens*, *Postia placenta*, *Selaginella moellendorffii* and *Microdochium nivale*. WO2005/012316 describes a $\Delta 12$ -desaturase from

Thalassiosira pseudonana and other fungi. WO2003/099216 describes genes encoding fungal $\Delta 12$ -desaturases and $\Delta 15$ -desaturases isolated from *Neurospora crassa*, *Aspergillus nidulans*, *Botrytis cinerea* and *Mortierella alpina*. WO2007133425 describes fungal $\Delta 15$ desaturases isolated from: *Saccharomyces kluyveri*, *Mortierella*
 5 *alpina*, *Aspergillus nidulans*, *Neurospora crassa*, *Fusarium graminearum*, *Fusarium moniliforme* and *Magnaporthe grisea*. A preferred $\Delta 12$ desaturase is from *Phytophthora sojae* (Ruiz-Lopez et al., 2012).

A distinct subclass of fungal $\Delta 12$ -desaturases, and of fungal $\Delta 15$ -desaturases, are the bifunctional fungal $\Delta 12/\Delta 15$ -desaturases. Genes encoding these have been
 10 cloned from *Fusarium moniliforme* (Accession No. DQ272516, Damude et al., 2006), *Acanthamoeba castellanii* (Accession No. EF017656, Sayanova et al., 2006), *Perkinsus marinus* (WO2007042510), *Claviceps purpurea* (Accession No. EF536898, Meesapyodsuk et al., 2007) and *Coprinus cinereus* (Accession No. AF269266, Zhang et al., 2007).

15 In another embodiment, the $\omega 3$ -desaturase has at least some activity on, preferably greater activity on, an acyl-CoA substrate than a corresponding acyl-PC substrate. As used herein, a "corresponding acyl-PC substrate" refers to the fatty acid esterified at the *sn*-2 position of phosphatidylcholine (PC) where the fatty acid is the same fatty acid as in the acyl-CoA substrate. For example, the acyl-CoA substrate may
 20 be ARA-CoA and the corresponding acyl-PC substrate is *sn*-2 ARA-PC. In an embodiment, the activity is at least two-fold greater. Preferably, the $\omega 3$ -desaturase has at least some activity on both an acyl-CoA substrate and its corresponding acyl-PC substrate and has activity on both C18 and C20 substrates. Examples of such $\omega 3$ -desaturases are known amongst the cloned fungal desaturases listed above.

25 In a further embodiment, the $\omega 3$ -desaturase comprises amino acids having a sequence as provided in SEQ ID NO:6, a biologically active fragment thereof, or an amino acid sequence which is at least 60% identical to SEQ ID NO:6, preferably at least 90% or at least 95% identical to SEQ ID NO:6.

In yet a further embodiment, a desaturase for use in the present invention has
 30 greater activity on an acyl-CoA substrate than a corresponding acyl-PC substrate. In another embodiment, a desaturase for use in the present invention has greater activity on an acyl-PC substrate than a corresponding acyl-CoA substrate, but has some activity on both substrates. As outlined above, a "corresponding acyl-PC substrate" refers to the fatty acid esterified at the *sn*-2 position of phosphatidylcholine (PC) where the fatty
 35 acid is the same fatty acid as in the acyl-CoA substrate. In an embodiment, the greater activity is at least two-fold greater. In an embodiment, the desaturase is a $\Delta 5$ or $\Delta 6$ -

desaturase, or an ω 3-desaturase, examples of which are provided, but not limited to, those listed in Table 2. To test which substrate a desaturase acts on, namely an acyl-CoA or an acyl-PC substrate, assays can be carried out in yeast cells as described in Domergue et al. (2003 and 2005). Acyl-CoA substrate capability for a desaturase can also be inferred when an elongase, when expressed together with the desaturase, has an enzymatic conversion efficiency in plant cells of at least about 90% where the elongase catalyses the elongation of the product of the desaturase. On this basis, the Δ 5-desaturase and Δ 4-desaturases expressed from the GA7 construct (Examples 2 and 3) and variants thereof (Example 4) are capable of desaturating their respective acyl-CoA substrates, ETA-CoA and DPA-CoA.

Elongases

Biochemical evidence suggests that the fatty acid elongation consists of 4 steps: condensation, reduction, dehydration and a second reduction. In the context of this invention, an "elongase" refers to the polypeptide that catalyses the condensing step in the presence of the other members of the elongation complex, under suitable physiological conditions. It has been shown that heterologous or homologous expression in a cell of only the condensing component ("elongase") of the elongation protein complex is required for the elongation of the respective acyl chain. Thus, the introduced elongase is able to successfully recruit the reduction and dehydration activities from the transgenic host to carry out successful acyl elongations. The specificity of the elongation reaction with respect to chain length and the degree of desaturation of fatty acid substrates is thought to reside in the condensing component. This component is also thought to be rate limiting in the elongation reaction.

As used herein, a " Δ 5-elongase" is at least capable of converting EPA to DPA. Examples of Δ 5-elongases include those disclosed in WO2005/103253. In one embodiment, the Δ 5-elongase has activity on EPA to produce DPA with an efficiency of at least 60%, more preferably at least 65%, more preferably at least 70% or most preferably at least 80% or 90%. In a further embodiment, the Δ 5-elongase comprises an amino acid sequence as provided in SEQ ID NO:25, a biologically active fragment thereof, or an amino acid sequence which is at least 47% identical to SEQ ID NO:25. In a further embodiment, the Δ 6-elongase is from *Ostreococcus taurii* or *Ostreococcus lucimarinus* (US2010/088776).

As used herein, a " Δ 6-elongase" is at least capable of converting SDA to ETA. Examples of Δ 6-elongases include those listed in Table 1. In one embodiment, the elongase comprises amino acids having a sequence as provided in SEQ ID NO:16, a

biologically active fragment thereof (such as the fragment provided as SEQ ID NO:17), or an amino acid sequence which is at least 55% identical to one or both of SEQ ID NO:16 or SEQ ID NO:17. In an embodiment, the $\Delta 6$ -elongase is from *Physcomitrella patens* (Zank et al., 2002; Accession No. AF428243) or *Thalassiosira pseudonana* (Ruiz-Lopez et al., 2012).

As used herein, a " $\Delta 9$ -elongase" is at least capable of converting ALA to ETrA. Examples of $\Delta 9$ -elongases include those listed in Table 1. In one embodiment, the $\Delta 9$ -elongase comprises amino acids having a sequence as provided in SEQ ID NO:29, a biologically active fragment thereof, or an amino acid sequence which is at least 80% identical to SEQ ID NO:29. In another embodiment, the $\Delta 9$ -elongase comprises amino acids having a sequence as provided in SEQ ID NO:31, a biologically active fragment thereof, or an amino acid sequence which is at least 81% identical to SEQ ID NO:31. In another embodiment, the $\Delta 9$ -elongase comprises amino acids having a sequence as provided in SEQ ID NO:33, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:33. In another embodiment, the $\Delta 9$ -elongase comprises amino acids having a sequence as provided in SEQ ID NO:35, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:35. In a further embodiment, the $\Delta 9$ -elongase has greater activity on an $\omega 6$ substrate than the corresponding $\omega 3$ substrate, or the converse.

As used herein, the term "has greater activity on an $\omega 6$ substrate than the corresponding $\omega 3$ substrate" refers to the relative activity of the enzyme on substrates that differ by the action of an $\omega 3$ desaturase. Preferably, the $\omega 6$ substrate is LA and the $\omega 3$ substrate is ALA.

An elongase with $\Delta 6$ -elongase and $\Delta 9$ -elongase activity is at least capable of (i) converting SDA to ETA and (ii) converting ALA to ETrA and has greater $\Delta 6$ -elongase activity than $\Delta 9$ -elongase activity. In one embodiment, the elongase has an efficiency of conversion on SDA to produce ETA which is at least 50%, more preferably at least 60%, and/or an efficiency of conversion on ALA to produce ETrA which is at least 6% or more preferably at least 9%. In another embodiment, the elongase has at least about 6.5 fold greater $\Delta 6$ -elongase activity than $\Delta 9$ -elongase activity. In a further embodiment, the elongase has no detectable $\Delta 5$ -elongase activity.

Other enzymes

The transgenes introduced into the recombinant cell such as a microbial cell, or transgenic plant or part thereof may also encode an LPAAT. As used herein, the term

"1-acyl-glycerol-3-phosphate acyltransferase" (LPAAT), also termed lysophosphatidic acid-acyltransferase or acylCoA-lysophosphatidate-acyltransferase, refers to a protein which acylates *sn*-1-acyl-glycerol-3-phosphate (*sn*-1 G-3-P) at the *sn*-2 position to form phosphatidic acid (PA). Thus, the term "1-acyl-glycerol-3-phosphate acyltransferase activity" refers to the acylation of (*sn*-1 G-3-P) at the *sn*-2 position to produce PA (EC 2.3.1.51). Preferred LPAATs are those that can use a polyunsaturated C22 acyl-CoA as substrate to transfer the polyunsaturated C22 acyl group to the *sn*-2 position of LPA, forming PA. In an embodiment, the polyunsaturated C22 acyl-CoA is DHA-CoA and/or DPA-CoA. Such LPAATs are exemplified in Example 7 and can be tested as described therein. In an embodiment, an LPAAT useful for the invention comprises amino acids having a sequence as provided in any one of SEQ ID NOs: 40 to 46, a biologically active fragment thereof, or an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NOs: 40 to 46. In another embodiment, the LPAAT does not have amino acids having a sequence as provided in any one of SEQ ID NO: 44. In a preferred embodiment, an LPAAT useful for the invention which can use a C22 polyunsaturated fatty acyl-CoA substrate, preferably DHA-CoA and/or DPA-CoA, comprises amino acids having a sequence as provided in any one of SEQ ID NOs: 41, 42 and 44, a biologically active fragment thereof, or an amino acid sequence which is at least 40% identical to any one or more of SEQ ID NOs: 41, 42 and 44. In a preferred embodiment, an LPAAT useful for the invention which can use a C22 polyunsaturated fatty acyl-CoA substrate, preferably DHA-CoA and/or DPA-CoA, comprises amino acids having a sequence as provided in any one of SEQ ID NOs: 41 or 42, a biologically active fragment thereof, or an amino acid sequence which is at least 40% identical to any one or both of SEQ ID NOs: 41 and 42. In an embodiment in which the genetic construct expresses a $\Delta 4$ -desaturase in the transgenic cell and/or the transgenic cell produces DHA, the LPAAT is preferably an LPAAT other than the *Mortierella alpina* LPAAT whose amino acid sequence is set forth as SEQ ID NO: 44. Alternatively, if the genetic construct does not express a $\Delta 4$ -desaturase in the transgenic cell and/or the transgenic cell produces DPA but not DHA, the LPAAT is preferably the *Mortierella alpina* LPAAT whose amino acid sequence is set forth as SEQ ID NO: 44 or another LPAAT which is capable of using DPA-CoA as a substrate to transfer the DPA to LPA, forming DAG having DPA at the *sn*-2 position.

The transgenes introduced into the recombinant cell, transgenic plant or part thereof may also encode a DGAT. As used herein, the term "diacylglycerol acyltransferase" (EC 2.3.1.20; DGAT) refers to a protein which transfers a fatty acyl group from acyl-CoA to a diacylglycerol substrate to produce a triacylglycerol. Thus,

the term "diacylglycerol acyltransferase activity" refers to the transfer of acyl-CoA to diacylglycerol to produce triacylglycerol. There are three known types of DGAT referred to as DGAT1, DGAT2 and DGAT3 respectively. DGAT1 polypeptides typically have 10 transmembrane domains, DGAT2 typically have 2 transmembrane domains, whilst DGAT3 is typically soluble. Examples of DGAT1 polypeptides include polypeptides encoded by DGAT1 genes from *Aspergillus fumigatus* (Accession No. XP_755172), *Arabidopsis thaliana* (CAB44774), *Ricinus communis* (AAR11479), *Vernicia fordii* (ABC94472), *Vernonia galamensis* (ABV21945, ABV21946), *Euonymus alatus* (AAV31083), *Caenorhabditis elegans* (AAF82410), *Rattus norvegicus* (NP_445889), *Homo sapiens* (NP_036211), as well as variants and/or mutants thereof. Examples of DGAT2 polypeptides include polypeptides encoded by DGAT2 genes from *Arabidopsis thaliana* (Accession No. NP_566952), *Ricinus communis* (AAY16324), *Vernicia fordii* (ABC94474), *Mortierella ramanniana* (AAK84179), *Homo sapiens* (Q96PD7, Q58HT5), *Bos taurus* (Q70VD8), *Mus musculus* (AAK84175), *Micromonas* CCMP1545, as well as variants and/or mutants thereof. Examples of DGAT3 polypeptides include polypeptides encoded by DGAT3 genes from peanut (*Arachis hypogaea*, Saba, et al., 2006), as well as variants and/or mutants thereof.

20 Polypeptides/Peptides

The terms "polypeptide" and "protein" are generally used interchangeably.

A polypeptide or class of polypeptides may be defined by the extent of identity (% identity) of its amino acid sequence to a reference amino acid sequence, or by having a greater % identity to one reference amino acid sequence than to another. The % identity of a polypeptide to a reference amino acid sequence is typically determined by GAP analysis (Needleman and Wunsch, 1970; GCG program) with parameters of a gap creation penalty=5, and a gap extension penalty=0.3. The query sequence is at least 15 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 15 amino acids. More preferably, the query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns two sequences over their entire length. The polypeptide or class of polypeptides may have the same enzymatic

activity as, or a different activity than, or lack the activity of, the reference polypeptide. Preferably, the polypeptide has an enzymatic activity of at least 10%, at least 50%, at least 75% or at least 90%, of the activity of the reference polypeptide.

As used herein a "biologically active" fragment is a portion of a polypeptide
5 defined herein which maintains a defined activity of a full-length reference polypeptide, for example possessing desaturase and/or elongase activity or other enzyme activity. Biologically active fragments as used herein exclude the full-length polypeptide. Biologically active fragments can be any size portion as long as they maintain the defined activity. Preferably, the biologically active fragment maintains at
10 least 10%, at least 50%, at least 75% or at least 90%, of the activity of the full length protein.

With regard to a defined polypeptide or enzyme, it will be appreciated that % identity figures higher than those provided herein will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is
15 preferred that the polypeptide/enzyme comprises an amino acid sequence which is at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%, more preferably at least 76%, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least
20 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%, more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more
25 preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

Amino acid sequence variants/mutants of the polypeptides of the defined herein can be prepared by introducing appropriate nucleotide changes into a nucleic acid defined herein, or by *in vitro* synthesis of the desired polypeptide. Such variants/mutants include, for example, deletions, insertions or substitutions of residues
30 within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final peptide product possesses the desired enzyme activity.

Mutant (altered) peptides can be prepared using any technique known in the art. For example, a polynucleotide defined herein can be subjected to *in vitro* mutagenesis
35 or DNA shuffling techniques as broadly described by Harayama (1998). Products

derived from mutated/altered DNA can readily be screened using techniques described herein to determine if they possess, for example, desaturase or elongase activity.

In designing amino acid sequence mutants, the location of the mutation site and the nature of the mutation will depend on characteristic(s) to be modified. The sites for mutation can be modified individually or in series, e.g., by (1) substituting first with conservative amino acid choices and then with more radical selections depending upon the results achieved, (2) deleting the target residue, or (3) inserting other residues adjacent to the located site.

Amino acid sequence deletions generally range from about 1 to 15 residues, more preferably about 1 to 10 residues and typically about 1 to 5 contiguous residues.

Substitution mutants have at least one amino acid residue in the polypeptide molecule removed and a different residue inserted in its place. The sites of greatest interest for substitutional mutagenesis include sites which are not conserved amongst naturally occurring desaturases or elongases. These sites are preferably substituted in a relatively conservative manner in order to maintain enzyme activity. Such conservative substitutions are shown in Table 3 under the heading of "exemplary substitutions".

In a preferred embodiment a mutant/variant polypeptide has only, or not more than, one or two or three or four conservative amino acid changes when compared to a naturally occurring polypeptide. Details of conservative amino acid changes are provided in Table 3. As the skilled person would be aware, such minor changes can reasonably be predicted not to alter the activity of the polypeptide when expressed in a recombinant cell.

Polynucleotides

The invention also provides for the use of polynucleotides which may be, for example, a gene, an isolated polynucleotide, a chimeric genetic construct such as a T-DNA molecule, or a chimeric DNA. It may be DNA or RNA of genomic or synthetic origin, double-stranded or single-stranded, and combined with carbohydrate, lipids, protein or other materials to perform a particular activity defined herein. The term "polynucleotide" is used interchangeably herein with the term "nucleic acid molecule".

In an embodiment, the polynucleotide is non-naturally occurring. Examples of non-naturally occurring polynucleotides include, but are not limited to, those that have been mutated (such as by using methods described herein), and polynucleotides where an open reading frame encoding a protein is operably linked to a promoter to which it is not naturally associated (such as in the constructs described herein).

Table 3. Exemplary substitutions.

Original Residue	Exemplary Substitutions
Ala (A)	val; leu; ile; gly
Arg (R)	lys
Asn (N)	gln; his
Asp (D)	glu
Cys (C)	ser
Gln (Q)	asn; his
Glu (E)	asp
Gly (G)	pro; ala
His (H)	asn; gln
Ile (I)	leu; val; ala
Leu (L)	ile; val; met; ala; phe
Lys (K)	arg
Met (M)	leu; phe
Phe (F)	leu; val; ala
Pro (P)	gly
Ser (S)	thr
Thr (T)	ser
Trp (W)	tyr
Tyr (Y)	trp; phe
Val (V)	ile; leu; met; phe, ala

As used herein, the term "gene" is to be taken in its broadest context and includes the deoxyribonucleotide sequences comprising the transcribed region and, if translated, the protein coding region, of a structural gene and including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of at least about 2 kb on either end and which are involved in expression of the gene. In this regard, the gene includes control signals such as promoters, enhancers, termination and/or polyadenylation signals that are naturally associated with a given gene, or heterologous control signals in which case the gene is referred to as a "chimeric gene". The sequences which are located 5' of the protein coding region and which are present

on the mRNA are referred to as 5' non-translated sequences. The sequences which are located 3' or downstream of the protein coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains
5 the coding region which may be interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA). Introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA
10 (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide. The term "gene" includes a synthetic or fusion molecule encoding all or part of the proteins described herein and a complementary nucleotide sequence to any one of the above.

As used herein, a "chimeric DNA" or "chimeric genetic construct" or similar
15 refers to any DNA molecule that is not a native DNA molecule in its native location, also referred to herein as a "DNA construct". Typically, a chimeric DNA or chimeric gene comprises regulatory and transcribed or protein coding sequences that are not found operably linked together in nature i.e. that are heterologous with respect to each other. Accordingly, a chimeric DNA or chimeric gene may comprise regulatory
20 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.

An "endogenous gene" refers to a native gene in its natural location in the genome of an organism. As used herein, "recombinant nucleic acid molecule",
25 "recombinant polynucleotide" or variations thereof refer to a nucleic acid molecule which has been constructed or modified by recombinant DNA technology. The terms "foreign polynucleotide" or "exogenous polynucleotide" or "heterologous polynucleotide" and the like refer to any nucleic acid which is introduced into the genome of a cell by experimental manipulations. Foreign or exogenous genes may be
30 genes that are inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. The terms "genetically modified", "transgenic" and variations thereof include introducing genes into cells by transformation or transduction, mutating genes in cells and altering or
35 modulating the regulation of a gene in a cell or organisms to which these acts have been done or their progeny. A "genomic region" as used herein refers to a position

within the genome where a transgene, or group of transgenes (also referred to herein as a cluster), have been inserted into a cell, or an ancestor thereof. Such regions only comprise nucleotides that have been incorporated by the intervention of man such as by methods described herein.

- 5 The term "exogenous" in the context of a polynucleotide refers to the polynucleotide when present in a cell in an altered amount compared to its native state. In one embodiment, the cell is a cell that does not naturally comprise the polynucleotide. However, the cell may be a cell which comprises a non-endogenous polynucleotide resulting in an altered amount of production of the encoded polypeptide.
- 10 An exogenous polynucleotide includes polynucleotides which have not been separated from other components of the transgenic (recombinant) cell, or cell-free expression system, in which it is present, and polynucleotides produced in such cells or cell-free systems which are subsequently purified away from at least some other components. The exogenous polynucleotide (nucleic acid) can be a contiguous stretch of nucleotides
- 15 existing in nature, or comprise two or more contiguous stretches of nucleotides from different sources (naturally occurring and/or synthetic) joined to form a single polynucleotide. Typically such chimeric polynucleotides comprise at least an open reading frame encoding a polypeptide operably linked to a promoter suitable of driving transcription of the open reading frame in a cell of interest.
- 20 With regard to the defined polynucleotides, it will be appreciated that % identity figures higher than those provided above will encompass preferred embodiments. Thus, where applicable, in light of the minimum % identity figures, it is preferred that the polynucleotide comprises a polynucleotide sequence which is at least 60%, more preferably at least 65%, more preferably at least 70%, more preferably at least 75%,
- 25 more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, more preferably at least 91%, more preferably at least 92%, more preferably at least 93%, more preferably at least 94%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, more preferably at least 99.1%, more preferably at least 99.2%,
- 30 more preferably at least 99.3%, more preferably at least 99.4%, more preferably at least 99.5%, more preferably at least 99.6%, more preferably at least 99.7%, more preferably at least 99.8%, and even more preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

- Polynucleotides may possess, when compared to naturally occurring molecules,
- 35 one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Polynucleotides which have mutations relative to a reference sequence can be

either naturally occurring (that is to say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis or DNA shuffling on the nucleic acid as described above). It is thus apparent that polynucleotides can be either from a naturally occurring source or recombinant. Preferred polynucleotides are those which
5 have coding regions that are codon-optimised for translation in plant cells, as is known in the art.

Recombinant Vectors

Recombinant expression can be used to produce recombinant cells, or plants or
10 plant parts of the invention. Recombinant vectors contain heterologous polynucleotide sequences, that is, polynucleotide sequences that are not naturally found adjacent to polynucleotide molecules defined herein that preferably are derived from a species other than the species from which the polynucleotide molecule(s) are derived. The vector can be either RNA or DNA and typically is a plasmid. Plasmid vectors typically
15 include additional nucleic acid sequences that provide for easy selection, amplification, and transformation of the expression cassette in prokaryotic cells, e.g., pUC-derived vectors, pSK-derived vectors, pGEM-derived vectors, pSP-derived vectors, pBS-derived vectors, or preferably binary vectors containing one or more T-DNA regions. Additional nucleic acid sequences include origins of replication to provide for
20 autonomous replication of the vector, selectable marker genes, preferably encoding antibiotic or herbicide resistance, unique multiple cloning sites providing for multiple sites to insert nucleic acid sequences or genes encoded in the nucleic acid construct, and sequences that enhance transformation of prokaryotic and eukaryotic (especially plant) cells. The recombinant vector may comprise more than one polynucleotide
25 defined herein, for example three, four, five or six polynucleotides defined herein in combination, preferably a chimeric genetic construct described herein, each polynucleotide being operably linked to expression control sequences that are operable in the cell of interest. Preferably the expression control sequences include, or are all, heterologous promoters i.e. are heterologous with respect to the coding regions they
30 control. More than one polynucleotide defined herein, for example 3, 4, 5 or 6 polynucleotides, preferably 7 or 8 polynucleotides each encoding a different polypeptide, are preferably covalently joined together in a single recombinant vector, preferably within a single T-DNA molecule, which may then be introduced as a single molecule into a cell to form a recombinant cell according to the invention, and
35 preferably integrated into the genome of the recombinant cell, for example in a transgenic plant. The integration into the genome may be into the nuclear genome or

into a plastid genome in the transgenic plant. Thereby, the polynucleotides which are so joined will be inherited together as a single genetic locus in progeny of the recombinant cell or plant. The recombinant vector or plant may comprise two or more such recombinant vectors, each containing multiple polynucleotides, for example wherein
5 each recombinant vector comprises 3, 4, 5 or 6 polynucleotides.

"Operably linked" as used herein refers to a functional relationship between two or more nucleic acid (e.g., DNA) segments. Typically, it refers to the functional relationship of transcriptional regulatory element (promoter) to a transcribed sequence. For example, a promoter is operably linked to a coding sequence, such as a
10 polynucleotide defined herein, if it stimulates or modulates the transcription of the coding sequence in an appropriate cell. Generally, promoter transcriptional regulatory elements that are operably linked to a transcribed sequence are physically contiguous to the transcribed sequence, i.e., they are *cis*-acting. However, some transcriptional regulatory elements, such as enhancers, need not be physically contiguous or located in
15 close proximity to the coding sequences whose transcription they enhance.

When there are multiple promoters present, each promoter may independently be the same or different. Preferably, at least 3 and up to a maximum of 6 different promoter sequences are used in the recombinant vector to control expression of the exogenous polynucleotides.

20 Recombinant molecules such as the chimeric DNAs or genetic constructs may also contain (a) one or more secretory signals which encode signal peptide sequences, to enable an expressed polypeptide defined herein to be secreted from the cell that produces the polypeptide or which provide for localisation of the expressed polypeptide, for example for retention of the polypeptide in the endoplasmic reticulum
25 (ER) in the cell or transfer into a plastid, and/or (b) contain fusion sequences which lead to the expression of nucleic acid molecules as fusion proteins. Examples of suitable signal segments include any signal segment capable of directing the secretion or localisation of a polypeptide defined herein. Recombinant molecules may also include intervening and/or untranslated sequences surrounding and/or within the
30 nucleic acid sequences of nucleic acid molecules defined herein.

To facilitate identification of transformants, the nucleic acid construct desirably comprises a selectable or screenable marker gene as, or in addition to, the foreign or exogenous polynucleotide. By "marker gene" is meant a gene that imparts a distinct phenotype to cells expressing the marker gene and thus allows such transformed cells
35 to be distinguished from cells that do not have the marker. A selectable marker gene confers a trait for which one can "select" based on resistance to a selective agent (e.g., a

herbicide, antibiotic, radiation, heat, or other treatment damaging to untransformed cells). A screenable marker gene (or reporter gene) confers a trait that one can identify through observation or testing, i.e., by "screening" (e.g., β -glucuronidase, luciferase, GFP or other enzyme activity not present in untransformed cells). The marker gene and
5 the nucleotide sequence of interest do not have to be linked. The actual choice of a marker is not crucial as long as it is functional (i.e., selective) in combination with the cells of choice such as a plant cell.

Examples of selectable markers are markers that confer antibiotic resistance such as ampicillin, erythromycin, chloramphenicol or tetracycline resistance, preferably
10 kanamycin resistance. Exemplary selectable markers for selection of plant transformants include, but are not limited to, a *hyg* gene which encodes hygromycin B resistance; a neomycin phosphotransferase (*nptII*) gene conferring resistance to kanamycin, paromomycin, G418; a glutathione-S-transferase gene from rat liver conferring resistance to glutathione derived herbicides as, for example, described in EP
15 256223; a glutamine synthetase gene conferring, upon overexpression, resistance to glutamine synthetase inhibitors such as phosphinothricin as, for example, described in WO 87/05327, an acetyltransferase gene from *Streptomyces viridochromogenes* conferring resistance to the selective agent phosphinothricin as, for example, described in EP 275957, a gene encoding a 5-enolshikimate-3-phosphate synthase (EPSPS)
20 conferring tolerance to N-phosphonomethylglycine as, for example, described by Hinchee et al. (1988), or preferably a *bar* gene conferring resistance against bialaphos as, for example, described in WO91/02071.

Preferably, the nucleic acid construct is stably incorporated into the genome of the cell, such as the plant cell. Accordingly, the nucleic acid may comprise appropriate
25 elements which allow the molecule to be incorporated into the genome, preferably the right and left border sequences of a T-DNA molecule, or the construct is placed in an appropriate vector which can be incorporated into a chromosome of the cell.

Expression

30 As used herein, an expression vector is a DNA vector that is capable of transforming a host cell and of effecting expression of one or more specified polynucleotide molecule(s). Expression vectors of the present invention can direct gene expression in plant cells or in recombinant cells such as microbial cells. Expression vectors useful for the invention contain regulatory sequences such as
35 transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with the recombinant cell and that

control the expression of polynucleotide molecules of the present invention. In particular, polynucleotides or vectors useful for the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly
5 important transcription control sequences are those which control transcription initiation, such as promoter and enhancer sequences. Suitable transcription control sequences include any transcription control sequence that can function in at least one of the recombinant cells of the present invention. The choice of the regulatory sequences used depends on the target organism such as a plant and/or target organ or tissue of
10 interest. Such regulatory sequences may be obtained from any eukaryotic organism such as plants or plant viruses, or may be chemically synthesized. A variety of such transcription control sequences are known to those skilled in the art. Particularly preferred transcription control sequences are promoters active in directing transcription in plants, either constitutively or stage and/or tissue specific, depending on the use of
15 the plant or parts thereof.

A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., *Cloning Vectors: A Laboratory Manual*, 1985, supp. 1987; Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academic Press, 1989; and Gelvin et al., *Plant Molecular
20 Biology Manual*, Kluwer Academic Publishers, 1990. Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-
25 regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

A number of constitutive promoters that are active in plant cells have been described. Suitable promoters for constitutive expression in plants include, but are not
30 limited to, the cauliflower mosaic virus (CaMV) 35S promoter, the Figwort mosaic virus (FMV) 35S, and the light-inducible promoter from the small subunit of the ribulose-1,5-bis-phosphate carboxylase.

For the purpose of expression in source tissues of the plant, such as the leaf, seed, root or stem, it is preferred that the promoters utilized in the present invention
35 have relatively high expression in these specific tissues. Many examples are well known in the art. A variety of plant gene promoters that are regulated in response to

environmental, hormonal, chemical, and/or developmental signals, also can be used for expression of genes in plant cells, or it may also be advantageous to employ organ-specific promoters.

As used herein, the term "seed specific promoter" or variations thereof refer to a promoter that preferentially, when compared to other plant tissues, directs gene transcription in a developing seed of a plant, preferably a *Brassica sp.*, *Camelina sativa* or *G. max* plant. In an embodiment, the seed specific promoter is expressed at least 5-fold more strongly in the developing seed of the plant relative to the leaves and/or stems of the plant, and is preferably expressed more strongly in the embryo of the developing seed compared to other plant tissues. Preferably, the promoter only directs expression of a gene of interest in the developing seed, and/or expression of the gene of interest in other parts of the plant such as leaves is not detectable by Northern blot analysis and/or RT-PCR. Typically, the promoter drives expression of genes during growth and development of the seed, in particular during the phase of synthesis and accumulation of storage compounds in the seed. Such promoters may drive gene expression in the entire plant storage organ or only part thereof such as the seedcoat, or cotyledon(s), preferably in the embryos, in seeds of dicotyledonous plants or the endosperm or aleurone layer of a seeds of monocotyledonous plants.

Preferred promoters for seed-specific expression include i) promoters from genes encoding enzymes involved in fatty acid biosynthesis and accumulation in seeds, such as fatty acid desaturases and elongases, ii) promoters from genes encoding seed storage proteins, and iii) promoters from genes encoding enzymes involved in carbohydrate biosynthesis and accumulation in seeds. Seed specific promoters which are suitable are the oilseed rape napin gene promoter (US5,608,152), the *Vicia faba* USP promoter (Baumlein et al., 1991), the *Arabidopsis* oleosin promoter (WO98/45461), the *Phaseolus vulgaris* phaseolin promoter (US5,504,200), the *Brassica* Bee4 promoter (WO91/13980) or the legumin LeB4 promoter from *Vicia faba* (Baumlein et al., 1992), and promoters which lead to the seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. Notable promoters which are suitable are the barley lpt2 or lpt1 gene promoter (WO95/15389 and WO95/23230) or the promoters described in WO99/16890 (promoters from the barley hordein gene, the rice glutelin gene, the rice oryzin gene, the rice prolamin gene, the wheat gliadin gene, the wheat glutelin gene, the maize zein gene, the oat glutelin gene, the sorghum kasirin gene, the rye secalin gene). Other promoters include those described by Broun et al. (1998), Potenza et al. (2004), US20070192902 and US20030159173. In an embodiment, the seed specific promoter is preferentially expressed in defined parts of

the seed such as the embryo, cotyledon(s) or the endosperm. Examples of such specific promoters include, but are not limited to, the FP1 promoter (Ellerstrom et al., 1996), the pea legumin promoter (Perrin et al., 2000), the bean phytohemagglutinin promoter (Perrin et al., 2000), the conlinin 1 and conlinin 2 promoters for the genes encoding the
 5 flax 2S storage proteins (Cheng et al., 2010), the promoter of the FAE1 gene from *Arabidopsis thaliana*, the BnGLP promoter of the globulin-like protein gene of *Brassica napus*, the LPXR promoter of the peroxiredoxin gene from *Linum usitatissimum*.

The 5' non-translated leader sequence can be derived from the promoter selected
 10 to express the heterologous gene sequence of the polynucleotide of the present invention, or preferably is heterologous with respect to the coding region of the enzyme to be produced, and can be specifically modified if desired so as to increase translation of mRNA. For a review of optimizing expression of transgenes, see Koziel et al. (1996). The 5' non-translated regions can also be obtained from plant viral RNAs
 15 (Tobacco mosaic virus, Tobacco etch virus, Maize dwarf mosaic virus, Alfalfa mosaic virus, among others) from suitable eukaryotic genes, plant genes (wheat and maize chlorophyll a/b binding protein gene leader), or from a synthetic gene sequence. The present invention is not limited to constructs wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence.
 20 The leader sequence could also be derived from an unrelated promoter or coding sequence. Leader sequences useful in context of the present invention comprise the maize Hsp70 leader (US5,362,865 and US5,859,347), and the TMV omega element.

The termination of transcription is accomplished by a 3' non-translated DNA sequence operably linked in the chimeric vector to the polynucleotide of interest. The 3'
 25 non-translated region of a recombinant DNA molecule contains a polyadenylation signal that functions in plants to cause the addition of adenylate nucleotides to the 3' end of the RNA. The 3' non-translated region can be obtained from various genes that are expressed in plant cells. The nopaline synthase 3' untranslated region, the 3' untranslated region from pea small subunit Rubisco gene, the 3' untranslated region
 30 from soybean 7S seed storage protein gene or a flax conlinin gene are commonly used in this capacity. The 3' transcribed, non-translated regions containing the polyadenylate signal of *Agrobacterium* tumor-inducing (Ti) plasmid genes are also suitable.

Recombinant DNA technologies can be used to improve expression of a transformed polynucleotide molecule by manipulating, for example, the number of
 35 copies of the polynucleotide molecule within a host cell, the efficiency with which those polynucleotide molecules are transcribed, the efficiency with which the resultant

transcripts are translated, and the efficiency of post-translational modifications. Recombinant techniques useful for increasing the expression of polynucleotide molecules defined herein include, but are not limited to, integration of the polynucleotide molecule into one or more host cell chromosomes, addition of stability
5 sequences to mRNAs, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of polynucleotide molecules to correspond to the codon usage of the host cell, and the deletion of sequences that destabilize transcripts.

10

Transgenic Plants

The term "plant" as used herein as a noun refers to whole plants, but as used as an adjective refers to any substance which is present in, obtained from, derived from, or related to a plant, such as for example, plant organs (e.g. leaves, stems, roots, flowers),
15 single cells (e.g. pollen), seeds, plant cells and the like. The term "plant part" refers to all plant parts that comprise the plant DNA, including vegetative structures such as, for example, leaves or stems, roots, floral organs or structures, pollen, seed, seed parts such as an embryo, endosperm, scutellum or seed coat, plant tissue such as, for example, vascular tissue, cells and progeny of the same, as long as the plant part synthesizes lipid
20 according to the invention.

A "transgenic plant", "genetically modified plant" or variations thereof refers to a plant that contains a gene construct ("transgene") not found in a wild-type plant of the same species, variety or cultivar. Transgenic plants as defined in the context of the present invention include plants and their progeny which have been genetically
25 modified using recombinant techniques to cause production of the lipid or at least one polypeptide defined herein in the desired plant or plant organ. Transgenic plant cells and transgenic plant parts have corresponding meanings. A "transgene" as referred to herein has the normal meaning in the art of biotechnology and includes a genetic sequence which has been produced or altered by recombinant DNA or RNA technology
30 and which has been introduced into a plant cell. The transgene may include genetic sequences derived from a plant cell which may be of the same species, variety or cultivar as the plant cell into which the transgene is introduced or of a different species, variety or cultivar, or from a cell other than a plant cell. Typically, the transgene has been introduced into the cell, such as a plant, by human manipulation such as, for
35 example, by transformation but any method can be used as one of skill in the art recognizes.

