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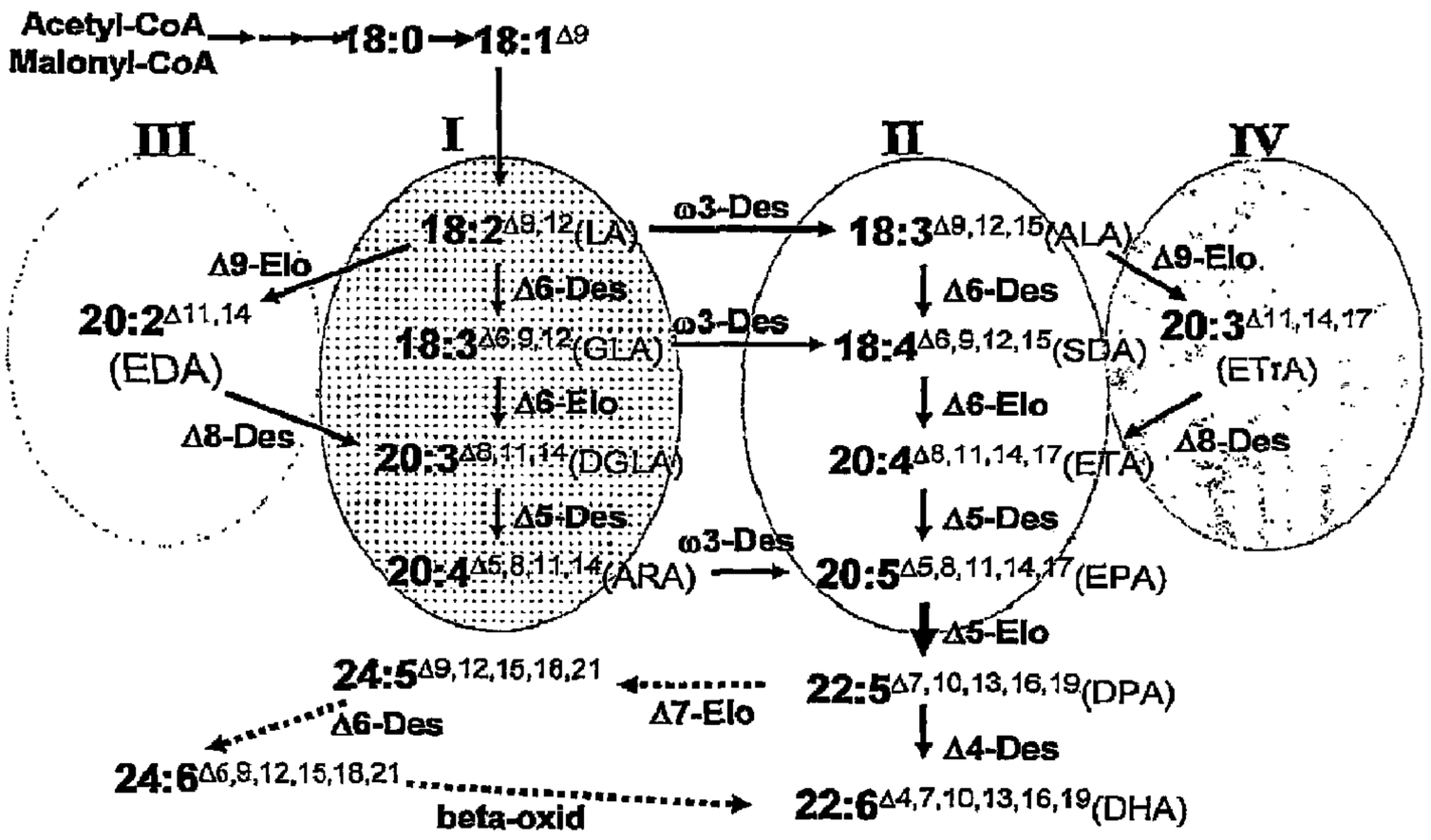
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(72) Inventeurs/Inventors:
SINGH, SURINDER PAL, AU;
ROBERT, STANLEY SURESH, AU;
NICHOLS, PETER DAVID, AU;
BLACKBURN, SUSAN IRENE ELLIS, AU;
...

(54) Titre : SYNTHESE D'ACIDES GRAS POLYINSATURES A CHAINE LONGUE PAR DES CELLULES DE RECOMBINAISON

(54) Title: SYNTHESIS OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS BY RECOMBINANT CELLS



(57) Abrégé/Abstract:
The present invention relates to methods of synthesizing long-chain polyunsaturated fatty acids, especially eicosapentaenoic acid, docosapentaenoic acid and docosahexaneoic acid, in recombinant cells such as yeast or plant cells. Also provided are

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(72) **Inventeurs(suite)/Inventors(continued)**: ZHOU, XUE-RONG, AU; PETRIE, JAMES ROBERTSON, AU; GREEN, ALLAN GRAHAM, AU

(73) **Propriétaires(suite)/Owners(continued)**:
COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION, AU

(74) **Agent**: SMART & BIGGAR LLP

(57) **Abrégé(suite)/Abstract(continued)**:
recombinant cells or plants which produce long-chain polyunsaturated fatty acids. Furthermore, the present invention relates to a group of new enzymes which possess desaturase or elongase activity that can be used in methods of synthesizing long-chain polyunsaturated fatty acids.

Abstract

The present invention relates to methods of synthesizing long-chain polyunsaturated fatty acids, especially eicosapentaenoic acid, docosapentaenoic acid and docosahexaneoic acid, in recombinant cells such as yeast or plant cells. Also provided are recombinant cells or plants which produce long-chain polyunsaturated fatty acids. Furthermore, the present invention relates to a group of new enzymes which possess desaturase or elongase activity that can be used in methods of synthesizing long-chain polyunsaturated fatty acids.

SYNTHESIS OF LONG-CHAIN POLYUNSATURATED FATTY ACIDS BY RECOMBINANT CELLS

This is a division of Canadian Patent Application Serial No. 2,884,237 filed on April 22, 2005, which is a division of Canadian Patent Application Serial No. 2,563,875 filed on April 22, 2005, now patented.

It is to be understood that the expression "the present invention" or the like used in this specification encompasses not only the subject-matter of this divisional application but that of the parent also.

FIELD OF THE INVENTION

The present invention relates to methods of synthesizing long-chain polyunsaturated fatty acids, especially eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid, in recombinant cells such as yeast or plant cells. Also provided are recombinant cells or plants which produce long-chain polyunsaturated fatty acids. Furthermore, the present invention relates to a group of new enzymes which possess desaturase or elongase activity that can be used in methods of synthesizing long-chain polyunsaturated fatty acids.

BACKGROUND OF THE INVENTION

Omega-3 long-chain polyunsaturated fatty acid(s) (LC-PUFA) are now widely recognized as important compounds for human and animal health. These fatty acids may be obtained from dietary sources or by conversion of linoleic (LA, omega-6) or α-linolenic (ALA, omega-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 LC-PUFA, they carry out this conversion at a very low rate. Moreover, most modern societies have imbalanced diets in which at least 90% of polyunsaturated fatty acid(s) (PUFA) consist of omega-6 fatty acids, instead of the 4:1 ratio or less for omega-6: omega-3 fatty acids that is regarded as ideal (Trautwein, 2001). The immediate dietary source of LC-PUFA such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) for humans is mostly from fish or 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. However, due to a decline in global and national fisheries, alternative sources of these beneficial health-enhancing oils are needed.

Inclusion of omega-3 LC-PUFA such as EPA and DHA in the human diet has been linked with numerous health-related benefits. These include prevention or reduction of coronary heart disease, hypertension, type-2 diabetes, renal disease, rheumatoid arthritis, ulcerative colitis and chronic obstructive pulmonary disease, and aiding brain development and growth (Simopoulos, 2000). More recently, a number of studies have also indicated that omega-3 PUFA may be beneficial in infant nutrition and development

and against various mental disorders such as schizophrenia, attention deficit hyperactive disorder and Alzheimer's disease.

Higher 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 omega-3 fatty acids such as EPA, DPA and DHA that are derived from ALA. An important goal in plant biotechnology is therefore the engineering of crop plants, particularly oilseed crops, that produce substantial quantities of LC-PUFA, thus providing an alternative source of these compounds.

Pathways of LC-PUFA synthesis

Biosynthesis of LC-PUFA from linoleic and α-linolenic fatty acids in organisms such as microalgae, mosses and fungi may occur by a series of alternating oxygen-dependent desaturations and elongation reactions as shown schematically in Figure 1. In one pathway (Figure 1, II), the desaturation reactions are catalysed by Δ6, Δ5, and Δ4 desaturases, each of which adds an additional double bond into the fatty acid carbon chain, while each of a Δ6 and a Δ5 elongase reaction adds a two-carbon unit to lengthen the chain. The conversion of ALA to DHA in these organisms therefore requires three desaturations and two elongations. Genes encoding the enzymes required for the production of DHA in this aerobic pathway have been cloned from various microorganisms and lower plants including microalgae, mosses, fungi. Genes encoding some of the enzymes including one that catalyses the fifth step, the Δ5 elongase, have been isolated from vertebrate animals including mammals (reviewed in Sayanova and Napier, 2004). However, the Δ5 elongase isolated from human cells is not specific for the EPA to DPA reaction, having a wide specificity for fatty acid substrates (Leonard et al., 2002).

Alternative routes have been shown to exist for two sections of the ALA to DHA pathway in some groups of organisms. The conversion of ALA to ETA may be carried out by a combination of a Δ9 elongase and a Δ8 desaturase (the so-called Δ8 desaturation route, see Figure 1, IV) in certain protists and thraustochytrids, as evidenced by the isolated of genes encoding such enzymes (Wallis and Browse, 1999; Qi et al., 2002). In mammals, the so-called "Sprecher" pathway converts DPA to DHA by three reactions, independent of a Δ4 desaturase (Sprecher et al., 1995).

Besides these desaturase/elongase systems, EPA and DHA can also be synthesized through an anaerobic pathway in a number of organisms such as *Shewanella, Mortiella* and *Schizochytrium* (Abbadi et al., 2001). The operons encoding these polyketide

synthase (PKS) enzyme complexes have been cloned from some bacteria (Morita et al., 2000; Metz et al., 2001; Tanaka et al., 1999; Yazawa, 1996; Yu et al., 2000; WO 00/42195). The EPA PKS operon isolated from *Shewanella spp* has been expressed in *Synechococcus* allowing it to synthesize EPA (Takeyama et al., 1997). The genes encoding these enzymes are arranged in relatively large operons, and their expression in transgenic plants has not been reported. Therefore it remains to be seen if the anaerobic PKS-like system is a possible alternative to the more classic aerobic desaturase/elongase for the transgenic synthesis of LC-PUFA.

Desaturases

The desaturase enzymes that have been shown to participate in LC-PUFA biosynthesis all belong to the group of so-called "front-end" desaturases which are characterised by the presence of a cytochrome b_5 domain at the N-terminus of each protein. The cyt b_5 domain presumably acts as a receptor of electrons required for desaturation (Napier et al., 1999; Sperling and Heinz, 2001).

The enzyme Δ5 desaturase catalyses the further desaturation of C20 LC-PUFA leading to arachidonic acid (ARA, 20:4ω6) and EPA (20:5ω3). Genes encoding this enzyme have been isolated from a number of organisms, including algae (*Thraustochytrium* sp. Qiu et al., 2001), fungi (*M. alpine*, *Pythium irregulare*, Michaelson et al., 1998; Hong et al., 2002), *Caenorhabditis elegans* and mammals. A gene encoding a bifunctional Δ5-/Δ6- desaturase has also been identified from zebrafish (Hasting et al., 2001). The gene encoding this enzyme might represent an ancestral form of the "front-end desaturase" which later duplicated and evolved distinct functions. The last desaturation step to produce DHA is catalysed by a Δ4 desaturase 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).

Elongases

Several genes encoding PUFA-elongation enzymes have also been isolated (Sayanova and Napier, 2004). The members of this gene family were unrelated to the elongase genes present in higher plants, such as FAE1 of *Arabidopsis*, that are involved in the extension of saturated and monounsaturated fatty acids. An example of the latter is erucic acid (22:1) in *Brassicas*. In some protist species, LC-PUFA are synthesized by elongation of linoleic or α-linolenic acid with a C2 unit, before desaturation with Δ8 desaturase (Figure 1 part IV; "Δ8-desaturation" pathway). Δ6 desaturase and Δ6

elongase activities were not detected in these species. Instead, a Δ9-elongase activity would be expected in such organisms, and in support of this, a C18 Δ9-elongase gene has recently been isolated from *Isochrysis galbana* (Qi et al., 2002).

Engineered production of LC-PUFA

Transgenic oilseed crops that are engineered to produce major LC-PUFA by the insertion of these genes have been suggested as a sustainable source of nutritionally important fatty acids. However, the requirement for coordinate expression and activity of five new enzymes encoded by genes from possibly diverse sources has made this goal difficult to achieve and the proposal remained speculative until now.

The LC-PUFA oxygen-dependent biosynthetic pathway to form EPA (Figure 1) has been successfully constituted in yeast by the co-expression of a Δ6-elongase with Δ6- and Δ5 fatty acid desaturases, resulting in small but significant accumulation of ARA and EPA from exogenously supplied linoleic and α-linolenic acids (Beaudoin et al., 2000; Zank et al., 2000). This demonstrated the ability of the genes belonging to the LC-PUFA synthesis pathway to function in heterologous organisms. However, the efficiency of producing EPA was very low. For example, three genes obtained from *C. elegans*, *Borago officinalis* and *Mortierella alpina* were expressed in yeast (Beaudoin et al., 2000). When the transformed yeast were supplied with 18:2ω-3 (LA) or 18:3ω-3 (ALA), there was slight production of 20:4ω-6 or 20:5ω-3, at conversion efficiencies of 0.65% and 0.3%, respectively. Other workers similarly obtained very low efficiency production of EPA by using genes expressing two desaturases and one elongase in yeast (Domergue et al., 2003a; Zank et al., 2002). There remains, therefore, a need to improve the efficiency of production of EPA in organisms such as yeast, let alone the production of the C22 PUFA which requires the provision of additional enzymatic steps.

Some progress has been made in the quest for introducing the aerobic LC-PUFA biosynthetic pathway into higher plants including oilseed crops (reviewed by Sayanova and Napier, 2004; Drexler et al., 2003; Abbadi et al., 2001). A gene encoding a Δ6-fatty acid desaturase isolated from borage (*Borago officinalis*) was expressed in transgenic tobacco and *Arabidopsis*, resulting in the production of GLA (18:3ω6) and SDA (18:4ω3), the direct precursors for LC-PUFA, in the transgenic plants (Sayanova et al., 1997; 1999). However, this provides only a single, first step.

Domergue et al. (2003a) used a combination of three genes, encoding Δ6- and Δ5 fatty acid desaturases and a Δ6-elongase in both yeast and transgenic linseed. The desaturase genes were obtained from the diatom *Phaeodactylum tricornutum* and the elongase gene from the moss *Physcomitrella patens*. Low elongation yields were

obtained for endogenously produced Δ6-fatty acids in yeast cells (i.e. combining the first and second enzymatic steps), and the main C20 PUFA product formed was $20:2^{\Delta 11,14}$, representing an unwanted side reaction. Domergue et al. (2003a) also state, without presenting data, that the combination of the three genes were expressed in transgenic linseed which consequently produced ARA and EPA, but that production was inefficient. They commented that the same problem as had been observed in yeast existed in the seeds of higher plants and that the "bottleneck" needed to be circumvented for production of LC-PUFA in oil seed crops.

WO 2004/071467 (DuPont) reported the expression of various desaturases and elongases in soybean cells but did not show the synthesis of DHA in regenerated plants or in seeds.

Abbadi et al. (2004) described attempts to express combinations of desaturases and elongases in transgenic linseed, but achieved only low levels of synthesis of EPA. Abbadi et al. (2004) indicated that their low levels of EPA production were also due to an unknown "bottleneck".

Qi et al. (2004) achieved synthesis in leaves but did not report results in seeds. This is an important issue as the nature of LC-PUFA synthesis can vary between leaves and seeds. In particular, oilseeds store lipid in seeds mostly as TAG while leaves synthesize the lipid mostly as phosphatidyl lipids. Furthermore, Qi et al. (2004) only produced AA and EPA.

As a result, there is a need for further methods of producing long-chain polyunsaturated, particularly EPA, DPA and DHA, in recombinant cells.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a recombinant cell which is capable of synthesising a long chain polyunsaturated fatty acid(s) (LC-PUFA), comprising one or more polynucleotides which encode at least two enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein said recombinant cell is derived from a cell that is not capable of synthesising said LC-PUFA.

In a second aspect, the present invention provides a recombinant cell with an enhanced capacity to synthesize a LC-PUFA relative to an isogenic non-recombinant cell, comprising one or more polynucleotides which encode at least two enzymes each of

which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of expressing said polynucleotides in said recombinant cell.

In one embodiment, at least one of the enzymes is a Δ5 elongase.

The present inventors are the first to identify an enzyme which has greater Δ5 elongase activity than Δ6 elongase activity. As a result, this enzyme provides an efficient means of producing DPA in a recombinant cell as the Δ5 elongation of EPA is favoured over the Δ6 elongation of SDA. Thus, in an embodiment, the Δ5 elongase is relatively specific, that is, where the Δ5 elongase also has Δ6 elongase activity the elongase is more efficient at synthesizing DPA from EPA than it is at synthesizing ETA from SDA.

In another embodiment, the Δ5 elongase comprises

i) an amino acid sequence as provided in SEQ ID NO:2,

ii) an amino acid sequence which is at least 50%, more preferably at least 80%, even more preferably at least 90%, identical to SEQ ID NO:2, or

iii) a biologically active fragment of i) or ii).

In another embodiment, the Δ5 elongase can be purified from algae.

In another embodiment, at least one of the enzymes is a Δ9 elongase.

The present inventors are the first to identify an enzyme which has both Δ9 elongase activity and Δ6 elongase activity. When expressed in a cell with a Δ6 desaturase and a Δ8 desaturase this enzyme can use the two available pathways to produce ETA from ALA, DGLA from LA, or both (see Figure 1), thus increasing the efficiency of ETA and/or DGLA production. Thus, in an embodiment, the Δ9 elongase also has Δ6 elongase activity. Preferably, the Δ9 elongase is moro efficient at synthesizing ETrA from ALA than it is at synthesizing ETA from SDA. Furthermore, in another embodiment the Δ9 elongase is able to elongate SDA to ETA, GLA to DGLA, or both, in a yeast cell.

In a further embodiment, the Δ9 elongase comprises

i) an amino acid sequence as provided in SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86,

ii) an amino acid sequence which is at least 50%, more preferably at least 80%, even more preferably at least 90%, identical to SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86, or

iii) a biologically active fragment of i) or ii).

Preferably, the Δ9 elongase can be purified from algae or fungi.

It is well known in the art that the greater the number of transgenes in an organism, the greater the likelihood that at least one fitness parameter of the organism, such as expression level of at least one of the transgenes, growth rate, oil production, reproductive capacity etc, will be compromised. Accordingly, it is desirable to minimize the number of transgenes in a recombinant cell. To this end, the present inventors have devised numerous strategies for producing LC-PUFA's in a cell which avoid the need for a gene to each step in the relevant pathway.

Thus, in another embodiment, at least one of the enzymes is a Δ5/Δ6 bifunctional desaturase or a Δ5/Δ6 bifunctional elongase. The Δ5/Δ6 bifunctional desaturase may be naturally produced by a freshwater species of fish.

In a particular embodiment, the Δ5/Δ6 bifunctional desaturase comprises

i) an amino acid sequence as provided in SEQ ID NO:15,

ii) an amino acid sequence which is at least 50%, more preferably at least 80%, even more preferably at least 90%, identical to SEQ ID NO:15, or

iii) a biologically active fragment of i) or ii).

Preferably, the Δ5/Δ6 bifunctional desaturase is naturally produced by a freshwater species of fish.

Preferably, the Δ5/Δ6 bifunctional elongase comprises

i) an amino acid sequence as provided in SEQ ID NO:2 or SEQ ID NO:14,

ii) an amino acid sequence which is at least 50%, more preferably at least 80%, even more preferably at least 90%, identical to SEQ ID NO:2 or SEQ ID NO:14, or

iii) a biologically active fragment of i) or ii).

In another embodiment, at least one of the enzymes is a Δ5 desaturase.

In a further embodiment, at least one of the enzymes is a Δ8 desaturase.

In another embodiment, the LC-PUFA is docosahexaenoic acid (DHA).

Preferably, the introduced polynucleotide(s) encode three or four enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, or Δ4 desaturase. More preferably, the enzymes are any one of the following combinations;

i) a Δ5/Δ6 bifunctional desaturase, a Δ5/Δ6 bifunctional elongase, and a Δ4 desaturase,

ii) a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, a Δ6 elongase, and a Δ4 desaturase, or

iii) a Δ5 desaturase, a Δ6 desaturase, a Δ5/Δ6 bifunctional elongase, and a Δ4 desaturase.

In another embodiment, the LC-PUFA is DHA and the introduced polynucleotide(s) encode five enzymes wherein the enzymes are any one of the following combinations;

i) a Δ4 desaturase, a Δ5 desaturase, a Δ6 desaturase, a Δ5 elongase and a Δ6 elongase, or

ii) a Δ4 desaturase, a Δ5 desaturase, a Δ8 desaturase, a Δ5 elongase and a Δ9 elongase.

In a further embodiment, the cell is of an organism suitable for fermentation, and the enzymes are at least a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, a Δ6 elongase, and a Δ4 desaturase.

In another embodiment, the LC-PUFA is docosapentaenoic acid (DPA).

Preferably, the introduced polynucleotide(s) encode two or three enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, or Δ6 elongase. More preferably, the enzymes are any one of the following combinations;

i) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase,

ii) a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, and a Δ6 elongase, or

iii) a Δ5 desaturase, a Δ6 desaturase, and a Δ5/Δ6 bifunctional elongase.

In a further embodiment, the LC-PUFA is DPA and the introduced polynucleotide(s) encode four enzymes wherein the enzymes are any one of the following combinations;

i) a Δ5 desaturase, a Δ6 desaturase, a Δ5 elongase and a Δ6 elongase; or

ii) a Δ5 desaturase, a Δ8 desaturase, a Δ5 elongase and a Δ9 elongase.

In another embodiment, the cell is of an organism suitable for fermentation, and the enzymes are at least a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, and a Δ6 elongase.

In a further embodiment, the LC-PUFA is eicosapentaenoic acid (EPA).

Preferably, the introduced polynucleotide(s) encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase.

In another embodiment, the introduced polynucleotide(s) encode three enzymes wherein the enzymes are any one of the following combinations;

i) a Δ5 desaturase, a Δ6 desaturase, and a Δ6 elongase, or

ii) a Δ5 desaturase, a Δ8 desaturase, and a Δ9 elongase.