The terms "seed" and "grain" are used interchangeably herein. "Grain" refers to mature grain such as harvested grain or grain which is still on a plant but ready for harvesting, but can also refer to grain after imbibition or germination, according to the context. Mature grain or seed commonly has a moisture content of less than about 18-
5 20%, preferably less than 10%. Brassica seed such as canola seed typically has a moisture content of about 4-8% or 6-8% when mature, preferably between about 4% to about 6%. "Developing seed" as used herein refers to a seed prior to maturity, typically found in the reproductive structures of the plant after fertilisation or anthesis, but can also refer to such seeds prior to maturity which are isolated from a plant.

10 As used herein, the term "obtaining a plant part" or "obtaining a seed" refers to any means of obtaining a plant part or seed, respectively, including harvesting of the plant parts or seed from plants in the field or in containment such as a glasshouse or growth chamber, or by purchase or receipt from a supplier of the plant parts or seed. Standard growth conditions in a glasshouse include 22-24°C daytime temperature and
15 16-18°C night-time temperature, with natural sunlight. The seed may be suitable for planting i.e. able to germinate and produce progeny plants, or alternatively has been processed in such a way that it is no longer able to germinate, e.g. cracked, polished or milled seed which is useful for food or feed applications, or for extraction of lipid of the invention.

20 As used herein, the term "plant storage organ" refers to a part of a plant specialized to storage energy in the form of, for example, proteins, carbohydrates, fatty acids and/or oils. Examples of plant storage organs are seed, fruit, tuberous roots, and tubers. A preferred plant storage organ is seed.

The plants or plant parts of the invention or used in the invention are preferably
25 phenotypically normal. As used herein, the term "phenotypically normal" refers to a genetically modified plant or plant organ, particularly a storage organ such as a seed, tuber or fruit not having a significantly reduced ability to grow and reproduce when compared to an unmodified plant or plant organ. In an embodiment, the genetically modified plant or plant organ which is phenotypically normal has an ability to grow or
30 reproduce which is essentially the same as an isogenic plant or organ not comprising the exogenous polynucleotide(s). Preferably, the biomass, growth rate, germination rate, storage organ size, pollen viability, male and female fertility, seed size and/or the number of viable seeds produced is not less than 90% of that of a plant lacking said exogenous polynucleotide when grown under identical conditions. Preferably the
35 pollen viability of the plant of the invention, or plants produced from seed of the invention, is about 100% relative to the pollen viability of a corresponding wild-type

plant. This term does not encompass features of the plant which may be different to the wild-type plant but which do not affect the usefulness of the plant for commercial purposes such as, for example, a ballerina phenotype of seedling leaves.

Plants provided by or contemplated for use in the practice of the present invention include both monocotyledons and dicotyledons. In preferred embodiments, the plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, or pea), or other legumes. The plants may be grown for production of edible roots, tubers, leaves, stems, flowers or fruit. The plants may be vegetables or ornamental plants. The plants of, or useful for, the invention may be: corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), mustard (*Brassica juncea*), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annuus*), wheat (*Triticum aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Lopmoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avocado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, or barley.

In a preferred embodiment, the plant is an angiosperm.

In an embodiment, the plant is an oilseed plant, preferably an oilseed crop plant. As used herein, an "oilseed plant" is a plant species used for the commercial production of oils from the seeds of the plant. The oilseed plant may be oil-seed rape (such as canola), maize, sunflower, soybean, sorghum, flax (linseed) or sugar beet. Furthermore, the oilseed plant may be other *Brassic*as, cotton, peanut, poppy, mustard, castor bean, sesame, sunflower, safflower, *Camelina*, *Crambe* or nut producing plants. The plant may produce high levels of oil in its fruit, such as olive, oil palm or coconut. Horticultural plants to which the present invention may be applied are lettuce, endive, or vegetable brassicas including cabbage, broccoli, or cauliflower. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, tomato, or pepper.

In a further preferred embodiment, the non-transgenic plant used to produce a transgenic plant of the invention produces oil, especially in the seed, which has i) less than 20%, less than 10% or less than 5% 18:2 fatty acids and/or ii) less than 10% or less than 5% 18:3 fatty acids.

In a preferred embodiment, the transgenic plant or part thereof is homozygous for each and every gene (exogenous polynucleotide) that has been introduced (transgene) so that its progeny do not segregate for the desired phenotype. The transgenic plant may also be heterozygous for the introduced transgene(s), preferably
 5 uniformly heterozygous for the transgene, such as for example in F1 progeny which have been grown from hybrid seed. Such plants may provide advantages such as hybrid vigour, well known in the art, or may be used in plant breeding or backcrossing.

Where relevant, the transgenic plant or part thereof may also comprise additional transgenes encoding enzymes involved in the production of LC-PUFAs such
 10 as, but not limited to, a $\Delta 6$ -desaturase, a $\Delta 9$ -elongase, a $\Delta 8$ -desaturase, a $\Delta 6$ -elongase, a $\Delta 5$ -desaturase, an $\omega 3$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 5$ -elongase, diacylglycerol acyltransferase, LPAAT, a $\Delta 17$ -desaturase, a $\Delta 15$ -desaturase and/or a $\Delta 12$ desaturase. Examples of such enzymes with one of more of these activities are known in the art and include those described herein. In specific examples, the transgenic plant at least
 15 comprises a set of exogenous polynucleotides encoding;

- a) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -elongase and a $\Delta 6$ -elongase,
- b) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -elongase and a $\Delta 9$ -elongase,
- 20 c) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -elongase, a $\Delta 6$ -elongase, and a $\Delta 15$ -desaturase,
- d) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -elongase, a $\Delta 9$ -elongase, and a $\Delta 15$ -desaturase,
- e) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -elongase, a $\Delta 6$ -
 25 elongase, and a $\Delta 17$ -desaturase,
- f) a $\Delta 4$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -elongase, a $\Delta 9$ -elongase, and a $\Delta 17$ -desaturase,
- g) an $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and a $\Delta 5$ -elongase,
- 30 h) an $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and a $\Delta 5$ -elongase,
- i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,
- j) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase or a $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -
 35 desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

k) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

l) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

m) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

n) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase and a Δ 5-elongase and optionally a Δ 4-desaturase,

o) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

p) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase,

q) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase, or

r) an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT), a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase, a Δ 5-elongase and optionally a Δ 4-desaturase.

In an embodiment, the exogenous polynucleotides encode set of polypeptides which are a *Pythium irregulare* Δ 6-desaturase, a *Thraustochytrid* Δ 5-desaturase or an *Emiliana huxleyi* Δ 5-desaturase, a *Physcomitrella patens* Δ 6-elongase, a *Thraustochytrid* Δ 5-elongase or an *Ostreococcus taurii* Δ 5-elongase, a *Phytophthora infestans* ω 3-desaturase or a *Pythium irregulare* ω 3-desaturase, and a *Thraustochytrid* Δ 4-desaturase.

In an embodiment, plants of, or used for, the invention are grown in the field, preferably as a population of at least 1,000, 1,000,000 or 2,000,000 plants that are essentially the same, or in an area of at least 1 hectare or 2 hectares. Planting densities differ according to the plant species, plant variety, climate, soil conditions, fertiliser rates and other factors as known in the art. For example, canola is typically grown at a planting density of 1.2-1.5 million plants per hectare. Plants are harvested as is known

in the art, which may comprise swathing, windrowing and/or reaping of plants, followed by threshing and/or winnowing of the plant material to separate the seed from the remainder of the plant parts often in the form of chaff. Alternatively, seed may be harvested from plants in the field in a single process, namely combining.

5

Transformation of plants

Transgenic plants can be produced using techniques known in the art, such as those generally described in A. Slater et al., *Plant Biotechnology - The Genetic Manipulation of Plants*, Oxford University Press (2003), and P. Christou and H. Klee, *Handbook of Plant Biotechnology*, John Wiley and Sons (2004).

As used herein, the terms “stably transforming”, “stably transformed” and variations thereof refer to the integration of the exogenous nucleic acid molecules into the genome of the cell such that they are transferred to progeny cells during cell division without the need for positively selecting for their presence. Stable transformants, or progeny thereof, can be selected by any means known in the art such as Southern blots on chromosomal DNA or *in situ* hybridization of genomic DNA. Preferably, plant transformation is performed as described in the Examples herein.

Agrobacterium-mediated transfer is a widely applicable system for introducing genes into plant cells because DNA can be introduced into cells in whole plant tissues or plant organs or explants in tissue culture, for either transient expression or for stable integration of the DNA in the plant cell genome. The use of *Agrobacterium*-mediated plant integrating vectors to introduce DNA into plant cells is well known in the art (see, for example, US 5177010, US 5104310, US 5004863 or US 5159135) including floral dipping methods using *Agrobacterium* or other bacteria that can transfer DNA into plant cells. The region of DNA to be transferred is defined by the border sequences, and the intervening DNA (T-DNA) is usually inserted into the plant genome. Further, the integration of the T-DNA is a relatively precise process resulting in few rearrangements. In those plant varieties where *Agrobacterium*-mediated transformation is efficient, it is the method of choice because of the facile and defined nature of the gene transfer. Preferred *Agrobacterium* transformation vectors are capable of replication in *E. coli* as well as *Agrobacterium*, allowing for convenient manipulations as described (Klee et al., In: *Plant DNA Infectious Agents*, Hohn and Schell, eds., Springer-Verlag, New York, pp. 179-203 (1985).

Acceleration methods that may be used include, for example, microprojectile bombardment and the like. One example of a method for delivering transforming nucleic acid molecules to plant cells is microprojectile bombardment. This method has

been reviewed by Yang et al., Particle Bombardment Technology for Gene Transfer, Oxford Press, Oxford, England (1994). Non-biological particles (microprojectiles) that may be coated with nucleic acids and delivered into cells by a propelling force. Exemplary particles include those comprised of tungsten, gold, platinum, and the like.

- 5 A particular advantage of microprojectile bombardment, in addition to it being an effective means of reproducibly transforming monocots, is that neither the isolation of protoplasts, nor the susceptibility of *Agrobacterium* infection are required.

In another alternative embodiment, plastids can be stably transformed. Methods disclosed for plastid transformation in higher plants include particle gun delivery of
10 DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination (US5, 451,513, US5,545,818, US5,877,402, US5,932,479, and WO99/05265).

Other methods of cell transformation can also be used and include but are not limited to introduction of DNA into plants by direct DNA transfer into pollen, by direct
15 injection of DNA into reproductive organs of a plant, or by direct injection of DNA into the cells of immature embryos followed by the rehydration of desiccated embryos.

The regeneration, development, and cultivation of plants from single plant protoplast transformants or from various transformed explants is well known in the art (Weissbach et al., In: Methods for Plant Molecular Biology, Academic Press, San
20 Diego, Calif., (1988). This regeneration and growth process typically includes the steps of selection of transformed cells, 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.

25 The development or regeneration of plants containing the foreign, exogenous gene is well known in the art. 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
30 plants. A transgenic plant of the present invention containing a desired exogenous nucleic acid is cultivated using methods well known to one skilled in the art.

To confirm the presence of the transgenes in transgenic cells and plants, a polymerase chain reaction (PCR) amplification or Southern blot analysis can be performed using methods known to those skilled in the art. Expression products of the
35 transgenes can be detected in any of a variety of ways, depending upon the nature of the product, and include Western blot and enzyme assay. Once transgenic plants have

been obtained, they may be grown to produce plant tissues or parts having the desired phenotype. The plant tissue or plant parts, may be harvested, and/or the seed collected. The seed may serve as a source for growing additional plants with tissues or parts having the desired characteristics.

- 5 A transgenic plant formed using *Agrobacterium* or other transformation methods typically contains a single genetic locus on one chromosome. Such transgenic plants can be referred to as being hemizygous for the added gene(s). More preferred is a transgenic plant that is homozygous for the added gene(s); i.e., a transgenic plant that contains two added genes, one gene at the same locus on each chromosome of a
- 10 chromosome pair. A homozygous transgenic plant can be obtained by self-fertilising a hemizygous transgenic plant, germinating some of the seed produced and analyzing the resulting plants for the gene of interest.

- It is also to be understood that two different transgenic plants that contain two independently segregating exogenous genes or loci can also be crossed (mated) to
- 15 produce offspring that contain both sets of genes or loci. Selfing of appropriate F1 progeny can produce plants that are homozygous for both exogenous genes or loci. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in Fehr, In: Breeding
- 20 Methods for Cultivar Development, Wilcox J. ed., American Society of Agronomy, Madison Wis. (1987).

Enhancing Exogenous RNA Levels and Stabilized Expression

Silencing Suppressors

- 25 In an embodiment, a plant cell, plant or plant part comprises an exogenous polynucleotide encoding a silencing suppressor protein.

- Post-transcriptional gene silencing (PTGS) is a nucleotide sequence-specific defense mechanism that can target both cellular and viral mRNAs for degradation. PTGS occurs in plants or fungi stably or transiently transformed with foreign
- 30 (heterologous) or endogenous DNA and results in the reduced accumulation of RNA molecules with sequence similarity to the introduced nucleic acid.

- It has widely been considered that co-expression of a silencing suppressor with a transgene of interest will increase the levels of RNA present in the cell transcribed from the transgene. Whilst this has proven true for cells *in vitro*, significant side-effects
- 35 have been observed in many whole plant co-expression studies. More specifically, as described in Mallory et al. (2002), Chapman et al. (2004), Chen et al. (2004), Dunoyer

et al. (2004), Zhang et al. (2006), Lewsey et al. (2007) and Meng et al. (2008) plants expressing silencing suppressors, generally under constitutive promoters, are often phenotypically abnormal to the extent that they are not useful for commercial production.

5 Recently, it has been found that RNA molecule levels can be increased, and/or RNA molecule levels stabilized over numerous generations, by limiting the expression of the silencing suppressor to a seed of a plant or part thereof (WO2010/057246). As used herein, a “silencing suppressor protein” or SSP is any polypeptide that can be expressed in a plant cell that enhances the level of expression product from a different
10 transgene in the plant cell, particularly over repeated generations from the initially transformed plant. In an embodiment, the SSP is a viral silencing suppressor or mutant thereof. A large number of viral silencing suppressors are known in the art and include, but are not limited to P19, V2, P38, Pe-Po and RPV-P0. In an embodiment, the viral silencing suppressor comprises amino acids having a sequence as provided in SEQ ID
15 NO:38, a biologically active fragment thereof, or an amino acid sequence which is at least 50% identical to SEQ ID NO:38 and which has activity as a silencing suppressor.

As used herein, the terms “stabilising expression”, “stably expressed”, “stabilised expression” and variations thereof refer to level of the RNA molecule being essentially the same or higher in progeny plants over repeated generations, for example
20 at least three, at least five or at least 10 generations, when compared to isogenic plants lacking the exogenous polynucleotide encoding the silencing suppressor. However, this term(s) does not exclude the possibility that over repeated generations there is some loss of levels of the RNA molecule when compared to a previous generation, for example not less than a 10% loss per generation.

25 The suppressor can be selected from any source e.g. plant, viral, mammal etc. See WO2010/057246 for a list of viruses from which the suppressor can be obtained and the protein (eg B2, P14 etc) or coding region designation for the suppressor from each particular virus. Multiple copies of a suppressor may be used. Different suppressors may be used together (e. g., in tandem).

30

RNA Molecules

Essentially any RNA molecule which is desirable to be expressed in a plant seed can be co-expressed with the silencing suppressor. The encoded polypeptides may be involved in metabolism of oil, starch, carbohydrates, nutrients, etc., or may be
35 responsible for the synthesis of proteins, peptides, fatty acids, lipids, waxes, oils, starches, sugars, carbohydrates, flavors, odors, toxins, carotenoids, hormones,

polymers, flavonoids, storage proteins, phenolic acids, alkaloids, lignins, tannins, celluloses, glycoproteins, glycolipids, etc, preferably the biosynthesis or assembly of TAG.

In a particular example, the plants produced increased levels of enzymes for oil
5 production in plants such as Brassicas, for example canola or sunflower, safflower, flax, cotton, soya bean, *Camelina* or maize.

Levels of LC-PUFA Produced

The levels of the LC-PUFA or combination of LC-PUFAs that are produced in
10 the recombinant cell or plant part such as seed are of importance. The levels may be expressed as a composition (in percent) of the total fatty acid that is a particular LC-PUFA or group of related LC-PUFA, for example the $\omega 3$ LC-PUFA or the $\omega 6$ LC-PUFA, or the VLC-PUFA, or other which may be determined by methods known in the art. The level may also be expressed as a LC-PUFA content, such as for example the
15 percentage of LC-PUFA in the dry weight of material comprising the recombinant cells, for example the percentage of the weight of seed that is LC-PUFA. It will be appreciated that the LC-PUFA that is produced in an oilseed may be considerably higher in terms of LC-PUFA content than in a vegetable or a grain that is not grown for oil production, yet both may have similar LC-PUFA compositions, and both may be
20 used as sources of LC-PUFA for human or animal consumption.

The levels of LC-PUFA may be determined by any of the methods known in the art. In a preferred method, total lipid is extracted from the cells, tissues or organisms and the fatty acid converted to methyl esters before analysis by gas chromatography (GC). Such techniques are described in Example 1. The peak position in the
25 chromatogram may be used to identify each particular fatty acid, and the area under each peak integrated to determine the amount. As used herein, unless stated to the contrary, the percentage of particular fatty acid in a sample is determined as the area under the peak for that fatty acid as a percentage of the total area for fatty acids in the chromatogram. This corresponds essentially to a weight percentage (w/w). The identity
30 of fatty acids may be confirmed by GC-MS. Total lipid may be separated by techniques known in the art to purify fractions such as the TAG fraction. For example, thin-layer chromatography (TLC) may be performed at an analytical scale to separate TAG from other lipid fractions such as DAG, acyl-CoAs or phospholipid in order to determine the fatty acid composition specifically of TAG.

35 In one embodiment, the sum total of ARA, EPA, DPA and DHA in the fatty acids in the extracted lipid is between about 21% and about 40% of the total fatty acids

in the cell. In a further embodiment, the total fatty acid in the cell has less than 1% C20:1. In preferred embodiments, the extractable TAG in the cell comprises the fatty acids at the levels referred to herein. Each possible combination of the features defining the lipid as described herein is also encompassed.

- 5 The level of production of LC-PUFA in the recombinant cell, plant or plant part such as seed may also be expressed as a conversion percentage of a specific substrate fatty acid to one or more product fatty acids, which is also referred to herein as a “conversion efficiency” or “enzymatic efficiency”. This parameter is based on the fatty acid composition in the lipid extracted from the cell, plant, plant part or seed, i.e., the amount of the LC-PUFA formed (including other LC-PUFA derived therefrom) as a percentage of one or more substrate fatty acids (including all other fatty acids derived therefrom). The general formula for a conversion percentage is: $100 \times (\text{the sum of percentages of the product LC-PUFA and all products derived therefrom}) / (\text{the sum of the percentages of the substrate fatty acid and all products derived therefrom})$. With regard to DHA, for example, this may be expressed as the ratio of the level of DHA (as a percentage in the total fatty acid content in the lipid) to the level of a substrate fatty acid (e.g. OA, LA, ALA, SDA, ETA or EPA) and all products including DHA derived from the substrate. The conversion percentage or efficiency of conversion can be expressed for a single enzymatic step in a pathway, or for part or the whole of a pathway.

Specific conversion efficiencies are calculated herein according to the formulae:

1. OA to DHA = $100 \times (\% \text{DHA}) / (\text{sum } \% \text{ for OA, LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
2. LA to DHA = $100 \times (\% \text{DHA}) / (\text{sum } \% \text{ for LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
3. ALA to DHA = $100 \times (\% \text{DHA}) / (\text{sum } \% \text{ for ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
4. EPA to DHA = $100 \times (\% \text{DHA}) / (\text{sum } \% \text{ for EPA, DPA and DHA})$.
5. DPA to DHA ($\Delta 4$ -desaturase efficiency) = $100 \times (\% \text{DHA}) / (\text{sum } \% \text{ for DPA and DHA})$.
6. $\Delta 12$ -desaturase efficiency = $100 \times (\text{sum } \% \text{ for LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA}) / (\text{sum } \% \text{ for OA, LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
7. $\omega 3$ -desaturase efficiency = $100 \times (\text{sum } \% \text{ for ALA, SDA, ETrA, ETA, EPA, DPA and DHA}) / (\text{sum } \% \text{ for LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.

8. OA to ALA = $100 \times (\text{sum \% for ALA, SDA, ETrA, ETA, EPA, DPA and DHA}) / (\text{sum \% for OA, LA, GLA, DGLA, ARA, EDA, ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
9. $\Delta 6$ -desaturase efficiency (on $\omega 3$ substrate ALA) = $100 \times (\text{sum \% for SDA, ETA, EPA, DPA and DHA}) / (\% \text{ALA, SDA, ETrA, ETA, EPA, DPA and DHA})$.
10. $\Delta 6$ -elongase efficiency (on $\omega 3$ substrate SDA) = $100 \times (\text{sum \% for ETA, EPA, DPA and DHA}) / (\text{sum \% for SDA, ETA, EPA, DPA and DHA})$.
11. $\Delta 5$ -desaturase efficiency (on $\omega 3$ substrate ETA) = $100 \times (\text{sum \% for EPA, DPA and DHA}) / (\text{sum \% for ETA, EPA, DPA and DHA})$.
- 10 12. $\Delta 5$ -elongase efficiency (on $\omega 3$ substrate EPA) = $100 \times (\text{sum \% for DPA and DHA}) / (\text{sum \% for EPA, DPA and DHA})$.

The fatty acid composition of the lipid, preferably seedoil, of the invention, is also characterised by the ratio of $\omega 6$ fatty acids: $\omega 3$ fatty acids in the total fatty acid content, for either total $\omega 6$ fatty acids:total $\omega 3$ fatty acids or for new $\omega 6$ fatty acids:new $\omega 3$ fatty acids. The terms total $\omega 6$ fatty acids, total $\omega 3$ fatty acids, new $\omega 6$ fatty acids and new $\omega 3$ fatty acids have the meanings as defined herein. The ratios are calculated from the fatty acid composition in the lipid extracted from the cell, plant, plant part or seed, in the manner as exemplified herein. It is desirable to have a greater level of $\omega 3$ than $\omega 6$ fatty acids in the lipid, and therefore an $\omega 6$: $\omega 3$ ratio of less than 1.0 is preferred. A ratio of 0.0 indicates a complete absence of the defined $\omega 6$ fatty acids; a ratio of 0.03 was achieved. Such low ratios can be achieved through the combined use of a $\Delta 6$ -desaturase which has an $\omega 3$ substrate preference together with an $\omega 3$ -desaturase, particularly a fungal $\omega 3$ -desaturase such as the *Pichia pastoris* $\omega 3$ -desaturase as exemplified herein.

The yield of LC-PUFA per weight of seed may also be calculated based on the total oil content in the seed and the %DHA and/or DPA in the oil. For example, if the oil content of canola seed is about 40% (w/w) and about 12% of the total fatty acid content of the oil is DHA, the DHA content of the seed is about 4.8% or about 48mg per gram of seed. As described in Example 2, the DHA content of *Arabidopsis* seed having about 9% DHA, which has a lower oil content than canola, was about 25mg/g seed. At a DHA content of about 21%, canola seed or *Camelina sativa* seed has a DHA content of about 84mg per gram of seed. The present invention therefore provides *Brassica napus*, *B. juncea* and *Camelina sativa* plants, and seed obtained therefrom, comprising at least about 80mg or at least about 84mg DHA per gram seed. The seed has a moisture content as is standard for harvested mature seed after drying down (4-15% moisture). The invention also provides a process for obtaining oil, comprising

obtaining the seed and extracting the oil from the seed, and uses of the oil and methods of obtaining the seed comprising harvesting the seeds from the plants according to the invention.

The amount of DHA and/or DPA produced per hectare can also be calculated if the seed yield per hectare is known or can be estimated. For example, canola in Australia typically yields about 2.5 tonnes seed per hectare, which at 40% oil content yields about 1000kg of oil. At 20.1% DHA and/or DPA in the total oil, this provides about 200kg of DHA and/or DPA per hectare. If the oil content is reduced by 50%, this still provides about 100kg DHA and/or DPA/ha.

Evidence to date suggests that some desaturases expressed heterologously in yeast or plants have relatively low activity in combination with some elongases. This may be alleviated by providing a desaturase with the capacity of to use an acyl-CoA form of the fatty acid as a substrate in LC-PUFA synthesis, and this is thought to be advantageous in recombinant cells particularly in plant cells. A particularly advantageous combination for efficient DHA and/or DPA synthesis is a fungal ω 3-desaturase, for example such as the *Pichia pastoris* ω 3-desaturase (SEQ ID NO: 6), with a Δ 6-desaturase which has a preference for ω 3 acyl substrates such as, for example, the *Micromonas pusilla* Δ 6-desaturase (SEQ ID NO: 9), or variants thereof which have at least 95% amino acid sequence identity.

As used herein, the term "essentially free" means that the composition (for example lipid or oil) comprises little (for example, less than about 0.5%, less than about 0.25%, less than about 0.1%, or less than about 0.01%) or none of the defined component. In an embodiment, "essentially free" means that the component is undetectable using a routine analytical technique, for example a specific fatty acid (such as ω 6-docosapentaenoic acid) cannot be detected using gas chromatography as outlined in Example 1.

Production of Oils

Techniques that are routinely practiced in the art can be used to extract, process, and analyze the oils produced by cells, plants, seeds, etc of the instant invention. Typically, plant seeds are cooked, pressed, and extracted to produce crude oil, which is then degummed, refined, bleached, and deodorized. Generally, techniques for crushing seed are known in the art. For example, oilseeds can be tempered by spraying them with water to raise the moisture content to, e.g., 8.5%, and flaked using a smooth roller with a gap setting of 0.23 to 0.27 mm. Depending on the type of seed, water may not be added prior to crushing. Application of heat deactivates enzymes, facilitates further

cell rupturing, coalesces the oil droplets, and agglomerates protein particles, all of which facilitate the extraction process.

In an embodiment, the majority of the seed oil is released by passage through a screw press. Cakes expelled from the screw press are then solvent extracted, e.g., with
5 hexane, using a heat traced column. Alternatively, crude oil produced by the pressing operation can be passed through a settling tank with a slotted wire drainage top to remove the solids that are expressed with the oil during the pressing operation. The clarified oil can be passed through a plate and frame filter to remove any remaining fine solid particles. If desired, the oil recovered from the extraction process can be
10 combined with the clarified oil to produce a blended crude oil.

Once the solvent is stripped from the crude oil, the pressed and extracted portions are combined and subjected to normal oil processing procedures. As used herein, the term "purified" when used in connection with lipid or oil of the invention typically means that the extracted lipid or oil has been subjected to one or more
15 processing steps of increase the purity of the lipid/oil component. For example, a purification step may comprise one or more or all of the group consisting of: degumming, deodorising, decolourising, drying and/or fractionating the extracted oil. However, as used herein, the term "purified" does not include a transesterification process or other process which alters the fatty acid composition of the lipid or oil of the
20 invention so as to increase the DHA content as a percentage of the total fatty acid content. Expressed in other words, the fatty acid composition of the purified lipid or oil is essentially the same as that of the unpurified lipid or oil.

Degumming

25 Degumming is an early step in the refining of oils and its primary purpose is the removal of most of the phospholipids from the oil, which may be present as approximately 1-2% of the total extracted lipid. Addition of ~2% of water, typically containing phosphoric acid, at 70–80°C to the crude oil results in the separation of most of the phospholipids accompanied by trace metals and pigments. The insoluble material
30 that is removed is mainly a mixture of phospholipids and triacylglycerols and is also known as lecithin. Degumming can be performed by addition of concentrated phosphoric acid to the crude seedoil to convert non-hydratable phosphatides to a hydratable form, and to chelate minor metals that are present. Gum is separated from the seedoil by centrifugation.

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Alkali refining

Alkali refining is one of the refining processes for treating crude oil, sometimes also referred to as neutralization. It usually follows degumming and precedes bleaching. Following degumming, the seedoil can be treated by the addition of a sufficient amount of an alkali solution to titrate all of the fatty acids and phosphoric acids, and removing the soaps thus formed. Suitable alkaline materials include sodium hydroxide, potassium hydroxide, sodium carbonate, lithium hydroxide, calcium hydroxide, calcium carbonate and ammonium hydroxide. This process is typically carried out at room temperature and removes the free fatty acid fraction. Soap is removed by centrifugation or by extraction into a solvent for the soap, and the neutralised oil is washed with water. If required, any excess alkali in the oil may be neutralized with a suitable acid such as hydrochloric acid or sulphuric acid.

Bleaching

Bleaching is a refining process in which oils are heated at 90–120°C for 10–30 minutes in the presence of a bleaching earth (0.2–2.0%) and in the absence of oxygen by operating with nitrogen or steam or in a vacuum. This step in oil processing is designed to remove unwanted pigments (carotenoids, chlorophyll, gossypol etc), and the process also removes oxidation products, trace metals, sulphur compounds and traces of soap.

Deodorization

Deodorization is a treatment of oils and fats at a high temperature (200–260°C) and low pressure (0.1–1 mm Hg). This is typically achieved by introducing steam into the seedoil at a rate of about 0.1 ml/minute/100 ml of seedoil. After about 30 minutes of sparging, the seedoil is allowed to cool under vacuum. The seedoil is typically transferred to a glass container and flushed with argon before being stored under refrigeration. This treatment improves the colour of the seedoil and removes a majority of the volatile substances or odorous compounds including any remaining free fatty acids, monoacylglycerols and oxidation products.

Winterisation

Winterization is a process sometimes used in commercial production of oils for the separation of oils and fats into solid (stearin) and liquid (olein) fractions by crystallization at sub-ambient temperatures. It was applied originally to cottonseed oil

to produce a solid-free product. It is typically used to decrease the saturated fatty acid content of oils.

Transesterification

- 5 As used herein, "transesterification" means a process that exchanges the fatty acids within and between TAGs or transfers the fatty acids to another alcohol to form an ester. This may initially involve releasing fatty acids from the TAGs as free fatty acids or it may directly produce fatty acid esters, preferably fatty acid methyl esters or ethyl esters. In a transesterification reaction of the TAG with an alcohol such as
- 10 methanol or ethanol, the alkyl group of the alcohol forms an ester linkage with the acyl groups (including the DHA) of the TAG. When combined with a fractionation process, transesterification can be used to modify the fatty acid composition of lipids (Marangoni et al., 1995). Transesterification can use either chemical (e.g. strong acid or base catalysed) or enzymatic means, the latter using lipases which may be position-
- 15 specific (*sn*-1/3 or *sn*-2 specific) for the fatty acid on the TAG, or having a preference for some fatty acids over others (Speranza et al, 2012). The fatty acid fractionation to increase the concentration of LC-PUFA in an oil can be achieved by any of the methods known in the art, such as, for example, freezing crystallization, complex formation using urea, molecular distillation, supercritical fluid extraction, counter
- 20 current chromatography and silver ion complexing. Complex formation with urea is a preferred method for its simplicity and efficiency in reducing the level of saturated and monounsaturated fatty acids in the oil (Gamez et al., 2003). Initially, the TAGs of the oil are split into their constituent fatty acids, often in the form of fatty acid esters, by hydrolysis under either acid or base catalysed reaction conditions, whereby one mol of
- 25 TAG is reacted with at least 3 mol of alcohol (e.g. ethanol for ethyl esters or methanol for methyl esters) with excess alcohol used to enable separation of the formed alkyl esters and the glycerol that is also formed, or by lipases. These free fatty acids or fatty acid esters, which are usually unaltered in fatty acid composition by the treatment, may then be mixed with an ethanolic solution of urea for complex formation. The saturated
- 30 and monounsaturated fatty acids easily complex with urea and crystallize out on cooling and may subsequently be removed by filtration. The non-urea complexed fraction is thereby enriched with LC-PUFA.

Feedstuffs

- 35 The present invention includes compositions which can be used as feedstuffs. For purposes of the present invention, "feedstuffs" include any food or preparation for

human or animal consumption which when taken into the body (a) serve to nourish or build up tissues or supply energy; and/or (b) maintain, restore or support adequate nutritional status or metabolic function. Feedstuffs of the invention include nutritional compositions for babies and/or young children such as, for example, infant formula,
5 and seedmeal of the invention.

Feedstuffs of the invention comprise, for example, a cell of the invention, a plant of the invention, the plant part of the invention, the seed of the invention, an extract of the invention, the product of the method of the invention, the product of the fermentation process of the invention, or a composition along with a suitable carrier(s).
10 The term "carrier" is used in its broadest sense to encompass any component which may or may not have nutritional value. As the skilled addressee will appreciate, the carrier must be suitable for use (or used in a sufficiently low concentration) in a feedstuff such that it does not have deleterious effect on an organism which consumes the feedstuff.

15 The feedstuff of the present invention comprises an oil, fatty acid ester, or fatty acid produced directly or indirectly by use of the methods, cells or plants disclosed herein. The composition may either be in a solid or liquid form. Additionally, the composition may include edible macronutrients, protein, carbohydrate, vitamins, and/or minerals in amounts desired for a particular use. The amounts of these ingredients will
20 vary depending on whether the composition is intended for use with normal individuals or for use with individuals having specialized needs, such as individuals suffering from metabolic disorders and the like.

Examples of suitable carriers with nutritional value include, but are not limited to, macronutrients such as edible fats, carbohydrates and proteins. Examples of such
25 edible fats include, but are not limited to, coconut oil, borage oil, fungal oil, black current oil, soy oil, and mono- and diglycerides. Examples of such carbohydrates include (but are not limited to): glucose, edible lactose, and hydrolyzed starch. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include (but are not limited to) soy proteins, electro dialysed whey,
30 electro dialysed skim milk, milk whey, or the hydrolysates of these proteins.

With respect to vitamins and minerals, the following may be added to the feedstuff compositions of the present invention: calcium, phosphorus, potassium, sodium, chloride, magnesium, manganese, iron, copper, zinc, selenium, iodine, and Vitamins A, E, D, C, and the B complex. Other such vitamins and minerals may also
35 be added.

The components utilized in the feedstuff compositions of the present invention can be of semi-purified or purified origin. By semi-purified or purified is meant a material which has been prepared by purification of a natural material or by *de novo* synthesis.

5 A feedstuff composition of the present invention may also be added to food even when supplementation of the diet is not required. For example, the composition may be added to food of any type, including (but not limited to): margarine, modified butter, cheeses, milk, yogurt, chocolate, candy, snacks, salad oils, cooking oils, cooking fats, meats, fish and beverages.

10 Additionally, fatty acids produced in accordance with the present invention or host cells transformed to contain and express the subject genes may also be used as animal food supplements to alter an animal's tissue, egg or milk fatty acid composition to one more desirable for human or animal consumption. Examples of such animals include sheep, cattle, horses, poultry such as chickens and the like.

15 Furthermore, feedstuffs of the invention can be used in aquaculture to increase the levels of fatty acids in fish or crustaceans such as, for example, prawns for human or animal consumption. Preferred fish are salmon.

Preferred feedstuffs of the invention are the plants, seed and other plant parts such as leaves and stems which may be used directly as food or feed for humans or
20 other animals. For example, animals may graze directly on such plants grown in the field or be fed more measured amounts in controlled feeding. The invention includes the use of such plants and plant parts as feed for increasing the LC-PUFA levels in humans and other animals.

25 Compositions

The present invention also encompasses compositions, particularly pharmaceutical compositions, comprising one or more of the fatty acids and/or resulting oils produced using the methods of the invention, preferably in the form of ethyl esters of the fatty acids.

30 A pharmaceutical composition may comprise one or more of the fatty acids and/or oils, in combination with a standard, well-known, non-toxic pharmaceutically-acceptable carrier, adjuvant or vehicle such as phosphate-buffered saline, water, ethanol, polyols, vegetable oils, a wetting agent or an emulsion such as a water/oil emulsion. The composition may be in either a liquid or solid form. For example, the
35 composition may be in the form of a tablet, capsule, ingestible liquid or powder, injectible, or topical ointment or cream. Proper fluidity can be maintained, for

example, by the maintenance of the required particle size in the case of dispersions and by the use of surfactants. It may also be desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Besides such inert diluents, the composition can also include adjuvants, such as wetting agents, emulsifying and
5 suspending agents, sweetening agents, flavoring agents and perfuming agents.

Suspensions, in addition to the active compounds, may comprise suspending agents such as ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar, and tragacanth or mixtures of these substances.

10 Solid dosage forms such as tablets and capsules can be prepared using techniques well known in the art. For example, fatty acids produced in accordance with the present invention can be tableted with conventional tablet bases such as lactose, sucrose, and cornstarch in combination with binders such as acacia, cornstarch or gelatin, disintegrating agents such as potato starch or alginic acid, and a lubricant
15 such as stearic acid or magnesium stearate. Capsules can be prepared by incorporating these excipients into a gelatin capsule along with antioxidants and the relevant fatty acid(s).

For intravenous administration, the fatty acids produced in accordance with the present invention or derivatives thereof may be incorporated into commercial
20 formulations.

A typical dosage of a particular fatty acid is from 0.1 mg to 20 g, taken from one to five times per day (up to 100 g daily) and is preferably in the range of from about 10 mg to about 1, 2, 5, or 10 g daily (taken in one or multiple doses). As known in the art, a minimum of about 300 mg/day of fatty acid, especially LC-PUFA, is desirable.
25 However, it will be appreciated that any amount of fatty acid will be beneficial to the subject.

Possible routes of administration of the pharmaceutical compositions of the present invention include, for example, enteral (e.g., oral and rectal) and parenteral. For example, a liquid preparation may be administered orally or rectally. Additionally,
30 a homogenous mixture can be completely dispersed in water, admixed under sterile conditions with physiologically acceptable diluents, preservatives, buffers or propellants to form a spray or inhalant.

The dosage of the composition to be administered to the patient may be determined by one of ordinary skill in the art and depends upon various factors such as
35 weight of the patient, age of the patient, overall health of the patient, past history of the patient, immune status of the patient, etc.

Additionally, the compositions of the present invention may be utilized for cosmetic purposes. It may be added to pre-existing cosmetic compositions such that a mixture is formed or a fatty acid produced according to the subject invention may be used as the sole "active" ingredient in a cosmetic composition.

5

EXAMPLES

Example 1. Materials and Methods

Expression of genes in plant cells in a transient expression system

Exogenous genetic constructs were expressed in plant cells in a transient expression system essentially as described by Voinnet et al. (2003) and Wood et al. (2009).

Gas chromatography (GC) analysis of fatty acids

FAME were analysed by gas chromatography using an Agilent Technologies 7890A GC (Palo Alto, California, USA) equipped with a 30 m SGE-BPX70 column (70 % cyanopropyl polysilphenylene-siloxane, 0.25 mm inner diameter, 0.25 mm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7693 Series auto sampler and injector. Helium was used as the carrier gas. Samples were injected in split mode (50:1 ratio) at an oven temperature of 150 °C. After injection, the oven temperature was held at 150 °C for 1 min then raised to 210 °C at 3 °C. min⁻¹, again raised to 240 °C at 50 °C. min⁻¹ and finally holding for 1.4 min at 240 °C. Peaks were quantified with Agilent Technologies ChemStation software (Rev B.04.03 (16), Palo Alto, California, USA) based on the response of the known amount of the external standard GLC-411 (Nuccheck) and C17:0-ME internal standard.

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Liquid Chromatography-Mass Spectrometry (LC-MS) analysis of lipids

Total lipids were extracted from freeze-dried developing seeds, twelve days after flowering (daf), and mature seeds after adding a known amount of tri-C17:0-TAG as an internal quantitation standard. The extracted lipids were dissolved into 1 mL of 10 mM butylated hydroxytoluene in butanol:methanol (1:1 v/v) per 5 mg dry material and analysed using an Agilent 1200 series LC and 6410b electrospray ionisation triple quadrupole LC-MS. Lipids were chromatographically separated using an Ascentis Express RP-Amide column (50 mm x 2.1 mm, 2.7 µm, Supelco) operating a binary gradient with a flow rate of 0.2 mL/min. The mobile phases were: A. 10 mM ammonium formate in H₂O:methanol:tetrahydrofuran (50:20:30 v/v/v); B. 10 mM ammonium formate in H₂O:methanol:tetrahydrofuran (5:20:75, v/v/v). Multiple

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reaction monitoring (MRM) lists were based on the following major fatty acids: 16:0, 18:0, 18:1, 18:2, 18:3, 18:4, 20:1, 20:2, 20:3, 20:4, 20:5, 22:4, 22:5, 22:6 using a collision energy of 30 V and fragmentor of 60 V. Individual MRM TAG was identified based on ammoniated precursor ion and product ion from neutral loss of 22:6. TAG
5 was quantified using a 10 μ M tristearin external standard.

Lipid profiling with LC-MS

The extracted total lipids were analysed using an Agilent 1200 series LC coupled to an Agilent 6410B electrospray ionisation QQQ-MS (Agilent, Palo Alto, California, USA). A 5 μ L injection of each total lipid extract was chromatographically separated with an Ascentis Express RP-Amide 50 mm x 2.1 mm, 2.7 μ m HPLC column (Sigma-Aldrich, Castle Hill, Australia) using a binary gradient with a flow rate of 0.2 mL/min. The mobile phases were: A. 10 mM ammonium formate in H₂O:methanol:tetrahydrofuran (50:20:30, v/v/v.); B. 10 mM ammonium formate in
15 H₂O:methanol:tetrahydrofuran (5:20:75, v/v/v.). Selected neutral lipids (TAG and DAG) and phospholipids (PL, including PC, PE, PI, PS, PA, PG) were analysed by multiple reaction monitoring (MRM) using a collision energy of 30 V and fragmentation energy of 60 V. Neutral lipids were targeted on the following major fatty acids: 16:0 (palmitic acid), 18:0 (stearic acid), 18:1 ω 9 (oleic acid, OA), 18:2 ω 6
20 (linoleic acid, LA), 18:3 ω 3 (α -linolenic acid, ALA), 18:4 ω 3 (stearidonic acid, SDA), 20:1, 20:2, 20:3, 20:4 ω 3, 20:5 ω 3, 22:4 ω 3, 22:5 ω 3, 22:6 ω 3, while phospholipids were scanned containing C₁₆, C₁₈, C₂₀ and C₂₂ species with double bonds of 0-3, 0-4, 0-5, 4-6 respectively.

Individual MRM TAG was identified based on ammoniated precursor ion and
25 product ion from neutral loss of 20:1, SDA, EPA and DHA. TAG and DAG were quantified using the 50 μ M tristearin and distearin as external standards. PL were quantified with 10 μ M of di-18:0-PC, di-17:0-PA, di-17:0-PE, 17:0-17:1-PG, di-18:1-PI and di-17:0-PS external standards (Avanti Polar Lipids, Alabaster, Alabama, USA). Selected TAG, DAG and PL species were further confirmed by Agilent 6520 Q-TOF
30 MS/MS.

Determination of seed fatty acid profile and oil content

Where seed oil content was to be determined, seeds were dried in a desiccator for 24 h and approximately 4 mg of seed was transferred to a 2 ml glass vial containing
35 Teflon-lined screw cap. 0.05 mg triheptadecanoic acid dissolved in 0.1 ml toluene was added to the vial as internal standard.

Seed FAME were prepared by adding 0.7 ml of 1N methanolic HCl (Supelco) to the vial containing seed material, vortexed briefly and incubated at 80 °C for 2h. After cooling to room temperature, 0.3 ml of 0.9% NaCl (w/v) and 0.1 ml hexane was added to the vial and mixed well for 10 min in Heidolph Vibramax 110. The FAME was
5 collected into 0.3 ml glass insert and analysed by GC with a flame ionization detector (FID) as mentioned earlier.

The peak area of individual FAME were first corrected on the basis of the peak area responses of known amount of the same FAMES present in a commercial standard GLC-411 (NU-CHEK PREP, INC., USA). GLC-411 contains equal amounts of 31
10 fatty acids (% by wt), ranging from C8:0 to C22:6. In case of fatty acids, which were not present in the standard, the inventors took the peak area responses of the most similar FAME. For example, peak area response of FAMES of 16:1d9 was used for 16:1d7 and FAME response of C22:6 was used for C22:5. The corrected areas were used to calculate the mass of each FAME in the sample by comparison to the internal
15 standard mass. Oil is stored mainly in the form of TAG and its weight was calculated based on FAME weight. Total moles of glycerol was determined by calculating moles of each FAMES and dividing total moles of FAMES by three. TAG was calculated as the sum of glycerol and fatty acyl moieties using a relation: % oil by weight= $100 \times ((41 \times \text{total mol FAME}/3) + (\text{total g FAME} - (15 \times \text{total mol FAME}))) / \text{g seed}$, where 41
20 and 15 are molecular weights of glycerol moiety and methyl group, respectively.

Analysis of the sterol content of oil samples

Samples of approximately 10mg of oil together with an added aliquot of C24:0 monol as an internal standard were saponified using 4mL 5% KOH in 80% MeOH and
25 heating for 2h at 80°C in a Teflon-lined screw-capped glass tube. After the reaction mixture was cooled, 2mL of Milli-Q water were added and the sterols were extracted into 2 mL of hexane: dichloromethane (4:1 v/v) by shaking and vortexing. The mixture was centrifuged and the sterol extract was removed and washed with 2mL of Milli-Q water. The sterol extract was then removed after shaking and centrifugation. The
30 extract was evaporated using a stream of nitrogen gas and the sterols silylated using 200mL of BSTFA and heating for 2h at 80°C.

For GC/GC-MS analysis of the sterols, sterol-OTMSi derivatives were dried under a stream of nitrogen gas on a heat block at 40°C and then re-dissolved in chloroform or hexane immediately prior to GC/GC-MS analysis. The sterol-OTMS
35 derivatives were analysed by gas chromatography (GC) using an Agilent Technologies 6890A GC (Palo Alto, California, USA) fitted with an Supelco EquityTM-1 fused silica

capillary column (15 m x 0.1 mm i.d., 0.1µm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was the carrier gas. Samples were injected in splitless mode at an oven temperature of 120°C. After injection, the oven temperature was raised to 270°C at 10°C min⁻¹ and
5 finally to 300°C at 5°C min⁻¹. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA). GC results are subject to an error of ±5% of individual component areas.

GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-MS and a Finnigan Thermo Electron Corporation GC-MS; both
10 systems were fitted with an on-column injector and Thermoquest Xcalibur software (Austin, Texas, USA). Each GC was fitted with a capillary column of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. A full procedural blank analysis was performed concurrent to the
15 sample batch.

RT-PCR conditions

Reverse transcription-PCR (RT-PCR) amplification was typically carried out using the Superscript III One-Step RT-PCR system (Invitrogen) in a volume of 25 µL
20 using 10 pmol of the forward primer and 30 pmol of the reverse primer, MgSO₄ to a final concentration of 2.5 mM, 400 ng of total RNA with buffer and nucleotide components according to the manufacturer's instructions. Typical temperature regimes were: 1 cycle of 45°C for 30 minutes for the reverse transcription to occur; then 1 cycle of 94°C for 2 minutes followed by 40 cycles of 94°C for 30 seconds, 52°C for 30
25 seconds, 70°C for 1 minute; then 1 cycle of 72°C for 2 minutes before cooling the reaction mixtures to 5°C.

Determination of copy-number of transgenes by digital PCR

To determine the copy-number of transgenes in a transgenic plant, a digital PCR
30 method was used as follows. This method could also be used to determine whether a plant was transgenic for the genetic constructs described herein. About a centimetre square of leaf tissue was harvested from each individual plant and placed in a collection microtube (Qiagen). The samples were then freeze dried for 24 to 48hr. For breaking up the samples for DNA extraction, stainless steel ball bearings were added to each
35 dried sample and the tubes shaken on a Qiagen Tissue lyser. 375µL of extraction buffer (0.1M Tris-HCl pH8, 0.05M EDTA pH8 and 1.25% SDS) was added to each tube, the

mixtures incubated at 65°C for 1hr, and then cooled before 187µL of 6M ammonium acetate (4°C) was added to each tube with thorough mixing. The samples were then centrifuged for 30 min at 3000 rpm. The supernatant from each tube was removed into new microtubes each containing 220µL of isopropanol for precipitation of the DNA at room temperature for 5min. DNA was collected by centrifuging the tubes at 3000rpm for 30min, the DNA pellets washed with 320µL of 70% ethanol and dried before resuspension of the DNA in 225µL of water. Non-dissolved material was pelleted by centrifugation at 3000 rpm for 20min, and 150µL of each supernatant transferred to 96-well plates for long term storage.

10 For efficient and quantitative digital PCR (ddPCR) the DNA was digested with restriction enzymes prior to amplification reactions, to ensure that multiple copies of the transgenes or multiple insertions were physically separated. Aliquots of the DNA preparations were therefore digested with *EcoRI* and *BamHI*, together, in 20µL volumes using 10x *EcoRI* buffer, 5µL of DNA and about 4 units of each enzyme per sample, incubated overnight at 37°C.

The primers used in these PCR reactions were designed using Primer3 software to confirm that primers for the reference and target genes were not predicted to interact, or such interaction would not be a problem under the conditions used. The reference gene used in the assay was the canola *Hmg* (high mobility group) gene, present at one gene per canola genome (Weng et al., 2004). Since canola is an allotetraploid, it was taken that there were 4 copies of the *Hmg* gene, i.e. 2 alleles of each of the two genes, in *Brassica napus*. The reference gene reactions used the pair of primers and a dual-labelled probe, as follows: Sense primer, Can11 GCGAAGCACATCGAGTCA (SEQ ID NO:50); Antisense primer, Can12 GGTTGAGGTGGTAGCTGAGG (SEQ ID NO:51); Probe, Hmg-P3 5'-Hex/TCTCTAC/zen/CCGTCTCACATGACGC/3IABkFQ/-3' (SEQ ID NO:52). The amplification product size was 73bp.

In one target gene amplification reaction which detected a region of the *PPT* selectable marker gene to screen all of the transgenic plants, the sense primer was Can17, ATACAAGCACGGTGGATGG (SEQ ID NO:53); the antisense primer, Can18 TGGTCTAACAGGTCTAGGAGGA (SEQ ID NO:54); the probe, PPT-P3 5'-/FAM/TGGCAAAGA/zen/GATTTTCGAGCTTCCTGC/3IABkFQ/-3' (SEQ ID NO:55). The size of this target gene amplification product was 82 bp. On some occasions, a second target gene assay was performed in parallel to detect partial insertions of the T-DNA. This second assay detected a region of the $\Delta 6$ -desaturase gene using a sense primer, Can23 CAAGCACCGTAGTAAGAGAGCA (SEQ ID NO:56),

the antisense primer, Can24 CAGACAGCCTGAGGTTAGCA (SEQ ID NO:57); the probe, D6des-P3 5'-/FAM/TCCCCACTT/zen/CTTAGCGAAAGGAACGA/3IABkFQ/-3' (SEQ ID NO:58). The size of this target gene amplification product was 89bp. Reactions

5 routinely used 2µL of the digested DNA preparations. Reaction composition per sample: reference sense primer (10pM), 1µL; reference antisense primer (10pM), 1µL; reference gene probe (10pM), 0.5µL; target gene sense primer (10pM), 1µL; target gene antisense primer (10pM), 1µL; target gene probe (10pM), 0.5µL; ddPCR reagent mix, 12.5µL; water 5.5µL in a total volume of 25µL.

- 10 The mixtures were then placed into a QX100 droplet generator, which partitioned each sample into 20000 nanoliter-sized droplets. This was done in 8-well cartridges until all of the samples were processed and transferred to a 96-well PCR plate. This plate was then heat sealed with a pierceable foil using a plate sealer machine. The samples were then treated under the following reaction conditions: 95°C,
- 15 10 min, ramping at 2.5°C/s; then 39 cycles of 94°C, 30s ramping at 2.5°C/s; 61°C, 1min, ramping at 2.5°C/s; 98°C, 10 min, followed by cooling to 12°C. Following the amplification reactions of the DNA in the droplets, the plate was placed in a QX100 droplet reader which analysed each droplet individually using a two-color detection system (set to detect FAM or Hex). The droplet digital PCR data were viewed as either
- 20 a 1-D plot with each droplet from a sample plotted on the graph of fluorescence intensity, or a 2-D plot in which fluorescence (FAM) was plotted against fluorescence (Hex) for each droplet. The software measured the number of positive and negatives droplets for each fluorophore (FAM or Hex) in each sample. The software then fitted the fraction of positive droplets to a Poisson algorithm to determine the concentration
- 25 of the target DNA molecule in units of copies/µL input. The copy number variation was calculated using the formula: $CNV = (A/B)^{Nb}$, where A= concentration of target gene, B= concentration of reference gene, and Nb = 4, the number of copies of the reference gene in the genome.

30 Assessment of pollen viability

Fluorescein diacetate (FDA) was dissolved in acetone at 2 mg/ml to provide a stock solution. FDA dilutions were prepared just before use by adding drops of the FDA stock solution to 2 ml of a sucrose solution (0.5 M) until saturation was reached as indicated by the appearance of persistent cloudiness.

- 35 Propidium iodide (PI) was dissolved in sterile distilled water at 1 mg/ml to provide a stock solution. Just before use, 100µl of the stock solution was added to 10ml

of sterile distilled water to make a working solution. To check the ratio of viable and non-viable pollen, PI and FDA stock solutions were mixed in 2:3 ratio.

Transgenic and wild-type canola and mustard plants were grown under standard conditions in a glasshouse at $22 \pm 2^\circ\text{C}$ with a 16hr photoperiod per day. Mature flower buds which were ready to open in the next day were labelled and collected on the following morning at 9-10 am. Pollen from opened flowers were stained with the FDA/PI mixture and visualized using a Leica MZFLIII fluorescence microscope. GFP-2, a 510 nm long pass emission filter (transmitting red and green light) with a 480/40 nm excitation filter was used to detect viable and non-viable pollen. Non-viable pollen which took up the PI stain appeared red under the fluorescence microscope whereas viable pollen appeared bright green when stained with PI and FDA.

Example 2. Stable Expression of Transgenic DHA Pathways in *Arabidopsis thaliana* Seeds

15 Binary vector construction

The binary vectors pJP3416-GA7 (also referred to herein as "GA7" described in WO 2013/185184) and pJP3404 each contained seven heterologous fatty acid biosynthesis genes, encoding 5 desaturases and 2 elongases, and a plant selectable marker between the left and right border repeats of the T-DNA present in each vector (Figures 2 and 3). SEQ ID NO:1 provides the nucleotide sequence of the T-DNA region of pJP3416-GA7 from the right to left border sequences. Both genetic constructs contained plant codon-optimised genes encoding a *Lachanea kluyveri* $\Delta 12$ -desaturase (comprising nucleotides 14143-16648 of SEQ ID NO:1), a *Pichia pastoris* $\omega 3$ -desaturase (comprising nucleotides 7654-10156 of SEQ ID NO:1), a *Micromonas pusilla* $\Delta 6$ -desaturase (comprising nucleotides 226-2309 of SEQ ID NO:1), *Pavlova salina* $\Delta 5$ - and $\Delta 4$ -desaturases (comprising nucleotides 4524-6485 and 10157-14142 of SEQ ID NO:1, respectively) and *Pyramimonas cordata* $\Delta 6$ - and $\Delta 5$ -elongases (comprising nucleotides 2310-4523 and 17825-19967 of SEQ ID NO:1, respectively).

The seven coding regions in the constructs were each under the control of a seed specific promoter- three different promoters were used, namely the truncated *Brassica napus* napin promoter (pBnFPI), the *Arabidopsis thaliana* FAE1 promoter (pAFAE1) and the *Linum usitatissimum* conlinin 1 promoter (pLuCn1). The seven fatty acid biosynthesis genes together coded for an entire DHA synthesis pathway that was designed to convert $18:1^{\Delta 9}$ (oleic acid) through to $22:6^{\Delta 4,7,10,13,16,19}$ (DHA). Both binary vectors contained a BAR plant selectable marker coding region operably linked to a Cauliflower Mosaic Virus (CaMV) 35S promoter with duplicated enhancer region and

A. tumefaciens nos3' polyadenylation region- transcription terminator. The plant selectable marker was situated adjacent to the left border of the T-DNA region, therefore distally located on the T-DNA with respect to the orientation of T-DNA transfer into the plant cells. This increased the likelihood that partial transfer of the T-DNA, which would be likely to not include the selectable marker gene, would not be selected. pJP3416-GA7 and pJP3404 each contained an RiA4 origin of replication from *Agrobacterium rhizogenes* (Hamilton, 1997).

The GA7 construct also included two *Nicotiana tabacum* Rb7 matrix attachment region (MAR) sequences, as described by Hall et al. (1991). MAR sequences, sometimes termed nuclear attachment regions, are known to bind specifically to the nuclear matrix *in vitro* and may mediate binding of chromatin to the nuclear matrix *in vivo*. MARs are thought to function to reduce transgene silencing. In pJP3416-GA7 the MARs were also inserted and positioned within the T-DNA region in order to act as DNA spacers to insulate transgenic expression cassettes. The pJP3416 vector prior to insertion of the GA7 region contained only the plant selectable marker cassette between the borders.

A. thaliana transformation and analysis of fatty acid composition

The chimeric vectors were introduced into *A. tumefaciens* strain AGL1 and cells from cultures of the transformed *Agrobacterium* used to treat *A. thaliana* (ecotypes Columbia and a *fad2* mutant) plants using the floral dip method for transformation (Clough and Bent, 1998). After maturation, the T₁ seeds from the treated plants were harvested and plated onto MS plates containing PPT for selection of plants containing the BAR selectable marker gene. Surviving, healthy T₁ seedlings were transferred to soil. After growth of the plants to maturity and allowing for self-fertilisation, T₂ seeds from these plants were harvested and the fatty acid composition of their seed lipid analysed by GC analysis as described in Example 1.

The pJP3416-GA7 construct resulted in the production of slightly higher levels of DHA, as a percentage of total fatty acid content, on average than the pJP3404 construct. The conversion efficiencies for each enzymatic step in the production of DHA from oleic acid were calculated as $(\% \text{products} \times 100) / (\% \text{remaining substrate} + \% \text{products})$, thereby expressed as a percentage.

The highest observed level of DHA produced in the pJP3416-GA7 T₂ transformed lines was 6.2%, additionally with 0.5% EPA and 0.2% DPA (line #14). These T₂ seeds were still segregating for the transgene i.e. were not yet uniformly homozygous. The level of ω3 fatty acids produced as a result of the transgenes in these

seeds (total new $\omega 3$ fatty acids, excluding the level of ALA which was produced endogenously in the Columbia background) was 10.7% while the level of $\omega 6$ fatty acids (total new $\omega 6$ fatty acids but excluding 18:2 ^{$\Delta 9,12$}) was 1.5%. This represents an extremely favourable ration of new $\omega 3$ fatty acids:new $\omega 6$ fatty acids, namely 7.3:1.

- 5 T_2 seeds of selected lines transformed with pJP3416-GA7, namely for lines designated 7, 10, 14, 22 and 34 in the Columbia background and for lines designated 18, 21 and 25 in the *fad2* mutant background, were plated onto MS media containing PPT for selection of transgenic seedlings *in vitro*. Twenty PPT-resistant seedlings for each line were transferred to soil and grown to maturity after self-fertilisation. These
- 10 plants were highly likely to be homozygous for the selectable marker gene, and therefore for at least one T-DNA insertion in the genome of the plants. T_3 seed from these plants were harvested and analysed for fatty acid composition in their seedoil by GC. This analysis revealed that the pJP3416-GA7 construct generated higher levels of the $\omega 3$ LC-PUFA DHA in T_3 seeds of the homozygous plants than in the segregating
- 15 T_2 seed. Up to about 13.9% DHA was observed in the T_3 pJP3416-GA7 transformed line designated 22.2 in the Columbia background, increased from about 5.5% in the hemizygous T_2 seed, with a sum level of about 24.3% of new $\omega 3$ fatty acids as a percentage of the total fatty acids in the seed lipid content. New $\omega 6$ fatty acids were at a level of 1.1% of total fatty acids, representing a very favourable ratio of new $\omega 3$ fatty
- 20 acids:new $\omega 6$ fatty acids, namely about 22:1. Similarly, transformants in the *fad2* mutant background yielded 20.6% as a sum of new $\omega 3$ fatty acids, including 11.5% DHA, as a percentage of the total fatty acids in the seed lipid content.

- Enzymatic conversion efficiencies for each enzyme step in the pathway for production of DHA from oleic acid are shown in Table 4 for the T_3 seeds with the
- 25 higher DHA levels. The $\Delta 12$ -desaturase conversion efficiency in seeds of line 22.2 was 81.6% and the $\omega 3$ -desaturase efficiency was 89.1%, both of them remarkably high and indicating that these fungal (yeast) enzymes were able to function well in developing seeds. The activities of the other exogenous enzymes in the DHA pathway were similarly high for $\omega 3$ substrates with the $\Delta 6$ -desaturase acting at 42.2% efficiency,
- 30 $\Delta 6$ -elongase at 76.8%, $\Delta 5$ -desaturase at 95.0%, $\Delta 5$ -elongase at 88.7% and $\Delta 4$ -desaturase at 93.3% efficiency. The $\Delta 6$ -desaturase activity on the $\omega 6$ substrate LA was much lower, with the $\Delta 6$ -desaturase acting at only 0.7% conversion efficiency on LA. GLA was present at a level of only 0.4% and was the only new $\omega 6$ product aside from 20:2 $\omega 6$ detected in the T_3 seeds with the highest DHA content. Compiled data from the
- 35 total seed lipid profiles from independent transgenic seed are shown in Table 5.

Table 4. Conversion efficiencies of the individual enzymatic steps for the production of DHA from oleic acid, observed in total seed lipid from transgenic *T₃ Arabidopsis* seeds.

		GA7_Col_7.2	GA7_Col_34.2	GA7_Col_10.13	GA7_Col_22.2	GA7_Col_14.19	GA7_FAD2-25.10	GA7_FAD2-21.2	GA7_FAD2-18.14	T ₄ Col_22.2 (mean)	T ₄ Col_22.2 best line
Omega-3	d6-des	30.7%	29.3%	28.2%	42.2%	30.2%	38.5%	40.0%	29.2%	41.0%	45.7%
	(d9-elo)	2.7%	2.7%	2.3%	2.4%	3.0%	2.3%	2.7%	2.9%	2.8%	3.1%
	d6-elo	79.0%	81.1%	79.0%	76.8%	70.9%	79.2%	73.2%	79.1%	77.5%	77.7%
	d5-des	94.0%	94.6%	94.5%	95.0%	97.9%	87.8%	93.3%	91.1%	95.0%	95.8%
	d5-elo	91.9%	91.7%	93.6%	88.7%	89.5%	89.9%	92.2%	91.6%	90.8%	90.2%
Omega-6	d4-des										
	d5-elo										
	d5-des										
	d6-elo										
	(d9-elo)	1.7%	1.7%	1.2%	1.2%	2.6%	1.1%	2.0%	1.3%	1.6%	1.5%
	d6-des	0.3%	0.3%	0.3%	0.7%	0.3%	0.6%	1.0%	0.2%	1.3%	0.7%
	d15-des	85.3%	84.4%	86.2%	89.1%	70.2%	87.5%	82.2%	87.6%	81.0%	90.9%
	d12-des	75.4%	73.1%	75.7%	81.6%	73.4%	66.6%	78.5%	63.1%	67.6%	82.7%

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	d4-des	93.2%	93.7%	94.4%	93.3%	93.7%	92.5%	95.0%	93.9%	92.2%	90.9%
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Table 5. Compiled data from the total seed lipid profiles from independent transgenic seed.

Parameter	GA7-Col_7.2	GA7-Col_34.2	GA7-Col_10.13	GA7-Col_22.2	GA7-Col_14.19	GA7-FAD2-25.10	GA7-FAD2-21.2	GA7-FAD2-18.14	T ₁ Col_22.2 (mean±SD)	T ₁ Col_22.2 best line
total w3 (% of total FA)	50.0	48.9	51.6	55.8	38.6	47.1	49.4	44.8	54.0	55.9
total w6 (% of total FA)	8.7	9.1	8.3	6.7	16.3	6.7	10.7	6.3	6.7	5.7
w3 / w6 ratio	5.75	5.37	6.22	8.33	2.37	7.03	4.62	7.11	8.06	9.81
w6 / w3 ratio	0.17	0.19	0.16	0.12	0.42	0.14	0.22	0.14	0.12	0.10
total novel w3 (% of total FA)	16.3	15.2	15.5	24.3	12.5	18.8	20.5	14.0	23.0	26.4
total novel w6 (% of total FA)	1.2	1.2	0.9	1.1	1.5	0.9	1.8	0.7	1.4	1.4
novel w3 / w6 ratio	13.58	12.67	17.22	22.09	8.33	20.89	11.39	20.00	16.43	18.86
novel w6 / w3 ratio	0.07	0.08	0.06	0.05	0.12	0.05	0.09	0.05	0.06	0.05
OA to EPA efficiency	14.1%	13.3%	13.4%	21.8%	10.2%	15.0%	16.8%	11.2%	20.4%	24.5%
OA to DHA efficiency	12.0%	11.4%	11.8%	18.0%	8.6%	12.6%	14.8%	9.6%	17.1%	20.1%
LA to EPA efficiency	18.9%	18.4%	17.9%	26.9%	14.2%	22.9%	21.8%	18.0%	26.2%	29.9%
LA to DHA efficiency	16.2%	15.9%	15.7%	22.2%	12.0%	19.1%	19.1%	15.5%	21.9%	24.5%
ALA to EPA efficiency	22.2%	21.9%	20.7%	30.1%	20.2%	26.1%	26.5%	20.5%	29.4%	32.9%
ALA to DHA efficiency	19.0%	18.8%	18.2%	24.9%	17.1%	21.9%	23.3%	17.6%	24.6%	27.0%
total saturates	16.0	14.7	15.4	16.0	16.2	13.4	16.5	12.9	16.0	17.8
total monounsaturates	23.7	25.8	23.4	19.2	26.5	30.9	21.3	34.3	21.1	18.1
total polyunsaturates	58.7	58.0	59.9	62.5	54.9	53.8	60.1	51.1	60.7	61.6
total C20	19	19.8	16.8	15.9	19.1	21.5	18.2	23.3	18	16.6
total C22	11.4	11	10.8	15.5	8.6	12.1	13.2	9.9	15.4	17.5
C20/C22 ratio	1.67	1.80	1.56	1.03	2.22	1.78	1.38	2.35	1.17	0.95

T₃ seeds from the pJP3416-GA7 line 22.2 in the Columbia background, which were progeny from T₂ line 22, were sown directly to soil and the fatty acid composition of mature seed from the resultant T₃ plants analysed by GC. The average DHA level of these seeds was 13.3% ± 1.6 (n=10) as a percentage of total fatty acids in the seed lipid. The line with the highest level of DHA contained 15.1% DHA in the total fatty acids of the seed lipid. The enzymatic conversion efficiencies are shown in Table 4 for each step in the production of DHA from oleic acid.

Southern blot hybridisation analysis was performed. The results showed that the high-accumulating DHA lines were either single- or double-copy for the T-DNA from the pJP3416-GA7 construct with the exception of transgenic line Columbia#22, which had three T-DNA insertions in the genome of the *Arabidopsis* plant. The T5 generation seed was also analysed and found to have up to 13.6% DHA in the total seed lipids. The GA7 construct was found to be stable across multiple generations in terms of DHA production capability.

Determination of oil content in transgenic *A. thaliana* DHA lines

The oil content of transgenic *A. thaliana* seeds with various levels of DHA was determined by GC as described in Example 1. The data are shown in Figure 4, graphing the oil content (% oil by weight of seed) against the DHA content (as a percentage of total fatty acids). Up to 26.5 mg of DHA per gram of seed was observed (Table 6). The oil content of the transgenic *Arabidopsis* seeds was found to be negatively correlated with DHA content. The amount of DHA per weight of seed was greater in the transformed seeds with a DHA level of about 9% relative to the seeds with about 14% DHA. Subsequent data from species other than *Arabidopsis* has shown that this negative correlation is more pronounced in *Arabidopsis* than in *C. sativa* or *Brassica* species (Example 8 below).

Table 6. Proportion and amount of DHA in GA7-transformed *Arabidopsis* seeds.

	DHA content (% of TFA)	Oil content (% oil per g seeds)	DHA content per weight (mg/g seed)
GA7/col 22.2-1	14.2	14.89	20.2
GA7/col 22.2-2	14.3	15.02	20.5
GA7/col 22.2-3	14.0	15.92	21.2
GA7/col 10.15-1	8.7	30.23	25.06
GA7/col 10.15-2	8.6	31.25	25.77
GA7/col 10.15-3	8.8	31.70	26.49

Example 3. Stable Expression of a Transgenic DHA Pathway in *Camelina sativa* Seeds

The binary vector pJP3416-GA7 as described above was introduced into *A. tumefaciens* strain AGL1 and cells from a culture of the transformed *Agrobacterium* used to treat *C. sativa* flowering plants using a floral dip method for transformation (Lu and Kang, 2008). After growth and maturation of the plants, the T₁ seeds from the treated plants were harvested, sown onto soil and the resultant plants treated by spraying with the herbicide BASTA to select for plants which were transgenic for, and expressing, the *bar* selectable marker gene present on the T-DNA of pJP3416-GA7. Surviving T₁ plants which were tolerant to the herbicide were grown to maturity after allowing them to self-fertilise, and the resultant T₂ seed harvested. Five transgenic plants were obtained, only three of which contained the entire T-DNA.

Lipid was extracted from a pool of approximately twenty seeds from each of the three plants that contained the entire T-DNA. Two of the pooled samples contained very low, barely detectable levels of DHA, but the third pool contained about 4.7% DHA. Therefore, lipid was extracted from 10 individual T₂ seeds from this plant and the fatty acid composition analysed by GC. The fatty acid composition data of the individual seeds for this transformed line is also shown in Table 7. Compiled data from the total seed lipid profiles (Table 7) are shown in Table 8.

DHA was present in six of the 10 individual seeds. The four other seeds did not have DHA and were presumed to be null segregants which did not have the T-DNA, based on hemizygosity of the T-DNA insertion in the parental plant. Extracted lipid from the single seed with the highest level of DHA had 9.0% DHA while the sum of the percentages for EPA, DPA and DHA was 11.4%.

Table 7. Fatty acid composition of total seed lipids from transgenic T₂ *Camelina sativa* seeds transformed with the T-DNA from pIP3416-GA7. The fatty acid composition is shown for a pooled seed batch (FD5.46) and for 10 single seeds ranked (left to right) from highest to lowest DHA.

Fatty acid	FD5.46 pooled	# 2	# 4	# 8	# 7	# 9	# 1	# 3	# 5	# 6	# 10
14:0	0	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.1	0.2	0.2
16:0	11.6	12.1	12.3	12.1	13.2	12.3	12.8	11.9	11.4	11.5	11.7
16:1	0.2	0.0	0.1	0.1	0.0	0.2	0.0	0.2	0.2	0.2	0.2
16:3	0.3	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
18:0	3.7	3.3	3.2	3.2	3.0	3.1	3.2	3.3	3.1	3.2	3.2
18:1	10.8	8.0	8.0	8.6	8.5	9.4	11.0	10.2	8.3	9.4	8.6
18:1d11	1.7	1.3	1.4	1.4	1.7	1.4	1.5	1.3	1.3	1.3	1.3
18:2	24.7	18.2	19.5	19.2	18.5	20.1	23.8	32.2	30.3	29.8	31.6
18:3o3	27.4	26.7	26.6	27.3	28.9	28.2	27.4	28.3	29.2	29.5	28.2
18:3o6	0.2	1.4	0.3	0.3	0.4	0.2	0.5	0.0	0.5	0.4	0.6
20:0	1.6	1.4	1.3	1.4	1.2	1.4	1.4	1.8	2.1	1.9	2.0
18:4o3	2.2	6.8	6.4	5.7	7.2	5.7	4.1	0.0	0.0	0.0	0.0
20:1d11	5.3	4.4	4.6	4.8	3.3	4.1	3.5	4.4	6.1	5.8	5.5
20:1iso	0.4	0.3	0.3	0.3	0.3	0.3	0.0	0.5	0.6	0.5	0.5

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20:206	0.8	0.8	0.9	0.8	0.6	0.8	0.7	1.3	1.5	1.4	1.4
20:303	0.6	0.8	0.8	0.8	0.7	0.8	0.7	0.6	0.7	0.7	0.6
22:0	0.4	0.5	0.5	0.5	0.4	0.5	0.5	0.6	0.6	0.6	0.6
20:403	0.2	0.3	0.3	0.3	0.4	0.4	0.5	0.0	0.0	0.0	0.0
22:1	1.1	1.1	1.2	1.1	0.5	0.9	0.8	1.6	2.2	1.9	2.0
20:503	0.7	1.3	1.6	1.5	1.6	1.1	1.7	0.0	0.0	0.0	0.1
22:206	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.3	0.2	0.2
22:406+22:303	0.3	0.2	0.3	0.3	0.0	0.3	0.0	0.4	0.6	0.5	0.5
24:0	0.3	0.3	0.3	0.3	0.0	0.3	0.0	0.4	0.4	0.4	0.4
24:1	0.3	0.4	0.4	0.3	0.0	0.3	0.0	0.5	0.6	0.5	0.5
22:503	0.3	1.1	1.2	1.1	1.1	0.9	0.8	0.0	0.0	0.0	0.0
22:603	4.7	9.0	8.5	8.3	8.3	7.1	4.9	0.0	0.0	0.0	0.0

Table 8. Compiled data from the total seed lipid profiles from transgenic seed as shown in Table 7. Calculations do not include the 'minor fatty acids' in Table 7.