Evidence to date suggests that desaturases expressed in at least some recombinant cells, particularly yeast, have relatively low activity. However, the present inventors have identified that this may be a function of the capacity of the desaturase to use acyl-

CoA as a substrate in LC-PUFA synthesis. In this regard, it has also been determined that desaturase of vertebrate origin are particularly useful for the production of LC-PUFA in recombinant cells, for example, plant cells, seeds, or yeast. Thus, in another preferred embodiment, the recombinant cell comprises either

i) at least one Δ5 elongase catalyses the conversion of EPA to DPA in the cell,
ii) at least one desaturase which is able to act on an acyl-CoA substrate,
iii) at least one desaturase from a vertebrate or a variant desaturase thereof, or
iv) any combination of i), ii) or iii).

In a particular embodiment, the Δ5 elongase comprises

i) an amino acid sequence as provided in SEQ ID NO:2,

ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:2, or

iii) a biologically active fragment of i) or ii).

The desaturase able to act on an acyl-CoA substrate or from a vertebrate may be a Δ5 desaturase, a Δ6 desaturase, or both. In a particular embodiment, the desaturase comprises

i) an amino acid sequence as provided in SEQ ID NO:16, SEQ ID NO:21 or SEQ ID NO:22,

ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:16, SEQ ID NO:21 or SEQ ID NO:22, or

iii) a biologically active fragment of i) or ii).

Preferably, the at least one desaturase is naturally produced by a vertebrate.

Alternatively, when the cell is a yeast cell, the LC-PUFA is DHA, and the enzymes are at least a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, a Δ6 elongase, and a Δ4 desaturase.

In a further alternative, when the cell is a yeast cell, the LC-PUFA is DPA, and the enzymes are at least a Δ5/Δ6 bifunctional desaturase, a Δ5 elongase, and a Δ6 elongase.

Although the cell may be any cell type, preferably, said cell is capable of producing said LC-PUFA from endogenously produced linoleic acid (LA), α-linolenic acid (ALA), or both. More preferably, the ratio of the endogenously produced ALA to LA is at least 1:1 or at least 2:1.

In one embodiment, the cell is a plant cell, a plant cell from an angiosperm, an oilseed plant cell, or a cell in a seed. Preferably, at least one promoter is a seed specific promoter.

In another embodiment, the cell is of a unicellular microorganism. Preferably, the unicellular microorganism is suitable for fermentation. Preferably, the microorganism is a yeast.

In a further embodiment, the cell is a non-human animal cell or a human cell *in vitro*.

In a further embodiment, the recombinant cell produces a LC-PUFA which is incorporated into triacylglycerols in said cell. More preferably, at least 50% of the LC-PUFA that is produced in said cell is incorporated into triacylglycerols.

In another embodiment, at least the protein coding region of one, two or more of the polynucleotides is obtained from an algal gene. Preferably, the algal gene is from the genus *Pavlova* such as from the species *Pavlova salina*.

In another aspect, the present invention provides a recombinant cell that is capable of producing DHA from a fatty acid which is ALA, LA, GLA, ARA, SDA, ETA, EPA, or any combination or mixture of these, wherein said recombinant cell is derived from a cell that is not capable of synthesising DHA.

In a further aspect, the present invention provides a recombinant cell that is capable of producing DPA from a fatty acid which is ALA, LA, GLA, ARA, SDA, ETA, EPA, or any combination or mixture of these, wherein said recombinant cell is derived from a cell that is not capable of synthesising DPA.

In yet a further aspect, the present invention provides a recombinant cell that is capable of producing EPA from a fatty acid which is ALA, LA, GLA, SDA, ETA or any combination or mixture of these, wherein said recombinant cell is derived from a cell that is not capable of synthesising EPA.

In another aspect, the present invention provides a recombinant cell that is capable of producing both ETrA from ALA and ETA from SDA, and which produces EPA from a fatty acid which is ALA, LA, GLA, SDA, ETA, or any combination or mixture of these, wherein said recombinant cell is derived from a cell that is not capable of synthesising ETrA, ETA or both.

In a further aspect, the present invention provides a recombinant cell of an organism useful in fermentation processes, wherein the cell is capable of producing DPA from LA, ALA, arachidonic acid (ARA), eicosatetraenoic acid (ETA), or any combination or mixture of these, wherein said recombinant cell is derived from a cell that is not capable of synthesising DPA.

In another aspect, the present invention provides a recombinant plant cell capable of producing DPA from LA, ALA, EPA, or any combination or mixture of these, wherein the plant cell is from an angiosperm.

In an embodiment, the plant cell is also capable of producing DHA.

In yet another aspect, the present invention provides a recombinant cell which is capable of synthesising DGLA, comprising a polynucleotide(s) encoding one or both of:

a) a polypeptide which is an Δ9 elongase, wherein the Δ9 elongase is selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86,

ii) a polypeptide comprising an amino acid sequence which is at least 40% identical to SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86, and

iii) a biologically active fragment of i) or ii), and/or

b) a polypeptide which is an Δ8 desaturase, wherein the Δ8 desaturase is selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:1,

ii) a polypeptide comprising an amino acid sequence which is at least 40% identical to SEQ ID NO:1, and

iii) a biologically active fragment of i) or ii),

wherein the polynucleotide(s) is operably linked to one or more promoters that are capable of directing expression of said polynucleotide(s) in the cell, and wherein said recombinant cell is derived from a cell that is not capable of synthesising DGLA.

In an embodiment, the cell is capable of converting DGLA to ARA.

In another embodiment, the cell further comprises a polynucleotide which encodes a Δ5 desaturase, wherein the polynucleotide encoding the Δ5 desaturase is operably linked to one or more promoters that are capable of directing expression of said polynucleotide in the cell, and wherein the cell is capable of producing ARA.

In a particular embodiment, the cell lacks ω3 desaturase activity and is not capable of producing ALA. Such cells may be naturally occurring, or produced by reducing the ω3 desaturase activity of the cell using techniques well known in the art.

Preferably, the cell is a plant cell or a cell of an organism suitable for fermentation.

In a further embodiment, a recombinant cell of the invention also possesses the enzyme required to perform the "Sprecher" pathway of converting EPA to DHA. These enzymes may be native to the cell or produced recombinantly. Such enzymes at least include a Δ7 elongase, Δ6 desaturase and enzymes required for the peroxisomal β-oxidation of tetracosahexaenoic acid to produce DHA.

The present inventors have also identified a group of new desaturases and elongases. As a result, further aspects of the invention relate to these enzymes, as well as homologs/variants/derivatives thereof.

The polypeptide may be a fusion protein further comprising at least one other polypeptide sequence.

The at least one other polypeptide may be a polypeptide that enhances the stability of a polypeptide of the present invention, or a polypeptide that assists in the purification of the fusion protein.

Also provided are isolated polynucleotides which, inter alia, encode polypeptides of the invention.

In a further aspect, the present invention provides a vector comprising or encoding a polynucleotide according to the invention. Preferably, the polynucleotide is operably linked to a seed specific promoter.

In another aspect, the present invention provides a recombinant cell comprising an isolated polynucleotide according to the invention.

In a further aspect, the present invention provides a method of producing a cell capable of synthesising one or more LC-PUFA, the method comprising introducing into the cell one or more polynucleotides which encode at least two enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell.

In another aspect, the present invention provides a method of producing a recombinant cell with an enhanced capacity to synthesize one or more LC-PUFA, the method comprising introducing into a first cell one or more polynucleotides which encode at least two enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the recombinant cell, and wherein said recombinant cell has an enhanced capacity to synthesize said one or more LC-PUFA relative to said first cell.

Naturally, it will be appreciated that each of the embodiments described herein in relation to the recombinant cells of the invention will equally apply to methods for the production of said cells.

In a further aspect, the present invention provides a cell produced by a method of the invention.

In another aspect, the present invention provides a transgenic plant comprising at least one recombinant cell according to the invention.

Preferably, the plant is an angiosperm. More preferably, the plant is an oilseed plant.

In a further embodiment, the transgenic plant, or part thereof including a transgenic seed, does not comprise a transgene which encodes an enzyme which preferentially converts an ω6 LC-PUFA into an ω3 LC-PUFA.

In yet a further embodiment, the transgenic plant, or part thereof including a transgenic seed, comprises a transgene encoding a Δ8 desaturase and/or a Δ9 elongase.

In a further aspect, the present invention provides a method of producing an oilseed, the method comprising

i) growing a transgenic oilseed plant according to the invention under suitable conditions, and

ii) harvesting the seed of the plant.

In a further aspect, the invention provides a part of the transgenic plant of the invention, wherein said part comprises an increased level of LC-PUFA in its fatty acid relative to the corresponding part from an isogenic non-transformed plant.

Preferably, said plant part is selected from, but not limited to, the group consisting of: a seed, leaf, stem, flower, pollen, roots or specialised storage organ (such as a tuber).

Previously, it has not been shown that LC-PUFA can be produced in plant seeds, nor that these LC-PUFA can be incorporated into plant oils such as triacylglycerol.

Thus, in another aspect the present invention provides a transgenic seed comprising a LC-PUFA.

Preferably, the LC-PUFA is selected from the group consisting of:

i) EPA,

ii) DPA,

iii) DHA,

iv) EPA and DPA, and

v) EPA, DHA, and DPA.

More preferably, the LC-PUFA is selected from the group consisting of:

i) DPA,

ii) DHA, or

iii) DHA and DPA.

Even more preferably, the LC-PUFA is EPA, DHA, and DPA.

Preferably, the seed is derived from an isogenic non-transgenic seed which produces LA and/or ALA. More preferably, the isogenic non-transgenic seed comprises a higher concentration of ALA than LA in its fatty acids. Even more preferably, the

isogenic non-transgenic seed comprises at least about 13% ALA or at least about 27% ALA or at least about 50% ALA in its fatty acid.

Preferably, the total fatty acid in the oil of the seed comprises at least 9% C20 fatty acids.

Preferably, the seed is derived from an oilseed plant. More preferably, the oilseed plant is oilseed rape (*Brassica napus*), maize (*Zea mays*), sunflower (*Helianthus annuus*), soybean (*Glycine max*), sorghum (*Sorghum bicolor*), flax (*Linum usitatissimum*), sugar (*Saccharum officinarum*), beet (*Beta vulgaris*), cotton (*Gossypium hirsutum*), peanut (*Arachis hypogaea*), poppy (*Papaver somniferum*), mustard (*Sinapis alba*), castor bean (*Ricinus communis*), sesame (*Sesamum indicum*), or safflower (*Carthamus tinctorius*).

It is preferred that the seed has a germination rate which is substantially the same as that of the isogenic non-transgenic seed.

It is further preferred that the timing of germination of the seed is substantially the same as that of the isogenic non-transgenic seed.

Preferably, at least 25%, or at least 50%, or at least 75% of the LC-PUFA in the seed form part of triacylglycerols.

Surprisingly, the present inventors have found that transgenic seeds produced using the methods of the invention have levels of ALA and LA which are substantially the same as those of an isogenic non-transgenic seed. As a result, it is preferred that the transgenic seed has levels of ALA and LA which are substantially the same as those of an isogenic non-transgenic seed. Furthermore, it was surprising to note that the levels of monounsaturated fatty acids were decreased in transgenic seeds produced using the methods of the invention. Accordingly, in a further preferred embodiment, the transgenic seed has decreased levels of monounsaturated fatty acids when compared to an isogenic non-transgenic seed.

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

i) introducing into a progenitor cell of a seed one or more polynucleotides which encode at least two enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, thereby producing a recombinant progenitor cell,

ii) culturing said recombinant progenitor cell to produce a plant which comprises said transgenic seed, and

iii) recovering the seed from the plant so produced.

In yet a further aspect, the present invention provides a method of producing a transgenic seed comprising cultivating a transgenic plant which produces the transgenic seed of the invention, and harvesting said transgenic seed from the plant.

In a further aspect, the invention provides an extract from the transgenic plant of the invention, or a plant part of the invention, or a seed of the invention, wherein said extract comprises an increased level of LC-PUFA in its fatty acid relative to a corresponding extract from an isogenic non-transformed plant.

Preferably, the extract is substantially purified oil comprising at least 50% triacylglycerols.

In a further aspect, the present invention provides a non-human transgenic animal comprising at least one recombinant cell according to the invention.

Also provided is a method of producing a LC-PUFA, the method comprising culturing, under suitable conditions, a recombinant cell according to the invention.

In one embodiment, the cell is of an organism suitable for fermentation and the method further comprises exposing the cell to at least one LC-PUFA precursor. Preferably, the LC-PUFA precursor is at least one of linoleic acid or α-linolenic acid. In a particular embodiment, the LC-PUFA precursor is provided in a vegetable oil.

In another embodiment, the cell is an algal cell and the method further comprises growing the algal cell under suitable conditions for production of said LC-PUFA.

In a further aspect, the present invention provides a method of producing one or more LC-PUFA, the method comprising cultivating, under suitable conditions, a transgenic plant of the invention.

In another aspect, the present invention provides a method of producing oil comprising at least one LC-PUFA, comprising obtaining the transgenic plant of the invention, or the plant part of the invention, or the seed of the invention, and extracting oil from said plant, plant part or seed.

Preferably, said oil is extracted from the seed by crushing said seed.

In another aspect, the present invention provides a method of producing DPA from EPA, the method comprising exposing EPA to a polypeptide of the invention and a fatty acid precursor, under suitable conditions.

In an embodiment, the method occurs in a cell which uses the polyketide-like system to produce EPA.

In yet another aspect, the present invention provides a fermentation process comprising the steps of:

i) providing a vessel containing a liquid composition comprising a cell of the invention and constituents required for fermentation and fatty acid biosynthesis; and

ii) providing conditions conducive to the fermentation of the liquid composition contained in said vessel.

Preferably, a constituent required for fermentation and fatty acid biosynthesis is LA.

Preferably, the cell is a yeast cell.

In another aspect, the present invention provides a composition comprising a cell of the invention, or an extract or portion thereof comprising LC-PUFA, and a suitable carrier.

In another aspect, the present invention provides a composition comprising the transgenic plant of the invention, or the plant part of the invention, or the seed of the invention, or an extract or portion thereof comprising LC-PUFA, and a suitable carrier.

In yet another aspect, the present invention provides a feedstuff comprising 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 of the invention.

Preferably, the feedstuff at least comprises DPA, wherein at least one enzymatic reaction in the production of DPA was performed by a recombinant enzyme in a cell.

Furthermore, it is preferred that the feedstuff comprises at least comprises DHA, wherein at least one enzymatic reaction in the production of DHA was performed by a recombinant enzyme in a cell.

In a further aspect, the present invention provides a method of preparing a feedstuff, the method comprising admixing 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 of the invention, with a suitable carrier. Preferably, the feedstuff is for consumption by a mammal or a fish.

In a further aspect, the present invention provides a method of increasing the levels of a LC-PUFA in an organism, the method comprising administering to the organism 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 of the invention, or a feedstuff of the invention.

Preferably, the administration route is oral.

Preferably, the organism is a vertebrate. More preferably, the vertebrate is a human, fish, companion animal or livestock animal.

In a further aspect, the present invention provides a method of treating or preventing a condition which would benefit from a LC-PUFA, the method comprising administering to a subject 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 of the invention, or a feedstuff of the invention.

Preferably, the condition is arrhythmia's, angioplasty, inflammation, asthma, psoriasis, osteoporosis, kidney stones, AIDS, multiple sclerosis, rheumatoid arthritis, Crohn's disease, schizophrenia, cancer, foetal alcohol syndrome, attention deficient hyperactivity disorder, cystic fibrosis, phenylketonuria, unipolar depression, aggressive hostility, adrenoleukodystophy, 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 encephalomyelitis, chronic fatigue after viral infections or ocular disease.

Whilst providing the subject with any amount of LC-PUFA will be beneficial to the subject, it is preferred that an effective amount to treat the condition is administered.

In another aspect, the present invention provides for the use of 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 of the invention, or a feedstuff of the invention, for the manufacture of a medicament for treating or preventing a condition which would benefit from a LC-PUFA.

The *Caenorhabditis elegans* Δ6 elongase has previously been expressed in yeast and been shown to convert octadecatetraenoic acid to eicosatetraenoic acid. However, the present inventors have surprisingly found that this enzyme also possesses Δ5 elongase activity, being able to convert eicosapentaenoic acid to docosapentaenoic acid.

In a further aspect, the present invention provides a method of producing an unbranched LC-PUFA comprising 22 carbon atoms, the method comprising incubating an unbranched 20 carbon atom LC-PUFA with a polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:2 or SEQ ID NO:14,

ii) a polypeptide comprising an amino acid sequence which is at least 50% identical to SEQ ID NO:2 or SEQ ID NO:14, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide also has Δ6 elongase activity.

Preferably, the unbranched LC-PUFA comprising 22 carbon atoms is DPA, and the unbranched 20 carbon atom LC-PUFA is EPA.

Preferably, the method is performed within a recombinant cell which produces the polypeptide and EPA.

In yet a further aspect, the present invention provides a substantially purified antibody, or fragment thereof, that specifically binds a polypeptide of the invention.

In another aspect, the present invention provides a method of identifying a recombinant cell, tissue or organism capable of synthesising one or more LC-PUFA, the method comprising detecting the presence in said cell, tissue or organism of one or more polynucleotides which encode at least two enzymes each of which is a Δ5/Δ6 bifunctional desaturase, Δ5 desaturase, Δ6 desaturase, Δ5/Δ6 bifunctional elongase, Δ5 elongase, Δ6 elongase, Δ4 desaturase, Δ9 elongase, or Δ8 desaturase, wherein the one or more polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, tissue or organism.

Preferably, the method comprises a nucleic acid amplification step, a nucleic acid hybridisation step, a step of detecting the presence of a transgene in the cell, tissue or organism, or a step of determining the fatty acid content or composition of the cell, tissue or organism.

Preferably, the organism is an animal, plant, angiosperm plant or microorganism.

In another aspect, the present invention provides a method of producing DPA from EPA, the method comprising exposing EPA to a Δ5 elongase of the invention and a fatty acid precursor, under suitable conditions.

Preferably, the method occurs in a cell which uses the polyketide-like system to produce EPA. Naturally, recombinant (transgenic) cells, plants, non-human animals comprising a new polynucleotide provided herein may also produce other elongase and/or desaturases such as those defined herein.

The present invention as claimed relates to:

- a recombinant plant cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode: a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase; wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes;

- a recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode: a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase; wherein the cell comprises oil having a total fatty acid content which comprises at least 2.1% EPA and less than 0.1% eicosatrienoic acid (w/w), and wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes;

- a method of producing a recombinant plant cell which synthesises one or more long-chain polyunsaturated fatty acids (LC-PUFA(s)), the method comprising introducing into the cell more than one heterologous polynucleotide, wherein said polynucleotides encode enzymes which comprise: a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or b) a Δ5/Δ6 bifunctional' desaturase and a Δ5/Δ6 bifunctional elongase; wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein said one or more LC-PUFAs comprise EPA, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes;

- a method of producing a long-chain polyunsaturated fatty acid (LC-PUFA) in a cell or a plant, the method comprising: i) culturing, under suitable conditions, a recombinant cell as described herein, and/or ii) cultivating, under suitable conditions, a transgenic plant comprising at least one cell as described herein, wherein the cell is a transgenic plant cell, wherein the cell or plant produces LC-PUFA which comprises EPA;

- a method of producing oil comprising long chain polyunsaturated fatty acid (LC-PUFA), wherein said LC-PUFA comprises eicosapentaenoic acid (EPA), the method comprising i) obtaining seed comprising a recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode: a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase; wherein the seed comprises oil having a total fatty acid content which comprises at least 1.5% EPA, and wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes, and ii) extracting the oil from said seed;

- a method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell as described herein, and extracting oil from said seed;

- a method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell as described herein, and extracting oil from said seed, and wherein the seed comprises more than one heterologous polynucleotide which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase;

- a method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell as described herein, and extracting oil from said seed, and wherein the seed comprises more than one heterologous polynucleotide which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

- a method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining *Brassica napus* or *Arabidopsis thaliana* seed comprising a cell as described herein, and extracting oil from said seed;

- a feedstuff comprising the recombinant cell as described herein;

- a feedstuff comprising the recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell as described herein;

- a method of preparing a feedstuff, the method comprising admixing a recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell as described herein with a suitable carrier;

- a method of preparing a feedstuff, the method comprising admixing oil produced using the method as described herein with a suitable carrier;

- a recombinant nucleic acid molecule comprising: a polynucleotide which encodes a Δ5/Δ6 bifunctional desaturase which catalyses desaturation of α-linolenic acid (ALA) to stearidonic acid (SDA) and desaturation of eicosatetraenoic acid (ETA) to eicosapentaenoic acid (EPA); and a polynucleotide which encodes a Δ6 elongase which catalyses elongation of SDA to ETA; wherein each polynucleotide is operably linked to a promoter which directs expression of the polynucleotide in a plant seed;

- a cell comprising the recombinant nucleic acid molecule as described herein;

- a process of producing oil, comprising: i) obtaining plant seed comprising a cell as described herein, and ii) extracting oil from the plant seed;

- a process for feeding fish comprising: a) obtaining feedstuff which comprises oil extracted from transgenic *Brassica* seeds which comprise eicosapentaenoic acid (EPA) and heterologous polynucleotides which encode enzymes that comprise: i) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or ii) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase; wherein the heterologous polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica* seed cells, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate; and b) providing the feedstuff to fish, thereby feeding fish;

- a process for feeding fish comprising: a) obtaining feedstuff which comprises oil extracted from transgenic *Brassica napus* seeds which comprise docosapentaenoic acid (DPA) and

docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols in the seeds, and heterologous polynucleotides which encode enzymes that comprise: i) a Δ5 elongase; ii) a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31; iii) a Δ4 desaturase whose amino acid sequence is at least 99% identical to the sequence set forth as SEQ ID NO:33; iv) a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18; and v) a Δ6 desaturase; wherein the heterologous polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seed cells; and b) providing the feedstuff to fish, thereby feeding fish;

- a *Brassica* plant cell comprising: a polynucleotide which encodes a Δ4 desaturase whose amino acid sequence is at least 99.5% identical to the sequence set forth as SEQ ID NO:33, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ5 elongase operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ6 desaturase operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and an exogenous desaturase which desaturates an acyl-CoA substrate;

- a *Brassica* plant cell for producing docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols in the plant cell comprising: a polynucleotide which encodes a Δ4 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ6 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and a polynucleotide which encodes a Δ5/Δ6 bifunctional elongase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

- a *Brassica* plant cell comprising: a polynucleotide which encodes a Δ4 desaturase which has the amino acid sequence encoded by a Δ4 desaturase gene from *Pavlova lutheri*, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO: 18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ6 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and a polynucleotide which encodes a Δ5/Δ6 bifunctional elongase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

- a *Brassica* plant cell comprising: a polynucleotide which encodes a Δ4 desaturase which has the amino acid sequence encoded by a Δ4 desaturase gene from *Pavlova lutheri*, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; a polynucleotide which encodes a Δ6 desaturase operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and a polynucleotide which encodes a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

- a process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising: i) obtaining *Brassica napus* seed comprising a cell as described herein, and ii) extracting triacylglycerols from the seed;

- a process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising: i) obtaining *Brassica napus* seed comprising a cell as described herein, and ii) extracting triacylglycerols from the seed;

- a process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising: i) obtaining *Brassica napus* seed comprising a cell as described herein, and ii) extracting triacylglycerols from the seed;

- an extract from a cell as described herein, wherein said extract comprises an increased level of EPA in its fatty acid relative to a corresponding extract from an isogenic non-transformed cell, and further comprises the exogenous polynucleotide(s) as described herein;

- a method of producing DHA in a cell or a plant, the method comprising: i) culturing, under suitable conditions, a recombinant cell as described herein, and/or ii) cultivating, under suitable conditions, a transgenic plant comprising at least one cell as described herein, wherein the cell is a transgenic plant cell, wherein the cell or plant produces DHA;

- a composition comprising a cell as described herein, and a suitable carrier;

- a feedstuff comprising a cell as described herein;

- a method of preparing a feedstuff, the method comprising admixing a cell as described herein with a suitable carrier; and

- use of a cell as described herein, or oil produced using the method as described herein, for the manufacture of a medicament for treating or preventing a condition which would benefit from at least one or more or all of EPA, DPA and DHA, wherein the cell comprises EPA.

As will be apparent, preferred features and characteristics of one aspect of the invention are applicable to many other aspects of the invention.

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.

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. Possible pathways of ω3 and ω6 LC-PUFA synthesis. The sectors labelled I, II, III, and IV correspond to the ω6 (Δ6), ω3 (Δ6), ω6 (Δ8), and ω3 (Δ) pathways, respectively. Compounds in sectors I and III are ω6 compounds, while those in sectors II and IV are ω3 compounds. "Des" refers to desaturase steps in the pathway catalysed by desaturases as indicated, while "Elo" refers to elongase steps catalysed by elongases as indicated. The thickened arrow indicates the Δ5 elongase step. The dashed arrows indicate the steps in the "Sprecher" pathway that operates in mammalian cells for the production of DHA from DPA.

Figure 2. Distribution of LC-PUFA in microalgal classes. Chlorophyceae and Prasinophyceae are described as "green algae", Eustigmatophyceae as "yellow-green algae", Rhodophyceae as "red algae", and Bacillariophyceae and Prymnesiophyceae as diatoms and golden brown algae.

Figure 3. Genetic construct for expression of LC-PUFA biosynthesis genes in plant cells.

Figure 4. PILEUP of desaturase enzymes. d8-atg – *Pavlova salina* Δ8 desaturase; euglena - AAD45877 (Δ8 desaturase, *Euglena gracilis*); rhizopus - AAP83964 (Δ6 desaturase, *Rhizopus sp.* NK030037); mucor - BAB69055 (Δ6 desaturase, *Mucor circinelloides*); mortierella - AAL73948 (Δ6 desaturase, *Mortierella isabellina*); malpina - BAA85588 (Δ6 desaturase, *Mortierella alpina*); physcomitrella - CAA11032 (Δ6 acyl-lipid desaturase, *Physcomitrella patens*); ceratadon - CAB94992 (Δ6 fatty acid acetylenase, *Ceratodon purpureus*).

Figure 5. Southern blot of PCR products, hybridized to Elo1 or Elo2 probes.

Figure 6. PILEUP of elongase enzymes.

Figure 7. Transgene constructs used to express genes encoding LC-PUFA biosynthetic enzymes in *Arabidopsis*. The "EPA construct" pSSP-5/6D.6E (also called pZebdesatCeloPWvec8 in Example 5) (Figure 7A) contained the zebra-fish dual function Δ5/Δ6-desaturase (D5/D6Des) and the nematode Δ6-elongase (D6Elo) both driven by the truncated napin promoter (Fp1), and the hygromycin resistance selectable marker gene (hph) driven by the CaMV-35S (35SP) promoter. The "DHA construct" pXZP355 (Figure 7B) comprised the *Pavlova salina* Δ4-desaturase (D4Des) and Δ5-elongase (D5Elo) genes both driven by the truncated napin promoter (Fp1), and the kanamycin resistance selectable marker gene (nptII) driven by the nopaline synthase promoter (NosP). All genes were flanked at the 3' end by the nopaline synthase terminator (NosT).

Figure 8. A. Gas chromatogram (GLC) showing fatty acid profile for *Arabidopsis thaliana* line DO11 carrying EPA and DHA gene constructs. B. Mass spectra for EPA and DHA obtained from *Arabidopsis thaliana* line DO11..

Figure 9. Autoradiograms of dot-blot hybridisations carried out under low stringency or high stringency conditions, as described in Example 12, to DNA from various microalgal species indicated at the top, using radiolabelled probes consisting of *P. salina* LC-PUFA gene coding regions as indicated on the right.

Figure 10. Amino acid sequence alignment of Δ6- and Δ8-desaturases from higher plants. The amino acid sequences of Δ6-desaturases from *E. plantagineum* (EplD6Des) (SEQ ID NO:64), *E. gentianoides* (EgeD6Des, accession number AY055117) (SEQ ID NO:65), *E. pitardii* (EpiD6Des, AY055118) (SEQ ID NO:66), *Borago officinalis* (BofD6Des, U79010) (SEQ ID NO:67) and Δ8-desaturases *from B. officinalis* (BofD8Des, AF133728) (SEQ ID NO:68), *Helianthus annus* (HanD8Des, S68358) (SEQ ID NO:69), and *Arabidopsis thaliana* (AtD8DesA, AAC62885.1; and AtD8DesB, CAB71088.1) (SEQ ID NO:70 and SEQ ID NO:71 respectively) were aligned by PILEUP (GCG, Wisconsin, USA). HBI, HBII, HBIII are three conserved histidine boxes. F1 and R1 are the corresponding regions for the degenerate primers EpD6Des-F1 and EpD6Des-R1 used to amplify the cDNA. The N-terminal cytochrome b_5 domain with conserved HPGG motif is also indicated.

Figure 11. Variant EplD6Des enzymes isolated and representative enzyme activities. EplD6Des with cytochrome b5, histidine boxes I, II, and III are shown as b5, HBI, HBII, HBIII respectively. Variants isolated are shown in panel A in the format: wild-type amino acid - position number – variant amino acid. Empty diamonds indicate mutants with significant reduction of enzyme activity, while solid diamonds indicate the variants with no significant effect on enzyme activity. Panel B shows the comparison of GLA and SDA production in transgenic tobacco leaves from two variants with that of wild-type enzyme.

Figure 12. Alternative pathways for synthesis of the ω3 LC-PUFA SDA (18:4), EPA (20:5) and DHA (22:6) from ALA (18:3). Desaturases, elongases and acyltransferases are shown as solid, open and dashed arrows respectively. Chain elongation occurs only on acyl-CoA substrates, whereas desaturation can occur on either acyl-PC [A&B] or acyl-CoA substrates [C]. The acyl-PC or acyl-CoA substrate preference of the final Δ4-desaturase step has not yet been determined. Pathways involving acyl-PC desaturases require acyltransferase-mediated shuttling of acyl groups between the PC and CoA substrates. Panels A and B show the "Δ6 pathway" and "Δ8 pathway" variants of the acyl-PC desaturase pathway respectively. Panel C shows the pathway expressed in the current study in which the acyl-CoA Δ6 and Δ5 desaturase activities were encoded by the

zebra-fish Δ6/Δ5 dual-function desaturase. Synthesis of ω6 LC-PUFA such as ARA (20:4) occurs by the same set of reactions but commencing with LA (18:2) as the initial substrate.

Figure 13. Growth rates of *Synechococcus* 7002 at 22°C, 25°C, 30°C.

Figure 14. *Synechococcus* 7002 linoleic and linolenic acid levels at various growth temperatures.

KEY TO THE SEQUENCE LISTING

SEQ ID NO:1 - Δ8 desaturase from *Pavlova salina.*

SEQ ID NO:2 - Δ5 elongase from *Pavlova salina.*

SEQ ID NO:3 - Δ9 elongase from *Pavlova salina.*

SEQ ID NO:4 - Δ4 desaturase from *Pavlova salina.*

SEQ ID NO:5 - cDNA encoding open reading frame of Δ8 desaturase from *Pavlova salina.*

SEQ ID NO:6 - Full length cDNA encoding of Δ8 desaturase from *Pavlova salina.*

SEQ ID NO:7 - cDNA encoding open reading frame of Δ5 elongase from *Pavlova salina.*

SEQ ID NO:8 - Full length cDNA encoding of Δ5 elongase from *Pavlova salina.*

SEQ ID NO:9 - cDNA encoding open reading frame of Δ9 elongase from *Pavlova salina.*

SEQ ID NO:10 - Full length cDNA encoding of Δ9 elongase from *Pavlova salina.*

SEQ ID NO:11 - Partial cDNA encoding N-terminal portion of Δ4 desaturase from *Pavlova salina.*

SEQ ID NO:12 - cDNA encoding open reading frame of Δ4 desaturase from *Pavlova salina.*

SEQ ID NO:13 - Full length cDNA encoding Δ4 desaturase from *Pavlova salina.*

SEQ ID NO:14 - Δ5/Δ6 bifunctional elongase from *Caenorhabditis elegans.*

SEQ ID NO:15 - Δ5/Δ6 bifunctional desaturase from *Danio rerio* (zebrafish).

SEQ ID NO:16 - Δ5 desaturase from humans (Genbank Accession No: AAF29378).

SEQ ID NO:17 - Δ5 desaturase from *Pythium irregulare* (Genbank Accession No: AAL13311).

SEQ ID NO:18 - Δ5 desaturase from *Thraustochytrium sp.* (Genbank Accession No: AAM09687).

SEQ ID NO:19 - Δ5 desaturase from *Mortierella alpina* (Genbank Accession No: O74212).

SEQ ID NO:20 - Δ5 desaturase from *Caenorhabditis elegans* (Genbank Accession No: T43319).

SEQ ID NO:21 - Δ6 desaturase from humans (Genbank Accession No: AAD20018).

SEQ ID NO:22 - Δ6 desaturase from mouse (Genbank Accession No: NP_062673).
SEQ ID NO:23 - Δ6 desaturase from *Pythium irregulare* (Genbank Accession No: AAL13310).
SEQ ID NO:24 - Δ6 desaturase from *Borago officinalis* (Genbank Accession No: AAD01410).
SEQ ID NO:25 - Δ6 desaturase from *Anemone leveillei* (Genbank Accession No: AAQ10731).
SEQ ID NO:26 - Δ6 desaturase from *Ceratodon purpureus* (Genbank Accession No: CAB94993).
SEQ ID NO:27 - Δ6 desaturase from *Physcomitrella patens* (Genbank Accession No: CAA11033).
SEQ ID NO:28 - Δ6 desaturase from *Mortierella alpina* (Genbank Accession No: BAC82361).
SEQ ID NO:29 - Δ6 desaturase from *Caenorhabditis elegans* (Genbank Accession No: AAC15586).
SEQ ID NO:30 - Δ5 elongase from humans (Genbank Accession No: NP_068586).
SEQ ID NO:31 - Δ6 elongase from *Physcomitrella patens* (Genbank Accession No: AAL84174).
SEQ ID NO:32 - Δ6 elongase from *Mortierella alpina* (Genbank Accession No: AAF70417).
SEQ ID NO:33 - Δ4 desaturase from *Thraustochytrium sp.* (Genbank Accession No: AAM09688).
SEQ ID NO:34 - Δ4 desaturase from *Euglena gracilis* (Genbank Accession No: AAQ19605).
SEQ ID NO:35 - Δ9 elongase from *Isochrysis galbana* (Genbank Accession No: AAL37626).
SEQ ID NO:36 - Δ8 desaturase from *Euglena gracilis* (Genbank Accession No: AAD45877).
SEQ ID NO:37 - cDNA encoding Δ5/Δ6 bifunctional elongase from *Caenorhabditis elegans*.
SEQ ID NO:38 - cDNA encoding Δ5/Δ6 bifunctional desaturase from *Danio rerio* (zebrafish).
SEQ ID NO's:39 to 42, 46, 47, 50, 51, 53, 54, 56, 57, 81, 82, 83, 84 and 87 - Oligonucleotide primers.
SEQ ID NO's:43 to 45, 48, 49 and 52 - Conserved motifs of various desaturases/elongases.

SEQ ID NO:55 - Partial cDNA encoding *Pavlova salina* FAE-like elongase.
SEQ ID NO:58 - Full length cDNA encoding Δ5 desaturase from *Pavlova salina.*
SEQ ID NO:59 - cDNA encoding open reading frame of Δ5 desaturase from *Pavlova salina.*
SEQ ID NO:60 - Δ5 desaturase from *Pavlova salina.*
SEQ ID NO's 61 and 62 - Fragments of *Echium pitardii* Δ6 desaturase.
SEQ ID NO:63 - cDNA encoding open reading frame of Δ6 desaturase from *Echium plantagineum.*
SEQ ID NO:64 - Δ6 desaturase from *Echium plantagineum.*
SEQ ID NO:65 - Δ6 desaturase from *Echium gentianoides* (Genbank Accession No: AY055117).
SEQ ID NO:66 - Δ6 desaturase from *Echium pitardii* (Genbank Accession No: AY055118).
SEQ ID NO:67 - Δ6 desaturase from *Borago officinalis* (Genbank Accession No: U79010).
SEQ ID NO:68 - Δ8 desaturase from *Borago officinalis* (Genbank Accession No: AF133728).
SEQ ID NO:69 - Δ8 desaturase from *Helianthus annus* (Genbank Accession No: S68358).
SEQ ID NO:70 - Δ8 desaturaseA from *Arabiposis thaliana* (Genbank Accession No: AAC62885.1).
SEQ ID NO:71 - Δ8 desaturaseB from *Arabiposis thaliana* (Genbank Accession No: CAB71088.1).
SEQ ID NO:72 and 73 - Conserved motifs of Δ6– and Δ8-desaturases.
SEQ ID NO:74 - Δ6 elongase from *Thraustochytrium sp.* (Genbank Accession No: AX951565).
SEQ ID NO:75 - Δ9 elongase from *Danio rerio* (Genbank Accession No: NM_199532).
SEQ ID NO:76 - Δ9 elongase from *Pavlova lutheri.*
SEQ ID NO:77 - Δ5 elongase from *Danio rerio* (Genbank Accession No: AF532782).
SEQ ID NO:78 - Δ5 elongase from *Pavlova lutheri.*
SEQ ID NO:79 - Partial gene sequence from *Heterocapsa niei* encoding an elongase.
SEQ ID NO:80 - Protein encoded by SEQ ID NO:79, presence of stop codon suggests an intron in SEQ ID NO:79.
SEQ ID NO:85 - Δ9 elongase from *Pavlova salina,* encoded by alternate start codon at position 31 of SEQ ID NO:9.

SEQ ID NO:86 - Δ9 elongase from *Pavlova salina*, encoded by alternate start codon at position 85 of SEQ ID NO:9.
SEQ ID NO:88 - Partial elongase amino acid sequence from *Melosira sp.*
SEQ ID NO:89 - cDNA sequence encoding partial elongase from *Melosira sp.*

DETAILED DESCRIPTION OF THE INVENTION

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, plant biology, molecular genetics, immunology, immunohistochemistry, protein chemistry, fatty acid synthesis, and biochemistry).

Unless otherwise indicated, the recombinant nucleic acid, recombinant 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, 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), and 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).

As used herein, the terms "long-chain polyunsaturated fatty acid", "LC-PUFA" or "C20+ polyunsaturated fatty acid" refer to a fatty acid which comprises at least 20 carbon atoms in its carbon chain and at least three carbon-carbon double bonds. As used herein, the term "very long-chain polyunsaturated fatty acid", "VLC-PUFA" or "C22+ polyunsaturated fatty acid" refers 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 ω3 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 ω6 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, 20:4Δ5,8,11,14; ω6), eicosatetraenoic acid (ETA, 20:4Δ8,11,14,17, ω3) eicosapentaenoic acid (EPA, 20:5Δ5,8,11,14,17; ω3), docosapentaenoic acid (DPA, 22:5Δ7,10,13,16,19, ω3), or docosahexaenoic acid (DHA, 22:6Δ4,7,10,13,16,19, ω3). The LC-PUFA may also be dihomo-γ-linoleic acid (DGLA) or eicosatrienoic acid (ETrA, 20:3Δ11,14,17, ω3). 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 ω3 fatty acid is EPA, DPA, or DHA, or even more preferably DPA or DHA.

Furthermore, as used herein the terms "long-chain polyunsaturated fatty acid" or "very long-chain polyunsaturated fatty acid" refer to the fatty acid being in a free state (non-esterified) or in an esterified form such as part of a triglyceride, diacylglyceride, monoacylglyceride, acyl-CoA 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. 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 85% triacylglycerols, with the remainder present as other forms of lipid such as those mentioned, with at least said triacylglycerols comprising the LC-PUFA. The oil may be further purified or treated, for example by hydrolysis with a strong base to release the free fatty acid, or by fractionation, distillation or the like.

As used herein, the abbreviations "LC-PUFA" and "VLC-PUFA" can refer to a single type of fatty acid, or to multiple types of fatty acids. For example, a transgenic plant of the invention which produces LC-PUFA may produce EPA, DPA and DHA.

The desaturase and elongase proteins and genes encoding them 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 the 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 which are characterised by the presence of a cytochrome b_5–like domain at the N-terminus of each protein. The

TABLE 1. Cloned genes involved in LC-PUFA biosynthesis.

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
Δ4-desaturase	Algae	*Euglena gracilis*	AY278558	541	Meyer et al., 2003
		Pavlova lutherii	AY332747	445	Tonon et al., 2003
		Thraustochytrium sp.	AF489589	519	Qiu et al., 2001
		Thraustochytrium aureum	AF391543-5	515	(NCBI)
Δ5-desaturase	Mammals	*Homo sapiens*	AF199596	444	Cho et al., 1999b Leonard et al., 2000b
	Nematode	*Caenorhabditis elegans*	AF11440, NM_069350	447	Michaelson et al., 1998b; Watts and Browse, 1999b
	Fungi	*Mortierella alpina*	AF067654	446	Michaelson et al., 1998a; Knutzon et al., 1998
		Pythium irregulare	AF419297	456	Hong et al., 2002a
		Dictyostelium discoideum	AB022097	467	Saito et al., 2000
		Saprolegnia diclina		470	WO02081668
	Diatom	*Phaeodactylum tricornutum*	AY082392	469	Domergue et al., 2002
	Algae	*Thraustochytrium sp*	AF489588	439	Qiu et al., 2001
		Thraustochytrium aureum		439	WO02081668
		Isochrysis galbana		442	WO02081668
	Moss	*Marchantia polymorpha*	AY583465	484	Kajikawa et al., 2004

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
Δ6-desaturase	Mammals	*Homo sapiens*	NM_013402	444	Cho et al., 1999a; Leonard et al., 2000
		Mus musculus	NM_019699	444	Cho et al., 1999a
	Nematode	*Caenorhabditis elegans*	Z70271	443	Napier et al., 1998
	Plants	*Borago officinales*	U79010	448	Sayanova et al., 1997
		Echium	AY055117 AY055118		Garcia-Maroto et al., 2002
		Primula vialii	AY234127	453	Sayanova et al., 2003
		Anemone leveillei	AF536525	446	Whitney et al., 2003
	Mosses	*Ceratodon purpureus*	AJ250735	520	Sperling et al., 2000
		Marchantia polymorpha	AY583463	481	Kajikawa et al., 2004
		Physcomitrella patens			Girke et al., 1998
	Fungi	*Mortierella alpina*	AF110510 AB020032	457	Huang et al., 1999; Sakuradani et al., 1999
		Pythium irregulare	AF419296	459	Hong et al., 2002a
		Mucor circinelloides	AB052086	467	NCBI*
		Rhizopus sp.	AY320288	458	Zhang et al., 2004
		Saprolegnia diclina		453	WO02081668
	Diatom	*Phaeodactylum tricornutum*	AY082393	477	Domergue et al., 2002
	Bacteria	*Synechocystis*	L11421	359	Reddy et al., 1993
	Algae	*Thraustochytrium aureum*		456	WO02081668
Bifunctional Δ5/Δ6 desaturase	Fish	*Danio rerio*	AF309556	444	Hastings et al., 2001
C20 Δ8-desaturase	Algae	*Euglena gracilis*	AF139720	419	Wallis and Browse, 1999
	Plants	*Borago officinales*	AF133728		

Enzyme	Type of organism	Species	Accession Nos.	Protein size (aa's)	References
Δ6-elongase	Nematode	**Caenorhabditis elegans**	NM_069288	288	Beaudoin et al., 2000
	Mosses	*Physcomitrella patens*	AF428243	290	Zank et al., 2002
		Marchantia polymorpha	AY583464	290	Kajikawa et al., 2004
	Fungi	*Mortierella alpina*	AF206662	318	Parker-Barnes et al., 2000
	Algae	*Pavlova lutheri***		501	WO 03078639
		Thraustochytrium	AX951565	271	WO 03093482
		*Thraustochytrium sp***	AX214454	271	WO 0159128
PUFA-elongase	Mammals	*Homo sapiens*	AF231981	299	Leonard et al., 2000b; Leonard et al., 2002
		Rattus norvegicus	AB071985	299	Inagaki et al., 2002
		*Rattus norvegicus***	AB071986	267	Inagaki et al., 2002
		Mus musculus	AF170907	279	Tvrdik et al., 2000
		Mus musculus	AF170908	292	Tvrdik et al., 2000
	Fish	*Danio rerio*	AF532782	291 (282)	Agaba et al., 2004
		*Danio rerio***	NM_199532	266	Lo et al., 2003
	Worm	*Caenorhabditis elegans*	Z68749	309	Abbott et al 1998 Beaudoin et al 2000
	Algae	*Thraustochytrium aureum***	AX464802	272	WO 0208401-A2
		*Pavlova lutheri***		?	WO 03078639
Δ9-elongase	Algae	*Isochrysis galbana*	AF390174	263	Qi et al., 2002

* http://www.ncbi.nlm.nih.gov/

** Function not proven/not demonstrated

cytochrome b_5–like domain presumably acts as a receptor of electrons required for desaturation (Napier et al., 1999; Sperling and Heinz, 2001).

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 yeast cell or a plant cell, and determining whether the cell has an increased capacity to produce LC-PUFA compared to a comparable cell in which the enzyme is not expressed.

Unless stated to the contrary, embodiments of the present invention which relate to cells, plants, seeds, etc, and methods for the production thereof, and that refer to at least "two enzymes" (or at least "three enzymes" etc) of the list that is provided means that the polynucleotides encode at least two "different" enzymes from the list provided and not two identical (or very similar with only a few differences as to not substantially alter the activity of the encoded enzyme) open reading frames encoding essentially the same enzyme.

As used herein, unless stated to the contrary, the term "substantially the same", or variations thereof, means that two samples being analysed, for example two seeds from different sources, are substantially the same if they only vary about +/-10% in the trait being investigated.

As used herein, the term "an enzyme which preferentially converts an ω6 LC-PUFA into an ω3 LC-PUFA" means that the enzyme is more efficient at performing said conversion than it is at performing a desaturation reaction outlined in pathways II or III of Figure 1.