Parameter	FDS.46 pooled	# 2	# 4	# 8	# 7	# 9	# 1	# 3	# 5	# 6	# 10
total w3 (% of total FA)	36.1	46	45.4	45	48.2	44.2	40.1	28.9	29.9	30.2	28.9
total w6 (% of total FA)	25.8	20.4	20.7	20.3	19.5	21.1	25	33.7	32.6	31.8	33.8
w3 / w6 ratio	1.40	2.25	2.19	2.22	2.47	2.09	1.60	0.86	0.92	0.95	0.86
w6 / w3 ratio	0.71	0.44	0.46	0.45	0.40	0.48	0.62	1.17	1.09	1.05	1.17
total novel w3 (% of total FA)	8.1	18.5	18	16.9	18.6	15.2	12	0	0	0	0.1
total novel w6 (% of total FA)	1.1	2.2	1.2	1.1	1	1	1.2	1.5	2.3	2	2.2
novel w3 / w6 ratio	7.36	8.41	15.00	15.36	18.60	15.20	10.00				0.05
novel w6 / w3 ratio	0.14	0.12	0.07	0.07	0.05	0.07	0.10				22.00
OA to EPA efficiency	8.2%	15.6%	15.5%	15.1%	15.1%	12.8%	10.5%	0.0%	0.0%	0.0%	0.1%
OA to DHA efficiency	6.7%	12.3%	11.6%	11.5%	11.4%	10.0%	7.0%	0.0%	0.0%	0.0%	0.0%
LA to EPA efficiency	9.2%	17.2%	17.1%	16.7%	16.2%	13.9%	11.4%	0.0%	0.0%	0.0%	0.2%
LA to DHA efficiency	7.6%	13.6%	12.9%	12.7%	12.3%	10.9%	7.5%	0.0%	0.0%	0.0%	0.0%
ALA to EPA efficiency	15.8%	24.8%	24.9%	24.2%	22.8%	20.6%	18.5%	0.0%	0.0%	0.0%	0.3%
ALA to DHA efficiency	13.0%	19.6%	18.7%	18.4%	17.2%	16.1%	12.2%	0.0%	0.0%	0.0%	0.0%
total saturates	17.6	17.8	17.8	17.6	18	17.8	18.1	18.2	17.7	17.8	18.1
total monounsaturates	19.8	15.5	16	16.6	14.3	16.6	16.8	18.7	19.3	19.6	18.6
total polyunsaturates	62.5	66.6	66.4	65.6	67.7	65.6	65.1	63	63.1	62.5	63.2
total C20	9.6	9.3	9.8	9.9	8.1	8.9	8.5	8.6	11	10.3	10.1
total C22	5.4	10.3	10	9.7	9.4	8.3	5.7	0.6	0.9	0.7	0.7
C20/C22 ratio	1.78	0.90	0.98	1.02	0.86	1.07	1.49	14.33	12.22	14.71	14.43

Homozygous seed from this line was obtained in the T₄ generation. Up to 10.3% DHA was produced in event FD5-46-18-110 with an average of 7.3% DHA observed across the entire T₄ generation. A subsequent generation (T₅) was established to further test the stability of PUFA production over multiple generations, particularly the DHA. The maximum DHA levels observed was found to be stable in the fifth generation, even though the pooled seed DHA content had not stabilised until the T₄ generation due to the presence of multiple transgenic loci. T₅ seed batches were also germinated on MS media *in vitro* alongside parental *C. sativa* seed with no obvious difference in germination efficiency or speed observed. Further generations of the transgenic line (T₆, T₇ generations etc) did not show any reduction in the seed DHA level. The transgenic plants were fully male and female fertile, and the pollen showed about 100% viability as for the wild-type plants. Analysis of the oil content of the seeds having different levels of DHA did not identify a correlation between DHA level and oil content, contrary to the correlation seen in *Arabidopsis thaliana*.

In several further transgenic lines, the DHA content of single seeds from independent events exceeded 12%. The transgenic:null ratio of these lines was found to be between approximately 3:1 and 15:1. Analysis of representative fatty acid profiles from the top DHA samples from each construct found only 1.2-1.4% GLA with no other new ω 6 PUFA detected. In contrast, new ω 3 PUFA (SDA) ω 3 LC-PUFA (ETA, EPA, DPA, DHA) were found to accumulate to 18.5% with a DHA level of 9.6% of the total fatty acid content. Δ 6-desaturation was 32% and EPA was 0.8% of the total fatty acid content. The Δ 5-elongation efficiency was 93% and Δ 6-elongation efficiency was 60%. DHA was detected in the polar seed lipid fraction of GA7 lines.

It was noted that the segregation ratios observed (~3:1 to ~15:1) indicated that one or, at most, two transgenic loci were required to produce fish oil-like levels of DHA in *C. sativa*. This had important implications for the ease with which the transgenic trait can be bred as well as for transgene stability.

Homozygous seed was planted out across several glasshouses to generate a total of over 600 individual plants. Oil was extracted from the seed using a variety of methods including soxhlet, acetone and hexane extractions.

¹³C NMR regiospecificity analysis was performed on the transgenic *C. sativa* seed oil to determine the positional distribution of the ω 3 LC-PUFA on TAG. An event with approximately equal EPA and DHA was selected to maximise response for these fatty acids and the ratio of *sn*-1,3 to *sn*-2 was found to be 0.75:0.25 for EPA and 0.86:0.14 for DHA where an unbiased distribution would be 0.66:0.33. That is, 75% of the EPA and 86% of the DHA were located at the *sn*-1,3 position of TAG. This

indicated that both fatty acids were preferentially located on the *sn*-1,3 positions in *C. sativa* TAG although the preference for EPA was weaker than for DHA. The finding that DHA was predominantly found on *sn*-1,3 was similar to results previously reported in *A. thaliana* seed (Petrie et al., 2012).

- 5 Since only a small number of independent transgenic lines were obtained in the transformation experiment described above, further *C. sativa* transformations were performed using the GA7-modB construct (Example 4). More transformants were obtained and homozygous lines producing in excess of 20.1% DHA are identified.

10 **Example 4. Modifications to T-DNAs Encoding DHA Pathways in Plant Seeds**

- In order to improve the DHA production level in *B. napus* beyond the levels described in WO2013/185184, the binary vectors pJP3416-GA7-modA, pJP3416-GA7-modB, pJP3416-GA7-modC, pJP3416-GA7-modD, pJP3416-GA7-modE and pJP3416-GA7-modF were constructed as described in WO2013/185184 and tested in transgenic
15 plants. These binary vectors were variants of the pJP3416-GA7 construct described in Example 2 and were designed to further increase the synthesis of DHA in plant seeds, particularly by improving $\Delta 6$ -desaturase and $\Delta 6$ -elongase functions. SDA had been observed to accumulate in some seed transformed with the GA7 construct due to a relatively low $\Delta 6$ elongation efficiency compared to the $\Delta 5$ -elongase, so amongst other
20 modifications, the two elongase gene positions were switched in the T-DNA.

- The two elongase coding sequences in pJP3416-GA7 were switched in their positions on the T-DNA to yield pJP3416-GA7-modA by first cloning a new *P. cordata* $\Delta 6$ -elongase cassette between the *Sbf*I sites of pJP3416-GA7 to replace the *P. cordata* $\Delta 5$ -elongase cassette. This construct was further modified by exchanging the
25 FP1 promoter driving the *M. pusilla* $\Delta 6$ -desaturase with a conlinin Cn12 promoter (pLuCn12) to yield pJP3416-GA7-modB. This modification was made in an attempt to increase the $\Delta 6$ -desaturase expression and thereby enzyme efficiency. It was thought that the Cn12 promoter might yield higher expression of the transgene in *B. napus* than the truncated napin promoter.

- 30 Eight transgenic pJP3416-GA7-modB *A. thaliana* events and 15 transgenic pJP3416-GA7-modG *A. thaliana* events were generated. Between 3.4% and 7.2% DHA in pooled pJP3416-GA7-modB seed was observed and between 0.6 and 4.1% DHA in pooled T2 pJP3416-GA7-modG seed was observed. Several of the highest pJP3416-GA7-modB events were sown out on selectable media and surviving seedlings taken to
35 the next generation. Seed is being analysed for DHA content. Since the pooled T1 seeds represented populations that were segregating for the transgenes and included any

null segregants, it is expected that the homozygous seeds from progeny plants would have increased levels of DHA, up to 30% of the total fatty acid content in the seed oil. The other modified constructs were used to transform *A. thaliana*. Although only a small number of transformed lines were obtained, none yielded higher levels of DHA than the modB construct.

The pJP3416-GA7-modB construct was also used to generate transformed *B. napus* plants of cultivar Oscar and of a series of breeding lines designated NX002, NX003, NX005, NX050, NX052 and NX054. A total of 1558 transformed plants were obtained including 77 independent transformed plants (T0) for the Oscar transformation, and 1480 independent plants for the breeding lines including 189 for NX005 which is a line having a high oleic acid content in its seedoil by virtue of mutations in FAD2 genes. The other breeding lines had higher levels of LA and ALA. Transgenic plants which exhibited more than 4 copies of the T-DNA as determined by a digital PCR method (Example 1) were discarded; about 25% of the T0 plants were discarded by this criterion. About 53% of the T0 transgenic plants had 1 or 2 copies of the T-DNA as determined by the digital PCR method, 12% had about 3 copies and 24% 4 or more copies. Seed (T1 seed) was harvested from about 450 of the transgenic lines after self-fertilisation, achieved by bagging the plants during flowering to avoid out-crossing. T1 seed are harvested from the remainder of the transgenic plants when mature. About 1-2% of the plant lines were either male or female sterile and produced no viable seeds, these T0 plants were discarded.

Pools of seed (20 T1 seeds in each pool) were tested for levels of DHA in the pooled seed oil, and lines which showed the highest levels were selected. In particular, lines having a DHA content of at least 2% of the total fatty content in the pooled T1 seeds were selected. About 15% of the transgenic lines were selected in this way; the other 85% were discarded. Some of these were designated lines CT132-5 (in cultivar Oscar), CT133-15, -24, -63, -77, -103, -129 and -130 (in NX005). Selected lines in NX050 included CT136-4, -8, -12, -17, -19, -25, -27, -49 and -51. Twenty seeds from selected lines including CT132.5 and 11 seeds from CT133.15 were imbibed and, after two days, oil was extracted from a half cotyledon from each of the individual seeds. The other half cotyledons with embryonic axes were kept and cultured on media to maintain the specific progeny lines. The fatty acid composition in the oil was determined; the data is shown in Table 9 for CT132.5. The DHA level in ten of the 20 seeds analysed was in the range of 7-20% of the total fatty acid content as determined by the GC analysis. Other seeds had less than 7% DHA and may have contained a partial (incomplete) copy of the T-DNA from pJP3416-GA7-modB. The transgenic line

appeared to contain multiple transgene insertions that were genetically unlinked. The seeds of transgenic line CT133.15 exhibited DHA levels in the range 0-5%. Seeds with no DHA were likely to be null segregants. These data confirmed that the modB construct performed well for DHA production in canola seed.

5 Twenty or 40 individual seeds (T2 seeds) obtained from each of multiple T1 plants, after self-fertilisation, from the selected transformed lines were tested individually for fatty acid composition. Seeds comprising DHA at levels greater than 20% were identified (Table 10). Two representative samples, CT136-27-18-2 and CT136-27-18-19 had 21.2% and 22.7% DHA, respectively. The total ω 3 fatty acid
10 content in these seeds was about 60% as a percentage of the total fatty acid content, and the ω 6 content was less than 10%. Further sets of 20 or 40 T2 seeds from each of the T1 plants were tested for fatty acid composition. Representative data for DHA levels in the total fatty acid content of seedoil from individual T2 seeds is shown in Figure 10. Seeds comprising up to 34.3% DHA were identified, for example in seed CT136-27-
15 47-25 (Table 12). The fatty acid composition for seedoil obtained from CT136-27-47-25 is shown in Table 12. The fatty acid composition included 34.3% DHA together with about 1.5% DPA, 0.6% EPA and 0.5% ETA. The SDA level was about 7.5%, ALA about 21.9% and LA about 6.9%. The new ω 6 PUFA exhibited 1.1% GLA but no detectable ω 6-C20 or -C22 LC-PUFA. Total saturated fatty acids: 9.6%;
20 monounsaturated fatty acids, 12.5%; total PUFA, 75.2%; total ω 6-PUFA (including LA), 7.2%; total ω 3-PUFA, 66.9%; the ratio of total ω 6: ω 3 fatty acids, 9.3:1; new ω 6:new ω 3 fatty acids, 37:1. The efficiencies of each of the enzymatic steps from oleic acid to DHA were as follows: Δ 12-desaturase, 90%; Δ 15/ ω 3-desaturase, 89%; Δ 6-desaturase, 67%; Δ 6-elongase, 83%; Δ 5-desaturase, 99%; Δ 5-elongase, 98%; Δ 4-
25 desaturase, 96%. The overall efficiency of conversion of oleic acid to DHA was about 50%. It was therefore clear that seeds producing DHA in the range of 20.1-35% of the total fatty acid content of the seedoil could be identified and selected, including seeds having between 20.1% and 30% DHA or between 30% and 35% DHA in the total fatty acid content.

30 The oil content in some seeds was decreased from about 44% in wild-type seeds to about 31-39% in some of the DHA producing seeds, but was similar to wild-type levels in other DHA producing seeds.

Various transformed plant lines which were producing DHA at levels of at least 10% in T2 seed are crossed and the F1 progeny selfed in order to produce F2 progeny
35 which are homozygous for multiple T-DNA insertions. Seedoil from homozygous seed is analysed and up to 30% or 35% of the total fatty acid content in the seed oil is DHA.

The TAG in the oil obtained from CT136-27-18-2 and CT136-27-18-19 was analysed by ^{13}C NMR regiospecificity assay for positional distribution of the DHA on the glycerol backbone of the TAG molecules. The DHA was preferentially linked at the *sn*-1,3 position. More than 70%, indeed more than 90% of the DHA was in the *sn*-1,3 position.

In several further transgenic lines, the DHA content of single seeds from independent events exceeded 12%. The transgenic:null ratio of these lines was found to be approximately 3:1, corresponding to a single transgenic locus, or 15:1, corresponding to two transgenic loci. Analysis of representative fatty acid profiles from the samples from each construct with the highest levels of DHA found only 1.2-1.4% GLA with no other new ω 6 PUFA detected. In contrast, new ω 3 PUFA (SDA) and ω 3 LC-PUFA (ETA, EPA, DPA, DHA) accumulated to a sum of 25.8% for the modF construct and 21.9% for the modG construct compared to 18.5% for the GA7-transformed seed. The DHA levels in the oil from these seeds were 9.6%, 12.4% and 11.5%, respectively. Δ 6-desaturation was found to be lower in the GA7-transformed seeds than the modF- and modG-transformed seeds (32% vs 47% and 43%) and this resulted in a reduction of ALA in the modF and modG seeds relative to GA7. Another noteworthy difference was the accumulation of EPA in the modF seed (3.3% vs 0.8% in the other two transgenic seeds) and this was reflected in the reduced Δ 5-elongation observed in modF (80%) seed relative to GA7 and modG seeds (93% and 94%). There was a slight increase in Δ 6-elongation in these seeds (66% vs 60% and 61%) although the amount of SDA actually increased due to the slightly more active Δ 6-desaturation. DHA was detected in the polar seed lipid fraction of GA7 lines.

The fatty acid composition was analysed of the lipid in the T1 seed of 70 independent transgenic plants of the *B. napus* breeding line NX54 transformed with the T-DNA of the modB construct. It was observed that one of these transgenic plants produced seed having DPA but no DHA in the seedoil. The T1 seed of this line (CT-137-2) produced about 4% DPA without any detectable DHA in the T1 pooled seed. The inventors concluded that this was caused by inactivation of the Δ 4-desaturase gene in that particular inserted T-DNA, perhaps through a spontaneous mutation. Around 50 T1 seeds from this transgenic line were germinated and one emerged cotyledon from each analysed for fatty acid composition in the remaining oil. Selected seedlings exhibiting more than 5% DPA were then grown to maturity and T2 seed harvested. Pooled seed fatty acid compositions are shown in Table 11, more than 7% DPA was observed in these lines.

Whilst the focus of this experiment was the demonstration of DHA production in an oilseed crop species, the differences noted above were also interesting from a construct design perspective. First, switching the $\Delta 6$ - and $\Delta 5$ -elongase coding region locations in the modF construct resulted in the intended profile change with more EPA
5 accumulated due to lower $\Delta 5$ -elongation. A concomitant increase in $\Delta 6$ -elongation was observed but this did not result in lower SDA levels. This was due to an increase in $\Delta 6$ -desaturation in the modF transformed seed, caused by adding an extra *M. pusilla* $\Delta 6$ -desaturase expression cassette as well as by replacing the truncated napin promoter (FP1) with a more highly active flax conlinin2 promoter. The somewhat lower increase
10 in $\Delta 6$ -desaturation observed with the modG construct was caused by capitalising on the highly expressed $\Delta 5$ -elongase cassette in GA7. Switching the positions of the $\Delta 6$ -desaturase and $\Delta 5$ -elongase coding regions resulted in greater $\Delta 6$ -desaturation. $\Delta 5$ -elongase activity was not reduced in this instance due to the replacement of the FP1 promoter with the Cnl2 promoter.

15 These data confirmed that the modB, modF and modG constructs performed well for DHA production in *Camelina* seed, as for *Arabidopsis* and canola.

The inventors considered that, in general, the efficiency of rate-limiting enzyme activities in the DHA pathway can be greater in multicopy T-DNA transformants compared to single-copy T-DNA transformants, or can be increased by inserting into
20 the T-DNA multiple genes encoding the enzyme which might be limiting in the pathway. Evidence for the possible importance of multi-copy transformants was seen in the *Arabidopsis* seeds transformed with the GA7 construct (Example 2), where the highest yielding DHA event had three T-DNAs inserted into the host genome. The multiple genes can be identical, or preferably are different variants that encode the
25 same polypeptide, or are under the control of different promoters which have overlapping expression patterns. For example, increased expression could be achieved by expression of multiple $\Delta 6$ -desaturase coding regions, even where the same protein is produced. In pJP3416-GA7-modF and pJP3416-GA7-modC, for instance, two versions of the *M. pusilla* $\Delta 6$ -desaturase were present and expressed by different promoters.
30 The coding sequences had different codon usage and therefore different nucleotide sequences, to reduce potential silencing or co-suppression effects but resulting in the production of the same protein.

Table 9. Fatty acid profiles of half cotyledons of germinating *B. napus* seeds containing the modB construct. Up to 18.1% DHA was observed with numerous samples containing greater than 10% DHA.

Seed	14:0	16:0	16:1d3?	16:1	16:3	18:0	18:1	18:1d11	18:2	18:3n6	18:3n3	20:0	18:4n3	C20:1d11	20:1d13	C20:2n6	C20:3n3	C22:0	20:4n3	20:5n3	22:3n3	C24:0	C24:1	22:5n3	C22:6n3
1	0.1	4.2	0.1	0.1	0.2	1.8	29.9	2.5	9.9	0.1	38.4	0.5	0.8	1.0	0.0	0.1	2.1	0.3	2.8	0.3	0.1	0.2	0.2	0.5	3.9
2	0.1	4.7	0.1	0.1	0.2	4.0	23.0	2.3	7.4	0.3	29.3	1.0	4.3	1.1	0.0	0.1	1.9	0.4	6.9	1.0	0.0	0.3	0.1	1.7	9.5
3	0.1	3.7	0.2	0.1	0.2	1.8	55.1	1.9	4.7	0.2	15.2	0.8	1.8	1.4	0.0	0.1	0.3	0.5	11.3	0.0	0.0	0.3	0.2	0.0	0.0
4	0.1	4.6	0.2	0.2	0.2	2.9	22.1	1.8	6.6	0.4	26.5	1.0	7.2	1.0	0.0	0.1	0.8	0.5	11.2	1.9	0.0	0.2	0.2	1.7	8.7
5	0.1	4.0	0.1	0.1	0.2	1.7	27.4	2.1	8.1	0.3	26.4	0.6	2.8	1.0	0.0	0.1	1.5	0.3	7.6	1.5	0.0	0.1	0.1	1.8	12.2
6	0.1	3.5	0.1	0.1	0.2	1.6	59.8	2.0	4.3	0.1	18.5	0.6	0.5	1.3	0.0	0.0	0.7	0.3	9.0	0.0	0.0	0.2	0.1	0.0	0.0
7	0.1	6.0	0.3	0.3	0.3	1.7	16.6	2.6	23.9	1.0	23.2	0.6	5.4	0.8	0.0	0.2	0.6	0.4	2.6	1.1	0.0	0.3	0.3	1.7	9.9
8	0.1	4.9	0.1	0.1	0.2	2.7	12.9	1.4	11.7	0.3	34.3	0.9	5.0	0.9	0.0	0.2	2.4	0.5	4.1	1.3	0.0	0.2	0.2	1.8	13.8
9	0.1	3.9	0.1	0.1	0.1	2.4	41.6	1.7	21.5	0.0	23.4	0.7	0.0	1.2	0.0	0.1	2.2	0.4	0.0	0.0	0.1	0.3	0.2	0.0	0.0
10	0.1	3.7	0.2	0.1	0.1	2.1	30.9	1.7	19.2	0.4	23.6	0.7	2.1	1.1	0.0	0.1	1.5	0.4	3.6	0.6	0.0	0.2	0.1	0.7	6.9
11	0.1	5.7	0.4	0.3	0.2	3.8	41.2	2.4	26.7	2.1	7.2	1.3	0.3	1.2	0.0	0.2	0.3	0.8	4.8	0.0	0.0	0.6	0.3	0.0	0.0
12	0.1	4.6	0.0	0.1	0.2	2.4	25.5	1.7	16.1	0.3	28.9	0.8	3.9	1.1	0.0	0.1	1.9	0.4	3.9	0.6	0.0	0.2	0.0	1.1	6.2
13	0.1	4.3	0.1	0.1	0.1	4.2	19.4	1.6	9.2	0.1	45.5	1.0	0.2	1.1	0.0	0.1	5.2	0.4	2.6	0.3	0.2	0.2	0.1	0.4	3.4
14	0.1	6.3	0.2	0.2	0.2	4.0	10.5	2.3	8.4	0.3	31.1	1.3	3.9	0.8	0.0	0.1	2.3	0.6	4.6	1.8	0.1	0.3	0.2	2.5	18.1
15	0.1	5.1	0.1	0.2	0.2	3.3	16.8	2.4	11.2	0.3	28.8	1.0	4.5	0.9	0.0	0.1	2.1	0.6	3.2	1.5	0.1	0.3	0.1	1.8	15.1
16	0.1	4.4	0.1	0.1	0.2	4.0	16.2	1.5	11.6	0.2	33.5	0.9	2.8	1.1	0.0	0.2	3.7	0.4	4.6	0.7	0.1	0.3	0.1	1.3	12.1
17	0.2	7.2	0.2	0.2	0.2	4.9	15.0	2.1	8.9	0.3	25.9	1.4	5.1	0.9	0.0	0.0	1.6	0.8	4.9	2.1	0.0	0.6	0.3	2.2	15.0
18	0.1	4.0	0.1	0.1	0.2	2.3	64.8	1.2	7.2	0.1	12.5	1.0	3.5	1.5	0.0	0.1	0.0	0.7	0.0	0.0	0.0	0.5	0.2	0.0	0.0
19	0.1	3.9	0.1	0.1	0.2	4.6	36.9	1.7	7.1	0.2	28.6	1.2	1.8	1.2	0.0	0.1	1.4	0.5	4.3	0.4	0.0	0.4	0.1	0.8	4.3
20	0.1	4.8	0.1	0.1	0.2	6.0	18.5	1.2	12.8	0.2	34.8	1.4	2.4	1.1	0.0	0.1	3.4	0.6	3.2	0.4	0.1	0.3	0.1	0.7	7.6

Table 10. Fatty acid profiles of T2 transgenic *B. napus* seeds containing the modB construct.

Sample (T2 seed)	C14:0	C16:0	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	18:4n3	C20:1d11	C20:2n6	C20:3n3	20:4n3	20:5n3	22:5n3	C22:6n3	Total ω3 (%)	Total ω6 (%)	Ratio ω6 to ω3	Total PUFA content (%)
CT136-27-18-1	0.1	5.0	2.6	25.4	3.6	6.7	0.2	37.5	1.4	1.0	0.1	2.1	0.8	0.4	0.9	10.2	53.4	7.1	0.13	60.5
CT136-27-18-2	0.2	7.1	2.8	16.9	4.3	5.5	0.4	29.1	5.4	0.8	0.1	1.2	0.5	0.5	1.9	21.2	59.8	6.1	0.10	66.0
CT136-27-18-3	0.1	5.4	2.5	26.5	3.8	6.4	0.4	26.4	4.7	1.0	0.1	0.7	1.1	0.6	1.2	17.3	52.0	6.9	0.13	58.9
CT136-27-18-4	0.1	5.3	2.4	34.7	4.0	5.9	0.3	30.3	1.3	1.1	0.1	1.1	1.5	0.3	0.4	9.3	44.4	6.3	0.14	50.7
CT136-27-18-5	0.1	4.8	2.7	34.5	3.8	5.6	0.3	23.5	3.9	1.2	0.1	0.7	1.1	0.5	1.1	14.2	45.1	6.0	0.13	51.1
CT136-27-18-6	0.1	5.0	2.1	54.3	3.8	5.7	0.2	18.2	0.6	1.5	0.1	1.1	0.7	0.1	0.2	4.4	25.5	6.1	0.24	31.5
CT136-27-18-7	0.1	5.3	2.1	43.8	4.2	5.6	0.4	18.3	2.2	1.3	0.2	0.6	1.5	0.4	0.5	11.6	35.2	6.2	0.18	41.4
CT136-27-18-8	0.1	5.4	2.7	25.8	4.1	6.7	0.4	26.6	5.7	1.0	0.1	0.6	1.3	0.6	1.2	15.8	51.9	7.1	0.14	59.0
CT136-27-18-9	0.1	4.6	1.6	53.8	3.7	17.5	0.5	9.2	0.5	1.6	0.3	0.6	0.4	0.1	0.1	3.7	14.5	18.3	1.26	32.8
CT136-27-18-10	0.1	4.8	2.4	44.1	3.7	5.4	0.4	19.1	2.3	1.1	0.1	0.6	1.5	0.5	0.8	11.4	36.1	5.9	0.16	42.0
CT136-27-18-11	0.1	5.1	2.2	48.3	4.1	10.9	0.7	12.5	1.2	1.3	0.2	0.5	1.5	0.3	0.3	9.1	25.3	11.8	0.47	37.1
CT136-27-18-12	0.1	5.3	2.7	23.3	3.7	6.0	0.4	27.9	4.9	0.9	0.1	0.7	1.3	0.8	1.5	18.5	55.7	6.6	0.12	62.2
CT136-27-18-13	0.1	5.5	3.4	30.7	5.6	5.1	0.4	23.1	3.5	1.1	0.1	1.2	1.1	0.6	1.2	14.9	45.8	5.5	0.12	51.3
CT136-27-18-14	0.1	5.4	2.3	23.9	3.5	6.0	0.4	30.1	3.7	1.0	0.1	1.0	0.7	0.6	1.2	18.2	55.5	6.6	0.12	62.1
CT136-27-18-15	0.1	5.0	2.3	45.4	4.0	5.3	0.4	16.2	2.3	1.2	0.1	0.5	1.9	0.6	0.7	12.3	34.4	5.8	0.17	40.3
CT136-27-18-16	0.1	4.8	2.7	37.9	4.1	6.2	0.4	22.0	2.4	1.0	0.1	0.7	1.4	0.5	0.8	13.1	41.0	6.7	0.16	47.7
CT136-27-18-17	0.1	4.5	2.3	38.8	3.3	7.6	0.3	26.8	0.9	1.4	0.2	1.6	0.9	0.2	0.7	8.6	39.9	8.0	0.20	47.9
CT136-27-18-18	0.1	5.1	2.3	29.0	3.6	5.7	0.4	26.5	3.8	1.1	0.2	0.8	0.8	0.6	1.0	17.4	50.8	6.3	0.12	57.1
CT136-27-18-19	0.1	5.8	2.3	19.7	4.2	6.7	0.7	23.7	7.7	0.9	0.1	0.4	0.7	0.6	1.7	22.7	57.6	7.5	0.13	65.1
CT136-27-18-20	0.1	5.7	2.9	23.2	4.0	5.6	0.3	35.8	2.4	1.0	0.1	1.3	1.1	0.5	1.0	13.0	55.1	6.1	0.11	61.2

ARA (C20:4n6) was not detected in any of the samples. The samples also contained about 0.2% or 0.3% C16:1, about 0.1 to 0.3% C16:3, between about 0.7% and 1.0% C20:0, about 0.3% C22:0, and some samples contained trace levels (<0.1%) of C20:1A13,

5 C22:3n3, C24:0 and C24:1

Table 11. Fatty acid composition of the lipid in T2 transgenic *B. napus* seeds transformed with the T-DNA of the modB construct, with a presumed mutation in the $\Delta 4$ -desaturase gene. The lipids also contained about 0.1% 14:0, 0.2% 16:3, 0.2-0.4% GLA, 0.1% 20:1 Δ 13, 0.3-0.4% 22:0, and 16:2 and 22:1 were not detected.

5

	16:0	16:1	16:1 ω 13 ω 1	18:0	18:1	18:1 Δ 11	18:2	18:3n3	20:0	18:4n3	20:1 Δ 11	20:2n6	20:3n6	20:4n6	20:3n3	20:4n3	20:5n3	22:2n6	22:3n3	24:0	24:1	22:5n3	22:6n3
CT-137- 2-34	5.3	0.0	0.2	3.7	26.8	3.1	12.4	29.1	0.8	2.5	0.8	0.1	0.0	0.0	1.1	1.7	0.8	0.0	0.1	0.1	0.1	10.0	0.0
CT-137- 2-38	5.3	0.0	0.2	4.2	24.4	3.0	12.6	29.4	0.9	2.5	0.8	0.1	0.0	0.0	1.3	2.2	0.9	0.0	0.1	0.2	0.1	10.8	0.0
CT-137- 2-48	5.0	0.0	0.2	4.2	24.1	3.1	11.9	31.0	0.9	2.4	0.9	0.1	0.0	0.0	1.5	2.0	1.0	0.0	0.1	0.1	0.1	10.5	0.0
CT-137- 2-51	5.7	0.0	0.2	4.6	22.3	3.4	12.3	34.5	1.0	2.0	0.8	0.1	0.0	0.0	1.9	1.2	0.5	0.0	0.1	0.2	0.2	7.9	0.0
CT-137- 2-59	5.4	0.0	0.2	3.9	25.7	3.4	12.9	27.8	0.9	2.6	0.8	0.1	0.0	0.0	1.0	1.9	0.9	0.0	0.1	0.2	0.1	11.0	0.0

Table 12. Fatty acid composition of seedoil from T2 seed of *B. napus* transformed with the T-DNA from GA7-modB.

C16:0	6.3	C18:0	2.4	C18:1n9	8.4	C18:1n7	3.1	C18:2n6	6.9	C18:3n6	1.1	C18:3n3	21.9	C20:0	0.7	C18:4n3	7.5	C20:1n9c	0.7	C20:2n6 + C21:0	0.1	C20:3n3	0.5	C20:4n3	0.5	C20:5n3	0.6	C22:5n6	0.2	C22:5n3	1.5	C22:6n3	34.3
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The seedoil samples also contained 0.1% C14:0; 0.2% C16:1; 0.1% C20:3n6; no C22:1 and C22:2n6; 0.1% C24:0 and 0.2% C24:1, 2.6% other fatty acids.

Example 5. Analysis of TAG from Transgenic *A. thaliana* Seeds Producing DHA

The positional distribution of DHA on the TAG from the transformed *A. thaliana* seed was determined by NMR. Total lipid was extracted from approximately 5 200 mg of seed by first crushing them under hexane before transferring the crushed seed to a glass tube containing 10 mL hexane. The tube was warmed at approximately 55°C in a water bath and then vortexed and centrifuged. The hexane solution was removed and the procedure repeated with a further 4 x 10 mL. The extracts were combined, concentrated by rotary evaporation and the TAG in the extracted lipid 10 purified away from polar lipids by passage through a short silica column using 20 mL of 7% diethyl ether in hexane. Acyl group positional distributions on the purified TAG were determined quantitatively as previously described (Petrie et al., 2010a and b).

The analysis showed that the majority of the DHA in the total seed oil was located at the *sn*-1/3 positions of TAG with little found at the *sn*-2 position (Figure 5). 15 This was in contrast to TAG from ARA producing seeds which demonstrated that 50% of the ARA (20:4^{Δ5,8,11,14}) was located at the *sn*-2 position of transgenic canola oil whereas only 33% would be expected in a random distribution (Petrie et al., 2012).

The total lipid from transgenic *A. thaliana* seeds was also analysed by triple quadrupole LC-MS to determine the major DHA-containing triacylglycerol (TAG) 20 species (Figure 6). The most abundant DHA-containing TAG species was found to be DHA-18:3-18:3 (TAG 58:12; nomenclature not descriptive of positional distribution) with the second-most abundant being DHA-18:3-18:2 (TAG 58:11). Tri-DHA TAG (TAG 66:18) was observed in total seed oil, albeit at low but detectable levels. Other major DHA-containing TAG species included DHA-34:3 (TAG 56:9), DHA-36:3 25 (TAG 58:9), DHA-36:4 (TAG 58:10), DHA-36:7 (TAG 58:13) and DHA-38:4 (TAG 60:10). The identities of the two major DHA-containing TAG were further confirmed by Q-TOF MS/MS.

Example 6. Assaying Sterol Content and Composition in Oils

30 The phytosterols from 12 vegetable oil samples purchased from commercial sources in Australia were characterised by GC and GC-MS analysis as O-trimethylsilyl ether (OTMSi-ether) derivatives as described in Example 1. Sterols were identified by retention data, interpretation of mass spectra and comparison with literature and laboratory standard mass spectral data. The sterols were quantified by use of a 5β(H)- 35 Cholan-24-ol internal standard. The basic phytosterol structure and the chemical structures of some of the identified sterols are shown in Figure 7 and Table 13.

The vegetable oils analysed were from: sesame (*Sesamum indicum*), olive (*Olea europaea*), sunflower (*Helianthus annuus*), castor (*Ricinus communis*), canola (*Brassica napus*), safflower (*Carthamus tinctorius*), peanut (*Arachis hypogaea*), flax (*Linum usitatissimum*) and soybean (*Glycine max*). In decreasing relative abundance, across all of the oil samples, the major phytosterols were: β -sitosterol (range 28-55% of total sterol content), Δ^5 -avenasterol (isofucosterol) (3-24%), campesterol (2-33%), Δ^5 -stigmasterol (0.7-18%), Δ^7 -stigmasterol (1-18%) and Δ^7 -avenasterol (0.1-5%). Several other minor sterols were identified, these were: cholesterol, brassicasterol, chalinasterol, campestanol and eburicol. Four C29:2 and two C30:2 sterols were also detected, but further research is required to complete identification of these minor components. In addition, several other unidentified sterols were present in some of the oils but due to their very low abundance, the mass spectra were not intense enough to enable identification of their structures.

Table 13. IUPAC/systematic names of identified sterols.

Sterol No.	Common name(s)	IUPAC / Systematic name
1	cholesterol	cholest-5-en-3 β -ol
2	brassicasterol	24-methylcholesta-5,22E-dien-3 β -ol
3	chalinasterol / 24-methylene cholesterol	24-methylcholesta-5,24(28)E-dien-3 β -ol
4	campesterol / 24-methylcholesterol	24-methylcholest-5-en-3 β -ol
5	campestanol / 24-methylcholestanol	24-methylcholestan-3 β -ol
7	Δ^5 -stigmasterol	24-ethylcholesta-5,22E-dien-3 β -ol
9	ergost-7-en-3 β -ol	24-methylcholest-7-en-3 β -ol
11	eburicol	4,4,14-trimethylergosta-8,24(28)-dien-3 β -ol
12	β -sitosterol / 24-ethylcholesterol	24-ethylcholest-5-en-3 β -ol
13	Δ^5 -avenasterol / isofucosterol	24-ethylcholesta-5,24(28)Z-dien-3 β -ol
19	Δ^7 -stigmasterol / stigmast-7-en-3 β -ol	24-ethylcholest-7-en-3 β -ol
20	Δ^7 -avenasterol	24-ethylcholesta-7,24(28)-dien-3 β -ol

The sterol contents expressed as mg/g of oil in decreasing amount were: canola oil (6.8 mg/g), sesame oil (5.8 mg/g), flax oil (4.8-5.2 mg/g), sunflower oil (3.7-4.1 mg/g), peanut oil (3.2 mg/g), safflower oil (3.0 mg/g), soybean oil (3.0 mg/g), olive oil

(2.4 mg/g), castor oil (1.9 mg/g). The % sterol compositions and total sterol content are presented in Table 14.