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

As used herein, a "Δ5/Δ6 bifunctional desaturase" or "Δ5/Δ6 desaturase" is at least capable of i) converting α-linolenic acid to octadecatetraenoic acid, and ii) converting eicosatetraenoic acid to eicosapentaenoic acid. That is, a Δ5/Δ6 bifunctional desaturase is both a Δ5 desaturase and a Δ6 desaturase, and Δ5/Δ6 bifunctional desaturases may be considered a sub-class of each of these. A gene encoding a bifunctional Δ5-/Δ6-desaturase has been identified from zebrafish (Hasting et al., 2001). The gene encoding this enzyme might represent an ancestral form of the "front-end desaturase" which later duplicated and the copies evolved distinct Δ5- and Δ6-desaturase functions. In one embodiment, the Δ5/Δ6 bifunctional desaturase is naturally produced by a freshwater species of fish. In a particular embodiment, the Δ5/Δ6 bifunctional desaturase comprises

i) an amino acid sequence as provided in SEQ ID NO:15,

ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:15, or

iii) a biologically active fragment of i) or ii).

As used herein, a "Δ5 desaturase" is at least capable of converting eicosatetraenoic acid to eicosapentaenoic acid. In one embodiment, the enzyme Δ5 desaturase catalyses the desaturation of C20 LC-PUFA, converting DGLA to arachidonic acid (ARA, 20:4ω6) and ETA to EPA (20:5ω3). Genes encoding this enzyme have been isolated from a number of organisms, including algae (*Thraustochytrium* sp. Qiu et al., 2001), fungi (*M. alpine, Pythium irregulare, P. tricornutum, Dictyostelium*), *Caenorhabditis elegans* and mammals (Table 1). In another embodiment, the Δ5 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or SEQ ID NO:60, (ii) an amino acid sequence which is at least 50% identical to any one of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20 or SEQ ID NO:60, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ5 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:60, (ii) an amino acid sequence which is at least 90% identical to any one of SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:20 or SEQ ID NO:60, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ5 desaturase is encoded by the protein coding region of one of the Δ5 desaturase genes listed in Table 1 or gene substantially identical thereto.

As used herein, a "Δ6 desaturase" is at least capable of converting α-linolenic acid to octadecatetraenoic acid. In one embodiment, the enzyme Δ6 desaturase catalyses the desaturation of C18 LC-PUFA, converting LA to GLA and ALA to SDA. In another embodiment, the Δ6 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:64 SEQ ID NO:65, SEQ ID NO:66 or SEQ ID NO:67, (ii) an amino acid sequence which is at least 50% identical to any one of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:64 SEQ ID NO:65, SEQ ID NO:66 or SEQ ID NO:67, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ6 desaturase comprises an amino acid sequence which is at least 90% identical to any one of SEQ ID NO:21, SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24, SEQ ID NO:25, SEQ ID NO:26, SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:64 SEQ ID NO:65, SEQ ID NO:66 or SEQ ID NO:67. In a further embodiment, the Δ6 desaturase is

encoded by the protein coding region of one of the Δ6 desaturase genes listed in Table 1 or gene substantially identical thereto

As used herein, a "Δ4 desaturase" is at least capable of converting docosapentaenoic acid to docosahexaenoic acid. The desaturation step to produce DHA from DPA is catalysed by a Δ4 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 Δ4 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:4, SEQ ID NO:33 or SEQ ID NO:34, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:4, SEQ ID NO:33 or SEQ ID NO:34, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ4 desaturase is encoded by the protein coding region of one of the Δ4 desaturase genes listed in Table 1 or gene substantially identical thereto.

As used herein, a "Δ8 desaturase" is at least capable of converting $20:3^{\Delta 11,14,17}\omega 3$ to eicosatetraenoic acid. In one embodiment, the Δ8 desaturase is relatively specific for Δ8 substrates. That is, it has greater activity in desaturating Δ8 substrates than other substrates, in particular Δ6 desaturated substrates. In a preferred embodiment, the Δ8 desaturase has little or no Δ6 desaturase activity when expressed in yeast cells. In another embodiment, the Δ8 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:1, SEQ ID NO:36, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 or SEQ ID NO:71, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:1, SEQ ID NO:36, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70 or SEQ ID NO:71, or (iii) a biologically active fragment of i) or ii). In further embodiment, the Δ8 desaturase comprises (i) an amino acid sequence as provided in SEQ ID NO:1, (ii) an amino acid sequence which is at least 90% identical to SEQ ID NO:1, or (iii) a biologically active fragment of i) or ii).

As used herein, an "ω3 desaturase" is at least capable of converting LA to ALA and/or GLA to SDA and/or ARA to EPA. Examples of ω3 desaturase include those described by Pereira et al. (2004), Horiguchi et al. (1998), Berberich et al. (1998) and Spychalla et al. (1997). In one embodiment, a cell of the invention is a plant cell which lacks ω3 desaturase activity. Such cells can be produced using gene knockout technology well known in the art. These cells can be used to specifically produce large quantities of ω6 LC-PUFA such as DGLA.

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.

Two groups of condensing enzymes have been identified so far. The first are involved in the extension of saturated and monounsaturated fatty acids (C18-22) such as, for example, the FAE1 gene of *Arabidopsis*. An example of a product formed is erucic acid (22:1) in *Brassicas*. This group are designated the FAE-like enzymes and do not appear to have a role in LC-PUFA biosynthesis. The other identified class of fatty acid elongases, designated the ELO family of elongases, are named after the ELO genes whose activities are required for the synthesis of the very long-chain fatty acids of sphingolipids in yeast. Apparent paralogs of the ELO-type elongases isolated from LC-PUFA synthesizing organisms like algae, mosses, fungi and nematodes have been shown to be involved in the elongation and synthesis of LC-PUFA. Several genes encoding such PUFA-elongation enzymes have also been isolated (Table 1). Such genes are unrelated in nucleotide or amino acid sequence to the FAE-like elongase genes present in higher plants.

As used herein, a "Δ5/Δ6 bifunctional elongase" or "Δ5/Δ6 elongase" is at least capable of i) converting octadecatetraenoic acid to eicosatetraenoic acid, and ii) converting eicosapentaenoic acid to docosapentaenoic acid. That is, a Δ5/Δ6 bifunctional elongase is both a Δ5 elongase and a Δ6 elongase, and Δ5/Δ6 bifunctional elongases may be considered a sub-class of each of these. In one embodiment, the Δ5/Δ6 bifunctional elongase is able to catalyse the elongation of EPA to form DPA in a plant cell such as, for example, a higher plant cell, when that cell is provided with a source of EPA. The EPA may be provided exogenously or preferably endogenously. A gene encoding such an elongase has been isolated from an invertebrate, *C. elegans* (Beaudoin et al., 2000) although it was not previously known to catalyse the Δ5-elongation step. In one

embodiment, the Δ5/Δ6 bifunctional elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:2 or SEQ ID NO:14, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:2 or SEQ ID NO:14, or (iii) a biologically active fragment of i) or ii).

As used herein, a "Δ5 elongase" is at least capable of converting eicosapentaenoic acid to docosapentaenoic acid. In one embodiment, the Δ5 elongase is from a non-vertebrate source such as, for example, an algal or fungal source. Such elongases can have advantages in terms of the specificity of the elongation reactions carried out (for example the Δ5 elongase provided as SEQ ID NO:2). In a preferred embodiment, the Δ5 elongase is relatively specific for C20 substrates over C22 substrates. For example, it may have at least 10-fold lower activity toward C22 substrates (elongated to C24 fatty acids) relative to the activity toward a corresponding C20 substrate when expressed in yeast cells. It is preferred that the activity when using C20 Δ5 desaturated substrates is high, such as for example, providing an efficiency for the conversion of 20:5ω3 into 22:5ω3 of at least 7% when expressed in yeast cells. In another embodiment, the Δ5 elongase is relatively specific for Δ5 desaturated substrates over Δ6 desaturated substrates. For example, it may have at least 10-fold lower activity toward Δ6 desaturated C18 substrates relative to Δ5 desaturated C20 substrates when expressed in yeast cells. In a further embodiment, the Δ5 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:2, SEQ ID NO:30, SEQ ID NO:77 or SEQ ID NO:78, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:2, SEQ ID NO:30, SEQ ID NO:77 or SEQ ID NO:78, or (iii) a biologically active fragment of i) or ii). In another embodiment, the Δ5 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:2, (ii) an amino acid sequence which is at least 90% identical to SEQ ID NO:2, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ5 elongase is encoded by the protein coding region of one of the Δ5 elongase genes listed in Table 1 or gene substantially identical thereto.

As used herein, a "Δ6 elongase" is at least capable of converting octadecatetraenoic acid to eicosatetraenoic acid. In one embodiment, the Δ6 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:74, SEQ ID NO:85, SEQ ID NO:86 or SEQ ID NO:88, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:31, SEQ ID NO:32, SEQ ID NO:74, SEQ ID NO:85, SEQ ID NO:86 or SEQ ID NO:88, or (iii) a biologically active fragment of i) or ii). In another embodiment, the Δ6 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:2, SEQ ID NO:3 or SEQ ID NO:32, SEQ ID NO:85, SEQ ID NO:86 or SEQ ID

NO:88, (ii) an amino acid sequence which is at least 90% identical to SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:32, SEQ ID NO:85, SEQ ID NO:86 or SEQ ID NO:88, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ6 elongase is encoded by the protein coding region of one of the Δ6 elongase genes listed in Table 1 or gene substantially identical thereto.

In some protist species, LC-PUFA are synthesized by elongation of linoleic or α-linolenic acid with a C2 unit, before desaturation with Δ8 desaturase (Figure 1 part IV; "Δ8-desaturation" pathway). Δ6 desaturase and Δ6 elongase activities were not detected in these species. Instead, a Δ9-elongase activity would be expected in such organisms, and in support of this, a C18 Δ9-elongase gene has recently been isolated from *Isochrysis galbana* (Qi et al., 2002). As used herein, a "Δ9 elongase" is at least capable of converting α-linolenic acid to $20:3^{\Delta 11,14,17}\omega 3$. In one embodiment, the Δ9 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:3, SEQ ID NO:35, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:85 or SEQ ID NO:86, (ii) an amino acid sequence which is at least 50% identical to SEQ ID NO:3, SEQ ID NO:35, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:85 or SEQ ID NO:86, or (iii) a biologically active fragment of i) or ii). In another embodiment, the Δ9 elongase comprises (i) an amino acid sequence as provided in SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86, (ii) an amino acid sequence which is at least 90% identical to SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86, or (iii) a biologically active fragment of i) or ii). In a further embodiment, the Δ9 elongase is encoded by the protein coding region of the Δ9 elongase gene listed in Table 1 or gene substantially identical thereto. In another embodiment, the Δ9 elongase also has Δ6 elongase activity. The elongase in this embodiment is able to convert SDA to ETA and/or GLA to DGLA (Δ6 elongase activity) in addition to converting ALA to ETrA (Δ9 elongase). In a preferred embodiment, such an elongase is from an algal or fungal source such as, for example, the genus *Pavlova*.

As used herein, a "Δ4 elongase" is at least capable of converting docosahexaenoic acid to $24:6^{\Delta 6,9,12,15,18,21}\omega 3$.

Cells

Suitable cells of the invention include any cell that can be transformed with a polynucleotide encoding a polypeptide/enzyme described herein, and which is thereby capable of being used for producing LC-PUFA. Host cells into which the polynucleotide(s) are introduced can be either untransformed cells or cells that are already transformed with at least one nucleic acid molecule. Such nucleic acid molecule may related to LC-PUFA synthesis, or unrelated. Host cells of the present invention

either can be endogenously (i.e., naturally) capable of producing proteins of the present invention or can be capable of producing such proteins only after being transformed with at least one nucleic acid molecule.

As used herein, the term "cell with an enhanced capacity to synthesize a long chain polyunsaturated fatty acid" is a relative term where the recombinant cell of the invention is compared to the native cell, with the recombinant cell producing more long chain polyunsaturated fatty acids, or a greater concentration of LC-PUFA such as EPA, DPA or DHA (relative to other fatty acids), than the native cell.

The cells may be prokaryotic or eukaryotic. Host cells of the present invention can be any cell capable of producing at least one protein described herein, and include bacterial, fungal (including yeast), parasite, arthropod, animal and plant cells. Preferred host cells are yeast and plant cells. In a preferred embodiment, the plant cells are seed cells.

In one embodiment, the cell is an animal cell or an algal cell. The animal cell may be of any type of animal such as, for example, a non-human animal cell, a non-human vertebrate cell, a non-human mammalian cell, or cells of aquatic animals such as fish or crustacea, invertebrates, insects, etc.

An example of a bacterial cell useful as a host cell of the present invention is *Synechococcus* spp. (also known as *Synechocystis* spp.), for example *Synechococcus elongatus*.

The cells may be of an organism suitable for fermentation. As used herein, the term the "fermentation process" refers to any fermentation process or any process comprising a fermentation step. A fermentation process includes, without limitation, fermentation processes used to produce alcohols (e.g., ethanol, methanol, butanol); organic acids (e.g., citric acid, acetic acid, itaconic acid, lactic acid, gluconic acid); ketones (e.g., acetone); amino acids (e.g., glutamic acid); gases (e.g., H_2 and CO_2); antibiotics (e.g., penicillin and tetracycline); enzymes; vitamins (e.g., riboflavin, beta-carotene); and hormones. Fermentation processes also include fermentation processes used in the consumable alcohol industry (e.g., beer and wine), dairy industry (e.g., fermented dairy products), leather industry and tobacco industry. Preferred fermentation processes include alcohol fermentation processes, as are well known in the art. 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 fermentation product. Examples of fermenting microorganisms include fungal organisms;

such as yeast. As used herein, "yeast" includes *Saccharomyces* spp., *Saccharomyces cerevisiae*, *Saccharomyces carlbergensis*, *Candida* spp., *Kluveromyces* spp., *Pichia* spp., *Hansenula* spp., *Trichoderma* spp., *Lipomyces starkey*, and *Yarrowia lipolytica*. Preferred yeast include strains of the *Saccharomyces spp.*, and in particular, *Saccharomyces cerevisiae*. Commercially available yeast include, e.g., Red Star/Lesaffre Ethanol Red (available from Red Star/Lesaffre, USA) FALI (available from Fleischmann's Yeast, a division of Burns Philp Food Inc., USA), SUPERSTART (available from Alltech), GERT STRAND (available from Gert Strand AB, Sweden) and FERMIOL (available from DSM Specialties).

Evidence to date suggests that some desaturases expressed heterologously in yeast have relatively low activity in combination with some elongases. However, the present inventors have identified that 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 other than yeast as well. In this regard, it has also been determined that desaturases of vertebrate origin are particularly useful for the production of LC-PUFA. Thus in embodiments of the invention, either (i) at least one of the enzymes is a Δ5 elongase that catalyses the conversion of EPA to DPA in the cell, (ii) at least one of the desaturases is able to act on an acyl-CoA substrate, (iii) at least one desaturase is from vertebrate or is a variant thereof, or (iv) a combination of ii) and iii).

In a particularly preferred embodiment, the host cell is a plant cell, such as those described in further detail herein.

As used herein, a "progenitor cell of a seed" is a cell that divides and/or differentiates into a cell of a transgenic seed of the invention, and/or a cell that divides and/or differentiates into a transgenic plant that produces a transgenic seed of the invention.

Levels of LC-PUFA produced

The levels of the LC-PUFA that are produced in the recombinant cell 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 ω3 LC-PUFA or the ω6 LC-PUFA, or the C22+ 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 percentage of LC-PUFA in the dry weight of material comprising the recombinant cells, for example the percentage of the dry 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 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. For example, total lipid may be 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 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 of fatty acids may be confirmed by GC-MS, as described in Example 1.

In certain embodiments, where the recombinant cell is useful in a fermentation process such as, for example, a yeast cell, the level of EPA that is produced may be at least 0.21% of the total fatty acid in the cell, preferably at least 0.82% or at least 2% and even more preferably at least 5%.

In other embodiments, the total fatty acid of the recombinant cell may comprise at least 1.5% EPA, preferably at least 2.1% EPA, and more preferably at least 2.5%, at least 3.1%, at least 4% or at least 5.1% EPA.

In further embodiments, where the recombinant cell is useful in a fermentation process or is a plant cell and DPA is produced, the total fatty acid in the cell may comprise at least 0.1% DPA, preferably at least 0.13% or at least 0.15% and more preferably at least 0.5% or at least 1% DPA.

In further embodiments, the total fatty acid of the cell may comprise at least 2% C20 LC-PUFA, preferably at least 3% or at least 4% C20 LC-PUFA, more preferably at least 4.7% or at least 7.9% C20 LC-PUFA and most preferably at least 10.2% C20 LC-PUFA.

In further embodiments, the total fatty acid of the cell may comprise at least 2.5% C20 ω3 LC-PUFA, preferably at least 4.1% or more preferably at least 5% C20 ω3 LC-PUFA.

In other embodiments, where both EPA and DPA are synthesized in a cell, the level of EPA reached is at least 1.5%, at least 2.1% or at least 2.5% and the level of DPA at least 0.13%, at least 0.5% or at least 1.0%.

In each of these embodiments, the recombinant cell may be a cell of an organism that is suitable for fermentation such as, for example, a unicellular microorganism which

may be a prokaryote or a eukaryote such as yeast, or a plant cell. In a preferred embodiment, the cell is a cell of an angiosperm (higher plant). In a further preferred embodiment, the cell is a cell in a seed such as, for example, an oilseed or a grain or cereal.

The level of production of LC-PUFA in the recombinant cell may also be expressed as a conversion ratio, i.e. the amount of the LC-PUFA formed as a percentage of one or more substrate PUFA or LC-PUFA. With regard to EPA, for example, this may be expressed as the ratio of the level of EPA (as a percentage in the total fatty acid) to the level of a substrate fatty acid (ALA, SDA, ETA or ETrA). In a preferred embodiment, the conversion efficiency is for ALA to EPA. In particular embodiments, the conversion ratio for production of EPA in a recombinant cell may be at least 0.5%, at least 1%, or at least 2%. In another embodiment, the conversion efficiency for ALA to EPA is at least 14.6%. In further embodiments, the conversion ratio for production of DPA from EPA in a recombinant cell is at least 5%, at least 7%, or at least 10%. In other embodiments, the total ω3 fatty acids produced that are products of Δ6 desaturation (i.e. downstream of 18:3ω3 (ALA), calculated as the sum of the percentages for 18:4ω3 (SDA), 20:4ω3 (ETA), 20:5ω3 (EPA) and 22:5ω3 (DPA)) is at least 4.2%. In a particular embodiment, the conversion efficiency of ALA to ω3 products through a Δ6 desaturation step and/or an Δ9 elongation step in a recombinant cell, preferably a plant cell, more preferably a seed cell, is at least 22% or at least 24%. Stated otherwise, in this embodiment the ratio of products derived from ALA to ALA (products:ALA) in the cell is at least 1:3.6.

The content of the LC-PUFA in the recombinant cell may be maximized if the parental cell used for introduction of the genes is chosen such that the level of fatty acid substrate that is produced or provided exogenously is optimal. In particular embodiments, the cell produces ALA endogenously at levels of at least 30%, at least 50%, or at least 66% of the total fatty acid. The level of LC-PUFA may also be maximized by growing or incubating the cells under optimal conditions, for example at a slightly lower temperature than the standard temperature for that cell, which is thought to favour accumulation of polyunsaturated fatty acid.

There are advantages to maximizing production of a desired LC-PUFA while minimizing the extent of side-reactions. In a particular embodiment, there is little or no ETrA detected (less than 0.1%) while the level of EPA is at least 2.1%.

Turning to transgenic plants of the invention, in one embodiment, at least one plant part synthesizes EPA, wherein the total fatty acid of the plant part comprises at least 1.5%, at least 2.1%, or at least 2.5% EPA.

In another embodiment, at least one plant part synthesizes DPA, wherein the total fatty acid of the plant part comprises at least 0.1%, at least 0.13%, or at least 0.5% DPA.

In a further embodiment, at least one plant part synthesizes DHA.

In another embodiment, at least one plant part synthesizes DHA, wherein the total fatty acid of the plant part comprises at least 0.1%, at least 0.2%, or at least 0.5% DHA.

In another embodiment, at least one plant part synthesizes at least one ω3 C20 LC-PUFA, wherein the total fatty acid of the plant part comprises at least 2.5%, or at least 4.1% ω3 C20 LC-PUFA.

In yet another embodiment, at least one plant part synthesizes EPA, wherein the efficiency of conversion of ALA to EPA in the plant part is at least 2% or at least 14.6%.

In a further embodiment, at least one plant part synthesizes ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, wherein the efficiency of conversion of ALA to said products in the plant part is at least 22% or at least 24%.

In yet another embodiment, at least one plant part synthesizes DPA from EPA, wherein the efficiency of conversion of EPA to DPA in the plant part is at least 5% or at least 7%.

With regard to transgenic seeds of the invention, in one embodiment EPA is synthesized in the seed and the total fatty acid of the seed comprises at least 1.5%, at least 2.1%, or at least 2.5% EPA.

In another embodiment, DPA is synthesized in the seed and the total fatty acid of the seed comprises at least 0.1%, at least 0.13%, or at least 0.5% DPA.

In a further embodiment, DHA is synthesized in the seed.

In another embodiment, DHA is synthesized in the seed and the total fatty acid of the seed comprises at least 0.1%, at least 0.2%, or at least 0.5% DHA.

In yet a further embodiment, at least one ω3 C20 LC-PUFA is synthesized in the seed and the total fatty acid of the seed comprises at least 2.5%, or at least 4.1% ω3 C20 LC-PUFA.

In a further embodiment, EPA is synthesized in the seed and the efficiency of conversion of ALA to EPA in the seed is at least 2% or at least 14.6%.

In another embodiment, ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, are synthesized in the seed, and the efficiency of conversion of ALA to said products in the seed is at least 22% or at least 24%.

In a further embodiment, DPA is synthesized from EPA in the seed and the efficiency of conversion of EPA to DPA in the seed is at least 5% or at least 7%.

Referring to extracts of the invention, in one embodiment, the total fatty acid content of the extract comprises at least 1.5%, at least 2.1%, or at least 2.5% EPA.

In another embodiment, the total fatty acid content of the extract comprises at least 0.1%, at least 0.13%, or at least 0.5% DPA.

In a further embodiment, the extract comprises DHA.

In another embodiment, the total fatty acid content of the extract comprises at least 0.1%, at least 0.2%, or at least 0.5% DHA.

In another embodiment, the total fatty acid content of the extract comprises at least 2.5%, or at least 4.1% ω3 C20 LC-PUFA.

In yet a further embodiment, the extract comprises ARA, EPA, DPA, DHA, or any mixture of these in the triacylglycerols.

With regard to methods of the invention for producing a LC-PUFA, in on embodiment, the cell comprises at least one C20 LC-PUFA, and the total fatty acid of the cell comprises at least 2%, at least 4.7%, or at least 7.9% C20 LC-PUFA.

In another embodiment, the cell comprises at least one ω3 C20 LC-PUFA, and the total fatty acid of the cell comprises at least 2.5%, or at least 4.1% ω3 C20 LC-PUFA.

In a further embodiment, the cell comprises ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, and the efficiency of conversion of ALA to said products in the cell is at least 22% or at least 24%.

In yet another embodiment, the cell comprises DPA, and the total fatty acid of the cell comprises at least 0.1%, at least 0.13%, or at least 0.5% DPA.

In a further embodiment, the cell comprises DPA, and the efficiency of conversion of EPA to DPA in the cell is at least 5% or at least 7%.

In another embodiment, the cell comprises EPA, and wherein the total fatty acid of the cell comprises at least 1.5%, at least 2.1%, or at least 2.5% EPA.

In a further embodiment, the cell comprises EPA, and the efficiency of conversion of ALA to EPA in the cell is at least 2% or at least 14.6%.

Polypeptides

In one aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:1,

ii) a polypeptide comprising an amino acid sequence which is at least 40% identical to SEQ ID NO:1, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ8 desaturase activity.

Preferably, the Δ8 desaturase does not also have Δ6 desaturase activity.

In another aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:2,

ii) a polypeptide comprising an amino acid sequence which is at least 60% identical to SEQ ID NO:2, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ5 elongase and/or Δ6 elongase activity.

Preferably, the polypeptide has Δ5 elongase and Δ6 elongase activity, and wherein the polypeptide is more efficient at synthesizing DPA from EPA than it is at synthesizing ETA from SDA. More preferably, the polypeptide can be purified from algae. Furthermore, when expressed in yeast cells, is more efficient at elongating C20 LC-PUFA than C22 LC-PUFA.

In another aspect, the invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86,

ii) a polypeptide comprising an amino acid sequence which is at least 40% identical to SEQ ID NO:3, SEQ ID NO:85 or SEQ ID NO:86, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ9 elongase and/or Δ6 elongase activity.

Preferably, the polypeptide has Δ9 elongase and Δ6 elongase activity. Preferably, the polypeptide is more efficient at synthesizing ETrA from ALA than it is at synthesizing ETA from SDA. Further, it is preferred that the polypeptide can be purified from algae or fungi.

In yet another aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:4,

ii) a polypeptide comprising an amino acid sequence which is at least 70% identical to SEQ ID NO:4, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ4 desaturase activity.

In a further aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:60,

ii) a polypeptide comprising an amino acid sequence which is at least 55% identical to SEQ ID NO:60, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ5 desaturase activity.

In yet another aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:64,

ii) a polypeptide comprising an amino acid sequence which is at least 90% identical to SEQ ID NO:64, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ6 desaturase activity.

In yet another aspect, the present invention provides a substantially purified polypeptide selected from the group consisting of:

i) a polypeptide comprising an amino acid sequence as provided in SEQ ID NO:88,

ii) a polypeptide comprising an amino acid sequence which is at least 76% identical to SEQ ID NO:88, and

iii) a biologically active fragment of i) or ii),

wherein the polypeptide has Δ6 elongase activity.

Preferably, in relation to any one of the above aspects, it is preferred that the polypeptide can be isolated from a species selected from the group consisting of *Pavlova* and *Melosira*.

By "substantially purified polypeptide" we mean a polypeptide that has been at least partially separated from the lipids, nucleic acids, other polypeptides, and other contaminating molecules with which it is associated in its native state. Preferably, the substantially purified polypeptide is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated. Furthermore, the term "polypeptide" is used interchangeably herein with the term "protein".

The % identity of a polypeptide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Unless stated otherwise, 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. Even 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.

With regard to the defined polypeptides/enzymes, 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 polypeptide 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 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 preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

In a further embodiment, the present invention relates to polypeptides which are substantially identical to those specifically described herein. As used herein, with reference to a polypeptide the term "substantially identical" means the deletion, insertion and/or substitution of one or a few (for example 2, 3, or 4) amino acids whilst maintaining at least one activity of the native protein.

As used herein, the term "biologically active fragment" refers to a portion of the defined polypeptide/enzyme which still maintains desaturase or elongase activity (whichever is relevant). Such biologically active fragments can readily be determined by serial deletions of the full length protein, and testing the activity of the resulting fragment.

Amino acid sequence mutants/variants of the polypeptides/enzymes defined herein can be prepared by introducing appropriate nucleotide changes into a nucleic acid encoding the polypeptide, or by *in vitro* synthesis of the desired polypeptide. Such mutants include, for example, deletions, insertions or substitutions of residues 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 protein product possesses the desired characteristics.

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 30 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 identified as the active or binding site(s). Other sites of interest are those in which particular residues obtained from various strains or species are identical. These positions may be important for biological activity. These sites, especially those falling within a sequence of at least three other identically conserved sites, are preferably substituted in a relatively conservative manner. Such conservative substitutions are shown in Table 2.

Furthermore, if desired, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into the polypeptides of the present invention. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general.

Also included within the scope of the invention are polypeptides of the present invention which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. These modifications may serve to increase the stability and/or bioactivity of the polypeptide of the invention.

TABLE 2. 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

Polypeptides of the present invention can be produced in a variety of ways, including production and recovery of natural proteins, production and recovery of recombinant proteins, and chemical synthesis of the proteins. In one embodiment, an isolated polypeptide of the present invention is produced by culturing a cell capable of expressing the polypeptide under conditions effective to produce the polypeptide, and recovering the polypeptide. A preferred cell to culture is a recombinant cell of the present invention. Effective culture conditions include, but are not limited to, effective media, bioreactor, temperature, pH and oxygen conditions that permit protein production. An effective medium refers to any medium in which a cell is cultured to produce a

polypeptide of the present invention. Such medium typically comprises an aqueous medium having assimilable carbon, nitrogen and phosphate sources, and appropriate salts, minerals, metals and other nutrients, such as vitamins. Cells of the present invention can be cultured in conventional fermentation bioreactors, shake flasks, test tubes, microtiter dishes, and petri plates. Culturing can be carried out at a temperature, pH and oxygen content appropriate for a recombinant cell. Such culturing conditions are within the expertise of one of ordinary skill in the art.

Polynucleotides

In one aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:5 or SEQ ID NO:6;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 50% identical to SEQ ID NO:5 or SEQ ID NO:6; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:7 or SEQ ID NO:8;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 51% identical to SEQ ID NO:7 or SEQ ID NO:8; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In yet another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:9 or SEQ ID NO:10;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 51% identical to SEQ ID NO:9 or SEQ ID NO:10; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In a preferred embodiment, the sequence encoding a polypeptide of the invention is nucleotides 31 to 915 or SEQ ID NO:9 or nucleotides 85 to 915 of SEQ ID NO:9.

In a further aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:13;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 70% identical to SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:13; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:58 or SEQ ID NO:59;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 55% identical to SEQ ID NO:58 or SEQ ID NO:59; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:63;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 90% identical to SEQ ID NO:63; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

In another aspect, the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:89;

ii) a sequence encoding a polypeptide of the invention;

iii) a sequence of nucleotides which is at least 76% identical to SEQ ID NO:89; and

iv) a sequence which hybridizes to any one of i) to iii) under high stringency conditions.

The present inventors are also the first to isolate polynucleotide encoding a keto-acyl synthase-like fatty acid elongase from a non-higher plant.

Accordingly, in a further aspect the present invention provides an isolated polynucleotide comprising a sequence of nucleotides selected from the group consisting of:

i) a sequence of nucleotides as provided in SEQ ID NO:55;

ii) a sequence of nucleotides which is at least 40% identical to SEQ ID NO:55; and

iii) a sequence which hybridizes to i) or ii) under high stringency conditions.

By an "isolated polynucleotide", including DNA, RNA, or a combination of these, single or double stranded, in the sense or antisense orientation or a combination of both, dsRNA or otherwise, we mean a polynucleotide which is at least partially separated from the polynucleotide sequences with which it is associated or linked in its native state. Preferably, the isolated polynucleotide is at least 60% free, preferably at least 75% free, and most preferably at least 90% free from other components with which they are naturally associated. Furthermore, the term "polynucleotide" is used interchangeably herein with the term "nucleic acid molecule".

The % identity of a polynucleotide is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension penalty=0.3. Unless stated otherwise, the query sequence is at least 45 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 45 nucleotides. Preferably, the query sequence is at least 150 nucleotides in length, and the GAP analysis aligns the two sequences over a region of at least 150 nucleotides. More preferably, the query sequence is at least 300 nucleotides in length and the GAP analysis aligns the two sequences over a region of at least 300 nucleotides.

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 nucleotide 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 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 preferably at least 99.9% identical to the relevant nominated SEQ ID NO.

In a further embodiment, the present invention relates to polynucleotides which are substantially identical to those specifically described herein. As used herein, with reference to a polynucleotide the term "substantially identical" means the substitution of one or a few (for example 2, 3, or 4) nucleotides whilst maintaining at least one activity of the native protein encoded by the polynucleotide. In addition, this term includes the addition or deletion of nucleotides which results in the increase or decrease in size of the encoded native protein by one or a few (for example 2, 3, or 4) amino acids whilst maintaining at least one activity of the native protein encoded by the polynucleotide.

Oligonucleotides of the present invention can be RNA, DNA, or derivatives of either. The minimum size of such oligonucleotides is the size required for the formation of a stable hybrid between an oligonucleotide and a complementary sequence on a nucleic acid molecule of the present invention. Preferably, the oligonucleotides are at least 15 nucleotides, more preferably at least 18 nucleotides, more preferably at least 19 nucleotides, more preferably at least 20 nucleotides, even more preferably at least 25 nucleotides in length. The present invention includes oligonucleotides that can be used as, for example, probes to identify nucleic acid molecules, or primers to produce nucleic acid molecules. Oligonucleotide of the present invention used as a probe are typically conjugated with a label such as a radioisotope, an enzyme, biotin, a fluorescent molecule or a chemiluminescent molecule.

Polynucleotides and oligonucleotides of the present invention include those which hybridize under stringent conditions to a sequence provided as SEQ ID NO's: 5 to 13. As used herein, stringent conditions are those that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% $NaDodSO_4$ at 50^0C; (2) employ during hybridisation a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42^0C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS and 10% dextran sulfate at 42^0C in 0.2 x SSC and 0.1% SDS.

Polynucleotides of the present invention may possess, when compared to naturally occurring molecules, one or more mutations which are deletions, insertions, or substitutions of nucleotide residues. Mutants can be either naturally occurring (that is to

say, isolated from a natural source) or synthetic (for example, by performing site-directed mutagenesis on the nucleic acid).

Also provided are antisense and/or catalytic nucleic acids (such as ribozymes) which hybridize to a polynucleotide of the invention, and hence inhibit the production of an encoded protein. Furthermore, provided are dsRNA molecules, particularly small dsRNA molecules with a double stranded region of about 21 nucleotides, which can be used in RNA interference to inhibit the production of a polypeptide of the invention in a cell. Such inhibitory molecules can be used to alter the types of fatty acids produced by a cell, such an animal cell, moss, or algael cell. The production of such antisense, catalytic nucleic acids and dsRNA molecules is well with the capacity of the skilled person (see for example, G. Hartmann and S. Endres, Manual of Antisense Methodology, Kluwer (1999); Haseloff and Gerlach, 1988; Perriman et al., 1992; Shippy et al., 1999; Waterhouse et al. (1998); Smith et al. (2000); WO 99/32619, WO 99/53050, WO 99/49029, and WO 01/34815).

Gene Constructs and Vectors

One embodiment of the present invention includes a recombinant vector, which includes at least one isolated polynucleotide molecule encoding a polypeptide/enzyme defined herein, inserted into any vector capable of delivering the nucleic acid molecule into a host cell. Such a vector contains heterologous nucleic acid sequences, that is nucleic acid sequences that are not naturally found adjacent to nucleic acid molecules of the present invention and that preferably are derived from a species other than the species from which the nucleic acid molecule(s) are derived. The vector can be either RNA or DNA, either prokaryotic or eukaryotic, and typically is a virus or a plasmid.

One type of recombinant vector comprises a nucleic acid molecule of the present invention operatively linked to an expression vector. As indicated above, the phrase operatively linked refers to insertion of a nucleic acid molecule into an expression vector in a manner such that the molecule is able to be expressed when transformed into a host cell. As used herein, an expression vector is a DNA or RNA vector that is capable of transforming a host cell and effecting expression of a specified nucleic acid molecule. Preferably, the expression vector is also capable of replicating within the host cell. Expression vectors can be either prokaryotic or eukaryotic, and are typically viruses or plasmids. Expression vectors of the present invention include any vectors that function (i.e., direct gene expression) in recombinant cells of the present invention, including in bacterial, fungal, endoparasite, arthropod, other animal, and plant cells. Preferred

expression vectors of the present invention can direct gene expression in yeast, animal or plant cells.

In particular, expression vectors of the present invention contain regulatory sequences such as 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 nucleic acid molecules of the present invention. In particular, recombinant molecules of the present invention include transcription control sequences. Transcription control sequences are sequences which control the initiation, elongation, and termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor 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. A variety of such transcription control sequences are known to those skilled in the art.

Another embodiment of the present invention includes a recombinant cell comprising a host cell transformed with one or more recombinant molecules of the present invention. Transformation of a nucleic acid molecule into a cell can be accomplished by any method by which a nucleic acid molecule can be inserted into the cell. Transformation techniques include, but are not limited to, transfection, electroporation, microinjection, lipofection, adsorption, and protoplast fusion. A recombinant cell may remain unicellular or may grow into a tissue, organ or a multicellular organism. Transformed nucleic acid molecules can remain extrachromosomal or can integrate into one or more sites within a chromosome of the transformed (i.e., recombinant) cell in such a manner that their ability to be expressed is retained.

Transgenic Plants and Parts Thereof

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), single cells (e.g. pollen), seeds, plant cells and the like. 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 the invention may be: corn (*Zea mays*), canola (*Brassica napus, Brassica rapa* ssp.), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolour, Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Tritium 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 one 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 *Brassicas*, cotton, peanut, poppy, mustard, castor bean, sesame, safflower, 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.

When the production of ω3 LC-PUFA is desired it is preferable that the plant species which is to be transformed has an endogenous ratio of ALA to LA which is at least 1:1, more preferably at least 2:1. Examples include most, if not all, oilseeds such as linseed. This maximizes the amount of ALA substrate available for the production of SDA, ETA, ETrA, EPA, DPA and DHA.

The plants produced using the methods of the invention may already be transgenic, and/or transformed with additional genes to those described in detail herein. In one embodiment, the transgenic plants of the invention also produce a recombinant ω3 desaturase. The presence of a recombinant ω3 desaturase increases the ratio of ALA to LA in the plants which, as outlined in the previous paragraph, maximizes the production of LC-PUFA such as SDA, ETA, ETrA, EPA, DPA and DHA.

Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Leguminous plants include beans and peas. Beans include guar, locust bean,

fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava bean, lentils, chickpea, etc.

The term "extract or portion thereof" refers to any part of the plant. "Portion" generally refers to a specific tissue or organ such as a seed or root, whereas an "extract" typically involves the disruption of cell walls and possibly the partial purification of the resulting material. Naturally, the "extract or portion thereof" will comprise at least one LC-PUFA. Extracts can be prepared using standard techniques of the art.

Transgenic plants, as defined in the context of the present invention include plants and their progeny which have been genetically modified using recombinant techniques. This would generally be to cause or enhance production of at least one protein/enzyme defined herein in the desired plant or plant organ. Transgenic plant parts include all parts and cells of said plants such as, for example, cultured tissues, callus, protoplasts. Transformed plants contain genetic material that they did not contain prior to the transformation. The genetic material is preferably stably integrated into the genome of the plant. The introduced genetic material may comprise sequences that naturally occur in the same species but in a rearranged order or in a different arrangement of elements, for example an antisense sequence. Such plants are included herein in "transgenic plants". A "non-transgenic plant" is one which has not been genetically modified with the introduction of genetic material by recombinant DNA techniques. In a preferred embodiment, the transgenic plants are homozygous for each and every gene that has been introduced (transgene) so that their progeny do not segregate for the desired phenotype.

Several techniques exist for introducing foreign genetic material into a plant cell. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (see, for example, US 4,945,050 and US 5,141,131). Plants may be transformed using *Agrobacterium* technology (see, for example, US 5,177,010, US 5,104,310, US 5,004,863, US 5,159,135). Electroporation technology has also been used to transform plants (see, for example, WO 87/06614, US 5,472,869, 5,384,253, WO 92/09696 and WO 93/21335). In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue type I and II, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during development and/or differentiation using appropriate techniques described herein.

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 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-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.

Examples of plant promoters include, but are not limited to ribulose-1,6-bisphosphate carboxylase small subunit, beta-conglycinin promoter, phaseolin promoter, high molecular weight glutenin (HMW-GS) promoters, starch biosynthetic gene promoters, ADH promoter, heat-shock promoters and tissue specific promoters. Promoters may also contain certain enhancer sequence elements that may improve the transcription efficiency. Typical enhancers include but are not limited to Adh-intron 1 and Adh-intron 6.

Constitutive promoters direct continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S). Tissue specific promoters are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these promoters may also be used. Promoters may also be active during a certain stage of the plants' development as well as active in plant tissues and organs. Examples of such promoters include but are not limited to pollen-specific, embryo specific, corn silk specific, cotton fibre specific, root specific, seed endosperm specific promoters and the like.

In a particularly preferred embodiment, the promoter directs expression in tissues and organs in which lipid and oil biosynthesis take place, particularly in seed cells such as endosperm cells and cells of the developing embryo. Promoters which are suitable are the oilseed rape napin gene promoter (US 5,608,152), the *Vicia faba* USP promoter (Baumlein et al., 1991), the *Arabidopsis* oleosin promoter (WO 98/45461), the *Phaseolus vulgaris* phaseolin promoter (US 5,504,200), the *Brassica* Bce4 promoter (WO 91/13980) or the legumin B4 promoter (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 (WO 95/15389 and WO 95/23230) or the promoters described in WO 99/16890 (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) and US 20030159173.

Under certain circumstances it may be desirable to use an inducible promoter. An inducible promoter is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; and stress. Other desirable transcription and translation elements that function in plants may be used.

In addition to plant promoters, promoters from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoters of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S) and the like may be used.

It will be apparent that transgenic plants adapted for the production of LC-PUFA as described herein, in particular DHA, can either be eaten directly or used as a source for the extraction of essential fatty acids, of which DHA would be a constituent.

As used herein, "germination" refers to the emergence of the root tip from the seed coat after imbibition. "Germination rate" refers to the percentage of seeds in a population which have germinated over a period of time, for example 7 or 10 days, after imbibition. A population of seeds can be assessed daily over several days to determine the germination percentage over time.

With regard to seeds of the present invention, as used herein the term "germination rate which is substantially the same" means that the germination rate of the transgenic seeds is at least 60%, more preferably at least 80%, and even more preferably at least 90%, that of isogenic non-transgenic seeds. Gemination rates can be calculated using techniques known in the art.

With further regard to seeds of the present invention, as used herein the term "timing of germination of the seed is substantially the same" means that the timing of germination of the transgenic seeds is at least 60%, more preferably at least 80%, and even more preferably at least 90%, that of isogenic non-transgenic seeds. Timing of gemination can be calculated using techniques known in the art.

The present inventors have found that at least in some circumstances the production of LC-PUFA in recombinant plant cells is enhanced when the cells are homozygous for the transgene. As a result, it is preferred that the recombinant plant cell, preferably the transgenic plant, is homozygous for at least one desaturase and/or elongase gene. In one embodiment, the cells/plant are homozygous for the zebrafish Δ6/Δ5 desaturase and/or the *C. elegans* elongase.

Transgenic non-human animals

Techniques for producing transgenic animals are well known in the art. A useful general textbook on this subject is Houdebine, Transgenic animals - Generation and Use (Harwood Academic, 1997).

Heterologous DNA can be introduced, for example, into fertilized mammalian ova. For instance, totipotent or pluripotent stem cells can be transformed by microinjection, calcium phosphate mediated precipitation, liposome fusion, retroviral infection or other means, the transformed cells are then introduced into the embryo, and the embryo then develops into a transgenic animal. In a highly preferred method, developing embryos are infected with a retrovirus containing the desired DNA, and transgenic animals produced from the infected embryo. In a most preferred method, however, the appropriate DNAs are coinjected into the pronucleus or cytoplasm of embryos, preferably at the single cell stage, and the embryos allowed to develop into mature transgenic animals.

Another method used to produce a transgenic animal involves microinjecting a nucleic acid into pro-nuclear stage eggs by standard methods. Injected eggs are then cultured before transfer into the oviducts of pseudopregnant recipients.

Transgenic animals may also be produced by nuclear transfer technology. Using this method, fibroblasts from donor animals are stably transfected with a plasmid incorporating the coding sequences for a binding domain or binding partner of interest under the control of regulatory. Stable transfectants are then fused to enucleated oocytes, cultured and transferred into female recipients.

Feedstuffs

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 (including for enteral and/or parenteral 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.

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). 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.

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, vitamins, and/or minerals in amounts desired for a particular use. The amounts of these ingredients will 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 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 search. Additionally, examples of proteins which may be utilized in the nutritional composition of the invention include (but are not limited to) soy proteins, electrodialysed whey, electrodialysed 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 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.

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.

The genus *Saccharomyces spp* is used in both brewing of beer and wine making and also as an agent in baking, particularly bread. Yeast is a major constituent of vegetable extracts. Yeast is also used as an additive in animal feed. It will be apparent that genetically engineered yeast strains can be provided which are adapted to synthesise LC-PUFA as described herein. These yeast strains can then be used in food stuffs and in

wine and beer making to provide products which have enhanced fatty acid content and in particular DHA content.

Additionally, LC-PUFA 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 or milk fatty acid composition to one more desirable for human or animal consumption. Examples of such animals include sheep, cattle, horses and the like.

Furthermore, feedstuffs of the invention can be used in aquaculture to increase the levels of LC-PUFA in fish for human or animal consumption.

In mammals, the so-called "Sprecher" pathway converts DPA to DHA by three reactions, independent of a Δ7 elongase, Δ4 desaturase, and a beta-oxidation step (Sprecher et al., 1995) (Figure 1). Thus, in feedstuffs for mammal consumption, for example formulations for the consumption by human infants, it may only be necessary to provide DPA produced using the methods of the invention as the mammalian subject should be able to fulfill its nutritional needs for DHA by using the "Sprecher" pathway to convert DPA to DHA. As a result, in an embodiment of the present invention, a feedstuff described herein for mammalian consumption at least comprises DPA, wherein at least one enzymatic reaction in the production of DPA was performed by a recombinant enzyme in a cell.

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.

A pharmaceutical composition may comprise one or more of the LC-PUFA 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 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 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.

Solid dosage forms such as tablets and capsules can be prepared using techniques well known in the art. For example, LC-PUFA 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 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 LC-PUFA(s).

For intravenous administration, the PUFA produced in accordance with the present invention or derivatives thereof may be incorporated into commercial 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 LC-PUFA is desirable. However, it will be appreciated that any amount of LC-PUFA 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, 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 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 LC-PUFA produced according to the subject invention may be used as the sole "active" ingredient in a cosmetic composition.