Among all the seed oil samples, the major phytosterol was generally β -sitosterol (range 30-57% of total sterol content). There was a wide range amongst the oils in the proportions of the other major sterols: campesterol (2-17%), Δ^5 -stigmasterol (0.7-18%), Δ^5 -avenasterol (4-23%), Δ^7 -stigmasterol (1-18%). Oils from different species had a different sterol profile with some having quite distinctive profiles. In the case of canola oil, it had the highest proportion of campesterol (33.6%), while the other species samples generally had lower levels, e.g. up to 17% in peanut oil. Safflower oil had a relatively high proportion of Δ^7 -stigmasterol (18%), while this sterol was usually low in the other species oils, up to 9% in sunflower oil. Because they were distinctive for each species, sterol profiles can therefore be used to help in the identification of specific vegetable or plant oils and to check their genuineness or adulteration with other oils.

Two samples each of sunflower and safflower were compared, in each case one was produced by cold pressing of seeds and unrefined, while the other was not cold-pressed and refined. Although some differences were observed, the two sources of oils had similar sterol compositions and total sterol contents, suggesting that processing and refining had little effect on these two parameters. The sterol content among the samples varied three-fold and ranged from 1.9 mg/g to 6.8 mg/g. Canola oil had the highest and castor oil the lowest sterol content.

Example 7. Increasing Accumulation of DHA at the *sn*-2 TAG Position

The present inventors considered that DHA and/or DPA accumulation at the *sn*-2 position in TAG could be increased by co-expressing an 1-acyl-glycerol-3-phosphate acyltransferase (LPAAT) together with the DHA or DPA biosynthesis pathway such as conferred by the GA7 construct or its variants. Preferred LPAATs are those which can act on polyunsaturated C22 fatty acyl-CoA as substrate, preferably DHA-CoA and/or DPA-CoA, resulting in increased insertion of the polyunsaturated C22 chain at the *sn*-2 position of LPA to form PA, relative to the endogenous LPAAT. Cytoplasmic LPAAT enzymes often display varied substrate preferences, particularly where the species synthesises and accumulates unusual fatty acids in TAG. A LPAAT2 from *Limnanthes douglasii* was shown to use erucoyl-CoA (C22:1-CoA) as a substrate for PA synthesis, in contrast to an LPAAT1 from the same species that could not utilise the C22 substrate (Brown et al., 2002).

Table 14. Sterol content and composition of assayed plant oils.

Sterol number*	Sterol common name	Sesame	Olive	Sun-flower	Sun-flower	Sun-flower	Castor	Canola	Saf-flower	Saf-flower	Saf-flower	Peanut	Flax (linseed)	Flax (linseed)	Soy-bean
				cold-pressed					cold-pressed						
1	cholesterol	0.2	0.8	0.2	0.0	0.1	0.3	0.3	0.2	0.2	0.1	0.2	0.4	0.4	0.2
2	brassicasterol	0.1	0.0	0.0	0.0	0.3	0.1	0.1	0.0	0.0	0.0	0.0	0.2	0.2	0.0
3	chalinasterol /24-methylene cholesterol	1.5	0.1	0.3	0.1	1.1	2.4	2.4	0.2	0.2	0.1	0.9	1.5	1.4	0.8
4	campesterol /24-methylcholesterol	16.2	2.4	7.4	7.9	8.4	33.6	33.6	12.1	12.1	8.5	17.4	15.7	14.4	16.9
5	campestanol /24-methylcholestanol	0.7	0.3	0.3	0.1	0.9	0.2	0.2	0.8	0.8	0.8	0.3	0.2	0.2	0.7
6	C29:2*	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.5	0.5	0.5	0.0	1.2	1.3	0.1
7	Δ^5 -stigmasterol	6.4	1.2	7.4	7.2	18.6	0.7	0.7	7.0	7.0	4.6	6.9	5.1	5.8	17.6
8	unknown	0.5	1.3	0.7	0.6	0.8	0.7	0.7	0.7	0.7	1.3	0.4	0.7	0.6	1.3
9	ergost-7-en-3 β -ol	0.1	0.1	1.9	1.8	0.2	0.4	0.4	2.7	2.7	4.0	1.4	1.4	1.4	1.0
10	unknown	0.0	1.3	0.9	0.8	1.2	0.9	0.9	1.8	1.8	0.7	1.2	0.7	0.5	0.7
11	eburicol	1.6	1.8	4.1	4.4	1.5	1.0	1.0	1.9	1.9	2.9	1.2	3.5	3.3	0.9
12	β -sitosterol / 24-ethylcholesterol	55.3	45.6	43.9	43.6	37.7	50.8	50.8	40.2	40.2	35.1	57.2	29.9	28.4	40.2

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13	Δ^5 -avenasterol / isofucosterol	8.6	16.9	7.2	4.1	19.3	4.4	7.3	6.3	5.3	23.0	24.2	3.3
14	triterpenoid alcohol	0.0	2.4	0.9	1.1	0.0	0.0	1.6	1.9	0.0	0.0	0.0	0.9
15	triterpenoid alcohol	0.0	0.0	0.7	0.6	0.0	0.0	2.8	1.8	0.0	0.0	0.3	0.0
16	C29:2*	0.0	0.5	0.7	0.7	1.5	1.2	2.8	1.9	2.0	1.0	0.7	0.5
17	C29:2*	1.0	0.9	2.3	2.4	0.6	0.4	1.3	1.9	0.9	1.0	1.0	1.0
18	C30:2*	0.0	0.0	0.0	0.0	1.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0
19	Δ^7 -stigmasterol / stigmast-7-en-3 β - ol	2.2	7.1	9.3	10.9	2.3	0.9	10.5	18.3	1.1	7.9	8.7	5.6
20	Δ^7 -avenasterol	1.3	0.1	4.0	3.6	0.6	0.2	2.0	4.7	0.7	0.4	0.4	0.6
21	unknown	0.7	7.1	0.9	0.8	0.0	0.4	0.3	0.4	0.0	3.0	3.6	0.0
22	unknown	0.3	0.0	0.3	0.9	0.0	0.0	1.2	1.3	0.2	0.1	0.0	0.3
23	unknown	0.2	0.2	0.3	0.3	0.2	0.1	0.3	0.2	0.2	0.1	0.2	0.5
24	unknown	0.0	3.1	0.9	1.3	0.6	0.4	0.2	0.4	0.7	1.7	1.9	0.8
25	unknown	0.9	0.4	0.3	0.5	0.3	0.1	0.5	0.7	0.3	0.1	0.1	0.6
26	C30:2	2.2	6.0	4.6	5.7	1.4	0.6	1.0	1.2	1.2	1.2	1.1	5.2
27	unknown	0.0	0.4	0.4	0.3	0.3	0.2	0.1	0.2	0.3	0.1	0.0	0.3
	Sum	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	Total sterol (mg/g oil)	5.8	2.4	4.1	3.7	1.9	6.8	3.2	3.0	3.2	4.8	5.2	3.0

C29:2* and C30:2* denotes a C29 sterol with two double bonds and a C30 sterol with two double bonds, respectively

Known LPAATs were considered and a number were selected for testing, including some which were not expected to increase DHA incorporation at the *sn*-2 position, as controls. The known LPAATs included: *Arabidopsis thaliana* LPAAT2: (SEQ ID NO: 40, Accession No. ABG48392, Kim et al., 2005), *Limnanthes alba* LPAAT (SEQ ID NO: 41, Accession No. AAC49185, Lassner et al., 1995), *Saccharomyces cerevisiae* Slc1p (SEQ ID NO: 42, Accession No. NP_010231, Zou et al., 1997), *Mortierella alpina* LPAAT1 (SEQ ID NO: 44, Accession No. AED33305; US 7879591) and *Brassica napus* LPAATs (SEQ ID NO: 45 and SEQ ID NO: 46, Accession Nos ADC97479 and ADC97478 respectively).

The *Arabidopsis* LPAAT2 (also designated LPAT2) is an endoplasmic reticulum-localised enzyme shown to have activity on C16 and C18 substrates, however activity on C20 or C22 substrates was not tested (Kim et al., 2005). *Limnanthes alba* LPAAT2 was demonstrated to insert a C22:1 acyl chain into the *sn*-2 position of PA, although the ability to use DHA or DPA as a substrate was not tested (Lassner et al., 1995). The selected *S. cerevisiae* LPAAT Slc1p was shown to have activity using 22:1-CoA in addition to 18:1-CoA as substrates, indicating a broad substrate specificity with respect to chain length (Zou et al., 1997). Again, DHA-CoA, DPA-CoA and other LC-PUFAs were not tested as substrates. The *Mortierella* LPAAT had previously been shown to have activity on EPA and DHA fatty acid substrates in transgenic *Yarrowia lipolytica* (US 7879591) but its activity in plant cells was unknown.

Additional LPAATs were identified by the inventors. *Micromonas pusilla* is a microalga that produces and accumulates DHA in its oil, although the positional distribution of the DHA on TAG in this species has not been confirmed. The *Micromonas pusilla* LPAAT (SEQ ID NO: 43, Accession No. XP_002501997) was identified by searching the *Micromonas pusilla* genomic sequence using the *Arabidopsis* LPAAT2 as a BLAST query sequence. Several candidate sequences emerged and the sequence XP_002501997 was synthesised for testing on C22 LC-PUFA. The *Ricinus communis* LPAAT was annotated as a putative LPAAT in the castor genome sequence (Chan et al., 2010). Four candidate LPAATs from the castor genome were synthesised and tested in crude leaf lysates of infiltrated *N. benthamiana* leaf tissue. The candidate sequence described here showed LPAAT activity.

A number of candidate LPAATs were aligned with known LPAATs on a phylogenetic tree (Figure 8). It was noted that the putative *Micromonas* LPAAT did not cluster with the putative C22 LPAATs but was a divergent sequence.

As an initial test of various LPAATs for their ability to use DHA-CoA as substrate, chimeric genetic constructs were made for constitutive expression of exogenous LPAATs in *N. benthamiana* leaves, each under the control of the 35S promoter, as follows: 35S:Arath-LPAAT2 (*Arabidopsis* ER LPAAT); 35S:Limal-LPAAT (*Limnanthes alba* LPAAT); 35S:Sacce-Slc1p (*S. cerevisiae* LPAAT); 35S:Micpu-LPAAT (*Micromonas pusilla* LPAAT); 35S:Moral-LPAAT1 (*Mortierella alpina* LPAAT); 35S:Brana-LPAAT1.13 (*Brassica napus* LPAAT1.13); 35S:Brana-LPAAT1.5 (*Brassica napus* LPAAT1.5). A 35S:p19 construct lacking an exogenous LPAAT was used as a control in the experiment. Each of these constructs was introduced via *Agrobacterium* into *N. benthamiana* leaves as described in Example 1, and 5 days after infiltration, the treated leaf zones were excised and ground to make leaf lysates. Each lysate included the exogenous LPAAT as well as the endogenous enzymes for synthesizing LPA. *In vitro* reactions were set up by separately adding ¹⁴C-labelled-OA and -DHA to the lysates. Reactions were incubated at 25°C and the level of incorporation of the ¹⁴C labelled fatty acids into PA determined by TLC. The ability of each LPAAT to use DHA relative to ARA and the C18 fatty acids were assessed. The meadowfoam (*Limnanthes alba*), *Mortierella* and *Saccharomyces* LPAATs were found to have activity on DHA substrate, with radiolabelled PA appearing for these but not the other LPAATs. All LPAATs were confirmed active by the oleic acid control feed.

To test LPAAT activity in seeds, several of the protein coding sequences or LPAATs were inserted into a binary vector under the control of a conlinin (pLuCn11) promoter. The resultant genetic constructs containing the chimeric genes, Cn11:Arath-LPAAT (negative control), Cn11:Limal-LPAAT, Cn1:Sacce-Slc1p, and Cn11:Moral-LPAAT, respectively, are then used to transform *A. thaliana* plants producing DHA in their seed to generate stable transformants expressing the LPAATs and the transgenic DHA pathway in a seed-specific manner to test whether there would be an increased incorporation of DHA at the *sn*-2 position of TAG. The constructs are also used to transform *B. napus* and *C. sativa* plants that already contain the GA7 construct and variants thereof (Examples 2 to 4) to generate progeny carrying both the parental and LPAAT genetic constructs. Increased incorporation of DHA at the *sn*-2 position of TAG is tested relative to the incorporation in plants lacking the LPAAT encoding transgenes. Oil content is also improved in the seeds, particularly for seeds producing higher levels of DHA, counteracting the trend seen in *Arabidopsis* seed as described in Example 2.

The 35S:Moral-LPAAT1 construct was used to transform an already transgenic *Arabidopsis thaliana* line which was homozygous for the T-DNA from the GA7 construct and whose seed contained approximately 15% DHA in seed lipids (Petric et al., 2012). For this, use was made of the kanamycin selectable marker gene in the 5 35S:Moral-LPAAT1 construct which was different to the *bar* selectable marker gene already present in the transgenic line. Transgenic seedlings were selected which were resistant to kanamycin and grown to maturity in a glasshouse. T2 seeds were harvested and the fatty acid composition of their total seed lipids analysed by GC (Table 15). Three phenotypes were observed amongst the 33 independently transformed lines. In a 10 first group (6/33 lines), DPA increased significantly to a level substantially greater than the level of DHA, up to about 10.6% of total seed lipids. This came at the expense of DHA which was strongly decreased in this group of lines. In two of the lines in this first group, the sum of DPA + DHA was reduced, but not in the other 4 lines. In a second group (5/33), the levels of DPA and DHA were about equal, with the sum of 15 DPA + DHA about the same as for the parental seed. In the third group, the levels of DPA and DHA were similar to those in the parental seeds. One possible explanation for the increased level of DPA in the first and second groups is that the LPAAT out-competes the $\Delta 4$ -desaturase for DPA-CoA substrate and preferentially incorporates the DPA into PA and thence into TAG, relative to the $\Delta 4$ -desaturation. A second possible 20 explanation is that the $\Delta 4$ -desaturation is partially inhibited.

Seed from the *Arabidopsis* plants transformed with the T-DNA of the GA7 construct which had been further transformed with the Cn1::Moral-LPAAT vector were harvested and oil extracted from the seed. The TAG fraction was then isolated from the extracted oil by TLC methods and recovered from the TLC plate. These TAG 25 samples and samples of the seedoil prior to the fractionation were analysed by digestion with *Rhizopus* lipase to determine the positional distribution of the DHA. The lipase is specific for acyl groups esterified at the sn-1 or sn-3 position of TAG. This was performed by emulsifying each lipid sample in 5% gum arabic using an ultrasonicator, adding the *Rhizopus* lipase solution in 0.1M Tris-HCl pH 7.7 containing 5 mM CaCl_2 30 and incubating the mixtures at 30°C with continuous shaking. Each reaction was stopped by adding chloroform: methanol (2/1, v/v) and one volume of 0.1M KCl to each mixture. The lipid was extracted into the chloroform fraction and the relative amounts determined of the sn-2 MAG, sn-1/3 FFA, DAG and TAG components of the resulting lipid by separation on 2.3% boric acid impregnated TLC using 35 hexane/diethylether/acetic acid (50/50/1, v/v). Lipid bands were visualized by spraying 0.01% primuline in acetone/water (80/20, v/v) onto the TLC plate and visualisation

under UV light. Individual lipid bands were identified on the basis of lipid standard spots, resolved on the same TLC plate. TLC lipid bands were collected into glass vials and their fatty acid methyl esters were prepared using 1N methanolic-HCl (Supelco) and incubating at 80°C for 2h. Fatty acid composition of individual lipids were
5 analysed by GC.

This assay demonstrated that the DHA in the parental seeds transformed with the GA7 (lines 22-2-1-1 and 22-2-38-7) was preferentially esterified at the *sn*-1 or *sn*-3 position of the TAG. In contrast, the DHA in the NY11 and NY15 seed transformed with both the GA7 constructs and the transgene encoding LPAAT was enriched at the
10 *sn*-2 position, with 35% of the DHA in one of the lines and 48% of the DHA in the other line being esterified at the *sn*-2 position of TAG i.e. after lipase digestion the DHA was present as *sn*-2-MAG (Table 16). Analogous results are obtained for *B. napus* and *B. juncea* seeds transformed with both the T-DNA from the GA7-modB construct and the LPAAT-encoding gene and producing DHA.

15 In order to determine whether the *Mortierella* LPAAT or another LPAAT had preference for either DPA-CoA or DHA-CoA, *in vitro* reactions are set up by separately adding ¹⁴C-labelled-DPA-CoA or -DHA-CoA to lysates of *N. benthamiana* leaves transiently expressing the candidate LPAAT under control of a constitutive promoter as described above. Reactions are incubated at 25°C and the level of
20 incorporation of the ¹⁴C labelled fatty acids into PA determined by TLC analysis of the lipids. The ability of each LPAAT to use DHA-CoA relative to DPA-CoA is assessed. Genes encoding LPAATs which are confirmed to have good DHA incorporating LPAAT activity are used to produced transformed DHA-producing canola plants and seed.

25 Genes encoding LPAATs which have strong activity using DPA-CoA are used to transform DPA-producing plants and seed, to increase the amount of DPA esterified at the *sn*-2 position of TAG.

Table 15. Fatty acid composition (% of total fatty acids) of transgenic *A. thaliana* seeds transformed with an LPAAT1 construct as well as the GA7 construct for DHA production.

	C16:0	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4n3	C20:1d11	20:1d13	C20:2n6	C20:4n6	C20:3n3	C22:0	20:4n3	C22:1	20:5n3	22:5n3	C22:6n3
NY-1	9.3	3.2	9.1	6.8	9.4	0.5	23.8	1.6	4.1	7.9	5.1	0.6	0.0	0.9	0.4	0.6	0.6	1.2	7.9	4.5
NY-2	10.7	3.3	6.5	4.4	7.6	0.3	28.1	1.9	4.3	8.5	3.7	0.7	0.0	1.1	0.5	1.1	0.8	1.4	1.1	11.6
NY-3	9.3	2.8	6.3	3.4	10.3	0.2	32.8	2.2	2.7	6.2	3.6	1.1	0.0	1.9	0.5	1.4	0.9	0.7	1.0	10.7
NY-4	11.4	3.5	4.5	3.1	7.0	0.3	32.5	2.1	4.7	5.5	2.3	1.0	0.0	1.9	0.6	0.8	0.6	1.1	0.9	14.3
NY-5	14.6	4.5	7.0	7.7	6.7	0.3	20.7	2.2	5.7	5.4	4.8	0.4	0.0	0.9	0.9	0.8	0.4	1.2	1.0	11.7
NY-6	7.8	2.7	12.5	2.2	18.0	0.1	24.9	1.8	0.7	15.5	3.1	1.4	0.0	1.2	0.5	0.5	1.5	0.3	3.0	8.8
NY-7	9.3	2.9	6.7	3.8	9.2	0.2	31.5	2.1	3.2	7.5	3.7	0.9	0.0	1.6	0.3	1.3	0.8	0.8	1.1	10.9
NY-8	8.8	3.2	8.2	5.5	11.0	0.3	25.3	1.9	3.0	8.3	5.4	1.0	0.0	1.2	0.5	0.8	0.8	0.8	6.1	6.0
NY-9	12.3	3.7	5.0	4.6	7.1	0.2	28.3	2.3	4.2	5.6	3.8	0.8	0.0	1.6	0.7	0.7	0.6	1.1	1.2	13.8
NY-10	8.6	3.2	8.5	3.1	9.7	0.3	31.5	1.6	3.4	8.7	2.8	1.0	0.0	1.3	0.3	0.9	0.6	1.1	10.6	1.0
NY-11	11.5	3.2	4.5	2.5	7.1	0.3	33.3	2.1	3.9	5.7	1.9	0.9	0.0	2.0	0.5	0.7	0.7	0.8	1.0	15.6
NY-12	8.7	3.2	7.5	5.1	8.5	0.2	26.8	2.0	3.7	8.7	5.1	0.9	0.0	1.2	0.5	1.1	0.8	1.2	10.0	2.6
NY-13	11.5	3.4	5.2	3.4	8.3	0.3	30.0	2.2	5.0	6.2	3.2	0.9	0.0	1.7	0.6	1.5	0.8	1.1	1.0	11.6
NY-14	9.2	2.9	6.6	2.0	10.3	0.2	34.7	1.9	3.3	7.7	1.6	1.2	0.0	1.8	0.4	1.2	0.8	0.9	0.8	11.1
NY-15	10.9	3.3	4.6	2.7	7.0	0.3	34.1	1.9	5.1	5.5	2.0	0.9	0.0	1.8	0.5	0.8	0.5	1.0	1.0	14.7
NY-16	10.5	3.4	6.0	4.6	7.8	0.3	30.3	1.8	4.4	5.4	2.9	0.7	0.0	1.5	0.5	0.9	0.5	1.1	1.3	14.2
NY-17	9.1	2.4	5.9	2.5	10.4	0.2	35.4	1.6	3.6	6.4	2.1	1.1	0.0	1.9	0.4	1.2	0.7	1.0	0.9	11.7
NY-18	9.7	3.6	8.8	6.2	12.1	0.3	21.0	1.9	4.0	8.3	5.9	0.8	0.0	0.9	0.5	0.6	0.9	1.0	5.7	5.1
NY-19	8.4	3.1	12.0	3.1	14.6	0.2	28.8	1.7	1.6	11.3	3.2	1.0	0.0	1.4	0.4	0.6	1.0	0.6	3.9	1.2
NY-20	10.1	3.2	5.4	3.3	8.9	0.3	32.8	2.1	4.1	5.5	2.8	1.0	0.0	1.9	0.5	1.1	0.7	0.9	1.1	12.1
NY-21	10.5	3.6	5.6	3.8	8.2	0.3	31.9	2.0	4.6	5.9	2.8	0.9	0.0	1.7	0.5	0.8	0.6	1.0	0.9	12.5
NY-22	8.4	3.3	7.4	2.3	9.4	0.2	33.5	1.8	3.4	8.8	2.2	1.2	0.0	1.7	0.4	1.3	0.7	1.0	5.5	6.1
NY-23	8.3	2.8	7.0	1.9	11.0	0.2	34.6	1.9	2.6	9.3	1.7	1.4	0.0	2.0	0.4	1.2	1.0	0.7	0.7	9.9
NY-24	9.0	3.3	7.0	4.3	9.9	0.2	30.0	1.8	3.2	7.7	4.3	1.0	0.0	1.6	0.4	0.6	0.8	0.8	3.4	8.8
NY-25	9.4	3.3	6.0	3.6	8.2	0.2	32.6	1.8	4.0	6.8	3.6	1.0	0.0	1.7	0.4	0.6	0.7	0.9	4.8	8.7
NY-26	10.4	4.2	8.0	3.8	16.0	0.4	18.7	2.5	2.5	10.1	4.0	1.0	0.0	0.8	0.8	1.9	1.0	1.4	1.4	8.4
NY-27	9.4	3.2	7.5	5.3	11.4	0.2	28.6	2.0	2.3	7.5	5.5	1.0	0.0	1.8	0.5	0.6	0.9	0.6	1.5	7.6
NY-28	9.4	3.4	6.5	3.6	8.8	0.3	32.4	1.8	3.9	6.7	3.3	0.9	0.0	1.6	0.4	0.7	0.6	1.0	10.1	2.7

NY-29	10.2	3.7	7.6	4.3	8.0	0.4	28.8	1.7	4.8	7.6	2.9	0.7	10.0	1.1	10.4	0.7	10.5	1.4	1.9	11.6
NY-30	11.1	3.5	5.4	4.1	7.3	0.3	30.2	2.0	4.7	6.0	3.0	0.8	10.0	1.7	10.5	0.7	10.7	1.1	1.0	13.7
NY-31	9.6	3.0	5.6	2.1	8.5	0.2	35.4	2.0	3.9	7.1	1.7	1.2	0.0	2.1	10.4	0.9	0.8	0.8	0.8	12.3
NY-32	8.5	3.1	8.0	1.9	9.5	0.3	31.7	1.5	3.3	12.9	1.4	1.0	0.1	1.1	10.3	1.2	0.8	1.2	0.8	9.8
NY-33	10.3	3.8	7.7	6.3	8.1	0.3	24.4	2.0	4.4	7.5	4.8	0.7	0.0	1.1	10.5	0.6	0.6	1.1	2.8	10.7

Table 16. Presence of DHA at the sn-2 position of TAG in the lipid from transgenic *A. thaliana* seeds transformed with Cn11::Moral-LPAAT vector as well as the T-DNA of the GA7 construct, relative to the presence of DHA in TAG. The TAG and sn-2 MAG fatty acid compositions also contained 0-0.4% each of 14:0, 16:1w13t, 16:2, 16:3, 22:0, and 24:0.

Sample	C16:0	16:1d9	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4	C20:1d11	20:1d13	C20:2n6	C20:3n6	C20:4n6	C20:3n3	20:4n3	C22:1	20:5n3	C22:4n6	22:5n6	22:4n3	22:5n3	C22:6n3
22-2-1-1 TAG	12.2	0.4	4.4	6.4	3.9	7.2	0.8	28.8	1.6	4.3	9.7	2.3	0.7	0.1	0.1	0.1	1.3	1.0	0.6	2.1		0.0	0.7	10.1
2-MAG	0.6	0.1	0.3	8.3	2.5	10.1	0.7	53.9	0.2	6.5	0.3	0.1	0.1	0.0	0.0	0.3	0.2	0.0	9.8			0.0	2.3	9.1
DHA at sn-2 = 30%																								
22-2-38-7 oil	10.0	0.2	3.7	6.0	2.7	6.4	0.4	33.8	1.6	3.7	11.3	1.8	0.8				1.3	0.9	0.6	1.2	0.1		0.7	11.6
2-MAG	0.5	0.1	0.3	9.7	2.4	11.1	0.6	60.0	0.1	3.6	0.3	0.1	0.1	0.0	0.0	0.4	0.2	0.0	2.1			0.1	1.3	6.7
DHA at sn-2 = 19%																								
Transformation additionally with gene encoding <i>Mortierella alpina</i> LPAAT																								
NY11-TAG	11.0	0.2	3.4	6.0	2.8	9.2	0.3	34.8	1.6	3.6	6.3	1.8	1.0	0.0	0.0	1.8	0.7	0.6	0.9		0.0	0.1	0.6	12.2
2-MAG	0.7	0.1	0.2	6.7	1.1	11.8	0.3	49.8	0.2	3.7	0.5	1.5	0.3	0.0	0.0	1.5	0.6	0.1	0.8		0.1	0.2	1.5	17.8
DHA at sn-2 = 48%																								
NY-15-oil	11.0	0.0	3.3	4.6	2.8	6.9	0.3	33.6	2.0	5.1	5.5	2.1	0.9	0.0	0.0	1.9	0.7	0.6	0.9	0.1	0.4		0.9	14.9
2-MAG	0.8	0.1	0.3	6.4	1.3	11.4	0.3	50.2	0.2	4.9	0.4	1.4	0.2	0.0	0.0	1.5	0.6	0.1	0.9	0.0	0.0	0.2	1.5	16.7
DHA at sn-2 = 37%																								

Example 8. Further Analysis of Transgenic *Camelina sativa* Seeds**Total lipid content**

C. sativa seed which was homozygous for the T-DNA from the GA7 construct and containing DHA in its total fatty acid content was analysed for its total lipid content and composition as follows. Two consecutive solvent extraction steps were performed on the seeds, firstly using hexane and secondly using chloroform/methanol. No antioxidants were added during the extractions or analysis. The Soxhlet extraction method which is commonly used to extract seed lipids by prolonged heating and refluxing of the lipid/solvent mixture was not used here because of the potential for degradation or oxidation of the ω 3 PUFA such as DHA.

Hexane was used as the solvent in the first extraction since it is the industry standard for oilseeds. Also, it preferentially extracts TAG-containing oil due to its solvating properties and its relatively poor solubilization of polar lipids, particularly at room temperature. Transformed and control *Camelina* seeds (130g and 30g, respectively) were wetted with hexane and crushed using an electric agate mortar and pestle (Retsch Muhle, Germany). The mixtures were transferred to separatory funnels and extracted four times using a total of 800 mL hexane, including an overnight static extraction for the third extraction. For each extraction, extracts were filtered to remove fines through a GFC glass fiber filter under vacuum, and then rotary evaporated at 40°C under vacuum. The extracts were pooled and constituted the TAG-rich hexane extracts.

Following extraction with hexane, the remaining seed meals were further extracted using chloroform-methanol (CM, 1:1 v/v) using the procedure as for the hexane extraction. The meal was then removed by filtration and the combined extracts rotary evaporated. The pooled CM total crude lipid extracts were then dissolved using a one-phase methanol-chloroform-water mix (2:1:0.8 v/v/v). The phases were separated by the addition of chloroform-water (final solvent ratio, 1:1:0.9 v/v/v methanol-chloroform-water). The purified lipid in each extract was partitioned in the lower chloroform phase, concentrated using rotary evaporation and constituted the polar lipid-rich CM extracts. The lipid content in each of these extracts was determined gravimetrically.

For fatty acid compositional analysis, aliquots of the hexane and CM extracts were *trans*-methylated according to the method of Christie et al. (1982) to produce fatty acid methyl esters (FAME) using methanol-chloroform-conc. hydrochloric acid (3mL, 10:1:1, 80°C, 2h). FAME were extracted into hexane-chloroform (4:1, 3 × 1.8mL). Samples of the remaining seed meal (1-2g) after the hexane and CM extractions were also *trans*-methylated to measure any residual lipid as FAME by

gravimetry. The total lipid content of the seeds was calculated by adding the lipid contents of the hexane and CM extracts and the FAME content of the transmethylated meal after solvent extraction.

The transgenic seeds contained slightly less total lipid at 36.2% of seed weight compared to the wild-type *Camelina sativa* seeds at 40.9% of seed weight. For seeds including oilseeds, the total lipid was determined as the sum of solvent extractable lipid obtained by consecutive extractions with hexane, then chloroform-methanol, plus the residual lipid released by transmethylation of the extracted meal after the solvent extractions, as exemplified herein. This total lipid consisted mainly of fatty acid containing lipids such as triacylglycerols and polar lipids and small amounts of non-fatty acid lipids e.g. phytosterols and fatty alcohols which may be present in the free unesterified form or esterified with fatty acids. In addition, any sterol esters or wax esters and hydrocarbons such as carotenoids, for example β -carotene, were also included in the solvent extractable lipid if present. These were included in the overall gravimetric determination and were indicated in the TLC-FID analysis (Table 17).

Of the total lipid, 31%–38% of lipid per seed weight was extracted by hexane for the transgenic and control seeds, respectively, which accounted for 86% and 92% of the total lipid in the seeds. The CM extraction recovered a further 4.8% and 2.4% (of seed weight) mostly polar lipid-rich extract from the transgenic and control seeds, respectively. The residual lipid released by transmethylation of the remaining solvent extracted oilseed meal was 0.3% and 0.4% of seed weight, respectively. That is, the first and second solvent extractions together extracted 99% of the total lipid content of the seeds (i.e. of the 36.2% or 40.9% of the seed weight, which was mostly fatty acid containing lipid such as triglycerides and polar lipids consisting of glyco- and phospholipids (see next section- Lipid class analysis)).

Lipid class analysis

Lipid classes in the hexane and CM extracts were analyzed by thin-layer chromatography with flame-ionization detection (TLC-FID; Iatroscan Mark V, Iatron Laboratories, Tokyo, Japan) using hexane/diethyl ether/glacial acetic acid (70:10:0.1, v/v/v) as the developing solvent system in combination with Chromarod S-III silica on quartz rods. Suitable calibration curves were prepared using representative standards obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA). Data were processed using SIC-480II software (SISC Version: 7.0-E). Phospholipid species were separated by applying the purified phospholipid fraction obtained from silica column

chromatography and developing the rods in chloroform/methanol/glacial acetic acid/water (85:17:5:2, v/v/v) prior to FID detection.

To separate TAG, glycolipid and phospholipid fractions from the CM extracts, silica gel 60 (100–200 mesh) (0.3–1 g) in a short glass column or Pasteur pipette plugged with glass wool was used to purify 10 mg of the purified CM lipid extract. The residual TAG fraction in the CM extract was eluted using 20 mL of 10% diethyl ether in hexane, the glycolipids eluted with 20 mL of acetone and the phospholipids eluted in two steps, first 10 mL of methanol then 10 mL of methanol-chloroform-water (5:3:2). This second elution increased the recovery of phospholipids. The yield of each fraction was determined gravimetrically and the purity checked by TLC-FID. All extracts and fractions were stored in dichloromethane at –20°C until further analysis by GC and GC-MS.

The TAG-rich hexane extracts from each of the transgenic and control seeds contained about 96% TAG. The CM extracts contained residual TAG amounting to 44% and 13% by weight of the CM extracts, respectively, for the transgenic and wild-type seeds. In contrast to the hexane extracts, the CM extracts were rich in polar lipids, namely phospholipids and glycolipids, amounting to 50% and 76% by weight of the CM extracts, respectively, for the transgenic and control seeds (Table 17). The main phospholipid was phosphatidyl choline (PC) and accounted for 70%–79% of the total phospholipids followed by phosphatidyl ethanolamine (PE, 7%–13%) with relatively low levels of phosphatidic acid (PA, 2%–5%) and phosphatidyl serine (PS, <2%).

Fatty acid composition

Generally for seeds producing DHA and/or DPA, the inventors observed that the fatty acid composition of the total lipids in the seeds as determined by direct transmethylation of all of the lipid in the seed was similar to that of the TAG fraction. This was because more than 90% of the total lipids present in the seed occurred in the form of TAG.

The fatty acid composition of the different lipid classes in the hexane and CM extracts was determined by gas chromatography (GC) and GC-MS analysis using an Agilent Technologies 6890A GC instrument (Palo Alto, CA, USA) fitted with a Supelco Equity™-1 fused silica capillary column (15 m × 0.1 mm i.d., 0.1 µm film thickness, Bellefont, PA, USA), an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was the carrier gas. Samples were injected in split-less mode at an oven temperature of 120°C. After

injection, the oven temperature was raised to 270°C at 10°C min⁻¹ and finally to 300°C at 5°C min⁻¹. Eluted compounds were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA). GC results were subject to an error of not more than ±5% of individual component areas.

5

Table 17. Lipid class composition (% of total lipid obtained for each extraction step) of hexane and CM extracts from transgenic and control *Camelina sativa* seeds. SE, WE and HC were not separated from each other.

Lipid class	Transgenic seeds		Control seeds	
	Hexane	CM	Hexane	CM
SE/WE/HC*	1.0	1.4	1.0	1.4
TAG	95.6	44.2	96.0	13.1
FFA	0.9	1.3	0.8	1.4
UN**	0.9	1.1	0.8	1.2
ST	0.5	0.7	0.4	0.4
MAG	0.7	1.1	0.8	6.2
PL	0.3	50.3	0.3	76.3
Total	100.0	100.0	100.0	100.0

Abbreviations: sterol esters (SE), wax esters (WE), hydrocarbons (HC), triacylglycerols (TAG), free fatty acids (FFA), unknown (UN), sterols (ST), monoacylglycerols (MAG), polar lipids (PL) consisting of glycolipids and phospholipids; * SE, WE and HC co-elute with this system; ** May contain fatty alcohols and diacylglycerols (DAG).

GC-mass spectrometric (GC-MS) analyses were performed on a Finnigan Trace ultra Quadrupole GC-MS (model: ThermoQuest Trace DSQ, Thermo Electron Corporation). Data were processed with ThermoQuest Xcalibur software (Austin, TX, USA). The GC was fitted with an on-column injector and a capillary HP-5 Ultra Agilent J & W column (50m × 0.32mm i.d., 0.17µm film thickness, Agilent Technologies, Santa Clara, CA, USA) of similar polarity to that described above. Individual components were identified using mass spectral data and by comparing retention time data with those obtained for authentic and laboratory standards. A full procedural blank analysis was performed concurrent to the sample batch.

The data for the fatty acid composition in the different lipid classes in the extracts are shown in Table 18. In the DHA-producing *Camelina* seed, the DHA was distributed in the major lipid fractions (TAG, phospholipids and glycolipids) at a proportion ranging between 1.6% and 6.8% with an inverse relationship between the

proportions of DHA and ALA. The TAG-rich hexane extract from the transgenic seed contained 6.8% DHA and 41% ALA (Table 18). The polar lipid-rich CM extract contained 4.2% DHA and 50% ALA i.e. relatively less DHA and more ALA. Residual TAG from the polar lipid-rich CM extract contained 6% DHA and 40% ALA. The glycolipid fraction isolated from the CM extract contained 3% DHA and 39% ALA and the phospholipid fraction contained the lowest level of DHA (1.6%) and the highest levels of ALA (54%). The transgenic *Camelina* seed contained higher levels of ALA and lower levels of LA (linoleic acid, 18:2 ω 6) compared with the control seeds in the major lipid classes (TAG, glycolipids and phospholipids). The proportions of ALA and LA were: ALA 39%–54% and LA 4%–9% for transgenic seeds and ALA 12%–32% and LA 20%–29% for control seeds. The relative level of erucic acid (22:1 ω 9) was lower in all fractions in the transgenic seeds than in the control seeds, for example, in the hexane extracts 1.3% versus 2.7% (Table 18).