Medical, Veterinary, Agricultural and Aquacultural Uses

The present invention also includes the treatment of various disorders by use of the pharmaceutical and/or feedstuff compositions described herein. In particular, the compositions of the present invention may be used to treat restenosis after angioplasty. Furthermore, symptoms of inflammation, rheumatoid arthritis, asthma and psoriasis may also be treated with the compositions (including feedstuffs) of the invention. Evidence also indicates that LC-PUFA may be involved in calcium metabolism; thus, the compositions of the present invention may be utilized in the treatment or prevention of osteoporosis and of kidney or urinary tract stones.

Additionally, the compositions of the present invention may also be used in the treatment of cancer. Malignant cells have been shown to have altered fatty acid compositions. Addition of fatty acids has been shown to slow their growth, cause cell death and increase their susceptibility to chemotherapeutic agents. Moreover, the compositions of the present invention may also be useful for treating cachexia associated with cancer.

The compositions of the present invention may also be used to treat diabetes as altered fatty acid metabolism and composition have been demonstrated in diabetic animals.

Furthermore, the compositions of the present invention, comprising LC-PUFA produced either directly or indirectly through the use of the cells of the invention, may also be used in the treatment of eczema and in the reduction of blood pressure. Additionally, the compositions of the present invention may be used to inhibit platelet aggregation, to induce vasodilation, to reduce cholesterol levels, to inhibit proliferation of vessel wall smooth muscle and fibrous tissue, to reduce or to prevent gastrointestinal bleeding and other side effects of non-steroidal anti-inflammatory drugs (US 4,666,701), to prevent or to treat endometriosis and premenstrual syndrome (US 4,758,592), and to treat myalgic encephalomyelitis and chronic fatigue after viral infections (US 5,116,871).

Further uses of the compositions of the present invention include, but are not limited to, use in the treatment or prevention of cardiac arrhythmia's, angioplasty, AIDS, multiple sclerosis, Crohn's disease, schizophrenia, foetal alcohol syndrome, attention deficient hyperactivity disorder, cystic fibrosis, phenylketonuria, unipolar depression, aggressive hostility, adrenoleukodystophy, coronary heart disease, hypertension, obesity, Alzheimer's disease, chronic obstructive pulmonary disease, ulcerative colitis or an ocular disease, as well as for maintenance of general health.

Furthermore, the above-described pharmaceutical and nutritional compositions may be utilized in connection with animals (i.e., domestic or non-domestic, including

mammals, birds, reptiles, lizards, etc.), as well as humans, as animals experience many of the same needs and conditions as humans. For example, the oil or fatty acids of the present invention may be utilized in animal feed supplements, animal feed substitutes, animal vitamins or in animal topical ointments.

Compositions such as feedstuffs of the invention can also be used in aquaculture to increase the levels of LC-PUFA in fish for human or animal consumption.

Any amount of LC-PUFA will be beneficial to the subject. However, it is preferred that an "amount effective to treat" the condition of interest is administered to the subject. Such dosages to effectively treat a condition which would benefit from administration of a LC-PUFA are known those skilled in the art. As an example, a dose of at least 300 mg/day of LC-PUFA for at least a few weeks, more preferably longer would be suitable in many circumstances.

Antibodies

The invention also provides monoclonal and/or polyclonal antibodies which bind specifically to at least one polypeptide of the invention or a fragment thereof. Thus, the present invention further provides a process for the production of monoclonal or polyclonal antibodies to polypeptides of the invention.

The term "binds specifically" refers to the ability of the antibody to bind to at least one protein of the present invention but not other proteins present in a recombinant cell, particularly a recombinant plant cell, of the invention.

As used herein, the term "epitope" refers to a region of a protein of the invention which is bound by the antibody. An epitope can be administered to an animal to generate antibodies against the epitope, however, antibodies of the present invention preferably specifically bind the epitope region in the context of the entire protein.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide. Serum from the immunised animal is collected and treated according to known procedures. If serum containing polyclonal antibodies contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use as immunogens in animals or humans.

Monoclonal antibodies directed against polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making

monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

For the purposes of this invention, the term "antibody", unless specified to the contrary, includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and $F(ab')_2$ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Preferably, antibodies of the present invention are detectably labeled. Exemplary detectable labels that allow for direct measurement of antibody binding include radiolabels, fluorophores, dyes, magnetic beads, chemiluminescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a coloured or fluorescent product. Additional exemplary detectable labels include covalently bound enzymes capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. Further exemplary detectable labels include biotin, which binds with high affinity to avidin or streptavidin; fluorochromes (e.g., phycobiliproteins, phycoerythrin and allophycocyanins; fluorescein and Texas red), which can be used with a fluorescence activated cell sorter; haptens; and the like. Preferably, the detectable label allows for direct measurement in a plate luminometer, e.g., biotin. Such labeled antibodies can be used in techniques known in the art to detect proteins of the invention.

EXAMPLES

Example 1. Materials and Methods

Culturing *Pavlova salina*

Pavlova salina isolates including strain CS-49 from the CSIRO Collection of Living Microalgae was cultivated under standard culture conditions (http://www.marine.csiro.au/microalgae). A stock culture from the Collection was sub-cultured and scaled-up in a dilution of 1 in 10 over consecutive transfers in 1 L Erlenmeyer flasks and then into 10 L polycarbonate carboys. The culture medium was f/2, a modification of Guillard and Ryther's (1962) f medium containing half-strength nutrients, with a growth temperature of 20±1°C. Other culturing conditions included a light intensity of 100 µmol. photons $PAR.m^{-2}.s^{-1}$, 12:12 hour light:dark photoperiod, and bubbling with 1% CO_2 in air at a rate of 200 $mL.L^{-1}.min^{-}$.

Yeast culturing and feeding with precursor fatty acids

Plasmids were introduced into yeast by heat shock and transformants were selected on yeast minimal medium (YMM) plates containing 2% raffinose as the sole carbon source. Clonal inoculum cultures were established in liquid YMM with 2% raffinose as the sole carbon source. Experimental cultures in were inoculated from these, in YMM + 1% NP-40, to an initial OD_{600} of ~ 0.3. Cultures were grown at 30°C with shaking (~60 rpm) until OD_{600} was approximately 1.0. At this point galactose was added to a final concentration of 2% and precursor fatty acids were added to a final concentration of 0.5mM. Cultures were incubated at 20°C with shaking for a further 48 hours prior to harvesting by centrifugation. Cell pellets were washed with 1% NP-40, 0.5% NP-40 and water to remove any unincorporated fatty acids from the surface of the cells.

Gas chromatography (GC) analysis of fatty acids

Fatty acid preparation

Fatty acid methyl esters (FAME) were formed by transesterification of the centrifuged yeast pellet or *Arabidopsis* seeds by heating with MeOH-$CHCl_3$-HCl (10:1:1, v/v/v) at 90-100 °C for 2 h in a glass test tube fitted with a Teflon-lined screw-cap. FAME were extracted into hexane-dichloromethane (4:1, v/v) and analysed by GC and GC-MS.

Capillary gas-liquid chromatography (GC)

FAME were analysed with Hewlett Packard (HP) 5890 GC or Agilent 6890 gas chromatograph fitted with HP 7673A or 6980 series automatic injectors respectively and a flame-ionization detector (FID). Injector and detector temperatures were 290^{o}C and 310^{o}C respectively. FAME samples were injected at 50^{o}C onto a non-polar cross-linked methyl-silicone fused-silica capillary column (HP-5; 50 m x 0.32 mm i.d.; 0.17 μm film thickness.). After 1 min, the oven temperature was raised to 210^{o}C at 30^{o}C min^{-1}, then to a final temperature of 280^{o}C at 3^{o}C min^{-1} where it was kept for 5 min. Helium was the carrier gas with a column head pressure of 65 KPa and the purge opened 2 min after injection. Identification of peaks was based on comparison of relative retention time data with standard FAME with confirmation using mass-spectrometry. For quantification Empower software (Waters) or Chemstation (Agilent) was used to integrate peak areas.

Gas chromatography-mass spectrometry (GC-MS)

GC-MS was carried out on a Finnigan GCQ Plus GC-MS ion-trap fitted with on-column injection set at 4^{o}C. Samples were injected using an AS2000 auto sampler onto a retention gap attached to an HP-5 Ultra 2 bonded-phase column (50 m x 0.32 mm i.d. x 0.17 μm film thickness). The initial temperature of 45^{o}C was held for 1 min, followed by temperature programming at 30^{o}C.min^{-1} to 140^{o}C then at 3^{o}C.min^{-1} to 310^{o}C where it was held for 12 min. Helium was used as the carrier gas. Mass spectrometer operating conditions were: electron impact energy 70 eV; emission current 250 μamp, transfer line 310 ^{o}C; source temperature 240^{o}C; scan rate 0.8 scans.s $^{-1}$ and mass range 40-650 Dalton. Mass spectra were acquired and processed with Xcalibur™ software.

Construction of *P. salina* cDNA library

mRNA, for the construction of a cDNA library, was isolated from *P. salina* cells using the following method. 2 g (wet weight) of *P. salina* cells were powdered using a mortar and pestle in liquid nitrogen and sprinkled slowly into a beaker containing 22 ml of extraction buffer that was being stirred constantly. To this, 5% insoluble polyvinylpyrrolidone, 90mM 2-mercaptoethanol, and 10mM dithiotheitol were added and the mixture stirred for a further 10 minutes prior to being transferred to a Corex™ tube. 18.4 ml of 3M ammonium acetate was added and mixed well. The sample was then centrifuged at 6000xg for 20 minutes at 4°C. The supernatant was transferred to a new tube and nucleic acid precipitated by the addition of 0.1 volume of 3M NaAc (pH 5.2) and 0.5 volume of cold isopropanol. After a 1 hour incubation at –20°C, the sample was

centrifuged at 6000xg for 30 minutes in a swing rotor. The pellet was resuspended in 1 ml of water extracted with phenol/chloroform. The aqueous layer was transferred to a new tube and nucleic acids were precipitated once again by the addition of 0.1 volume 3M NaAc (pH 5.2) and 2.5 volume of ice cold ethanol. The pellet was resuspended in water, the concentration of nucleic acid determined and then mRNA was isolated using the Oligotex mRNA system (Qiagen).

First strand cDNA was synthesised using an oligo-dT primer supplied with the ZAP-cDNA synthesis kit (Stratagene – cat # 200400) and the reverse transcriptase SuperscriptIII (Invitrogen). Double stranded cDNA was ligated to *EcoRI/Xho*I adaptors and from this a library was constructed using the ZAP-cDNA synthesis kit as described in the accompanying instruction manual (Stratagene – cat # 200400). The titer of the primary library was 2.5 x 10^5 plaque forming units (pfu)/ ml and that of the amplified library was 2.5 x 10^9 pfu/ ml. The average insert size of cDNA inserts in the library was 1.3 kilobases and the percentage of recombinants in the library was 74%.

Example 2. Microalgae and Polyunsaturated Fatty Acid Contents Thereof

The CSIRO Collection of Living Microalgae

CSIRO established and maintained a Collection of Living Microalgae (CLM) containing over 800 strains from 140 genera representing the majority of marine and some freshwater microalgal classes (list of strains available downloadable from http://www.marine.csiro.au). Selected micro-heterotrophic strains were also maintained.

This collection is the largest and most diverse microalgal culture collection in Australia. The CLM focused on isolates from Australian waters - over 80% of the strains were isolated from diverse localities and climatic zones, from tropical northern Australia to the Australian Antarctic Territory, from oceanic, inshore coastal, estuarine, intertidal and freshwater environments. Additionally, emphasis has been placed on representation of different populations of a single species, usually by more than one strain. All strains in the culture collection were unialgal and the majority were clonal. A subset of strains were axenic. Another collection is the NIES-Collection (National Institute for Environmental Studies, Environment Agency) maintained in Japan.

Microalgae are known for their cosmopolitanism at the morphological species level, with very low endemicity being shown. However this morphological cosmopolitanism can hide a plethora of diversity at the intra-specific level. There have been a number of studies of genetic diversity on different microalgae using approaches such as interbreeding, isozymes, growth rates and a range of molecular techniques. The diversity identified by these studies ranges from large regional and global scales (Chinain

et al. 1997) to between and within populations (Gallagher, 1980; Medlin et al., 1996; Bolch et al., 1999a,b). Variation at the intra-specific level, between morphologically indistinguishable microalgae, can usually only be identified using strains isolated from the environment and cultured in the laboratory.

It is essential to have identifiable and stable genotypes within culture collections. While there are recorded instances of change or loss of particular characteristics in long term culture (Coleman, 1977), in general, culturing guarantees genetic continuity and stability of a particular strain. Cryopreservation strategies could also be used to limit the potential for genetic drift.

Microalgae and their use in aquaculture

Because of their chemical/nutritional composition including PUFAs, microalgae are utilized in aquaculture as live feeds for various marine organisms. Such microalgae must be of an appropriate size for ingestion and readily digested. They must have rapid growth rates, be amenable to mass culture, and also be stable in culture to fluctuations in temperature, light and nutrients as may occur in hatchery systems. Strains fulfilling these attributes and used widely in aquaculture include northern hemisphere strains such as *Isochrysis* sp. (T.ISO) CS-177, *Pavlova lutheri* CS-182, *Chaetoceros calcitrans* CS-178, *C. muelleri* CS-176, *Skeletonema costatum* CS-181, *Thalassiosira pseudonana* CS-173, *Tetraselmis suecica* CS-187 and *Nannochloropsis oculata* CS-189. Australian strains used include *Pavlova pinguis* CS-375, *Skeletonema* sp. CS-252, *Nannochloropsis* sp. CS-246, *Rhodomonas salina* CS-24 and *Navicula jeffreyi* CS-46. Biochemical assessment of over 50 strains of microalgae used (or of potential use) in aquaculture found that cells grown to late-logarithmic growth phase typically contained 30 to 40% protein, 10 to 20% lipid and 5 to 15% carbohydrate (Brown et al., 1997).

Lipid composition including PUFA content of microalgae

There is considerable interest in microalgae containing a high content of the nutritionally important long-chain polyunsaturated fatty acids (LC-PUFA), in particular EPA [eicosapentaenoic acid, 20:5(ω3)] and DHA [docosahexaenoic acid, 22:6(ω3)] as these are essential for the health of both humans and aquacultured animals. While these PUFA are available in fish oils, microalgae are the primary producers of EPA and DHA.

The lipid composition of a range of microalgae (46 strains) and particularly the proportion and content of important PUFA in the lipid of the microalgae were profiled. C_{18}-C_{22} PUFA composition of microalgal strains from different algal classes varied considerably across the range of classes of phototrophic algae (Table 3, Figure 2, see also

Dunstan et. al., 1994, Volkman et al., 1989; Mansour et al., 1999a). Diatoms and eustigmatophytes were rich in EPA and produced small amounts of the less common PUFA, ARA [arachidonic acid, 20:4(ω6)] with negligible amounts of DHA. In addition, diatoms made unusual C_{16} PUFA such as 16:4(ω1) and 16:3(ω4). In contrast, dinoflagellates had high concentrations of DHA and moderate to high proportions of EPA and precursor C_{18} PUFA [18:5(ω3) and 18:4(ω3) SDA, stearidonic acid]. Prymnesiophytes also contained EPA and DHA, with EPA the dominant PUFA. Cryptomonads were a rich source of the C_{18} PUFA 18:3(ω3) (ALA α-linolenic acid) and SDA, as well as EPA and DHA. Green algae (e.g. Chlorophytes such as *Dunaliella* spp. and *Chlorella* spp.) were relatively deficient in both C20 and C22 PUFA, although some species had small amounts of EPA (up to 3%) and typically contained abundant ALA and 18:2(ω6), and were also able to make 16:4(ω3). The biochemical or nutritional significance of uncommon C_{16} PUFA [e.g. 16:4(ω3), 16:4(ω1), 16:3(ω4)] and C_{18} PUFA (e.g. 18:5(ω3) and STA] is unclear. However there is current interest in C_{18} PUFA such as SDA that are now being increasingly recognized as precursors for the beneficial EPA and DHA, unlike ALA which has only limited conversion to EPA and DHA.

New strains of Australian thraustochytrids were isolated. When examined, these thraustochytrids showed great morphological diversity from single cells to clusters of cells, complex reticulate forms and motile stages. Thruastochytrids are a group of single cell organisms that produce both high oil and LC-PUFA content. They were initially thought to be primitive fungi, although more recently have been assigned to the subclass Thraustochytridae (Chromista, Heterokonta), which aligns them more closely with other heterokont algae (e.g. diatoms and brown algae). Under culture, thraustochytrids can achieve considerably higher biomass yield (>20 g/L) than other microalgae. In addition, thraustochytrids can be grown in fermenters with an organic carbon source and therefore represent a highly attractive, renewable and contaminant-free, source of omega-3 oils.

TABLE 3. Distribution of selected PUFA and LC-PUFA in microalgae and other groups, and areas of application.

Group	Genus / Species	PUFA	Application
Eustigmatophytes	*Nannochloropsis*	EPA	Aquaculture
Diatoms	*Chaetoceros*		
Dinoflagellates	*Crypthecodinium cohnii*	DHA	Aquaculture, health supplements, infant formula
Thraustochytrids	*Schizochytrium*		
Red algae	*Phorphyridium*	ARA	Aquaculture, infant formula
Thraustochytrids	undescribed species		Pharmaceutical industry (precursor to prostaglandins)
Fungi	*Mortiella*		
Blue green algae	*Spirulina*	GLA	health supplements

Abbreviations: γ–linolenic acid, GLA, 18:3ω6; 20:5ω3, eicosapentaenoic acid, EPA, 20:5ω3; docosahexaenoic acid, DHA, 22:6ω3; arachidonic acid, ARA, 20:4ω6.

Representative fatty acid profiles for selected Australian thraustochytrids are shown in Table 4. Strain O was particularly attractive as it contained very high levels of DHA (61%). Other PUFA were present at less than 5% each. High DHA-containing thraustochytrids often also contained high proportions of 22:5ω6, docosapentaenoic acid

(DPA), as was observed for strains A, C and H. DPA was only a minor component in strain O under the culture conditions employed, making this strain particularly interesting. Strain A contained both DHA (28%) and EPA (16%) as the main LC-PUFA. Strains C and H differed from the other strains with ARA (10-13%) also being present as a major LC-PUFA. A number of other LC-PUFA were present in the thraustochytrids including DPA(3) and 22:4ω6 and other components.

TABLE 4. Fatty acid composition (% of total) of thraustochytrid strains.

Fatty acid	Percentage composition			
	Strain			
	A	C	H	O
16:0	18.0	16.4	13.5	22.1
20:4ω6 ARA		4.0	10.5	13.4 0.7
20:5ω3 EPA	15.8	7.7	5.2	4.1
22:5ω6 DPA(6)	16.6	9.3	12.7	3.4
22:6ω3 DHA	28.2	21.6	19.2	61.0

The microalgal and thraustochytrid isolates in the CLM that may be used for isolation of genes involved in the synthesis of LC-PUFA are of the genera or species as follows:

Class Bacillariophyceae (Diatoms)

Attheya septentrionalis, Aulacoseira sp., *Chaetoceros affinis, Chaetoceros calcitrans, Chaetoceros calcitrans* f. *pumilum, Chaetoceros* cf. *mitra, Chaetoceros* cf. *peruvianus, Chaetoceros* cf. *radians, Chaetoceros didymus, Chaetoceros difficile, Chaetoceros gracilis, Chaetoceros muelleri, Chaetoceros simplex, Chaetoceros socialis, Chaetoceros* sp., *Chaetoceros* cf. *minus, Chaetoceros* cf. *tenuissimus, Coscinodiscus wailesii,* other *Coscinodiscus* spp., *Dactyliosolen fragilissimus, Detonula pumila, Ditylum brightwellii, Eucampia zodiacus, Extubocellulus spinifera, Lauderia annulata, Leptocylindrus danicus, Melosira moniliformis, Melosira* sp., *Minidiscus*

trioculatus, Minutocellus polymorphus, Odontella aurita, Odontella mobiliensis, Odontella regia, Odontella rhombus, Odontella sp., Papiliocellulus simplex, Planktosphaerium sp., *Proboscia alata, Rhizosolenia imbricata, Rhizosolenia setigera, Rhizosolenia* sp., *Skeletonema costatum, Skeletonema pseudocostatum, Skeletonema sp., Skeletonema tropicum,* other *Skeletonema* spp., *Stephanopyxis turris, Streptotheca sp., Streptotheca tamesis, Streptotheca* spp., *Striatella* sp., *Thalassiosira delicatula, Thalassiosira eccentrica, Thalassiosira mediterranea, Thalassiosira oceanica, Thalassiosira oestrupii, Thalassiosira profunda, Thalassiosira pseudonana, Thalassiosira rotula, Thalassiosira stellaris,* other *Thalassiosira* spp., *Achnanthes* cf. *amoena, Amphiprora* cf. *alata, Amphiprora hyalina, Amphora* spp., *Asterionella glacialis, Asterionellopsis glacialis, Biddulphia* sp., *Cocconeis* sp., *Cylindrotheca closterium, Cylindrotheca fusiformis, Delphineis* sp., *Diploneis* sp., *Entomoneis* sp., *Fallacia carpentariae, Grammatophora oceanica, Haslea ostrearia, Licmophora* sp., *Manguinea* sp., *Navicula* cf. *jeffreyi, Navicula jeffreyi,* other *Navicula* spp., *Nitzschia* cf. *bilobata, Nitzschia* cf. *constricta, Nitzschia* cf. *cylindrus , Nitzschia* cf. *frustulum, Nitzschia* cf *paleacea, Nitzschia closterium, Nitzschia fraudulenta, Nitzschia frustulum, Nitzschia* sp., *Phaeodactylum tricornutum, Pleurosigma delicatulum,* other *Pleurosigma* spp., *Pseudonitzschia australis, Pseudonitzschia delicatissima, Pseudonitzschia fraudulenta, Pseudonitzschia pseudodelicatissima, Pseudonitzschia pungens, Pseudonitzschia* sp., *Pseudostaurosira shiloi, Thalassionema nitzschioides, or Thalassiothrix heteromorpha.*

Class Chrysophyceae

Chrysolepidomonas cf. *marina, Hibberdia* spp., *Ochromonas danica, Pelagococcus subviridis, Phaeoplaca* spp., *Synura shagnicola* or other Chrysophyte spp.

Class Cryptophyceae

Chroomonas placoidea, Chroomonas sp., *Geminigera cryophila, Hemiselmis simplex, Hemiselmis sp., Rhodomonas baltica, Rhodomonas maculata, Rhodomonas salina, Rhodomonas* sp. or other Cryptomonad spp.

Class Dinophyceae (Dinoflagellates)

Alexandrium affine, Alexandrium catenella, Alexandrium margalefi, Alexandrium minutum, Alexandrium protogonyaulax, Alexandrium tamarense, Amphidinium carterae, Amphidinium cf britannicum, Amphidinium klebsii, Amphidinium sp., *Amphidinium steinii, Amylax tricantha, Cryptothecodinium cohnii, Ensiculifera*

sp., Fragilidium spp., Gambierdiscus toxicus, Gymnodinium catenatum, Gymnodinium galathaneum, Gymnodinium galatheanum, Gymnodinium nolleri, Gymnodinium sanguineum, or other *Gymnodinium spp., Gyrodinium pulchellum*, or other *Gyrodinium* spp., *Heterocapsa niei, Heterocapsa rotundata, Katodinium* cf. *rotundatum, Kryptoperidinium foliaceum, Peridinium balticum, Prorocentrum gracile, Prorocentrum mexicanum, Prorocentrum micans, Protoceratium reticulatum, Pyrodinium bahamense, Scrippsiella* cf. *precaria*, or other *Scrippsiella* spp. *Symbiodinium microadriaticum*, or *Woloszynskia* sp.

Class Euglenophyceae

Euglena gracilis.

Class Prasinophyceae

Pycnococcus sp., *Mantoniella squamata, Micromonas pusilla, Nephroselmis minuta, Nephroselmis pyriformes, Nephroselmis rotunda, Nephroselmis* spp., or other Prasinophyte spp., *Pseudoscourfieldia marina, Pycnococcus provasolii, Pyramimonas cordata, Pyramimonas gelidicola, Pyramimonas grossii, Pyramimonas oltmansii, Pyramimonas propulsa*, other *Pyramimonas* spp., *Tetraselmis antarctica, Tetraselmis chuii, Tetraselmis* sp., *Tetraselmis suecica*, or other *Tetraselmis* spp.

Class Prymnesiophyceae

Chrysochromulina acantha, Chrysochromulina apheles, Chrysochromulina brevifilum, Chrysochromulina camella, Chrysochromulina hirta, Chrysochromulina kappa, Chrysochromulina minor, Chrysochromulina pienaar, Chrysochromulina simplex, Chrysochromulina sp., *Chrysochromulina spinifera, Chrysochromulina strobilus*, and other Chrysophyte spp., *Chrysotila lamellosa, Cricosphaera carterae, Crystallolithus hyalinus, Diacronema vlkianum, Dicrateria inornata, Dicrateria* sp., *Emiliania huxleyi, Gephyrocapsa oceanica, Imantonia rotunda*, and other *Isochrysis spp., Ochrosphaera neapolitana, Pavlova* cf. *pinguis, Pavlova gyrans, Pavlova lutheri, Pavlova pinguis, Pavlova salina, Pavlova* sp., *Phaeocystis* cf. *pouchetii, Phaeocystis globosa, Phaeocystis pouchetii*, other *Phaeocystis spp., Pleurochrysis aff. carterae, Prymnesium parvum, Prymnesium patelliferum*, other *Prymnesium* spp., or *Pseudoisochrysis paradoxa.*

Class Raphidophyceae
Chattonella antiqua, other *Chattonella spp., Fibrocapsa japonica,* other *Fibrocapsa spp., Heterosigma akashiwo, Heterosigma carterae,* or other *Heterosigma* spp.

Class Thraustochytridae
Schizochytrium spp., *Thraustochytrium aureum, Thraustochytrium roseum,* or other *Thraustochytrium* spp.

Class Eustigmatophytae as a source of genes for EPA production:
Eustigmatos vischeri, Monodus subterraneus, Nannochloropsis oculata, Nannochloropsis salina, Vischeria helvetica, Vischeria punctata, Chloridella neglecta, Chloridella simplex, Chlorobotrys regularis, Ellipsoidon parvum, Ellipsoidon solitare, Eustigmatos magnus, Eustigmatos polyphem, Goniochloris sculpta, Monodus subterraneus, Monodus unipapilla, Nannochloropsis gaditana, Nannochloropsis granulata, Nannochloropsis limnetica, Pseudocharaciopsis, ovalis, Pseudocharaciopsis texensis, Pseudostaurastrum limneticum, or *Vischeria stellata*

Example 3. Isolation of Zebrafish Δ5/6 Desaturase and Functional Characterization in Yeast

As well as microalgae, some other organisms have the capacity to synthesise LC-PUFA from precursors such as α-linolenic acid (18:3, ALA) (see Figure 1) and some of the genes responsible for such synthesis have been isolated (see Sayanova and Napier, 2004). The genes involved in omega-3 C_{20} +PUFA biosynthesis have been cloned from various organisms including algae, fungi, mosses, plants, nematodes and mammals. Based on the current understanding of genes involved in the synthesis of omega-3 C_{20} +PUFA, synthesis of EPA in plants would require the transfer of genes encoding at least two desaturases and one PUFA elongase. The synthesis of DHA from EPA in plants would require the additional transfer of a further desaturase and a further elongase (Sayanova and Napier, 2004). These enzymes are: for the synthesis of EPA, the sequential activities of a Δ6 desaturase, Δ6 elongase and a Δ5 desaturase is required. Based on an alternative pathway operative in some algae, EPA may also be synthesised by the sequential activities of a Δ9 elongase, a Δ8 desaturase and a Δ5 desaturase (Wallis and Browse, 1999; Qi et al., 2002). For the further conversion of EPA to DHA in plants, a further transfer of a Δ5 elongase and Δ4 desaturase will be required (Sayanova and Napier, 2004).

Hastings et al. (2001) isolated a gene encoding a Δ5/Δ6 bifunctional desaturase from zebra fish (*Danio rerio*) and showed that, when expressed in yeast, the desaturase was able to catalyse the synthesis of both Δ6 (GLA and SDA) and Δ5 (20:4 and EPA) fatty acids. The desaturase was therefore able to act on both ω6 and ω3 substrates.

Isolation of the zebrafish Δ5/Δ6 desaturase

RNA was extracted using the RNAeasy system according to the manufacturers instructions (Qiagen) from freshly dissected zebrafish livers. Based on the published sequence (Hastings et al. 2001), primers, sense, 5'-CCCAAGCTTACTATGGGTGGCGGAGGACAGC-3' (SEQ ID NO:39) and antisense 5'-CCGCTGGAGTTATTTGTTGAGATACGC-3' (SEQ ID NO:40) at the 5' and 3' extremities of the zebrafish Δ5/6 ORF were designed and used in a one-step reverse transcription-PCR (RT-PCR, Promega) with the extracted RNA and using buffer conditions as recommended by the manufacturer. A single amplicon of size 1335bp was obtained, ligated into pGEM-T easy (Promega) and the sequence confirmed as identical to that published.

A fragment containing the entire coding region (SEQ ID NO:38) was excised and ligated into the yeast shuttle vector pYES2 (Invitrogen). The vector pYES2 carried the *URA3* gene, which allowed selection for yeast transformants based on uracil prototrophy. The inserted coding region was under the control of the inducible *GAL1* promoter and polyadenylation signal of pYES2. The resultant plasmid was designated pYES2-zfΔ5/6, for introduction and expression in yeast (*Saccharomyces cerevisiae*).

Expression of zebrafish Δ5/Δ6 desaturase in yeast

The gene construct pYES2-zfΔ5/6 was introduced into yeast strain S288. Yeast was a good host for analysing heterologous potential LC-PUFA biosynthesis genes including desaturases and elongases for several reasons. It was easily transformed. It synthesised no LC-PUFA of its own and therefore any new PUFA made was easily detectable without any background problems. Furthermore, yeast cells readily incorporated fatty acids from growth media into cellular lipids, thereby allowing the presentation of appropriate precursors to transformed cells containing genes encoding new enzymes, allowing for confirmation of their enzymatic activities.

Biochemical analyses

Yeast cells transformed with pYES2-zfΔ5/6 were grown in YMM medium and induced by the addition of galactose. The fatty acids 18:3ω3 (ALA, 0.5 mM) or 20:4ω3

(ETA, 0.5 mM) were added to the medium as described above. After 48 hours incubation, the cells were harvested and fatty acid analysis carried out by capillary gas-liquid chromatography (GC) as described in Example 1. The analysis showed that 18:4ω3 (1.9% of total fatty acid) was formed from 18:3ω3 and 20:5ω3 (0.24% of fatty acids) from 20:4ω3, demonstrating Δ6 desaturase activity and Δ5 desaturase activity, respectively. These data are summarized in Table 5 and confirm the results of Hastings et al (2001).

Example 4. Isolation of *C. elegans* Elongase and Functional Characterization in Yeast

Cloning of *C. elegans* elongase gene

Beaudoin and colleagues isolated a gene encoding an ELO-type fatty acid elongase from the nematode *Caenorhabditis elegans* (Beaudoin et al., 2000) and this gene was isolated as follows. Oligonucleotide primers having the sequences 5'-GCGGGTACCATGGCTCAGCATCCGCTC-3' (SEQ ID NO:41) (sense orientation) and 5'- GCGGGATCCTTAGTTGTTCTTCTTCTT-3' (SEQ ID NO:42) (antisense orientation) were designed and synthesized, based on the 5' and 3' ends of the elongase coding region. These primers were used in a PCR reaction to amplify the 867 basepair coding region from a *C. elegans* N2 mixed-stage gene library, using an annealing temperature of 58^0C and an extension time of 1 minute. The PCR amplification was carried out for 30 cycles. The amplification product was inserted into the vector pGEM™ T-easy (Promega) and the nucleotide sequence confirmed (SEQ. ID NO:37). An *Eco*RI/*Bam*HI fragment including the entire coding region was excised and inserted into the *Eco*RI/*Bgl*II sites of pSEC-TRP (Stratagene), generating pSEC-Ceelo, for introduction and expression in yeast. pSEC-TRP contains the TRP1 gene, which allowed for the selection of transformants in yeast by tryptophan prototrophy, and the GAL1 promoter for expression of the chimeric gene in an inducible fashion in the presence of galactose in the growth medium.

TABLE 5. Enzymatic activities in yeast and *Arabidopsis*

Clone	Precursor PUFA	Synthesised PUFA	% (of total FA)	Observed activity
pYES2-zfΔ5/6	18:3ω3	18:4ω3	1.9	Δ6 desaturase
pYES2-zfΔ5/6	20:4ω3	20:5ω3	0.24	Δ5 desaturase
pYES2zfΔ5/6, pSEC-Ceelo	18:3ω3	18:4ω3	0.82	Δ6 desaturase
		20:3ω3	0.20	Δ9 elongase
		20:4ω3	0.02	Δ6 elongase
pYES2-psΔ8	18:3ω3	18:4ω3	-	NOT Δ6 desaturase
pYES2-psΔ8	20:3ω3	20:4ω3	0.12	Δ8 desaturase
pYES2-psELO1	18:2ω6	20:2ω6	-	
pYES2-psELO1	18:3ω3		-	
pYES2-psELO1	20:3ω3	22:3ω3	-	
pYES2-psELO1	20:4ω3	22:4ω3	-	
pYES2-psELO1	20:5ω3	22:5ω3	0.82	Δ5 elongase
pYES-psELO2	18:2ω6	20:2ω6	0.12	Δ9 elongase
pYES-psELO2	18:3ω3	20:3ω3	0.20	Δ9 elongase
pYES-psELO2	20:3ω3	22:3ω3	-	
pYES-psELO2	20:4ω3	22:4ω3	-	
pYES-psELO2	20:5ω3	22:5ω3	-	
Arabidopsis + zfΔ5/6 & Ceelo (plant #1)	-	18:3ω6	0.32	Δ5/6 desaturase, Δ5/6/9 elongase
	-	18:4ω3	1.1	
	-	20:4ω6	1.1	
	-	20:5ω3	2.1	
	-	20:3ω6	1.1	
	-	20:4ω3	0.40	
	-	20:2ω6	3.2	
	-	20:3ω3	TR	
	-	22:4ω6	0.06	
	-	22:5ω3	0.13	
		22:3ω6	0.03	

TR, trace, not accurately determined.

Functional characterization of *C. elegans* elongase gene in yeast

Yeast strain S288 was transformed, using the method described in Example 1, with both vectors pYES2-zfΔ5/6 and pSEC-Ceelo simultaneously and double transformants were selected on YMM medium that lacked tryptophan and uracil. The transformants grew well on both minimal and enriched media, in contrast to transformants of strain S288 carrying pSEC-Ceelo alone, in the absence of pYES2-zfΔ5/6, which grew quite poorly. Double transformants were grown in YMM medium and induced by the addition of galactose. The fatty acid 18:3ω3 (ALA, 0.5 mM) was added to the medium and, after 48 hours incubation, cells were harvested and fatty acid analysis carried out by capillary gas-liquid chromatography (GC) as described in Example 1. The analysis showed that 18:4ω3 (0.82% of total fatty acid) and 20:3ω3 (0.20%) were formed from 18:3ω3, and 20:4ω3 (0.02% of fatty acids) from either of those, demonstrating the concerted action of an elongase activity in addition to the Δ6 desaturase activity and Δ5 desaturase activity of the zebrafish desaturase (Table 5). The concerted action of a bifunctional Δ5/6 desaturase gene and an elongase gene has not been reported previously. In particular, the use of a bifunctional enzyme, if showing the same activities in plant cells, would reduce the number of genes that would need to be introduced and expressed. This also has not been reported previously.

Example 5. Coordinate Expression of Fatty Acid Desaturase and Elongase in Plants

Genetic construct for co-expression of the zebrafish Δ6/Δ5 desaturase and *C. elegans* elongase in plant cells

Beaudoin and colleagues (2000) showed that the *C. elegans* Δ6 elongase protein, when expressed in yeast, could elongate the C18 Δ6 desaturated fatty acids GLA and SDA, i.e. that it had Δ6 elongase activity on C18 substrates. They also showed that the protein did not have Δ5 elongase activity on a C20 substrate in yeast. We tested, therefore, whether this elongase would be able to elongate the Δ6 desaturated fatty acids GLA and SDA in *Arabidopsis* seed. *Arabidopsis thaliana* seed have been shown to contain both omega-6 (18:2, LA) and omega-3 (18:3, ALA) fatty acids (Singh et al, 2001). The presence of 18:3 in particular makes *Arabidopsis* seed an excellent system to study the expression of genes that could lead to the synthesis of omega-3 C_{20}+PUFA like EPA and DHA.

The test for elongase activity in *Arabidopsis* required the coordinate expression of a Δ6 desaturase in the seed to first form GLA or SDA. We chose to express the elongase gene in conjunction with the zebrafish desaturase gene described above. There were no

previous reports of the expression of the zebra fish Δ6/Δ5 desaturase and *C .elegans* elongase genes in plant cells, either individually or together.

Seed–specific co-expression of the zebra fish Δ6/Δ5 desaturase and *C. elegans* elongase genes was achieved by placing the genes independently under the control of a -309 napin promoter fragment, designated Fp1 (Stalberg et al., 1993). For plant transformation, the genes were inserted into the binary vector pWvec8 that comprised an enhanced hygromycin resistance gene as selectable marker (Wang et al., 1997). To achieve this, the *C. elegans* elongase coding region from Example 4 was inserted as a blunt-end fragment between the Fp1 and Nos 3' polyadenylation/terminator fragment in the binary vector pWvec8, forming pCeloPWvec8. The zebrafish Δ5/Δ6 desaturase coding region from Example 3 was initially inserted as a blunt end fragment between the Fp1 and Nos 3' terminator sequences and this expression cassette assembled between the *Hin*dIII and *Apa*I cloning sites of the pBluescript cloning vector (Stratagene). Subsequently, the entire vector containing the desaturase expression cassette was inserted into the *Hin*dIII site of pCeloPWvec8, forming pZebdesatCeloPWvec8. The construct, shown schematically in Figure 3, was introduced into *Agrobacterium* strain AGLI (Valvekens et al., 1988) by electroporation prior to transformation into *Arabidopsis thaliana*, ecotype Columbia. The construct was also designated the "DO" construct, and plants obtained by transformation with this construct were indicated by the prefix "DO".

Plant transformation and analysis

Plant transformation was carried out using the floral dipping method (Clough and Bent, 1998). Seeds (T1 seeds) from the treated plants (T0 plants) were plated out on hygromycin (20 mg/l) selective media and transformed plants selected and transferred to soil to establish T1 plants. One hygromycin resistant plant was recovered from a first screen and established in soil. The transformation experiment was repeated and 24 further confirmed T1 transgenic plants were recovered and established in soil. Most of these T1 plants were expected to be heterozygous for the introduced transgenes.

T2 seed from the 25 transgenic plants were collected at maturity and analysed for fatty acid composition. As summarised in Table 6, seed of untransformed *Arabidopsis* (Columbia ecotype) contained significant amounts of both the ω6 and ω3, C18 fatty acid precursors LA and ALA but did not contain any Δ6-desaturated C18 (18:3ω6 or 18:4ω3), ω6-desaturated C20 PUFA or ω3-desaturated C20 PUFA. In contrast, fatty acids of the seed oil of the transformed plants comprising the zebra fish Δ5/Δ6 desaturase and *C. elegans* elongase gene constructs contained 18:3ω6, 18:4ω3 and a whole series of ω6-

TABLE 6. Fatty acid composition in transgenic seed (% of the total fatty acid in seed oil).

	Fatty acid										
Plant number	GLA 18:3ω6	SDA 18:4ω3	ARA 20:4ω6	EPA 20:5ω3	DGLA 20:3ω6	ETA 20:4ω3	EDA 20:2ω6	ETrA 20:3ω3	22:4ω6	DPA 22:5ω3	22:3ω6
Wt	-	-	-	-	-	-	-	-	-	-	-
DO1	0.32	1.10	1.10	2.10	1.10	0.40	3.20	TR	0.06	0.13	0.03
DO2	0.20	0.70	0.60	1.20	0.80	0.40	1.60	-	0.10	TR	-
DO3	0.20	0.50	0.40	0.80	0.60	0.30	1.90	-	TR	TR	-
DO4	0.30	0.90	0.80	1.30	1.10	0.50	1.90	-	-	0.10	-
DO5	0.10	0.50	0.20	0.40	0.40	-	0.30	-	TR	TR	-
DO6	0.30	1.00	1.00	1.70	1.20	0.50	2.50	-	0.10	0.10	-
DO7	0.10	0.40	0.40	0.70	0.70	0.30	1.60	-	TR	TR	-
DO8	0.30	1.20	1.10	2.10	1.40	0.60	2.80	-	0.10	0.10	-
DO9	0.30	1.30	0.90	2.20	1.30	0.60	3.10	-	0.10	0.10	-
DO10	0.10	0.40	0.30	0.70	0.50	0.30	0.10	-	TR	TR	-
DO11	0.30	1.00	1.40	2.30	1.50	0.60	3.20	-	0.10	0.20	-
DO12	0.40	1.40	1.10	1.90	1.20	0.60	2.30	-	0.10	0.10	-
DO13	0.20	0.60	0.60	0.90	0.80	0.40	0.40	-	TR	0.10	-
DO14	0.30	1.00	0.70	1.70	1.10	0.60	2.50	-	TR	TR	-
DO15	0.30	1.30	1.00	2.30	1.50	0.60	2.60	-	0.10	0.10	-
DO17	0.20	0.40	0.40	0.70	0.70	0.30	1.80	-	TR	TR	-
DO18	0.20	0.60	0.50	0.90	0.80	0.40	1.70	-	TR	TR	-
DO19	0.20	0.40	0.40	0.80	0.70	0.30	2.00	-	TR	0.10	
DO20	0.30	1.00	0.50	0.90	0.70	0.30	1.60	-	TR	TR	-
DO21	0.30	1.20	0.90	2.00	1.30	0.60	2.50	-	-	0.10	-
DO22	0.30	0.90	0.70	1.20	1.00	0.40	0.30	-	TR	TR	-
DO23	-	-	-	-	0.10	0.10	1.80	-	-	-	-
DO24	0.30	1.10	0.70	1.50	1.10	0.50	2.90	-	TR	0.10	-
DO25	0.10	0.50	0.30	0.70	0.50	0.20	1.60	-	TR	0.10	-

Wt = untransformed Arabidopsis (Columbia). TR indicates less than 0.05%. Dash (-) indicates not detected.

and ω3-C20 PUFA. These resulted from the sequential action of the desaturase and elongase enzymes on the respective C18 precursors. Most importantly and unexpectedly, the transgenic seed contained both 20:5ω3 (EPA), reaching at least 2.3% of the total fatty acid in the seedoil, and 22:5ω3 (DPA), reaching at least 0.2% of this omega-3 LC-PUFA in the fatty acid of the seedoil. The total C20 fatty acids produced in the transgenic seed oil reached at least 9.0%. The total ω3 fatty acids produced that were a product of Δ6 desaturation (i.e. downstream of 18:3ω3 (ALA), calculated as the sum of the percentages for 18:4ω3 (SDA), 20:4ω3 (ETA), 20:5ω3 (EPA) and 22:5w3 (DPA)) reached at least 4.2%. These levels represent a conversion efficiency of ALA, which is present in seed oil of the wild-type *Arabidopsis* plants used for the transformation at a level of about 13-15%, to ω3 products through a Δ6 desaturation step of at least 28%. Stated otherwise, the ratio of ALA products to ALA (products:ALA) in the seed oil was at least 1:3.6. Of significance here, *Arabidopsis* has a relatively low amount of ALA in its seed oil compared to some commercial oilseed crops.

The T2 lines described above included lines that were homozygous for the transgenes as well as heterozygotes. To distinguish homozygotes and heterozygotes for lines expressing the transgenes at the highest levels, T2 plants were established from the T2 seed for the 5 lines containing the highest EPA levels, using selection on MS medium containing hygromycin (15mg/L) to determine the presence of the transgenes. For example, the T2 seed was used from the T1 plant designated DO11, containing 2.3% EPA and showing a 3:1 segregation ratio of resistant to susceptible progeny on the hygromycin medium, indicating that DO11 contained the transgenes at a single genetic locus. Homozygous lines were identified. For example, T2 progeny plant DO11-5 was homozygous as shown by the uniformly hygromycin resistance in its T3 progeny. Other T2 plants were heterozygous for the hygromycin marker.

The fatty acid profiles of T3 seed lots from DO11-5 and other T2 progeny of DO11 were analysed and the data are presented in Table 7. As expected, the EPA contents reflected segregation of the DO construct. The levels of EPA in the fatty acid of the seedoil obtained from the T3 lines were in three groups: negligible (nulls for the DO construct), in the range 1.6-2.3% (heterozygotes for the DO construct) and reaching at least 3.1% (homozygotes for the DO construct). The levels obtained were higher in homozygotes than heterozygotes, indicating a gene dosage effect. T3 seed from the DO11-5 plant synthesized a total of 9.6% new ω3 and ω6 PUFAs, including 3.2% EPA, 1.6% ARA, 0.1% DPA, 0.6% SDA and 1.8% GLA (Table 7). This level of EPA synthesis in seed was four fold higher than the 0.8% level previously achieved in linseed

TABLE 7. Fatty acid composition in transgenic seed (% of the total fatty acid in seed oil).