15 Sterol composition in the seeds

To determine the sterol content and composition in the extracted lipids, samples of approximately 10 mg total lipid from the TAG-rich hexane extract and the polar lipid-rich CM extract were saponified using 4 mL 5% KOH in 80% MeOH and heated for 2h at 80°C in a Teflon-lined screw-capped glass test tube. After the reaction mixtures were cooled, 2mL of Milli-Q water was added and the sterols and alcohols were extracted three times into 2mL of hexane:dichloromethane (4:1, v/v) by shaking and vortexing. The mixtures were centrifuged and each extract in the organic phase was washed with 2mL of Milli-Q water by shaking and centrifugation. After taking off the top sterol-containing organic layer, the solvent was evaporated using a stream of nitrogen gas and the sterols and alcohols silylated using 200 μ L of Bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma-Aldrich) by heating for 2h at 80°C in a sealed GC vial. By this method, free hydroxyl groups were converted to their trimethylsilyl ethers. The sterol- and alcohol-OTMSi derivatives were dried under a stream of nitrogen gas on a heating block (40°C) and re-dissolved in dichloromethane (DCM) immediately prior to GC/GC-MS analysis as described above.

Table 18. Fatty acid composition (% of total fatty acids) of lipid extracts and fractions of transgenic and control *C. sativa* seeds.

Transgenic seeds							Control seeds					
	Hexane	CM				Meal	Hexane	CM				Meal
	TAG	Total	TAG	GL	PL	Residue	TAG	Total	TAG	GL	PL	Residue
Fatty acid												
16:1ω7	0.1	0.2	0.1	0.2	0.1	0.2	0.1	0.2	0.2	-	-	0.3
16:0	6.2	12.8	6.8	21.3	19.4	10.4	6.7	12.8	7.8	29.6	13.7	10.3
18:4ω3	3.7	3.3	3.4	2.1	2.9	3.6	-	-	-	-	-	-
18:2ω6	7.1	3.9	8.8	7.2	3.7	8.8	22.2	28.4	29.4	20.8	29.3	27.9
18:3ω3	41.9	50.3	39.9	38.6	54.1	38.9	32.0	20.6	19.7	13.0	12.3	20.0
18:1ω9	11.1	4.7	9.6	7.2	2.8	8.1	14.0	25.4	13.3	14.7	35.7	14.3
18:1ω7	1.4	2.3	2.1	3.7	3.4	2.8	1.0	1.5	2.2	4.0	2.8	2.2
18:0	3.2	4.0	3.0	4.5	5.7	3.1	3.0	2.7	2.9	5.7	3.6	2.7
20:5ω3	0.4	0.2	0.3	-	-	0.3	-	-	-	-	-	-
20:4ω3	0.4	0.4	0.4	-	0.2	0.3	-	-	-	-	-	-
20:2ω6	0.7	0.7	0.8	0.6	0.4	0.7	1.8	0.8	2.1	1.2	-	1.8
20:3ω3	0.8	1.2	0.9	0.6	1.3	0.5	0.9	0.3	-	-	-	0.4

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20:1 ω 9/11	11.6	6.1	10.9	5.1	1.3	8.4	12.5	3.0	11.1	4.2	1.7	9.4
20:1 ω 7	0.6	0.8	1.4	0.6	0.2	1.1	0.6	0.6	2.0	1.3	-	1.8
20:0	1.3	0.8	1.4	0.6	0.1	1.4	1.5	0.7	2.0	1.4	-	1.8
22:6 ω 3	6.8	4.2	6.1	3.0	1.6	5.4	-	-	-	-	-	-
22:5 ω 3	0.3	1.1	0.4	0.6	1.4	0.3	-	-	-	-	-	-
22:1 ω 9	1.3	1.0	1.8	0.6	0.1	1.5	2.7	0.7	3.6	0.9	-	2.9
22:0	0.3	0.2	0.3	0.6	0.1	0.7	0.3	0.2	0.7	0.8	-	0.8
24:1 ω 9	0.3	0.4	0.4	0.6	0.3	0.6	0.3	0.6	0.7	0.9	0.5	1.0
24:0	0.1	0.4	0.2	0.9	0.4	1.1	0.1	0.4	0.5	1.4	0.4	1.3
others [*]	0.4	1.0	1.0	1.4	0.5	1.8	0.3	1.1	0.9	0.1	-	1.1
Total	100	100	100	100	100	100	100	100	100	100	100	100

Abbreviations: triacylglycerols (TAG), glycolipids (GL), phospholipids (PL); Total: polar lipid-rich extract containing GL and PL from CM extraction; TAG, GL and PL were separated by silica column chromatography of the CM extracts; ^{*} Sum of minor fatty acids

The major sterols in both the transgenic and control seeds were 24-ethylcholesterol (sitosterol, 43%–54% of the total sterols), 24-methylcholesterol (campesterol, 20%–26%) with lower levels of cholesterol (5%–8%), brassicasterol (2%–7%), isofucosterol ($\Delta 5$ -avenasterol, 4%–6%), stigmasterol (0.5%–3%), cholest-7-en-3 β -ol, (0.2%–0.5%), 24-methylcholestanol (campestanol, 0.4%–1%) and 24-dehydrocholesterol (0.5%–2%) (Table 19). These nine sterols accounted for 86%–95% of the total sterols, with the remaining components being sterols only partially identified for the numbers of carbons and double bonds. The overall sterol profiles were similar between the transgenic and control seeds for both the hexane and CM extracts.

Fatty alcohol analysis

Fatty alcohols in the extracts were derivatised and analysed as for the sterols. A series of fatty alcohols from C₁₆–C₂₂, with accompanying iso-branched fatty alcohols, were identified in both the hexane and CM extracts. Similar profiles were observed for the transgenic and control seeds, with some variation in the proportions of individual components observed. Phytol, derived from chlorophyll, was the major aliphatic alcohol and accounted for 47% and 37% of the total fatty alcohols in the hexane fractions in the transgenic and control seeds, respectively. The odd-chain alcohols were present at higher levels in the CM extract (37%–38% of the total fatty alcohol content) than in the hexane extract (16%–23%). Iso-17:0 (16%–38%) predominated over 17:0 (0.3%–5.7%). Another odd-chain alcohol present was 19:0 (4.5%–6.5%). Other alcohols detected included iso-16:0, 16:0, iso-18:0, 18:1, 18:0, with minor levels of iso-20:0, 20:1, 20:0, iso-22:0, 22:1 and 22:0 also present.

Discussion

The results indicated that crushing using a motorized mortar and pestle with multiple extractions with hexane at room temperature was effective in recovering most of the TAG-containing oil from the transgenic seeds. In addition to the oil from the transgenic seeds containing moderate levels of DHA, the transgenic seeds also had markedly higher levels of ALA in the major lipid classes (triacylglycerols, glycolipids and phospholipids) compared with the control seeds. This showed that the $\Delta 15$ -desaturase activity was considerably enhanced in the transgenic seeds during seed development. Interestingly, there were some slight differences in the fatty acid composition and proportion of DHA in the various extracts and fractions with the DHA levels being higher in the TAG-rich hexane extract and TAG from CM extraction (6%–

6.8%) and lower in the polar lipid fractions (3% in glycolipids and 1.6% in phospholipids). The level of 16:0 was higher in the polar lipid fractions of glycolipids and phospholipids in the CM extracts (19%–21%) compared with the TAG-rich hexane extract and TAG from CM extraction (6%–7%).

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Table 19. Sterol composition (% of total sterols) of transgenic and control *Camelina* seeds.

Sterols	Transgenic seeds		Control seeds	
	Hexane	CM	Hexane	CM
24-dehydrocholesterol	0.8	1.8	0.5	1.4
cholesterol	5.7	7.6	4.7	7.2
brassicasterol	4.4	6.5	1.9	4.2
cholest-7-en-3 β -ol	0.2	0.5	0.3	0.4
campesterol	24.5	20.8	25.7	21.7
campestanol	0.4	1.1	0.4	0.9
stigmasterol	1.0	2.6	0.5	1.6
sitosterol	54.3	43.7	53.8	42.9
Δ 5-avenasterol (isofucosterol)	4.2	5.2	4.7	5.5
Sum	95.5	89.6	92.6	85.9
Others				
UN1 C28 1db	0.6	1.2	0.7	1.2
UN2 C29 1db	1.2	2.0	1.2	2.4
UN3 C29 2db	0.9	1.8	1.3	2.4
UN4 C28 1db	0.3	0.9	0.6	1.1
UN5 C30 2db	1.2	1.8	1.4	1.8
UN6 C29 1db + C30 2db	0.3	2.7	2.2	5.2
Sum of others	4.5	10.4	7.4	14.1
Total	100	100	100	100

Abbreviations: UN denotes unknown sterol, the number after C indicates the number of carbon atoms and db denotes number of double bonds

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The sterol composition of the transgenic seeds and control seeds were similar to that found in refined Camelina oil (Shukla et al., 2002) with the same major sterols present, indicating that the added genes did not affect sterol synthesis in the seeds. The level of cholesterol in Camelina oil was higher than occurred in most vegetable oils. Brassicasterol was present, which is a characteristic sterol found in the Brassicaceae family which includes *Camelina sativa*.

Example 9. Production of LC-PUFA in *Brassica juncea* Seeds

Transgenic *Brassica juncea* plants were produced using the GA7-modB construct (Example 4) for the production of DHA, as follows. *B. juncea* seeds of a long-daylength sensitive variety were sterilized using chlorine gas as described by Kereszt et al. (2007). Sterilized seeds were germinated on 1/2 strength MS media (Murashige and Skoog, 1962) solidified with 0.8% agar, adjusted to pH 5.8 and grown at 24°C under fluorescent lighting (50 $\mu\text{E}/\text{m}^2\text{s}$) with a 16/8 hour (light/dark) photoperiod for 6-7 days. Cotyledonary petioles with 2-4 mm stalk were isolated aseptically from these seedlings and used as explants. *Agrobacterium tumefaciens* strain AGL1 was transformed with the binary construct GA7. *Agrobacterium* culture was initiated and processed for infection as described by Belide et al. (2013). For all transformations, about 50 freshly-isolated cotyledonary petioles were infected with 10ml of *A. tumefaciens* culture for 6 minutes. The infected petioles were blotted on sterile filter paper to remove excess *A. tumefaciens* and transferred to co-cultivation media (MS containing 1.5mg/L BA, 0.01mg/L NAA and 100 μM acetosyringone, also supplemented with L-cysteine (50mg/L), ascorbic acid (15mg/L) and MES (250mg/L). All plates were sealed with micropore tape and incubated in the dark at 24°C for 48 hours of co-cultivation. The explants were then transferred to pre-selection medium (MS-agar containing 1.5mg/L BA, 0.01mg/L NAA, 3mg/L AgNO_3 , 250mg/L cefotaxime and 50mg/L timentin) and cultured for 4-5 days at 24°C with a 16/8 hour photoperiod before the explants were transferred to selection medium (MS-agar containing 1.5mg/L BA, 0.01mg/L NAA, 3mg/L AgNO_3 , 250mg/L cefotaxime, 50mg/L timentin and 5mg/L PPT) and cultured for 4 weeks at 24°C with 16/8 hour photoperiod. Explants with green callus were transferred to shoot regeneration medium (MS-agar containing 2.0 mg/L BA, 3mg/L AgNO_3 , 250mg/L cefotaxime, 50mg/L timentin and 5mg/L PPT) and cultured for another 2 weeks. Small regenerating shoot buds were transferred to hormone free MS medium (MS-agar containing 3mg/L AgNO_3 , 250mg/L cefotaxime, 50mg/L timentin and 5mg/L PPT) and cultured for another 2-3 weeks.

Potential transgenic shoots of at least 1.5cm in size were isolated and transferred to root induction medium (MS-agar containing 0.5mg/L NAA, 3mg/L AgNO₃, 250mg/L cefotaxime and 50mg/L timentin) and cultured for 2-3 weeks. Transgenic shoots confirmed by PCR and having prolific roots were transferred to soil in a greenhouse and grown under a photoperiod of 16/8 h (light/dark) at 22°C. Three confirmed transgenic plants were obtained. The transformed plants were grown in the greenhouse, allowed to self-fertilise, and T1 seed harvested. The fatty acid composition was analysed of the lipid from pools of T1 seeds from each T0 transformed plants, which showed the presence of 2.8% DPA and 7.2% DHA in one line designated JT1-4, whereas another line designated JT1-6 exhibited 2.6% DPA.

Seedoil from individual T1 seeds was analysed for fatty acid composition; some of the data is shown in Table 20. Several T1 seeds produced DHA at a level of 10% to about 21% of the total fatty acid content, including JT1-4-A-13, JT1-4-A -5, and JT1-4-B-13. Surprisingly and unexpectedly, some of the T1 seeds contained DPA at levels of 10% to about 18% of the total fatty acid content and no detectable DHA (<0.1%). One possible explanation for these seeds is that the $\Delta 4$ -desaturase gene in the T-DNA inserted in these plants was inactivated, perhaps through a spontaneous mutation. T1 seeds were germinated and one emerged cotyledon from each analysed for fatty acid composition in the remaining oil. The remainder of each seedling was maintained and grown to maturity to provide T2 seed.

Transgenic plants which were homozygous for single T-DNA insertions were identified and selected. Plants of one selected line designated JT1-4-17 had a single T-DNA insertion and produced DHA with only low levels of DPA, whereas those of a second selected line designated JT1-4-34 also had a single T-DNA insertion but produced DPA without producing DHA. The inventors concluded that the original transformant contained two separate T-DNAs, one which conferred production of DHA and the other which conferred production of DPA without DHA. The *B. juncea* plants producing DHA in their seeds were crossed with the plants producing DPA in their seeds. The F1 progeny included plants which were heterozygous for both of the T-DNA insertions. Seed from these progeny plants were observed to produce about 20% DHA and about 6% DPA, for a total DHA + DPA content of 26%. The F1 plants are self-fertilised and progeny which are homozygous for both of the T-DNA insertions are expected to produce up to 35% DHA and DPA.

About 18% DPA was observed in the lipid of pooled seed of the T3 progeny designated JT1-4-34-11. Similarly about 17.5% DHA was observed in the lipid from pooled seed in the progeny of T3 JT1-4-17-20. Fatty acid compositions of JT1-4 T1

pooled seed, T1 single seed, T2 pooled seed, T2 single seed, and T3 pooled seed, T3 single seed are in Tables 21 to 24. JT1-4 T3 segregant JT-1-4-34-11, had a pooled T3 seed DPA content of 18% and the single seed from this particular segregant had a DPA content of about 26%, each as a percentage of the total fatty acid content.

5 The following parameters were calculated for oil from a seed having 17.9% DPA: total saturated fatty acids, 6.8%; total monounsaturated fatty acids, 36.7%; total polyunsaturated fatty acids, 56.6%, total ω 6 fatty acids, 7.1%; new ω 6 fatty acids, 0.4% of which all was GLA; total ω 3 fatty acids, 46.5%; new ω 3 fatty acids, 24.0%; ratio of total ω 6: total ω 3 fatty acids, 6.5; ratio of new ω 6: new ω 3 fatty acids, 60; the
10 efficiency of conversion of oleic acid to LA by Δ 12-desaturase, 61%; the efficiency of conversion of ALA to SDA by Δ 6-desaturase, 51%; the efficiency of conversion of SDA to ETA acid by Δ 6-elongase, 90%; the efficiency of conversion of ETA to EPA by Δ 5-desaturase, 87%; the efficiency of conversion of EPA to DPA by Δ 5-elongase, 98%.

15 In order to produce more transgenic plants in *B. juncea* with the modB construct, the transformation was repeated five times and 35 presumed transgenic shoots/seedlings were regenerated. T1 seed analysis is carried out to determine DPA and DHA content.

20 In order to produce further seed containing DPA and no DHA, the Δ 4-desaturase gene was deleted from the modB construct and the resultant construct used to transform *B. juncea* and *B. napus*. Progeny seed with up to 35% DPA in the total fatty acid content of the seed lipid are produced.

25 When the oil extracted from the seeds of a plant producing DHA was examined by NMR, at least 95% of the DHA was observed to be present at the *sn*-1,3 position of the TAG molecules.

Table 20. Fatty acid composition of seedoil from T1 seeds of *B. juncea* transformed with the T-DNA from GA7.

T1 seed No.	C16:0	16:1d9	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4n3	C20:1d11	C20:2n6	C20:3n3	20:4n3	20:5n3	22:5n3	C22:6n3
JT1-4-A-1	5.0	0.2	2.7	23.5	3.4	17.0	0.7	24.8	0.7	2.0	1.1	0.2	0.8	4.0	0.6	2.4	9.9
JT1-4-A-2	4.3	0.3	2.6	37.2	3.2	11.0	0.3	22.1	0.7	0.9	1.3	0.2	1.4	3.2	0.3	9.4	0.0
JT1-4-A-3	5.6	0.3	2.7	20.8	3.7	16.0	0.6	24.4	0.7	2.0	0.9	0.2	1.1	4.5	0.7	3.1	11.4
JT1-4-A-4	4.6	0.4	2.8	36.2	3.4	10.6	0.3	24.5	0.8	9.9	1.7	0.2	0.3	0.5	0.0	2.5	0.0
JT1-4-A-5	5.0	0.2	3.2	20.3	3.6	13.7	0.7	25.9	0.7	2.0	0.9	0.2	1.3	4.4	1.5	1.6	13.5
JT1-4-A-6	4.8	0.4	3.4	37.9	3.7	7.4	0.4	19.9	0.9	1.4	1.4	0.1	0.8	1.9	0.4	13.9	0.0
JT1-4-A-7	5.6	0.3	3.0	26.2	4.0	8.9	0.3	26.6	0.6	1.8	1.0	0.1	1.8	3.7	1.3	2.2	11.3
JT1-4-A-8	4.8	0.4	2.9	40.3	3.4	7.8	0.3	22.2	0.8	1.4	1.3	0.1	0.8	2.4	0.4	9.6	0.0
JT1-4-A-9	7.1	0.3	3.6	17.7	4.3	17.9	0.7	23.1	1.0	2.1	0.8	0.2	1.5	3.6	0.8	2.0	11.9
JT1-4-A-10	5.1	0.2	4.2	22.3	3.4	19.5	0.7	21.7	0.8	1.5	0.9	0.2	1.7	7.8	0.9	1.0	6.5
JT1-4-A-11	5.0	0.5	2.8	37.6	4.0	7.1	0.4	19.2	0.7	1.9	1.4	0.2	0.5	1.6	0.3	15.5	0.0
JT1-4-A-12	5.2	0.3	3.0	28.2	4.0	9.2	0.3	27.4	0.6	1.9	0.9	0.1	1.5	3.2	1.1	1.8	10.2
JT1-4-A-13	5.4	0.2	3.0	16.7	4.1	9.9	0.6	29.9	0.7	2.2	1.0	0.2	1.7	2.0	1.1	2.0	17.9
JT1-4-A-14	5.1	0.4	3.1	30.0	4.0	11.5	0.3	27.7	0.7	2.2	1.0	0.1	0.6	2.4	0.8	1.3	7.8
JT1-4-A-15	5.1	0.4	2.5	34.2	3.6	6.9	0.6	20.4	0.7	1.6	1.1	0.2	0.6	4.7	0.9	15.2	0.0
JT1-4-B-1	5.5	0.2	2.7	18.9	4.0	17.6	0.8	24.1	0.8	2.2	1.0	0.2	1.2	4.6	0.9	2.2	11.5
JT1-4-B-2	5.5	0.2	2.7	20.2	4.0	14.3	0.5	25.5	0.7	1.7	0.9	0.2	1.6	8.7	1.3	2.2	8.5
JT1-4-B-3	5.3	0.3	3.6	34.1	3.5	35.0	0.6	9.3	0.8	0.2	1.4	0.4	0.6	0.9	0.1	0.3	2.1
JT1-4-B-4	5.3	0.3	3.1	25.2	3.6	17.0	0.7	24.1	0.7	1.9	1.0	0.2	0.8	4.3	0.5	2.3	7.8
JT1-4-B-5	5.5	0.5	2.2	30.1	4.6	10.2	0.5	21.7	0.6	1.4	1.1	0.2	0.9	2.4	0.5	16.1	0.0
JT1-4-B-6	5.6	0.3	2.5	19.5	3.8	15.2	0.5	27.7	0.6	2.1	0.9	0.2	1.1	3.7	0.6	3.3	11.1
JT1-4-B-7	5.9	0.5	2.0	29.9	4.0	11.2	0.3	26.2	0.6	11.5	1.4	0.2	0.3	0.4	0.0	4.1	0.1
JT1-4-B-8	6.2	0.5	1.9	33.1	4.0	30.0	0.5	12.7	0.6	0.3	1.3	0.4	1.4	0.9	0.1	4.4	0.0
JT1-4-B-9	4.9	0.2	3.4	24.6	3.0	18.5	0.3	26.2	0.8	1.3	1.1	0.2	2.0	5.5	0.6	0.8	5.2

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JT1-4-B-10	5.2	0.3	2.7	19.0	4.0	12.0	0.6	30.5	0.7	1.6	1.0	0.2	1.7	4.9	1.1	3.0	10.2
JT1-4-B-11	4.8	0.2	3.0	23.7	3.1	18.1	0.6	23.5	0.7	1.6	1.2	0.2	1.5	4.5	0.8	1.6	9.6
JT1-4-B-12	5.0	0.2	2.6	19.6	3.4	12.5	0.6	26.9	0.8	3.1	1.1	0.2	0.9	5.6	0.9	3.5	11.7
JT1-4-B-13	5.6	0.3	2.8	20.9	3.9	11.9	0.4	27.0	0.7	2.0	1.0	0.2	1.7	2.3	0.7	4.1	13.5
JT1-4-B-14	5.1	0.3	3.1	25.5	3.3	16.7	0.7	23.9	0.8	1.8	1.2	0.2	0.9	2.6	0.4	2.9	9.2
JT1-4-B-15	5.6	0.3	2.7	19.5	4.1	14.0	0.8	24.6	0.7	2.7	0.9	0.2	0.7	9.4	1.3	2.5	8.5

The seedoil samples also contained 0.1% C14:0; 0.1-0.2% C16:3; 0.0-0.1% of each of C20:1Δ13, C20:3ω6 and C20:4ω6; 0.3-0.4% C22:0; no C22:1 and C22:2ω6; 0.2% C24:0 and 0.2-0.4% C24:1.

Table 21. Fatty acid composition of lipid from T1 seeds (pooled) of *B. juncea* transformed with the T-DNA from GA7-modB. The lipids also contained about 0.1% of each of 14:0, 16:3, 20:1d13, and 16:2, 22:1 were not detected.

	C16:0	C16:1	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4n3	C20:1d11	C20:2n6	C20:3n6	C20:4n6	C20:3n3	C22:0	20:4n3	20:5n3	C22:2n6	22:3n3	C24:0	C24:1	22:5n3	C22:6n3
JT1-2	4.2	0.3	2.5	42.4	3.2	27.7	0.1	16.4	0.6	0.0	1.2	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.2	0.4	0.0	0.0
JT1-3	4.5	0.3	2.7	44.5	3.1	26.8	0.1	14.8	0.7	0.0	1.2	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.2	0.4	0.0	0.0
JT1-4	5.1	0.3	3.2	26.8	3.5	17.4	0.5	22.8	0.7	2.5	1.1	0.2	0.0	0.0	1.2	0.3	2.9	0.7	0.0	0.1	0.2	0.3	2.8	7.2
JT1-5	4.7	0.4	2.4	41.6	3.4	28.4	0.1	15.8	0.7	0.0	1.2	0.1	0.0	0.0	0.0	0.3	0.0	0.0	0.0	0.0	0.2	0.4	0.0	0.0
JT1-6	4.8	0.4	2.3	37.3	3.3	30.2	0.4	13.2	0.7	0.2	1.4	0.3	0.0	0.0	0.7	0.3	0.6	0.1	0.0	0.3	0.2	0.5	2.6	0.0

Table 22. Fatty acid composition of seed oil from T1(single) seeds of *B. juncea* transformed with the T-DNA from GA7-modB.

T1 seed No.	C16:0	16:1d9	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4n3	C20:1d11	C20:2n6	C20:3n3	20:4n3	20:5n3	22:5n3	C22:6n3
JT1-4-A-1	5.0	0.2	2.7	23.5	3.4	17.0	0.7	24.8	0.7	2.0	1.1	0.2	0.8	4.0	0.6	2.4	9.9
JT1-4-A-2	4.3	0.3	2.6	37.2	3.2	11.0	0.3	22.1	0.7	0.9	1.3	0.2	1.4	3.2	0.3	9.4	0.0
JT1-4-A-3	5.6	0.3	2.7	20.8	3.7	16.0	0.6	24.4	0.7	2.0	0.9	0.2	1.1	4.5	0.7	3.1	11.4
JT1-4-A-4	4.6	0.4	2.8	36.2	3.4	10.6	0.3	24.5	0.8	9.9	1.7	0.2	0.3	0.5	0.0	2.5	0.0
JT1-4-A-5	5.0	0.2	3.2	20.3	3.6	13.7	0.7	25.9	0.7	2.0	0.9	0.2	1.3	4.4	1.5	1.6	13.5

JT1-4-A-6	4.8	0.4	3.4	37.9	3.7	7.4	0.4	19.9	0.9	1.4	1.4	0.1	0.8	1.9	0.4	13.9	0.0
JT1-4-A-7	5.6	0.3	3.0	26.2	4.0	8.9	0.3	26.6	0.6	1.8	1.0	0.1	1.8	3.7	1.3	2.2	11.3
JT1-4-A-8	4.8	0.4	2.9	40.3	3.4	7.8	0.3	22.2	0.8	1.4	1.3	0.1	0.8	2.4	0.4	9.6	0.0
JT1-4-A-9	7.1	0.3	3.6	17.7	4.3	17.9	0.7	23.1	1.0	2.1	0.8	0.2	1.5	3.6	0.8	2.0	11.9
JT1-4-A-10	5.1	0.2	4.2	22.3	3.4	19.5	0.7	21.7	0.8	1.5	0.9	0.2	1.7	7.8	0.9	1.0	6.5
JT1-4-A-11	5.0	0.5	2.8	37.6	4.0	7.1	0.4	19.2	0.7	1.9	1.4	0.2	0.5	1.6	0.3	15.5	0.0
JT1-4-A-12	5.2	0.3	3.0	28.2	4.0	9.2	0.3	27.4	0.6	1.9	0.9	0.1	1.5	3.2	1.1	1.8	10.2
JT1-4-A-13	5.4	0.2	3.0	16.7	4.1	9.9	0.6	29.9	0.7	2.2	1.0	0.2	1.7	2.0	1.1	2.0	17.9
JT1-4-A-14	5.1	0.4	3.1	30.0	4.0	11.5	0.3	27.7	0.7	2.2	1.0	0.1	0.6	2.4	0.8	1.3	7.8
JT1-4-A-15	5.1	0.4	2.5	34.2	3.6	6.9	0.6	20.4	0.7	1.6	1.1	0.2	0.6	4.7	0.9	15.2	0.0
JT1-4-B-1	5.5	0.2	2.7	18.9	4.0	17.6	0.8	24.1	0.8	2.2	1.0	0.2	1.2	4.6	0.9	2.2	11.5
JT1-4-B-2	5.5	0.2	2.7	20.2	4.0	14.3	0.5	25.5	0.7	1.7	0.9	0.2	1.6	8.7	1.3	2.2	8.5
JT1-4-B-3	5.3	0.3	3.6	34.1	3.5	35.0	0.6	9.3	0.8	0.2	1.4	0.4	0.6	0.9	0.1	0.3	2.1
JT1-4-B-4	5.3	0.3	3.1	25.2	3.6	17.0	0.7	24.1	0.7	1.9	1.0	0.2	0.8	4.3	0.5	2.3	7.8
JT1-4-B-5	5.5	0.5	2.2	30.1	4.6	10.2	0.5	21.7	0.6	1.4	1.1	0.2	0.9	2.4	0.5	16.1	0.0
JT1-4-B-6	5.6	0.3	2.5	19.5	3.8	15.2	0.5	27.7	0.6	2.1	0.9	0.2	1.1	3.7	0.6	3.3	11.1
JT1-4-B-7	5.9	0.5	2.0	29.9	4.0	11.2	0.3	26.2	0.6	11.5	1.4	0.2	0.3	0.4	0.0	4.1	0.1
JT1-4-B-8	6.2	0.5	1.9	33.1	4.0	30.0	0.5	12.7	0.6	0.3	1.3	0.4	1.4	0.9	0.1	4.4	0.0
JT1-4-B-9	4.9	0.2	3.4	24.6	3.0	18.5	0.3	26.2	0.8	1.3	1.1	0.2	2.0	5.5	0.6	0.8	5.2
JT1-4-B-10	5.2	0.3	2.7	19.0	4.0	12.0	0.6	30.5	0.7	1.6	1.0	0.2	1.7	4.9	1.1	3.0	10.2
JT1-4-B-11	4.8	0.2	3.0	23.7	3.1	18.1	0.6	23.5	0.7	1.6	1.2	0.2	1.5	4.5	0.8	1.6	9.6
JT1-4-B-12	5.0	0.2	2.6	19.6	3.4	12.5	0.6	26.9	0.8	3.1	1.1	0.2	0.9	5.6	0.9	3.5	11.7
JT1-4-B-13	5.6	0.3	2.8	20.9	3.9	11.9	0.4	27.0	0.7	2.0	1.0	0.2	1.7	2.3	0.7	4.1	13.5
JT1-4-B-14	5.1	0.3	3.1	25.5	3.3	16.7	0.7	23.9	0.8	1.8	1.2	0.2	0.9	2.6	0.4	2.9	9.2
JT1-4-B-15	5.6	0.3	2.7	19.5	4.1	14.0	0.8	24.6	0.7	2.7	0.9	0.2	0.7	9.4	1.3	2.5	8.5

The seed oil samples also contained 0.1% C14:0; 0.1-0.2% C16:3; 0.0-0.1% of each of C20:1Δ13, C20:3Δ6 and C20:4Δ6; 0.3-0.4% C22:0; no C22:1 and C22:2Δ6; 0.2% C24:0 and 0.2-0.4% C24:1.

Table 23. Fatty acid composition of seed oil from T2 single seeds of *B. juncea* transformed with the T-DNA from GA7-modB.

Code	C16:0	16:1d9	16:3	C18:0	C18:1	C18:1d11	C18:2	C18:3n6	C18:3n3	C20:0	18:4	C20:1d11	C20:2n6	C20:3n6	C20:4n6	C20:3n3	C22:0	20:4n3	20:5n3	C22:2n6	C22:3n3	C24:0	22:5n6	22:4n3	C24:1	22:5n3	C22:6n3
JT-1-4-19	4.4	0.3	0.2	1.7	36.3	2.9	8.3	0.5	22.0	0.5	1.4	1.2	0.1	0.0	0.0	0.4	0.3	4.2	0.6	0.0	0.1	0.1	0.0	1.8	0.3	12.1	0.0
JT-1-4-19	5.6	0.4	0.1	1.9	39.1	3.1	8.4	0.4	18.9	0.8	1.2	1.3	0.1	0.0	0.0	0.5	0.3	2.5	0.4	0.0	0.1	0.2	0.0	1.5	0.4	12.6	0.0
JT-1-4-19	5.5	0.4	0.2	1.8	42.3	3.2	9.9	0.3	24.0	0.6	5.9	1.5	0.2	0.0	0.0	0.2	0.4	0.5	0.0	0.0	0.0	0.2	0.0	0.4	0.4	1.5	0.0
JT-1-4-19	4.7	0.3	0.1	2.3	42.1	2.8	10.0	0.2	27.1	0.6	4.1	1.6	0.2	0.0	0.0	0.2	0.3	0.5	0.0	0.0	0.1	0.2	0.0	0.6	0.3	1.4	0.0
JT-1-4-19	5.6	0.4	0.1	1.5	36.8	3.7	9.4	0.3	19.6	0.5	0.6	1.4	0.2	0.0	0.0	1.4	0.3	1.9	0.3	0.0	0.2	0.2	0.0	1.6	0.4	13.1	0.0
JT-1-4-19	4.6	0.3	0.1	1.7	36.3	2.7	7.2	0.3	22.6	0.5	1.0	1.5	0.1	0.0	0.0	0.7	0.3	2.1	0.3	0.0	0.1	0.2	0.0	2.2	0.3	14.4	0.0
JT-1-4-19	4.9	0.3	0.1	1.8	38.3	3.1	7.4	0.3	20.2	0.5	0.8	1.3	0.1	0.0	0.0	0.8	0.3	2.7	0.5	0.0	0.2	0.2	0.0	1.7	0.3	13.7	0.0
JT-1-4-19	4.7	0.3	0.1	1.7	36.2	3.0	8.2	0.4	20.9	0.5	0.7	1.3	0.2	0.0	0.0	0.9	0.3	2.9	0.5	0.0	0.2	0.2	0.0	2.0	0.3	14.2	0.0
JT-1-4-19	4.8	0.3	0.1	2.2	41.0	3.0	9.8	0.2	27.0	0.5	4.2	1.8	0.2	0.0	0.0	0.3	0.3	0.5	0.0	0.0	0.1	0.2	0.0	0.7	0.3	2.2	0.0
JT-1-4-19	5.8	0.5	0.1	1.7	36.6	3.7	9.1	0.3	21.3	0.6	0.9	1.4	0.2	0.0	0.0	0.8	0.3	1.5	0.3	0.0	0.1	0.2	0.0	1.2	0.4	12.7	0.0
JT-1-4-19	4.8	0.4	0.1	2.1	47.1	2.9	7.4	0.2	23.9	0.6	4.8	1.7	0.1	0.0	0.0	0.2	0.3	0.5	0.0	0.0	0.0	0.2	0.0	0.5	0.3	1.5	0.0
JT-1-4-19	5.1	0.4	0.1	1.7	37.4	3.3	7.7	0.3	20.7	0.6	0.9	1.4	0.1	0.0	0.0	0.8	0.3	2.5	0.4	0.0	0.1	0.2	0.0	1.6	0.4	13.6	0.0
JT-1-4-19	4.7	0.3	0.1	1.8	37.3	2.7	7.9	0.4	20.6	0.5	1.1	1.3	0.1	0.0	0.0	0.5	0.3	4.3	0.6	0.0	0.1	0.1	0.0	2.2	0.3	12.3	0.0
JT-1-4-19	4.9	0.3	0.2	2.0	37.9	3.0	7.1	0.4	20.1	0.5	1.1	1.3	0.1	0.0	0.0	0.6	0.3	4.1	0.5	0.0	0.1	0.1	0.0	2.1	0.3	12.6	0.0
JT-1-4-19	4.7	0.3	0.1	1.6	35.7	3.2	6.9	0.3	22.4	0.5	0.7	1.4	0.1	0.0	0.0	1.3	0.3	3.0	0.5	0.0	0.2	0.1	0.0	1.9	0.3	14.0	0.0
JT-1-4-34	4.7	0.4	0.1	1.8	37.6	3.4	7.8	0.3	23.7	0.5	0.6	1.5	0.2	0.0	0.0	1.2	0.2	1.7	0.3	0.0	0.2	0.1	0.0	1.8	0.3	11.4	0.0
JT-1-4-34	5.3	0.4	0.1	1.6	35.3	3.5	8.1	0.5	21.1	0.5	0.8	1.2	0.1	0.0	0.0	0.7	0.3	3.1	0.5	0.0	0.2	0.1	0.0	1.9	0.3	13.9	0.0
JT-1-4-34	4.9	0.3	0.1	1.7	39.4	3.3	7.7	0.3	21.1	0.5	0.7	1.4	0.2	0.0	0.0	0.8	0.3	2.0	0.3	0.0	0.2	0.1	0.0	1.7	0.3	12.3	0.0
JT-1-4-34	5.0	0.3	0.1	1.8	38.5	3.1	7.8	0.4	20.5	0.5	0.8	1.3	0.2	0.0	0.0	0.8	0.2	2.3	0.3	0.0	0.2	0.1	0.0	2.0	0.3	13.1	0.0
JT-1-4-34	5.1	0.3	0.1	1.8	39.5	2.9	9.0	0.2	22.2	0.6	0.6	1.5	0.2	0.0	0.0	1.0	0.3	1.7	0.2	0.0	0.1	0.2	0.0	1.6	0.3	10.2	0.0
JT-1-4-34	4.8	0.3	0.1	1.8	38.2	3.2	7.8	0.4	21.1	0.5	0.7	1.4	0.2	0.0	0.0	0.7	0.3	2.1	0.4	0.0	0.2	0.1	0.0	1.7	0.3	13.3	0.0
JT-1-4-34	5.0	0.3	0.1	2.0	39.7	2.9	7.9	0.4	20.2	0.5	0.7	1.3	0.1	0.0	0.0	0.7	0.3	2.3	0.3	0.0	0.2	0.1	0.0	1.9	0.3	12.2	0.0
JT-1-4-34	4.7	0.3	0.1	1.6	36.0	3.3	8.3	0.3	23.7	0.5	0.6	1.5	0.2	0.0	0.0	1.2	0.3	1.7	0.3	0.0	0.2	0.1	0.0	1.8	0.3	12.7	0.0
JT-1-4-34	6.2	0.5	0.2	2.1	32.0	4.4	7.2	0.6	19.4	0.6	1.2	1.2	0.2	0.0	0.0	0.6	0.4	2.2	0.5	0.0	0.3	0.2	0.0	1.6	0.4	17.6	0.0

Table 24. Fatty acid composition of seed oil from T3 single seeds of *B. juncea* transformed with the T-DNA from GA7-modB.

	C16:0	16:1n7	16:3	C18:0	C18:1	C18:1n11	C18:2	C18:3n6	C18:3n3	C20:0	18:4	C20:1n11	20:1n13	C20:2n6	C20:3n6	C20:4n6	C20:5n3	C22:0	20:4n3	20:5n3	C22:2n6	C22:3n3	22:5n6	22:4n3	C24:1	22:5n3	C22:6n3
JT-1-4-34-11	4.8	0.4	0.1	2.8	38.4	3.7	5.7	0.4	18.0	0.7	1.0	1.5	0.1	0.1	0.0	0.0	1.1	0.3	1.4	0.4	0.0	0.3	0.0	1.4	0.5	16.3	0.0
JT-1-4-34-11	4.3	0.4	0.1	3.0	43.3	3.6	5.2	0.2	18.5	0.7	0.8	1.7	0.1	0.1	0.0	0.0	1.4	0.3	1.2	0.3	0.0	0.2	0.0	1.2	0.3	12.4	0.0
JT-1-4-34-11	4.6	0.4	0.1	2.8	33.1	4.1	5.1	0.4	18.5	0.7	1.2	1.4	0.1	0.1	0.0	0.0	1.1	0.3	1.6	0.5	0.0	0.3	0.0	1.4	0.4	20.8	0.0
JT-1-4-34-11	4.5	0.4	0.1	2.9	39.5	3.3	6.3	0.4	18.5	0.8	1.2	1.5	0.1	0.1	0.0	0.0	1.0	0.3	1.7	0.3	0.0	0.2	0.0	1.8	0.3	14.2	0.0
JT-1-4-34-11	4.9	0.5	0.2	2.8	32.2	3.9	4.7	0.3	20.7	0.8	1.2	1.4	0.1	0.2	0.0	0.0	2.0	0.3	1.4	0.5	0.0	0.3	0.0	1.2	0.4	19.4	0.0
JT-1-4-34-11	4.3	0.3	0.1	3.0	38.1	3.2	5.8	0.3	19.4	0.7	1.1	1.5	0.1	0.1	0.0	0.0	1.2	0.3	1.5	0.4	0.0	0.2	0.0	1.3	0.4	16.0	0.0
JT-1-4-34-11	5.4	0.5	0.2	3.2	29.3	4.0	4.6	0.4	18.6	0.9	1.7	1.3	0.1	0.1	0.0	0.0	1.2	0.4	1.6	0.7	0.0	0.3	0.0	1.4	0.5	22.9	0.0
JT-1-4-34-11	5.2	0.5	0.2	3.7	34.5	4.1	4.5	0.3	17.2	1.0	1.4	1.4	0.1	0.1	0.0	0.0	1.5	0.4	1.4	0.6	0.0	0.3	0.0	1.2	0.5	19.4	0.0
JT-1-4-34-11	5.3	0.5	0.1	3.4	33.4	3.7	4.6	0.3	17.6	0.9	1.7	1.2	0.1	0.1	0.0	0.0	1.1	0.4	1.5	0.6	0.0	0.2	0.0	1.2	0.5	20.7	0.0
JT-1-4-34-11	4.6	0.4	0.1	3.0	39.5	3.5	5.1	0.3	17.8	0.8	0.8	1.6	0.1	0.1	0.0	0.0	1.4	0.4	1.3	0.4	0.0	0.3	0.0	1.3	0.4	16.1	0.0
JT-1-4-34-11	4.3	0.4	0.1	3.1	41.7	3.5	5.6	0.2	19.0	0.7	0.9	1.6	0.1	0.1	0.0	0.0	1.3	0.3	1.4	0.3	0.0	0.2	0.0	1.5	0.3	12.7	0.0
JT-1-4-34-11	4.8	0.5	0.2	2.8	33.8	4.0	5.3	0.4	18.2	0.7	1.4	1.3	0.1	0.1	0.0	0.0	1.2	0.3	1.6	0.6	0.0	0.3	0.0	1.3	0.4	20.1	0.0
JT-1-4-34-11	4.4	0.4	0.1	3.5	40.3	3.5	5.2	0.2	19.1	0.7	1.0	1.5	0.1	0.1	0.0	0.0	1.6	0.3	1.4	0.4	0.0	0.2	0.0	1.4	0.3	13.8	0.0
JT-1-4-34-11	4.8	0.4	0.1	3.2	36.1	3.7	5.9	0.3	19.9	0.7	1.4	1.3	0.1	0.1	0.0	0.0	1.1	0.3	1.9	0.5	0.0	0.2	0.0	1.7	0.3	15.4	0.0
JT-1-4-34-11	4.0	0.3	0.1	2.8	37.2	3.2	4.9	0.3	19.6	0.8	0.9	1.6	0.1	0.1	0.0	0.0	1.5	0.4	1.3	0.5	0.0	0.3	0.0	1.1	0.4	17.9	0.0
JT-1-4-34-11	4.5	0.4	0.1	3.8	36.7	3.2	4.5	0.2	19.0	0.9	1.1	1.4	0.1	0.1	0.0	0.0	1.8	0.4	1.2	0.5	0.0	0.2	0.0	1.0	0.5	17.8	0.0
JT-1-4-34-11	5.2	0.4	0.2	2.8	27.8	3.7	5.3	0.5	18.3	0.8	1.7	1.3	0.1	0.1	0.0	0.0	1.0	0.4	1.9	0.7	0.0	0.3	0.0	1.7	0.5	24.7	0.0
JT-1-4-34-11	5.4	0.6	0.2	2.8	31.7	4.1	4.6	0.3	18.5	0.8	1.3	1.3	0.1	0.1	0.0	0.0	1.4	0.4	1.4	0.6	0.0	0.2	0.0	1.3	0.4	21.8	0.0
JT-1-4-34-11	6.4	0.6	0.1	2.7	30.3	3.5	4.1	0.4	16.1	0.8	2.1	1.1	0.1	0.1	0.0	0.0	0.9	0.4	1.4	0.7	0.0	0.2	0.0	1.1	0.5	25.8	0.0
JT-1-4-34-11	4.3	0.3	0.1	3.2	39.2	3.3	5.7	0.2	20.1	0.7	0.9	1.6	0.1	0.1	0.0	0.0	1.7	0.3	1.3	0.3	0.0	0.2	0.0	1.3	0.3	14.1	0.0
JT-1-4-34-21	4.2	0.4	0.1	2.3	39.9	3.9	5.9	0.3	20.2	0.6	0.9	1.6	0.1	0.1	0.0	0.0	1.1	0.3	1.5	0.3	0.0	0.3	0.0	1.8	0.4	13.3	0.0
JT-1-4-34-21	4.4	0.4	0.1	2.7	38.8	3.9	5.7	0.4	18.5	0.6	1.3	1.3	0.1	0.1	0.0	0.0	0.8	0.3	1.8	0.4	0.0	0.3	0.0	1.7	0.3	15.6	0.0
JT-1-4-34-21	4.2	0.4	0.1	2.0	42.4	3.6	6.0	0.4	19.1	0.5	1.0	1.6	0.1	0.1	0.0	0.0	0.9	0.2	1.5	0.3	0.0	0.3	0.0	1.6	0.3	12.9	0.0

Example 10. Further Analysis of Transformed Plants and Field Trials

Southern blot hybridisation analysis was carried out on selected T2 *B. napus* plants transformed with the T-DNA from the GA7-modB construct. DNA extracted from samples of plant tissue were digested with several restriction enzymes for the

5 Southern blot hybridisation analysis. A radioactive probe corresponding to part of the T-DNA was hybridised to the blots, which were washed under stringent conditions, and the blots exposed to film to detect hybridising bands. Some of the samples exhibited single hybridising bands for each of the restriction digests, corresponding to single T-DNA insertions in the plants, while others showed two bands and others again showed

10 multiple T-DNA bands, corresponding to 4 to 6 insertions. The number of hybridising bands observed by Southern Blot analysis correlated well with the T-DNA copy number in the transgenic plants as determined by the digital PCR method, up to a copy number of about 3 or 4. At higher copy numbers than about 5, the digital PCR method was less reliable.

15 Some of the selected lines were used as pollen donors in crosses with a series of about 30 different *B. napus* varieties of different genetic backgrounds. Further backcrosses are carried out to demonstrate whether the multiple T-DNA insertions are genetic linked or not, and allowing segregation of genetically-unlinked transgenic loci. Thereby, lines containing single transgenic loci are selected.

20 Single-primer PCR reactions are carried out on the transgenic lines, using primers adjacent to the left- and right-borders of the T-DNA, and any lines that show the presence of inverted repeats of the T-DNAs are discarded.

Several of the transgenic lines showed delayed flowering, while others had reduced seed-set and therefore reduced seed yield per plant after growth in the

25 glasshouse, consistent with a reduced male or female fertility. Flower morphology was examined in these plants and it was observed that in some cases, dehiscence and release of pollen from the anthers was delayed so that styles had elongated before dehiscence occurred, thereby distancing the anthers from the stigmas. Full fertility could be restored by artificial pollination. Furthermore, pollen viability at dehiscence was

30 determined by staining with the vital stains FDA and PI (Example 1) and was shown to be reduced in some of the lines, whereas in most of the transgenic lines, pollen viability was about 100% as in the wild-type controls. As a further test for a possible cause of the reduced seed yield in some plants, the fatty acid content and composition of flower buds including the anthers and stigmas/styles of some T3 and T4 plants was tested. No

35 DHA was detected in the extracted lipids, indicating that the genes in the genetic

construct were not expressed in the flower buds during plant development, and ruling this out as a cause of the reduced seed yield.

The oil content was measured by NMR and the DHA level in the total fatty acid content was determined for T2 seeds. Transgenic lines having less than 6% DHA were
5 discarded. T-DNA copy number in leaf samples from plants of the T1, T2 and T3 generations were determined by the digital PCR method (Example 1).

Selected T3 and T4 seed lots were sown in the field at two sites in Victoria, Australia, each in 10m rows at a sowing density of about 10 seeds/m. The selected seed lots included a B003-5-14 derived line which showed pooled seed DHA levels of about
10 8-11% and individual T2 seed DHA levels of up to about 19%, with a T0 plant T-DNA copy number of 3. The selected seed lots also included B0050-27 derived lines which had shown T2 seed DHA levels in excess of 20%, and a T2 plant T-DNA copy number of 1 or 2. Seeds sown in the field germinated and plantlets emerged at the same rate as the wild-type seeds. Plants grown from most, but not all, of the sown seed lots were
15 phenotypically normal, for example had morphology, growth rate, plant height, male and female fertility, pollen viability (100%), seed set, silique size and morphology that was essentially the same as the wild-type control plants grown under the same conditions. Seed yield per plant was similar to that of wild-type controls grown under the same conditions. Other seed samples were sown in larger areas to bulk-up the
20 selected transgenic lines. The total DHA content in harvested seeds was at least 30mg/g seed.

It will be appreciated by persons skilled in the art that numerous variations
25 and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present application claims priority from AU 2013905033 filed 18 December
30 2013, and AU 2014902471 filed 27 June 2014, the entire contents of both of which are incorporated herein by reference. All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a
35 context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the

field relevant to the present invention as it existed before the priority date of each claim of this application.

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CLAIMS

1. Extracted plant lipid, comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA),
5 $\omega 3$ fatty acids which comprise α -linolenic acid (ALA) and docosahexaenoic acid (DHA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and eicosatetraenoic acid (ETA), wherein the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid
10 content of the extracted lipid, if present, is less than 1%, and wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%.

2. Extracted plant lipid or microbial lipid, comprising fatty acids in an esterified
15 form, the fatty acids comprising oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA) and docosapentaenoic acid (DPA), and optionally one or more of stearidonic acid (SDA), eicosapentaenoic acid (EPA), and eicosatetraenoic acid (ETA), wherein the level of DPA in the total fatty acid content of the extracted lipid is between 7% and 35%.

- 20 3. The lipid of claim 1 or claim 2 which has one or more of the following features
 - i) the level of palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 15%, or between about 3% and about 10%,
 - 25 ii) the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid is about 0.1%,
 - 30 iii) the level of oleic acid in the total fatty acid content of the extracted lipid is between about 1% and about 30%, between about 3% and about 30%, between about 6% and about 30%, between 1% and about 20%, between about 30% and about 60%, about 45% to about 60%, about 30%, or between about 15% and about 30%,
 - 35 iv) the level of linoleic acid (LA) in the total fatty acid content of the extracted lipid is between about 4% and about 35%, between about 4% and about 20%, between about 4% and about 17%, or between about 5% and about 10%,
 - v) the level of α -linolenic acid (ALA) in the total fatty acid content of the extracted lipid is between about 4% and about 40%, between about 7% and

- about 40%, between about 10% and about 35%, between about 20% and about 35%, between about 4% and 16%, or between about 2% and 16%,
- 5 vi) the level of γ -linolenic acid (GLA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 3%, less than about 2%, less than about 1%, less than about 0.5%, between 0.05% and 7%, between 0.05% and 4%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 10 vii) the level of stearidonic acid (SDA) in the total fatty acid content of the extracted lipid is less than about 10%, less than about 8%, less than about 7%, less than about 6%, less than about 4%, less than about 3%, between about 0.05% and about 7%, between about 0.05% and about 6%, between about 0.05% and about 4%, between about 0.05% and about 3%, between about 0.05% and about 10%, or between 0.05% and about 2%,
- 15 viii) the level of eicosatetraenoic acid (ETA) in the total fatty acid content of the extracted lipid is less than about 6%, less than about 5%, less than about 4%, less than about 1%, less than about 0.5%, between 0.05% and about 6%, between 0.05% and about 5%, between 0.05% and about 4%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 20 ix) the level of eicosatrienoic acid (ETrA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 2%, less than about 1%, between 0.05% and 4%, between 0.05% and 3%, or between 0.05% and about 2%, or between 0.05% and about 1%,
- 25 x) the level of eicosapentaenoic acid (EPA) in the total fatty acid content of the extracted lipid is between 4% and 15%, less than 4%, less than about 3%, less than about 2%, between 0.05% and 10%, between 0.05% and 5%, between 0.05% and about 3%, or between 0.05% and about 2%,
- 30 xi) if the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%, the level of docosapentaenoic acid (DPA) in the total fatty acid content of the extracted lipid is less than 4%, less than about 3%, less than about 2%, between 0.05% and 8%, between 0.05% and 5%, between 0.05% and about 3%, between 5% and 15%, between 5% and 10%, or between 0.05% and about 2%,
- 35 xii) the level of DHA in the total fatty acid content of the extracted lipid is about 22%, about 24%, about 26%, about 28%, about 31%, between 20.1% and 29%, between 20.1% and 28%, between 20.1% and about 27%, between 20.1% and about 26%, between 20.1% and about 25%, between 20.1% and

- about 24%, between 21% and 35%, between 21% and 30%, between 21% and 28%, between 21% and about 26%, or between 21% and about 24%,
- xiii) the lipid comprises ω 6-docosapentaenoic acid ($22:5^{\Delta 4,7,10,13,16}$) in its fatty acid content,
- 5 xiv) the lipid comprises less than 0.1% of ω 6-docosapentaenoic acid ($22:5^{\Delta 4,7,10,13,16}$) in its fatty acid content,
- xv) the lipid comprises less than 0.1% of one or more or all of SDA, EPA and ETA in its fatty acid content,
- 10 xvi) the level of total saturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 25%, between about 4% and about 20%, between about 6% and about 20%, or between about 6% and about 12%,
- xvii) the level of total monounsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 4% and about 40%, between about 4% and about 35%, between about 8% and about 25%, between 8% and about 22%, between about 15% and about 40% or between about 15% and about 35%,
- 15 xviii) the level of total polyunsaturated fatty acids in the total fatty acid content of the extracted lipid is between about 20% and about 75%, between 30% and 75%, between about 50% and about 75%, about 60%, about 65%, about 70%, about 75%, or between about 60% and about 75%,
- 20 xix) the level of total ω 6 fatty acids in the total fatty acid content of the extracted lipid is between about 35% and about 50%, between about 20% and about 35%, between about 6% and 20%, less than 20%, less than about 16%, less than about 10%, between about 1% and about 16%, between about 2% and about 10%, or between about 4% and about 10%,
- 25 xx) the level of new ω 6 fatty acids in the total fatty acid content of the extracted lipid is less than about 10%, less than about 8%, less than about 6%, less than 4%, between about 1% and about 20%, between about 1% and about 10%, between 0.5% and about 8%, or between 0.5% and 4%,
- 30 xxi) the level of total ω 3 fatty acids in the total fatty acid content of the extracted lipid is between 36% and about 65%, between 36% and about 70%, between 40% and about 60%, between about 30% and about 60%, between about 35% and about 60%, between 40% and about 65%, between about 30% and about 65%, between about 35% and about 65%, about 35%,
- 35

about 40%, about 45%, about 50%, about 55%, about 60%, about 65% or about 70%,

xxii) the level of new $\omega 3$ fatty acids in the total fatty acid content of the extracted lipid is between 21% and about 45%, between 21% and about 35%, between about 23% and about 35%, between about 25% and about 35%, between about 27% and about 35%, about 23%, about 25%, about 27%, about 30%, about 35%, about 40% or about 45%,

xxiii) the ratio of total $\omega 6$ fatty acids: total $\omega 3$ fatty acids in the fatty acid content of the extracted lipid is between about 1.0 and about 3.0, between about 0.1 and about 1, between about 0.1 and about 0.5, less than about 0.50, less than about 0.40, less than about 0.30, less than about 0.20, less than about 0.15, about 1.0, about 0.1, about 0.10 to about 0.4, or about 0.2,

xxiv) the ratio of new $\omega 6$ fatty acids: new $\omega 3$ fatty acids in the fatty acid content of the extracted lipid is between about 1.0 and about 3.0, between about 0.02 and about 0.1, between about 0.1 and about 1, between about 0.1 and about 0.5, less than about 0.50, less than about 0.40, less than about 0.30, less than about 0.20, less than about 0.15, about 0.02, about 0.05, about 0.1, about 0.2 or about 1.0,

xxv) the fatty acid composition of the lipid is based on an efficiency of conversion of oleic acid to LA by $\Delta 12$ -desaturase of at least about 60%, at least about 70%, at least about 80%, between about 60% and about 98%, between about 70% and about 95%, or between about 75% and about 90%,

xxvi) the fatty acid composition of the lipid is based on an efficiency of conversion of ALA to SDA by $\Delta 6$ -desaturase of at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 30% and about 70%, between about 35% and about 60%, or between about 50% and about 70%,

xxvii) the fatty acid composition of the lipid is based on an efficiency of conversion of SDA to ETA acid by $\Delta 6$ -elongase of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 95%, between about 70% and about 88%, or between about 75% and about 85%,

xxviii) the fatty acid composition of the lipid is based on an efficiency of conversion of ETA to EPA by $\Delta 5$ -desaturase of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 99%, between about 70% and about 99%, or between about 75% and about 98%,

- xxix) the fatty acid composition of the lipid is based on an efficiency of conversion of EPA to DPA by $\Delta 5$ -elongase of at least about 80%, at least about 85%, at least about 90%, between about 50% and about 99%, between about 85% and about 99%, between about 50% and about 95%, or between about 85% and about 95%,
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- xxx) if the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%, the fatty acid composition of the lipid is based on an efficiency of conversion of DPA to DHA by $\Delta 4$ -desaturase of at least about 80%, at least about 90%, at least about 93%, between about 50% and about 95%, between about 80% and about 95%, or between about 85% and about 95%,
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- xxxi) the fatty acid composition of the lipid is based on an efficiency of conversion of oleic acid to DPA and/or DHA of at least about 10%, at least about 15%, at least about 20%, at least about 25%, about 20%, about 25%, about 30%, between about 10% and about 50%, between about 10% and about 30%, between about 10% and about 25% or between about 20% and about 30%,
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- xxxii) the fatty acid composition of the lipid is based on an efficiency of conversion of LA to DPA and/or DHA of at least about 15%, at least about 20%, at least about 22%, at least about 25%, at least about 30%, at least about 40%, about 25%, about 30%, about 35%, about 40%, about 45%, about 50%, between about 15% and about 50%, between about 20% and about 40%, or between about 20% and about 30%,
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- xxxiii) the fatty acid composition of the lipid is based on an efficiency of conversion of ALA to DPA and/or DHA of at least about 17%, at least about 22%, at least about 24%, at least about 30%, about 30%, about 35%, about 40%, about 45%, about 50%, about 55%, about 60%, between about 22% and about 70%, between about 17% and about 55%, between about 22% and about 40%, or between about 24% and about 40%,
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- xxxiv) the total fatty acid in the extracted lipid has less than 1.5% C20:1, less than 1% C20:1 or about 1% C20:1,
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- xxxv) the triacylglycerol (TAG) content of the lipid is at least about 70%, at least about 80%, at least about 90%, at least 95%, between about 70% and about 99%, or between about 90% and about 99%,
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- xxxvi) the lipid comprises diacylglycerol (DAG), which DAG preferably comprises DHA and/or DPA,

- xxxvii) the lipid comprises less than about 10%, less than about 5%, less than about 1%, or between about 0.001% and about 5%, free (non-esterified) fatty acids and/or phospholipid, or is essentially free thereof,
- xxxviii) at least 70%, at least 72% or at least 80%, of the DHA and/or DPA esterified in the form of TAG is in the *sn*-1 or *sn*-3 position of the TAG,
- xxxix) the most abundant DHA-containing TAG species in the lipid is DHA/18:3/18:3 (TAG 58:12),
- xl) the most abundant DPA-containing TAG species in the lipid is DPA/18:3/18:3 (TAG 56:12),
- xli) the lipid comprises tri-DHA TAG (TAG 66:18), and
- xlii) the level of DPA in the total fatty acid content of the extracted lipid is about 7%, about 8%, about 9%, about 10%, about 12%, about 15%, about 18%, about 20%, about 22%, about 24%, about 26%, about 28%, about 31%, between about 7% and about 31%, between about 7% and about 28%, between about 10% and 35%, between about 10% and about 30%, between about 10% and about 25%, between about 10% and about 22%, between about 14% and 35%, between about 16% and 35%, between about 16% and about 30%, between about 16% and about 25%, or between about 16% and about 22%, optionally wherein the level of DHA is less than 0.5% of the total fatty acid content of the extracted lipid.

4. The lipid according to any one of claims 1 to 3, wherein the lipid is an oil, preferably oil from an oilseed, more preferably wherein the lipid comprises or is *Brassica* sp. oil such as *Brassica napus* oil or *Brassica juncea* oil, *Gossypium hirsutum* oil, *Linum usitatissimum* oil, *Helianthus* sp. oil, *Carthamus tinctorius* oil, *Glycine max* oil, *Zea mays* oil, *Elaeagnus guineensis* oil, *Nicotiana benthamiana* oil, *Lupinus angustifolius* oil, *Camelina sativa* oil, *Crambe abyssinica* oil, *Miscanthus x giganteus* oil, or *Miscanthus sinensis* oil.

5. A process for producing extracted plant lipid, comprising the steps of

i) obtaining a plant part comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA) and γ -linolenic acid (GLA), ω 3 fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally one or more of eicosapentaenoic acid (EPA) and eicosatetraenoic acid (ETA), wherein the level of

palmitic acid in the total fatty acid content of the extracted lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the extracted lipid, if present, is less than 1%, and wherein the level of DHA in the total fatty acid content of extractable lipid in the plant part is between 20.1% and 30% or between 20.1% and 35%, and

ii) extracting lipid from the plant part,

wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35%.

6. A process for producing extracted plant lipid or microbial lipid, comprising the steps of

i) obtaining a plant part or a microbial cell comprising lipid, the lipid comprising fatty acids in an esterified form, the fatty acids comprising oleic acid, palmitic acid, ω 6 fatty acids which comprise linoleic acid (LA), ω 3 fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA), and optionally one or more of eicosapentaenoic acid (EPA) and eicosatetraenoic acid (ETA), wherein the level of DPA in the total fatty acid content of extractable lipid in the plant part is between 7% and 35%, and

ii) extracting lipid from the plant part or microbial cell,

wherein the level of DPA in the total fatty acid content of the extracted lipid is between 7% and 35%.

7. The process of claim 5 or claim 6, wherein the extracted lipid has one or more of the features defined in claim 3 or claim 4.

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8. The process of any one of claims 5 to 7, wherein the plant part is a seed, preferably an oilseed such as *Brassica sp.* such as *Brassica napus* or *Brassica juncea*, *Gossypium hirsutum*, *Linum usitatissimum*, *Helianthus sp.*, *Carthamus tinctorius*, *Glycine max*, *Zea mays*, *Elaeisis guineensis*, *Nicotiana benthamiana*, *Lupinus angustifolius*, *Camelina sativa*, or *Crambe abyssinica*, preferably a *Brassica napus*, *B. juncea* or *C. sativa* seed.

9. The process according to any one of claims 5 to 8, wherein the plant part or microbial cell comprises exogenous polynucleotides encoding one of the following sets of enzymes;

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- i) an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- ii) a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- 5 iii) a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
- iv) a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
- v) an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- 10 vi) a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and a Δ 5-elongase,
- vii) a Δ 12-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- 15 viii) a Δ 12-desaturase, a ω 3-desaturase and/or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- ix) an ω 3-desaturase or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- x) an ω 3-desaturase or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase and a Δ 5-elongase,
- 20 xi) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 6-elongase and an Δ 5-elongase, or
- xii) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- 25 and wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in a cell of the plant part or microbial cell.

10. The process of claim 9, wherein the plant part or microbial cell has one or more or all of the following features

- i) the Δ 12-desaturase converts oleic acid to linoleic acid in one or more cells of the plant part or the microbial cell with an efficiency of at least about 60%, at least about 70%, at least about 80%, between about 60% and about 95%, between about 70% and about 90%, or between about 75% and about 85%,
- 35 ii) the ω 3-desaturase converts ω 6 fatty acids to ω 3 fatty acids in one or more cells of the plant part or the microbial cell with an efficiency of at least about 65%, at

least about 75%, at least about 85%, between about 65% and about 95%, between about 75% and about 91%, or between about 80% and about 91%,

iii) the $\Delta 6$ -desaturase converts ALA to SDA in one or more cells of the plant part or the microbial cell with an efficiency of at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, between about 30% and about 70%, between about 35% and about 60%, or between about 50% and about 70%,

iv) the $\Delta 6$ -desaturase converts linoleic acid to γ -linolenic acid in one or more cells of the plant part or the microbial cell with an efficiency of less than about 5%, less than about 2.5%, less than about 1%, between about 0.1% and about 5%, between about 0.5% and about 2.5%, or between about 0.5% and about 1%,

v) the $\Delta 6$ -elongase converts SDA to ETA in one or more cells of the plant part or the microbial cell with an efficiency of at least about 60%, at least about 70%, at least about 75%, between about 60% and about 95%, between about 70% and about 80%, or between about 75% and about 80%,

vi) the $\Delta 5$ -desaturase converts ETA to EPA in one or more cells of the plant part or the microbial cell with an efficiency of at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, between about 60% and about 95%, between about 70% and about 95%, or between about 75% and about 95%,

vii) the $\Delta 5$ -elongase converts EPA to DPA in one or more cells of the plant part or the microbial cell with an efficiency of at least about 80%, at least about 85%, at least about 90%, between about 50% and about 90%, or between about 85% and about 95%,

viii) the $\Delta 4$ -desaturase converts DPA to DHA in one or more cells of the plant part with an efficiency of at least about 80%, at least about 90%, at least about 93%, between about 50% and about 95%, between about 80% and about 95%, or between about 85% and about 95%,

ix) the efficiency of conversion of oleic acid to DHA or DPA in one or more cells of the plant part or the microbial cell is at least about 10%, at least about 15%, at least about 20%, at least about 25%, about 20%, about 25%, about 30%, between about 10% and about 50%, between about 10% and about 30%, between about 10% and about 25%, or between about 20% and about 30%,

x) the efficiency of conversion of LA to DHA or DPA in one or more cells of the plant part or the microbial cell is at least about 15%, at least about 20%, at least about 22%, at least about 25%, at least about 30%, about 25%, about 30%, about 35%,

between about 15% and about 50%, between about 20% and about 40%, or between about 20% and about 30%,

xi) the efficiency of conversion of ALA to DHA or DPA in one or more cells of the plant part or the microbial cell is at least about 17%, at least about 22%, at least about 24%, at least about 30%, about 30%, about 35%, about 40%, between about 17% and about 55%, between about 22% and about 35%, or between about 24% and about 35%,

xii) one or more cells of the plant part or the microbial cell comprise at least about 25%, at least about 30%, between about 25% and about 40%, or between about 27.5% and about 37.5%, more ω 3 fatty acids than corresponding cells lacking the exogenous polynucleotides,

xiii) the Δ 6-desaturase preferentially desaturates α -linolenic acid (ALA) relative to linoleic acid (LA),

xiv) the Δ 6-elongase also has Δ 9-elongase activity,

xv) the Δ 12-desaturase also has Δ 15-desaturase activity,

xvi) the Δ 6-desaturase also has Δ 8-desaturase activity,

xvii) the Δ 8-desaturase also has Δ 6-desaturase activity or does not have Δ 6-desaturase activity,

xviii) the Δ 15-desaturase also has ω 3-desaturase activity on GLA,

xix) the ω 3-desaturase also has Δ 15-desaturase activity on LA,

xx) the ω 3-desaturase desaturates both LA and/or GLA,

xxi) the ω 3-desaturase preferentially desaturates GLA relative to LA,

xxii) one or more or all of the desaturases have greater activity on an acyl-CoA substrate than a corresponding acyl-PC substrate,

xxiii) the Δ 6-desaturase has greater Δ 6-desaturase activity on ALA than LA as fatty acid substrate,

xxiv) the Δ 6-desaturase has greater Δ 6-desaturase activity on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxv) the Δ 6-desaturase has at least about a 2-fold greater Δ 6-desaturase activity, at least 3-fold greater activity, at least 4-fold greater activity, or at least 5-fold greater activity, on ALA as a substrate compared to LA,

xxvi) the Δ 6-desaturase has greater activity on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxvii) the Δ 6-desaturase has at least about a 5-fold greater Δ 6-desaturase activity or at least 10-fold greater activity, on ALA-CoA as fatty acid substrate than on ALA joined to the *sn*-2 position of PC as fatty acid substrate,

xxviii) the desaturase is a front-end desaturase, and

xxix) the $\Delta 6$ -desaturase has no detectable $\Delta 5$ -desaturase activity on ETA.

11. The process of claim 9 or claim 10, wherein the plant part or the microbial cell
5 further comprises an exogenous polynucleotide encoding a diacylglycerol
acyltransferase (DGAT), monoacylglycerol acyltransferase (MGAT), glycerol-3-
phosphate acyltransferase (GPAT), 1-acyl-glycerol-3-phosphate acyltransferase
(LPAAT) preferably an LPAAT which can use a C22 polyunsaturated fatty acyl-CoA
substrate such as DHA-CoA and/or DPA-CoA, acyl-CoA:lysophosphatidylcholine
10 acyltransferase (LPCAT), phospholipase A_2 (PLA₂), phospholipase C (PLC),
phospholipase D (PLD), CDP-choline diacylglycerol choline phosphotransferase
(CPT), phosphatidylcholine diacylglycerol acyltransferase (PDAT),
phosphatidylcholine:diacylglycerol choline phosphotransferase (PDCT), acyl-CoA
synthase (ACS), or a combination of two or more thereof.

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12. The process according to any one of claims 9 to 11, wherein the exogenous
polynucleotides are covalently linked in a DNA molecule, preferably a T-DNA
molecule, integrated into the genome of cells of the plant part or the microbial cell and
preferably where the number of such DNA molecules integrated into the genome of the
20 cells of the plant part or the microbial cell is not more than one, not more than two or
three, or is two or three.

13. The process according to any one of claims 5 to 12, wherein the total oil content
of the plant part or the microbial cell comprising the exogenous polynucleotides is at
25 least about 40%, at least about 50%, at least about 60%, at least about 70%, between
about 50% and about 80%, or between about 80% and about 100% of the total oil
content of a corresponding plant part or microbial cell lacking the exogenous
polynucleotides.

30 14. The process according to any one of claims 5 to 13 which further comprises
treating the lipid to increase the level of DHA and/or DPA as a percentage of the total
fatty acid content, wherein the treatment comprises one or more of fractionation,
distillation or transesterification such as the production of methyl- or ethyl-esters of
DHA and/or DPA.

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15. An oilseed plant or part thereof comprising

- a) lipid in its seed, the lipid comprising fatty acids in an esterified form, and
- b) exogenous polynucleotides encoding one of the following sets of enzymes;

- i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or
- 5 ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 4$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, wherein the fatty acids comprise oleic acid, palmitic acid,
 10 $\omega 6$ fatty acids which comprise linoleic acid (LA) and optionally γ -linolenic acid (GLA), $\omega 3$ fatty acids which comprise α -linolenic acid (ALA), stearidonic acid (SDA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and optionally eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level of DHA in the total fatty acid content of the lipid of the seed is between 20.1% and
 15 30%, or between 20.1% and 35%, and wherein the level of palmitic acid in the total fatty acid content of the lipid is between about 2% and 16%, and wherein the level of myristic acid (C14:0) in the total fatty acid content of the lipid, if present, is less than 1%.

- 20 16. An oilseed plant comprising lipid in its seed, or part thereof, or a microbial cell, comprising

- a) lipid comprising fatty acids in an esterified form, and
- b) exogenous polynucleotides encoding one of the following sets of enzymes;

- i) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -
 25 desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase,
- ii) a $\Delta 12$ -desaturase, a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,
- iii) a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 6$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 6$ -elongase and an $\Delta 5$ -elongase, or
- 30 iv) a $\omega 3$ -desaturase and/or $\Delta 15$ -desaturase, a $\Delta 8$ -desaturase, a $\Delta 5$ -desaturase, a $\Delta 9$ -elongase and an $\Delta 5$ -elongase,

wherein each polynucleotide is operably linked to one or more seed-specific promoters that are capable of directing expression of said polynucleotides in developing seed of the plant, or one or more promoters that are capable of directing
 35 expression of said polynucleotides in the microbial cell, wherein the fatty acids comprise oleic acid, palmitic acid, $\omega 6$ fatty acids which comprise linoleic acid (LA),

ω3 fatty acids which comprise α-linolenic acid (ALA), stearidonic acid (SDA), and docosapentaenoic acid (DPA), and optionally docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and/or eicosatetraenoic acid (ETA), and wherein the level of DPA in the total fatty acid content of the lipid of the seed or microbial cell is
 5 between 7% and 35%.

17. A *Brassica napus*, *B. juncea* or *Camelina sativa* plant which is capable of producing seed comprising DHA and/or DPA, wherein mature, harvested seed of the plant has a DHA and/or DPA content of at least about 28mg per gram seed, preferably
 10 at least about 32mg per gram seed, at least about 36mg per gram seed, at least about 40mg per gram seed, more preferably at least about 44mg per gram seed or at least about 48mg per gram seed, about 80 mg per gram seed, or between about 30mg and about 80mg per gram seed.

15 18. A plant cell of claim 15 or claim 16 comprising the exogenous polynucleotides.

19. A plant part, preferably a seed, or a microbial cell which has one or more of the following features
 20 i) is from a plant according to any one of claims 15 to 17,
 ii) comprises lipid as defined in any one of claims 1 to 4, or
 iii) can be used in a process according to any one of claims 5 to 14.

20. Mature, harvested *Brassica napus*, *B. juncea* or *Camelina sativa* seed comprising DHA and/or DPA and a moisture content of between about 4% and about
 25 15% by weight, preferably between about 6% and about 8% by weight or between about 4% and about 8% by weight, wherein the DHA and/or DPA content of the seed at least about 28mg per gram seed, preferably at least about 32mg per gram seed, at least about 36mg per gram seed, at least about 40mg per gram seed, more preferably at least about 44mg per gram seed or at least about 48mg per gram seed, about 80 mg per gram
 30 seed, or between about 30mg and about 80mg per gram seed.

21. A method of producing a plant or a microbial cell which can be used to produce extracted plant lipid or extracted microbial lipid according any one of claims 1 to 4, the method comprising
 35 a) assaying the level of DHA and/or DPA in lipid produced by one or more plant parts or microbial cells from a plurality of plants or microbial cells, each plant or

microbial cell comprising one or more exogenous polynucleotides encoding one of the following sets of enzymes;

- i) an ω 3-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- 5 ii) a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and a Δ 5-elongase,
- iii) a Δ 12-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
- iv) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 6-desaturase, a
10 Δ 5-desaturase, a Δ 4-desaturase, a Δ 6-elongase and an Δ 5-elongase,
- v) an ω 3-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- vi) a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a
15 Δ 9-elongase and a Δ 5-elongase,
- vii) a Δ 12-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- viii) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 8-desaturase, a
20 Δ 5-desaturase, a Δ 4-desaturase, a Δ 9-elongase and an Δ 5-elongase,
- ix) an ω 3-desaturase or a Δ 15-desaturase, a Δ 6-desaturase, a Δ 5-desaturase, a
20 Δ 6-elongase and a Δ 5-elongase,
- x) an ω 3-desaturase or a Δ 15-desaturase, a Δ 8-desaturase, a Δ 5-desaturase, a
20 Δ 9-elongase and a Δ 5-elongase,
- xi) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 6-desaturase, a
20 Δ 5-desaturase, a Δ 6-elongase and an Δ 5-elongase, or
- 25 xii) a Δ 12-desaturase, a ω 3-desaturase or a Δ 15-desaturase, a Δ 8-desaturase, a
25 Δ 5-desaturase, a Δ 9-elongase and an Δ 5-elongase,

wherein each polynucleotide is operably linked to one or more promoters that are capable of directing expression of said polynucleotides in a cell of a plant part or microbial cell, and

- 30 b) identifying a plant or microbial cell, from the plurality of plants or microbial cells, which can be used to produce extracted plant lipid or microbial lipid according to any one of claims 1 to 4 in one or more of its parts, and
- c) optionally, producing progeny plants or microbial cells from the identified plant or microbial cell, or seed therefrom.

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22. A method of producing seed, the method comprising,

- a) growing a plant according to any one of claims 15 to 17, or a plant which produces a plant part of claim 19, or which produces a seed of claim 20, preferably in a field as part of a population of at least 1000 or 2000 or 3000 such plants or in an area of at least 1 hectare or 2 hectares or 3 hectares planted at a standard planting density,
- 5 b) harvesting seed from the plant or plants, and
- c) optionally, extracting lipid from the seed, preferably to produce oil with a total DHA and/or DPA yield of at least 60kg or 70kg or 80kg DHA and/or DPA/hectare.
- 10 23. The plant, plant cell, plant part or seed, or microbial cell, of any one of claims 15 to 20, which has one or more of the following features
- i) comprises oil as defined in claim 3 or claim 4, and
- ii) the plant part or seed or microbial cell is capable of being used in a process according to any one of claims 5 to 14.
- 15 24. Lipid, or oil, produced by, or obtained from, using the process according to any one of claims 5 to 14, the cell according to claim 18, the oilseed plant of claim 15 or claim 16, the microbial cell of claim 16, the *Brassica napus*, *B. juncea* or *Camelina sativa* plant of claim 17, the plant part of claim 19, or the seed of claim 20.
- 20 25. Seedmeal obtained from seed of claim 20, or obtained from the plant of any one of claims 15 to 17.
26. A composition comprising one or more of the lipid or oil of claim 24, the cell according to claim 18, the plant cell or microbial cell of claim 23, the seed of claim 20, or the seedmeal of claim 25.
- 25 27. Feedstuffs, cosmetics or chemicals comprising one or more of the lipid or oil of claim 24, the cell according to claim 18, the oilseed plant of claim 15 or claim 16, the plant cell or microbial cell of claim 23, the *Brassica napus*, *B. juncea* or *Camelina sativa* plant of claim 17, the plant part of claim 23, the seed of claim 20, the seedmeal of claim 25, or the composition of claim 26.
- 30 28. A method of producing a feedstuff, the method comprising mixing one or more of the lipid or oil according to any one of claims 1 to 4, the cell according to claim 18, the oilseed plant of claim 15 or claim 16, the plant cell or microbial cell of claim 23,
- 35 29. A method of producing a feedstuff, the method comprising mixing one or more of the lipid or oil according to any one of claims 1 to 4, the cell according to claim 18, the oilseed plant of claim 15 or claim 16, the plant cell or microbial cell of claim 23,

the *Brassica napus*, *B. juncea* or *Camelina sativa* plant of claim 17, the plant part of claim 23, the seed of claim 20, the seedmeal of claim 25, or the composition of claim 26, with at least one other food ingredient.

- 5 29. Use of one or more of the lipid or oil according to any one of claims 1 to 4, the cell according to claim 18, the plant cell or microbial cell of claim 23, the plant part of claim 23, the seed of claim 20, the seedmeal of claim 25, or the composition of claim 26, for the manufacture of a medicament for treating or preventing a condition which would benefit from a PUFA.

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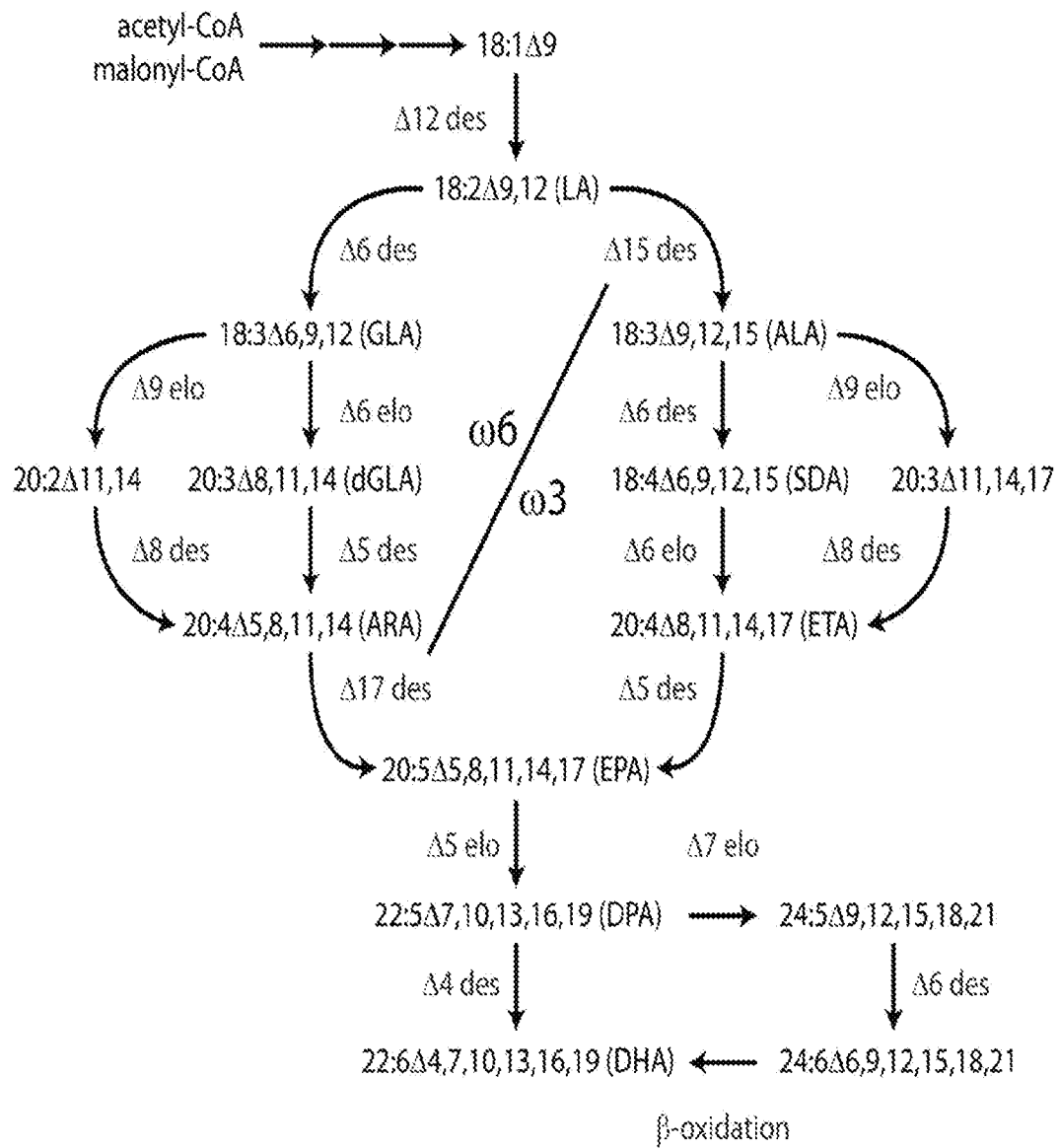


Figure 1

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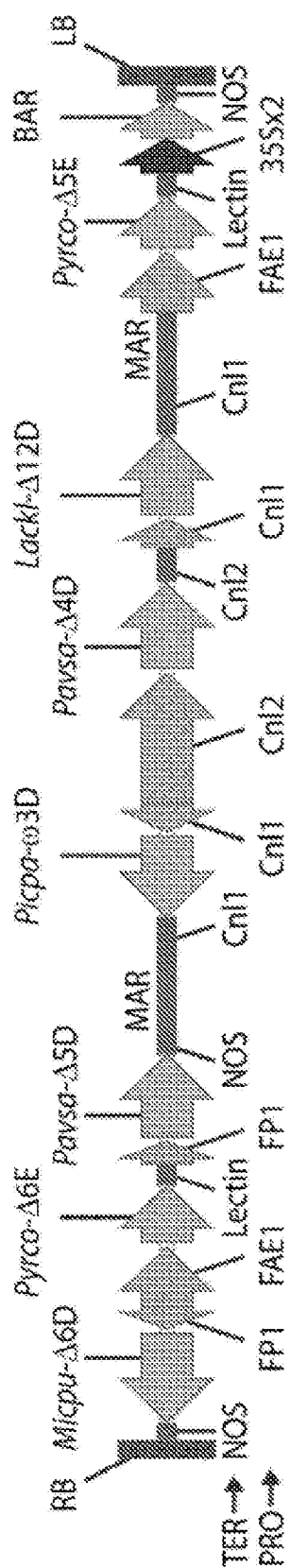


Figure 2

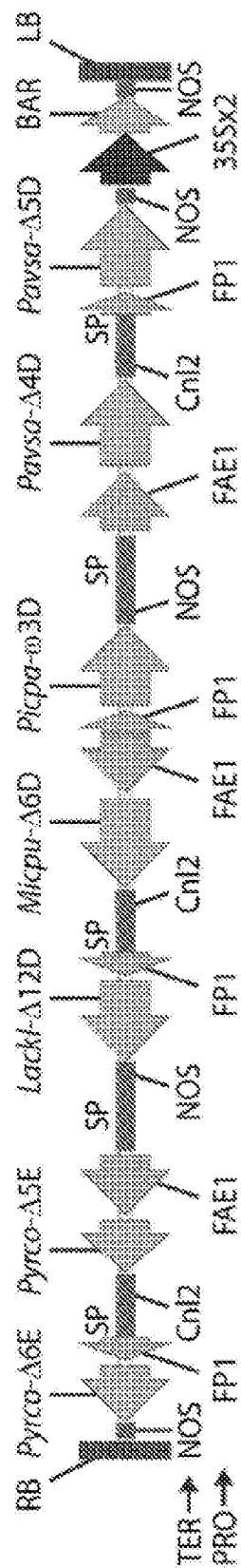
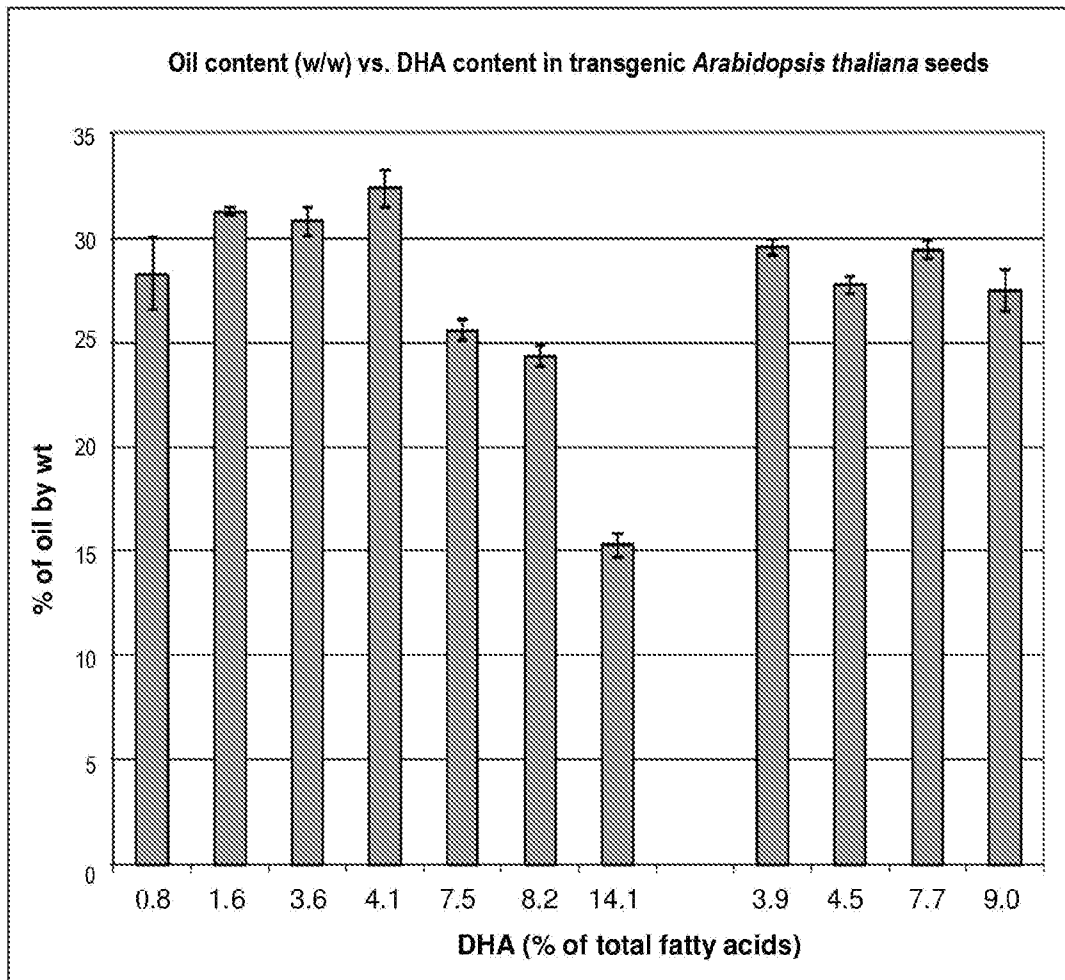


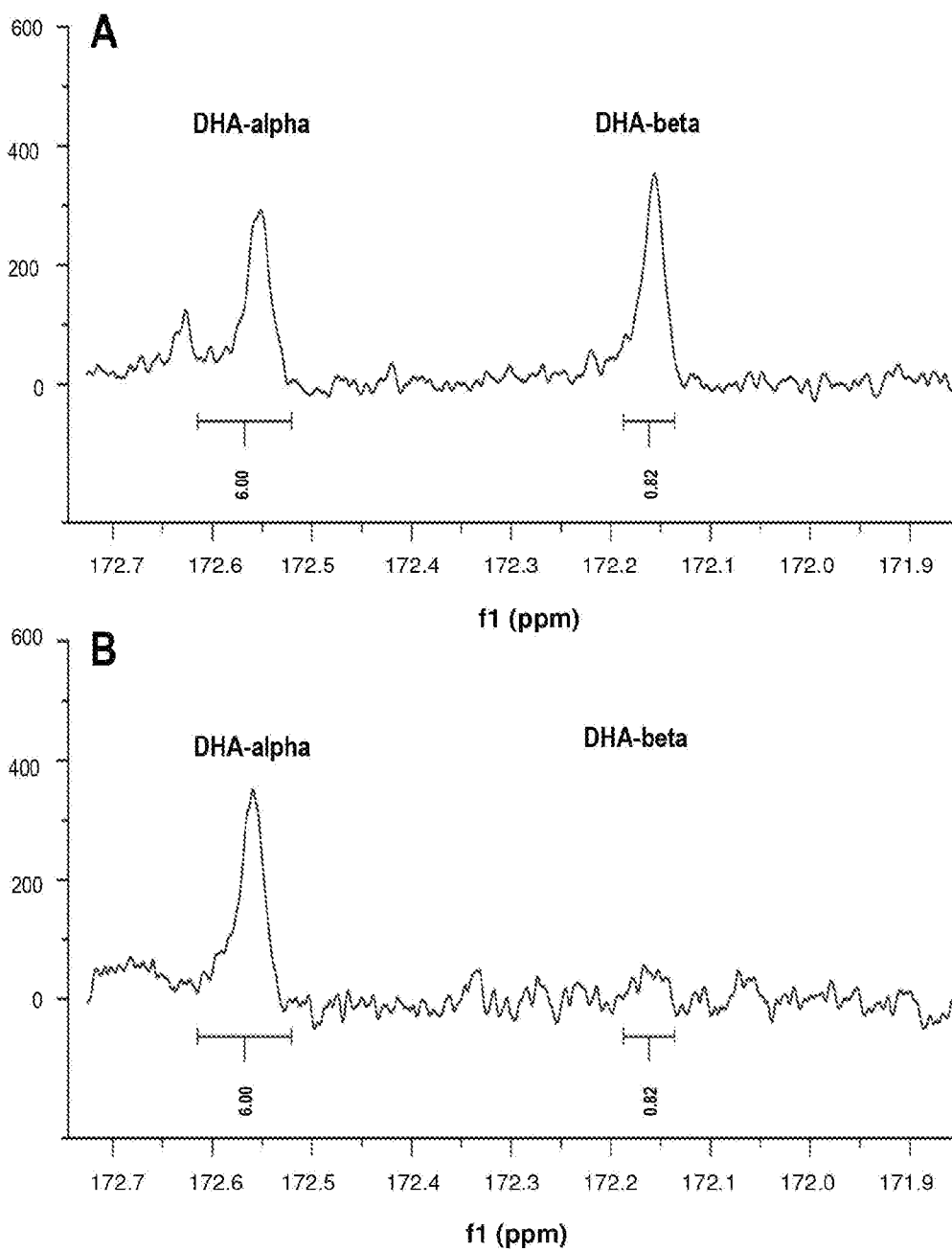
Figure 3

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**Figure 4**

Substitute Sheet
(Rule 26) RO/AU

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**Figure 5**

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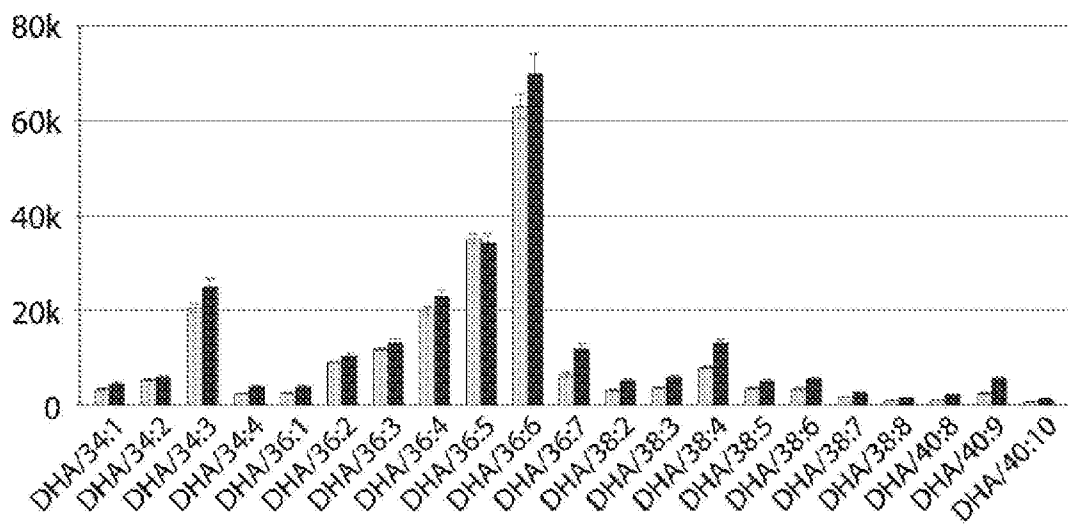
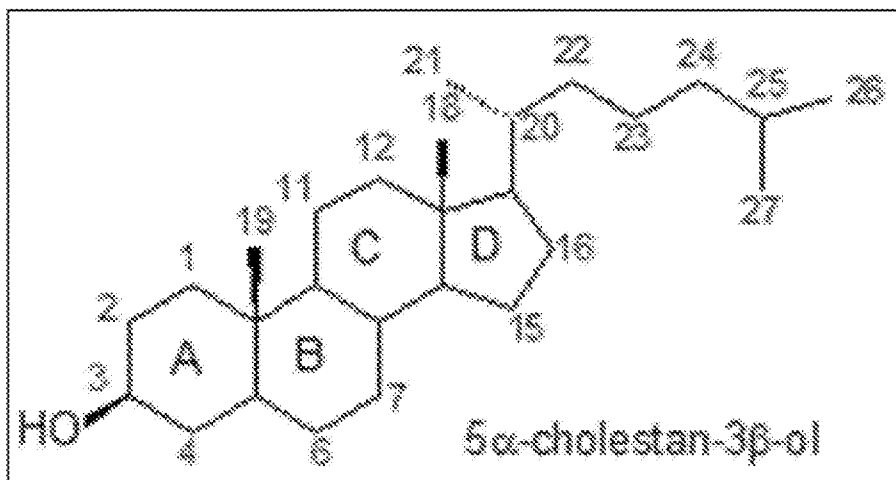


Figure 6

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A)



B)

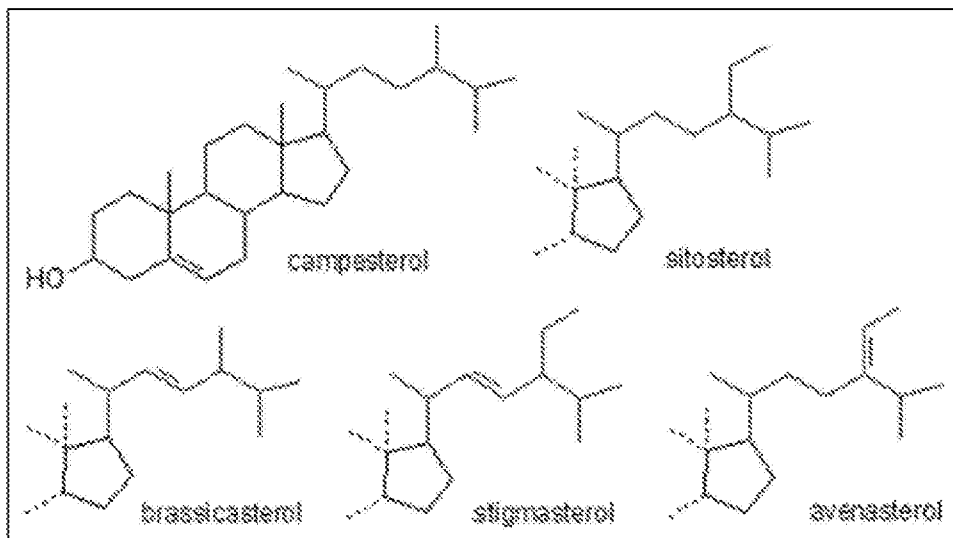


Figure 7

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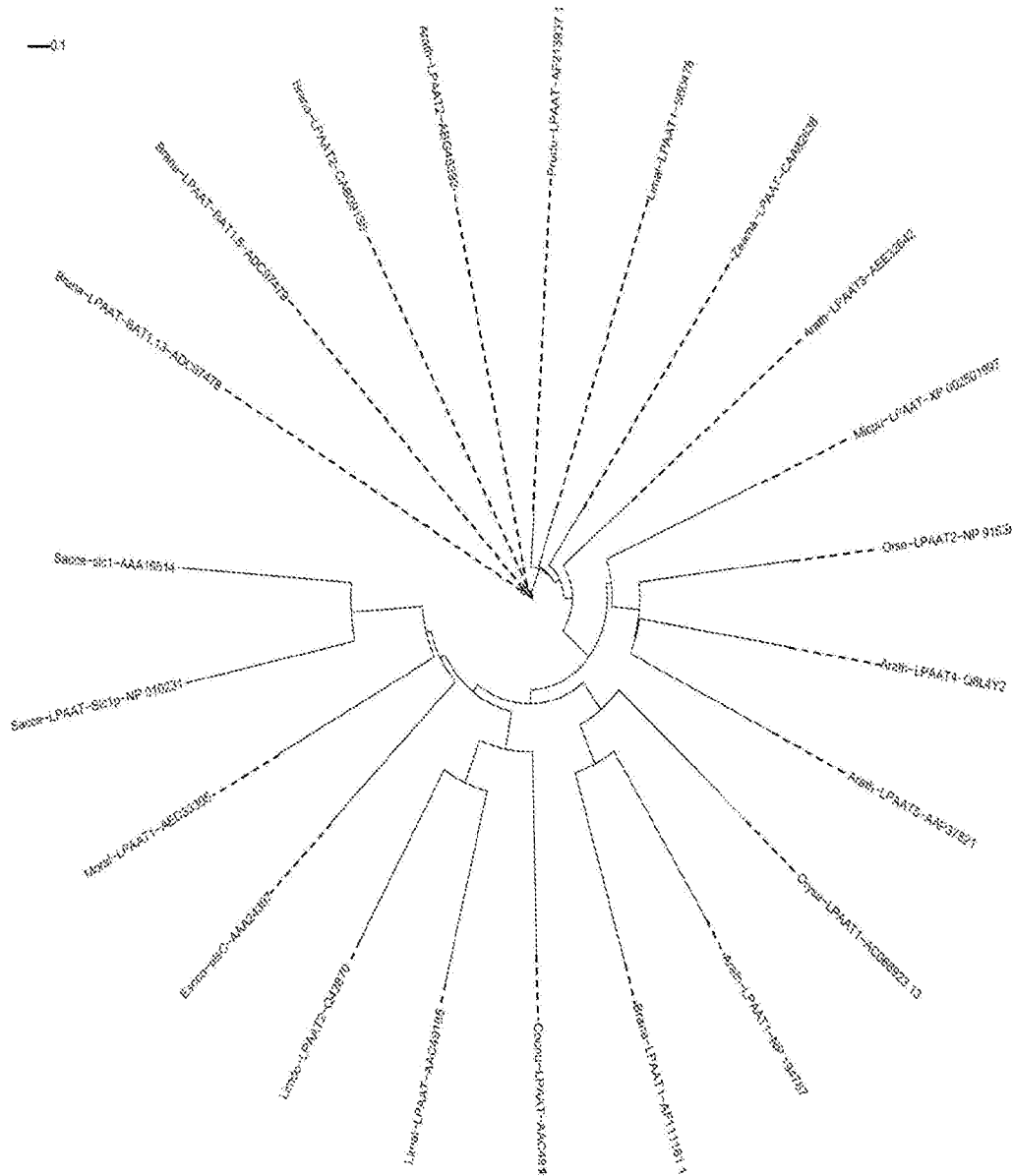
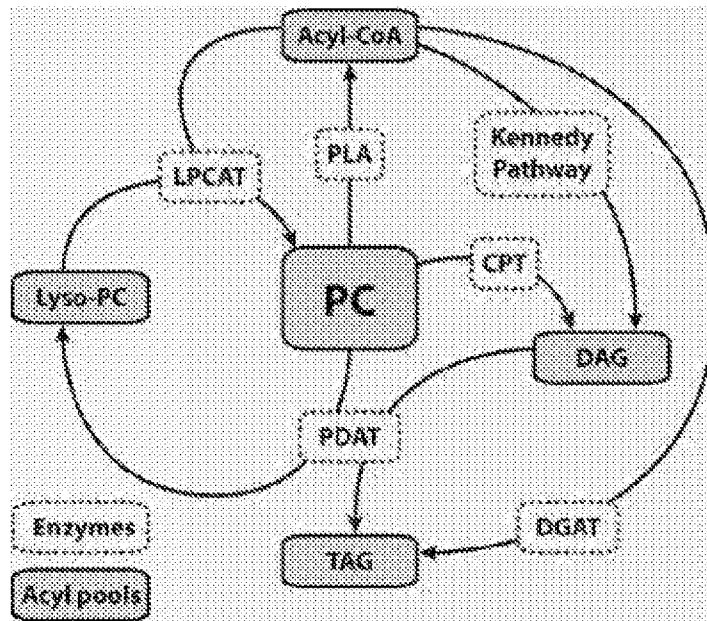


Figure 8

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**Figure 9**

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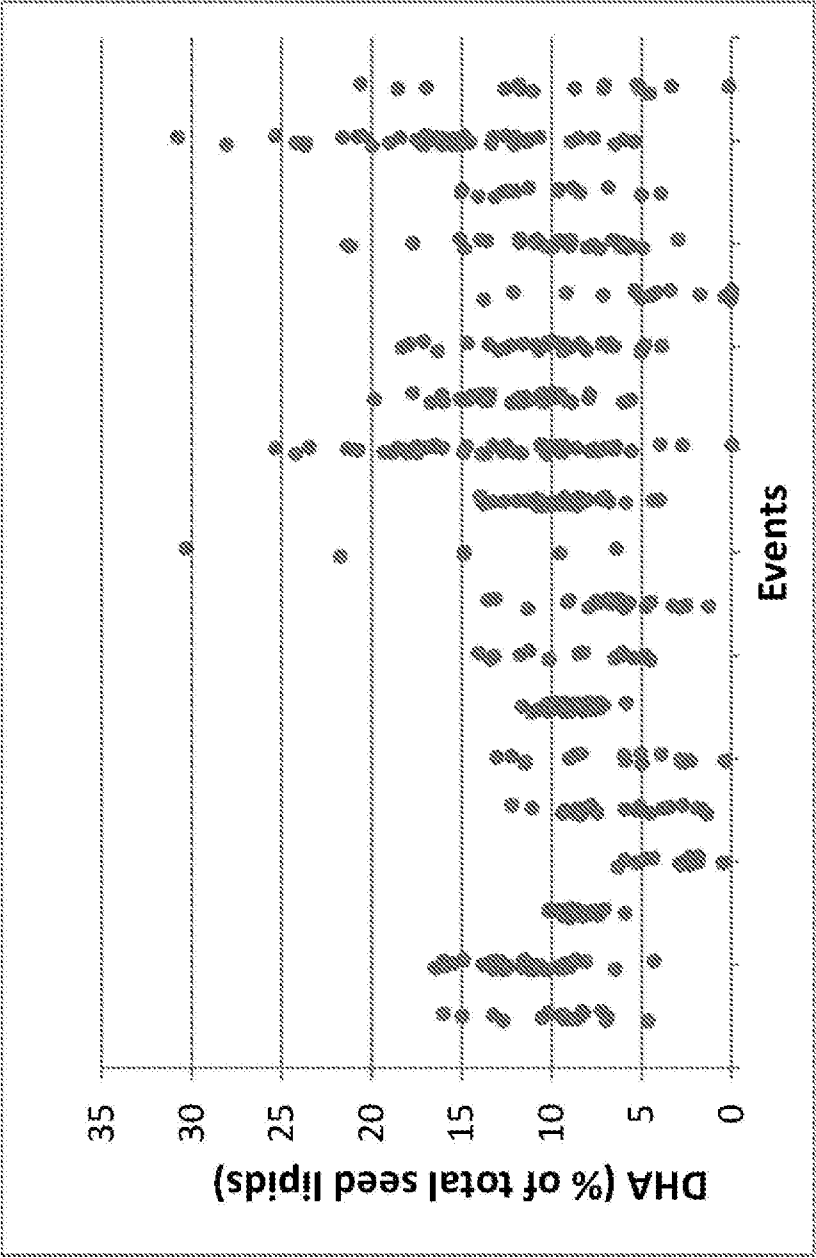


Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2014/050433

A. CLASSIFICATION OF SUBJECT MATTER

**A01H 5/00 (2006.01) C11B 1/10 (2006.01) C12N 15/52 (2006.01) C07H 21/04 (2006.01) C12N 15/82 (2006.01)
C12N 15/79 (2006.01)**

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, HCAPLUS, BIOSIS, AGRICOLA, EMBASE, WPIAP, EPODOC, TXTE: plant, seed, seed oil, oil-seed, Arabidopsis, crop, brassica, camelina, canola, rapeseed, lipid, fatty acid, PUFA, HUFA, oil, DHA, docosahexaeno, cervon, doconexent, 22-6-N-3, omega-3, Increase, improve, raise, alter, modify, optimize, enhance, high, engineer, transgenic, enzyme, pathway, metabolism, recombinant, exogenous, insert, express, encode, DPA, docosapentaenoic acid, docosa 4,7,10,13,16 pentaenoic acid), (4,7,10,13,16 docosapentaenoic acid, 22-5n-3, osbond acid, clupanodonic acid, osbond acid, microalgae, alga, microbial, microorganism, and like terms and synonyms. Applicant/inventor name search.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	



Further documents are listed in the continuation of Box C



See patent family annex

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		
Date of the actual completion of the international search 20 March 2015		Date of mailing of the international search report 20 March 2015	
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustalia.gov.au		Authorised officer Susan MacKenzie AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. 0262832690	

INTERNATIONAL SEARCH REPORT		International application No.
C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		PCT/AU2014/050433
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2010/147900 A1 (E.I. DUPONT DE NEMOURS AND COMPANY) 23 December 2010 Table 19, Table 20, page 109, Example 2, page 54	2-4, 6, 7, 9-14, 16, 21, 23, 24, 26-29
A	US 7550286 B2 (DAMUDE, H.G.. et al.) 23 June 2009 Table 10	1-29
A	US 2013/0309772 A1 (SAKAGUCHI, K. et al.) 21 November 2013 Fig. 30	1-29
A	WO 2005/103253 A1 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 03 November 2005 Table 4	1-29
A	PETRIE, J.R. et al., 'Metabolic engineering plant seeds with fish oil-like levels of DHA', PLoS One, 2012. Vol. 7, e49165, published online 7 November 2012 Whole document	1-29
A	RUIZ-LOPEZ, N. et al., 'Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants', Journal of Experimental Botany, 2012, Vol. 63, pages 2397-2410 Whole document	1-29
P,A	PETRIE, J.R. et al. 'Metabolic engineering <i>Camelina sativa</i> with fish oil-like levels of DHA', PLoS One, January 2014. Vol. 9, page e85061 Whole document	1-29
T	WO 2013/185184 A2 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION et al.) 19 December 2013 Examples	1-29
<p>Form PCT/ISA/210 (fifth sheet) (July 2009)</p>		

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2014/050433

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
the subject matter listed in Rule 39 on which, under Article 17(2)(a)(i), an international search is not required to be carried out, including
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Supplemental Box for Details

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☒ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box**Continuation of: Box III**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into distinct groups:

(1) Claims 1, 5, 15 (all fully), 3, 4, 7-14, 17-29 (all partially). The feature of extracted plant lipid wherein the level of DHA in the total fatty acid content of the extracted lipid is between 20.1% and 30% or between 20.1% and 35% and claims related thereto, is specific to this group of claims.

(2) Claims 2, 6, 16 (all fully), 3, 4, 7-14, 17-29 (all partially). The feature of extracted plant lipid or microbial lipid wherein the level of DPA in the total fatty acid content of the extracted lipid is between 7% and 35% and claims related thereto is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. The only feature common to all of the claimed inventions and which provides a technical relationship among them is the concept of the production modified plant oils with modified omega-3 LC PUFAS. However this feature does not make a contribution over the prior art because it is disclosed in many documents in the prior art of which the documents below are cited as one of many which disclose the concept of genetically altering plants to produce extracted lipids comprising fatty acids similar to fish oil derived lipids. Furthermore, the concept of increasing production of C22 polyunsaturated fatty acids such as DHA or DPA is disclosed in the 2nd cited document Petrie et al, 2012.

RUIZ-LOPEZ, N. et al., 'Metabolic engineering of the omega-3 long chain polyunsaturated fatty acid biosynthetic pathway into transgenic plants', Journal of Experimental Botany, 2012, Vol. 63, pages 2397-2410

PETRIE, J.R. et al., 'Metabolic engineering plant seeds with fish oil-like levels of DHA', PLoS One, 2012. Vol. 7, No. 11, e49165, published online 7 November 2012

Therefore in the light of this document this common feature cannot be a special technical feature. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a posteriori*.

INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2014/050433	
This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.			
Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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Form PCT/ISA/210 (Family Annex)(July 2009)

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Patent Document/s Cited in Search Report		Patent Family Member/s	
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INTERNATIONAL SEARCH REPORT		International application No.	
Information on patent family members		PCT/AU2014/050433	
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.			

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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. Form PCT/ISA/210 (Family Annex)(July 2009)			

INTERNATIONAL SEARCH REPORT		International application No.	
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Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	Publication Date	Publication Number	Publication Date
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