Fatty acid		Wildtype	DO 11-5	DO 11-6	DO11-7	DO11-8	DO11-10	DO11-11	DO11-12	DO11-13	DO11-16	DO11-18	DO11-19	DO11-20	DO11-21
14:0		0.3	0.0	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1
15:0		0.0	0.0	0.2	0.2	0.2	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.2	0.1
16:1ω7		0.5	0.4	0.6	0.7	0.6	0.5	0.4	0.6	0.5	0.6	0.4	0.4	0.7	0.5
16:0		8.1	7.1	7.9	7.8	7.6	7.0	7.1	7.8	7.7	7.6	6.8	6.7	7.6	7.3
17:1ω8		0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.0	0.1	0.1	0.0	0.1
17:0		0.3	0.1	0.0	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.1
18:3ω6	GLA	0.0	0.6	0.0	0.0	0.0	0.3	0.3	0.0	0.4	0.0	0.2	0.3	0.0	0.4
18:4ω3	SDA	0.0	1.8	0.0	0.0	0.0	1.0	1.1	0.0	1.3	0.0	0.7	1.1	0.0	1.2
18:2ω6	LA	26.6	25.8	29.8	28.6	28.8	25.6	25.4	28.6	25.6	29.0	25.7	25.2	29.4	27.3
18:1ω9		17.9	18.7	15.6	19.6	18.2	22.0	18.6	18.8	20.4	15.5	20.1	19.8	16.6	14.8
18:1ω7/ 18:3ω3	ALA	16.0	11.5	15.3	14.7	15.9	10.6	11.6	14.5	11.1	16.0	13.7	13.6	14.8	13.1
18:0		3.4	4.2	2.9	2.7	2.8	3.5	3.9	2.8	3.9	2.9	3.3	3.4	2.9	3.7
19:0		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.1
20:4ω6	ARA	0.0	1.6	0.0	0.0	0.0	0.9	0.9	0.0	1.3	0.0	0.4	0.8	0.0	1.3
20:5ω3	EPA	0.0	3.2	0.0	0.1	0.0	1.6	2.1	0.0	2.1	0.0	1.1	1.8	0.0	2.3
20:3ω6	DGLA	0.0	1.9	0.0	0.0	0.0	1.2	1.5	0.0	1.4	0.0	0.7	1.0	0.0	1.5
20:4ω3	ETA	0.0	0.4	0.0	0.0	0.0	0.4	0.6	0.0	0.2	0.0	0.3	0.4	0.0	0.5
20:2ω6		0.0	3.4	0.2	0.1	0.2	2.2	3.1	0.1	2.4	0.2	1.7	2.1	0.1	2.8
20:1ω9/ ω11		17.4	10.9	17.8	18.1	17.3	14.8	12.5	18.2	13.2	18.0	15.4	14.0	18.6	12.4
20:1ω7		1.9	2.7	2.2	1.9	2.2	2.2	2.3	2.0	2.0	2.3	2.2	2.2	2.3	2.7
20:0		1.8	1.8	2.1	1.8	2.0	2.0	2.0	2.0	1.9	2.2	2.0	2.0	2.3	2.1
22:4ω6		0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.1
22:5ω3	DPA	0.0	0.1	0.0	0.0	0.0	0.1	0.1	0.0	0.1	0.0	0.1	0.1	0.0	0.2
22:1ω11/ ω13		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
22:1ω9		1.3	0.8	1.9	1.7	1.7	1.5	1.1	1.7	1.1	2.0	1.6	1.4	2.1	1.5
22:1ω7		0.0	0.0	0.2	0.1	0.2	0.1	0.0	0.1	0.0	0.2	0.1	0.1	0.2	0.2
22:0		0.2	0.3	0.3	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.3	0.3	0.4	0.4
24:1ω9		0.6	0.4	0.2	0.2	0.3	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.3
24:1ω7		0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
24:0		0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3

Wild-type here refers to untransformed *Arabidopsis* thaliana, ecotype Columbia

(Abbadi et al., 2004). Considering also that the level of ALA precursor for EPA synthesis in *Arabidopsis* seed was less than a third of that present in linseed, it appeared that the LC-PUFA pathway as described above which included a desaturase that was capable of using an acyl-CoA substrate, was operating with significantly greater efficiency than the acyl-PC dependent desaturase pathway expressed in linseed.

The relative efficiencies of the individual enzymatic steps encoded by the EPA construct can be assessed by examining the percentage conversion of substrate fatty acid to product fatty acids (including subsequent derivatives) in DO11-5. The zebra-fish Δ5/Δ6 desaturase exhibited strong Δ5 desaturation, with 89% of 20:4ω3 being converted to EPA and DPA, and 45% of 20:3ω6 being converted to ARA, consistent with the previously reported preference of this enzyme for ω3 PUFA over ω6 PUFA substrates (Hastings et al., 2001). In comparison, Δ6-desaturation occurred at significantly lower levels, with 32% of ALA and 14% LA being converted to Δ6-desaturated PUFA. Given that previous studies in yeast showed this enzyme to actually have higher Δ6-desaturase activity than Δ5-desaturase activity, the lower Δ6-desaturation levels achieved in *Arabidopsis* seeds could be reflect a limited availability of ALA and LA substrates in the acyl-CoA pool (Singh et al., in press). The Δ6-elongase operated highly efficiently, with 86% of GLA and 67% of SDA being elongated, suggesting that this enzyme may have a slight preference for elongation of ω6-PUFA substrate.

The germination ability of the T2 (segregating) and T3 seed (homozygous population) was assessed on MS medium and on soil. Seed from the EPA and DPA containing lines DO11 and DO11-5 showed the same timing and frequency of germination as wild-type seed, and the T2 and T3 plants did not have any apparent abnormal morphological features. Plant growth rates *in vitro* or in soil and the quantities of seed obtained from the plants were also unaffected. Including the germination of the T1 seed from which plant DO11 was obtained, the normal germination of seed of the DO11 line was thus observed over three generations. In addition, normal germination rates and timing were also observed for the other EPA and DPA containing seed. This feature was both important and not predictable, as higher plants do not naturally produce EPA or DPA and their seed therefore has never previously contained these LC-PUFA. Germination requires the catabolism of stored seed oils and use for growth and as an energy supply. The observed normal germination rates showed that plant seed were able to carry out these processes using EPA and DPA, and that these compounds were not toxic.

It has been reported that a Δ4 desaturase encoded by a gene isolated from *Thraustochytrium spp* and expressed in *Brassica juncea* leaves was able to convert

exogenously supplied DPA to DHA (Qiu et al., 2001). DPA produced in the plant seed described herein can serve as a precursor for DHA production. This conversion of DPA to DHA may be achieved in plant cells by the introduction of a Δ4 desaturase gene into the DPA producing plant cells (Example 11).

Discussion

The presence of 22:5ω3 in the *Arabidopsis* seed oil implied that the *C. elegans* elongase gene not only had Δ6 elongase activity, but also Δ5 elongase activity in plant cells. This result was most surprising given that the gene had been demonstrated to lack Δ5 elongase activity in yeast. Furthermore, this demonstrated that only two genes could be used for the synthesis of DPA from ALA in plant cells. The synthesis of DPA in a higher plant has not previously been reported. Furthermore, the conversion efficiency of ALA to its ω3 products in seed, including EPA, DPA or both, of at least 28% was striking.

Synthesis of LC-PUFA such as EPA and DHA in cells such as plant cells by the Δ6 desaturation pathway required the sequential action of PUFA desaturases and elongases. The required desaturases in one pathway had Δ6, Δ5 and Δ4 desaturating activity, in that order, and the required PUFA elongases had elongating activity on Δ6 and Δ5 substrates. This conventional pathway operates in algae, mosses, fungi, diatoms, nematodes and some freshwater fish (Sayanova and Napier, 2004). The PUFA desaturases from algae, fungi, mosses and worms are selective for desaturation of fatty acids esterified to the *sn*-2 position of phosphatidylcholine (PC) while the PUFA elongases act on fatty acids in the form of acyl-CoA substrates represented in the acyl-CoA pool of tissues. In contrast, vertebrate Δ6 desaturases have been shown to be able to desaturate acyl-CoA substrates (Domergue et al., 2003a).

Attempts to reconstitute LC-PUFA pathways in plant cells and other cells have to take into account the different sites of action and substrate requirements of the desaturases and elongase enzymes. For example, PUFA elongases are membrane bound, and perhaps even integral membrane proteins, which use acyl-CoAs which are present as a distinct pool in the endoplasmic reticulum (ER). This acyl-CoA pool is physiologically separated from the PC component of the ER, hence for a PUFA fatty acid to be sequentially desaturated and elongated it has to be transferred between PC and acyl-CoA pools in the ER. Therefore, earlier reported attempts to constitute LC-PUFA biosynthesis in yeast using desaturases and elongase from lower and higher plants, fungi and worms, have been inefficient, at best. In addition, the constituted pathways have led to the synthesis of only C20 PUFA such as ARA and EPA. There is no previous report of the

synthesis of C22 PUFA such as DPA and DHA in yeast (Beaudoin et al., 2000, Domergue et al., 2003a).

The strategy described above of using a vertebrate desaturase, in this example a Δ5/Δ6 desaturase from zebra fish, with a Δ6 PUFA elongase from *C. elegans* had the advantage that both the desaturase and the elongase have activity on acyl-CoA substrates in the acyl-CoA pool. This may explain why this strategy was more efficient in the synthesis of LC-PUFA. Furthermore, using a bifunctional desaturase displaying dual Δ5/Δ6 desaturase activities allowed the synthesis of EPA by the action of only 2 genes instead of the 3 genes used by other researchers (Beaudoin et al., 2000, Domergue et al., 2003a). The use of a bifunctional Δ5/Δ6 elongase in plant cells also allowed the formation of DPA from ALA by the insertion of only three genes (one elongase and two desaturases) or, as exemplified, of only two genes (bifunctional elongase and bifunctional desaturase). Both of these aspects were surprising and unexpected.

Biochemical evidence suggests that the fatty acid elongation consists of 4 steps: condensation, reduction, dehydration and a second reduction. Two groups of condensing enzymes have been identified so far. The first are involved in the synthesis of saturated and monosaturated fatty acids (C18-22). These are the FAE-like enzymes and do not appear to have a role in LC-PUFA biosynthesis. The other class of elongases identified belong to the ELO family of elongases named after the ELO gene family whose activities are required for the synthesis of the very long-chain fatty acids of sphingolipids in yeast. Apparent paralogs of the ELO-type elongases isolated from LC-PUFA synthesizing organisms like algae, mosses, fungi and nematodes have been shown to be involved in the elongation and synthesis of LC-PUFA. It has been shown that only the expression of the condensing component of the elongase is required for the elongation of the respective acyl chain. Thus the introduced condensing component of the elongase is able to successfully recruit the reduction and dehydration activities from the transgenic host to carry out successful acyl elongations. Thus far, successful elongations of C16 and C18 PUFA have been demonstrated in yeast by the heterologous expression of ELO type elongases. In this regard, the *C. elegans* elongase used as described above was unable to elongate C20 PUFA when expressed in yeast (Beaudoin et al, 2000). Our demonstration that the *C. elegans* elongase, when expressed in plants, was able to elongate the C20:5 fatty acid EPA as evidenced by the production of DPA in *Arabidopsis* seed was a novel and unexpected result. One explanation as to why the *C. elegans* elongase was able to elongate C20 PUFA in plants, but not in yeast, might reside in its ability to interact successfully with the other components of the elongation machinery of plants to bind and act on C20 substrates.

This example showed that an ELO-type elongase from a non-verteberate organism was able to elongate C20 PUFA in plant cells. Leonard et al. (2002) reported that an ELO-type elongase gene isolated from humans, when expressed in yeast, was able to elongate EPA to DPA but in a non-selective fashion.

Example 6. Isolation of a Δ8 Desaturase Gene from *P. salina* and Functional Characterization in Yeast

Microalgae are the only organisms which have been reported to contain Δ8 desaturases, aside from the Δ8 sphingolipid desaturases in higher plants that are not involved in LC-PUFA biosynthesis. A gene encoding a Δ8 desaturase has been isolated from *Euglena gracilis* (Wallis and Browse, 1999). The existence of a Δ8 desaturase in *Isochrysis galbana* may be presumed because it contains a Δ9 elongase (Qi et al., 2002); the product of which, 20:3n-3, is the precursor for a Δ8 desaturase (see Figure 1). The fatty acid profiles of microalgae alone, however, do not provide sufficient basis for identifying which microalgae will contain Δ8 desaturase genes since multiple pathways may operate to produce the LC-PUFA.

Isolation of a Δ8 desaturase gene fragment

An alignment of Δ6 desaturase amino acid sequences with those from the following Genbank accession numbers, AF465283, AF007561, AAC15586 identified the consensus amino acid sequence blocks DHPGGS (SEQ ID NO:43), WWKDKHN (SEQ ID NO:44) and QIEHHLF (SEQ ID NO:45) corresponding to amino acid positions 204-210 and 394-400, respectively, of AF465283. DHPGSS corresponded to the "cytochrome b5 domain" block that had been identified previously (Mitchell and Martin, 1995). WWKDKHN was a consensus block that had not previously been identified or used to design degenerate primers for the isolation of desaturase genes. The QIEHHLF block, or variants thereof, corresponded to a required histidine-containing motif that was conserved in desaturases. It had been identified and used before as the "third His box" to design degenerate oligonucleotides for desaturase gene isolation (Michaelson et al., 1998). This combination of blocks had not been used previously to isolate desaturase genes.

Based on the second and third conserved amino acid blocks, the degenerate primers 5'-TGGTGGAARCAYAARCAYAAY-3' (SEQ ID NO:46) and 5'-GCGAGGGATCCAAGGRAANARRTGRTGYTC-3' (SEQ ID NO:47) were synthesised. Genomic DNA from *P. salina* was isolated using the DNAeasy system (Qiagen). PCR amplifications were carried out in reaction volumes of 20μL using

20pmol of each primer, 200ng of *P. salina* genomic DNA and Hotstar Taq DNA polymerase (Qiagen) with buffer and nucleotide components as specified. The cycling conditions were: 1 cycle of 95°C for 15 minutes; 5 cycles of 95°C 1min; 38°C, 1min; 72°C, 1min; followed by 35 cycles of 95°C, 35 sec; 52°C, 30 sec; 72°C, 1min; and finishing with 1 cycle of 72°C, 10min. A 515 basepair amplicon was generated, ligated into pGEM-T easy (Promega), sequenced and used as a probe to screen a *P. salina* cDNA library.

Isolation of a cDNA encoding a Δ8 desaturase from *P. salina*

A *P. salina* cDNA library in λ-bacteriophage was constructed using the Zap-cDNA Synthesis Kit (Stratagene) (see Example 1). The library was plated out at a concentration of ~50,000 plaques per plate and lifts taken with Hybond N+ membrane and treated using standard methods (Ausubel et al., 1988, *supra*). The 515bp desaturase fragment, generated by PCR, was radio-labelled with ^{32}P-dCTP and used to probe the lifts under the following high stringency conditions: Overnight hybridisation at 65°C in 6X SSC with shaking, a 5 minute wash with 2× SSC/0.1% SDS followed by two 10 minute washes with 0.2× SSC/0.1% SDS.

Fifteen primary library plates (150mm) were screened for hybridization to the labeled 515bp fragment. Forty strongly hybridizing plaques were identified and ten of these were carried through to a secondary screen. Plasmids from five secondary plaques hybridizing to the 515bp probe were excised with ExAssist Helper phage according to the suppliers protocol (Stratagene). The nucleotide sequences of the inserts were obtained using the ABI Prism Big Dye Terminator kit (PE Applied Biosystems). The nucleotide sequences were identical where they overlapped, indicating that all five inserts were from the same gene. One of the five inserts was shown to contain the entire coding region, shown below to be from a Δ8 desaturase gene. This sequence is provided as SEQ ID NO:6.

The full-length amino acid sequence (SEQ ID NO:1) revealed that the isolated cDNA encoded a putative Δ6 or Δ8 desaturase, based on BLAST analysis. These two types of desaturases are very similar at the amino acid level and it was therefore not possible to predict on sequence alone which activity was encoded. The maximum degree of identity between the *P. salina* desaturase and other desaturases (BLASTX) was 27-30%, while analysis using the GAP program which allows the insertions of "gaps" in the alignment showed that the maximum overall amino acid identity over the entire coding regions of the *P. salina* desaturase and AAD45877 from *Euglena gracilis* was 45%. A

Pileup diagram of other sequences similar to the *Pavlova salina* desaturase is provided in Figure 4.

The entire coding region of this clone, contained within an *Eco*RI/*Xho*I fragment, was inserted into pYES2 (Invitrogen), generating pYES2-psΔ8, for introduction and functional characterisation in yeast. Cells of yeast strain S288 were transformed with pYES2-psΔ8 as described in Example 1, and transformants were selected on medium without uracil. The yeast cells containing pYES2-psΔ8 were grown in culture and then induced by galactose. After the addition of 18:3ω3 or 20:3ω3 (0.5 mM) to the culture medium and 48 hours of further culturing at 30°C, the fatty acids in cellular lipids were analysed as described in Example 1. When 18:3ω3 (Δ9, 12, 15) was added to the medium, no 18:4ω3 (Δ6, 9, 12, 15) was detected. However, when 20:3ω3 (Δ11,14,17) was added to the medium, the presence of 20:4ω3 (Δ8,11,14,17) in the cellular lipid of the yeast transformants was detected (0.12%). It was concluded the transgene encoded a polypeptide having Δ8 but not Δ6 desaturase activity in yeast cells.

Isolation of a gene encoding a Δ8 fatty acid desaturase that does not also have Δ6 desaturase activity has not been reported previously. The only previously reported gene encoding a Δ8 desaturase that was isolated (from *Euglena gracilis*) was able to catalyse the desaturation of both 18:3ω3 and 20:3ω3 (Wallis and Browse, 1999). Moreover, expression of a gene encoding a Δ8 desaturase has not previously been reported in higher plants.

As shown in Figure 1, expression of a Δ8 desaturase in concert with a Δ9 elongase (for example the gene encoding ELO2 – see below) and a Δ5 desaturase (for example, the zebrafish Δ5/Δ6 gene or an equivalent gene from *P. salina* or other microalgae) would cause the synthesis of EPA in plants.

Aside from providing an alternative route for the production of EPA in cells, the strategy of using a Δ9 elongase in combination with the Δ8 desaturase may provide an advantage in that the elongation, which occurs on fatty acids coupled to CoA, precedes the desaturation, which occurs on fatty acids coupled to PC, thereby ensuring the availability of the newly elongated C20 PUFA on PC for subsequent desaturations by Δ8 and Δ5 desaturases, leading possibly to a more efficient synthesis of EPA. That is, the order of reactions - an elongation followed by two desaturations - will reduce the number of substrate linking switches that need to occur. The increased specificity provided by the *P. salina* Δ8 desaturase is a further advantage.

Example 7. Isolation of *P.salina* ELO1 and ELO2 Fatty Acid Elongases

ELO-type PUFA elongases from organisms such as nematodes, fungi and mosses have been identified on the basis of EST or genome sequencing strategies. A gene encoding a Δ9 elongase with activity on 18:3ω3 (ALA) was isolated from *Isochrysis galbana* using a PCR approach with degenerate primers, and shown to have activity in yeast cells that were supplied with exogenous 18:2ω6 (LA) or 18:3ω3 (ALA), forming C20 fatty acids 20:2ω6 and 20:3ω3 respectively. The coding region of the gene *IgASE1* encoded a protein of 263 amino acids with a predicted molecular weight of about 30kDa and with limited homology (up to 27% identity) to other elongating proteins.

Isolation of elongase gene fragments from *P. salina*

Based on multiple amino acid sequence alignments for fatty acid elongases the consensus amino acid blocks FLHXYH (SEQ ID NO:48) and MYXYYF (SEQ ID NO:49) were identified and the corresponding degenerate primers 5'-CAGGATCCTTYYTNCATNNNTAYCA-3' (SEQ ID NO:50) (sense) and 5'-GATCTAGARAARTARTANNNRTACAT-3' (SEQ ID NO:51) (antisense) were synthesised. Primers designed to the motif FLHXYH or their use in combination with the MYXYYF primer have not previously been described. These primers were used in PCR amplification reactions in reaction volumes of 20μL with 20pmol of each primer, 200ng of *P. salina* genomic DNA and Hotstar Taq DNA polymerase (Qiagen) with buffer and nucleotide components as specified by the supplier. The reactions were cycled as follows: 1 cycle of 95°C for 15 minutes, 5 cycles of 95°C, 1min, 38°C, 1min, 72°C, 1min, 35 cycles of 95°C, 35 sec, 52°C, 30 sec, 72°C, 1min, 1 cycle of 72°C, 10min. Fragments of approximately 150bp were generated and ligated into pGEM-Teasy for sequence analysis.

Of the 35 clones isolated, two clones had nucleotide or amino acid sequence with similarity to known elongases. These were designated Elo1 and Elo2. Both gene fragments were radio-labelled with ^{32}P-dCTP and used to probe the *P. salina* cDNA library under the following high stringency conditions: overnight hybridisation at 65°C in 6X SSC with shaking, 5 minute wash with 2× SSC/ 0.1% SDS followed by two 10 minute washes with 0.2× SSC/ 0.1% SDS. Ten primary library plates (150mm) were screened using the Elo1 or Elo2 probes. Elo1 hybridized strongly to several plaques on each plate, whilst Elo2 hybridised to only three plaques in the ten plates screened. All Elo1-hybridising plaques were picked from a single plate and carried through to a secondary screen, whilst all three Elo2-hybridising plaques were carried through to a secondary screen. Each secondary plaque was then used as a PCR template using the

forward and reverse primers flanking the multiple cloning site in the pBluescript phagemid and the PCR products electrophoresed on a 1% TAE gel. Following electrophoresis, the gel was blotted onto a Hybond N+ membrane and the membrane hybridised overnight with ^{32}P-labelled Elo1 and Elo2 probes. Six of the amplified Elo1 secondary plaques and one of the amplified Elo2 secondary plaques hybridised to the Elo1/2 probe (Figure 5).

Two classes of elongase-like sequences were identified in the *P. salina* cDNA library on the basis of their hybdridisation to the Elo1 and Elo2 probes. Phagemids that hybridised strongly to either labelled fragment were excised with ExAssist Helper phage (Stratagene), and sequenced using the ABI Prism Big Dye Terminator kit (PE Applied Biosystems). All of the 5 inserts hybridizing to the Elo1 probe were shown to be from the same gene. Similarly DNA sequencing of the 2 inserts hybridising to the Elo2 probe showed them to be from the same gene. The cDNA sequence of the Elo1 clone is provided as SEQ ID NO:8, and the encoded protein as SEQ ID NO:2, whereas the cDNA sequence of the Elo2 clone is provided as SEQ ID NO:10, and the encoded proteins as SEQ ID NO:3, SEQ ID NO:85 and SEQ ID NO:86 using three possible start methionines).

A comparison was performed of the Elo1 and Elo2 and other known PUFA elongases from the database using the PILEUP software (NCBI), and is shown in Figure 6.

The Elo1 cDNA was 1234 nucleotides long and had a open reading frame encoding a protein of 302 amino acid residues. According to the PILEUP analysis, Elo1 clustered with other Elo-type sequences associated with the elongation of PUFA including Δ6 desaturated fatty acids (Figure 6). The Elo1 protein showed the greatest degree of identity (33%) to an elongase from the moss, *P. patens* (Accession No. AF428243) across the entire coding regions. The Elo1 protein also displayed a conserved amino acids motifs found in all other Elo-type elongases.

The Elo2 cDNA was 1246 nucleotides long and had an open reading frame encoding a protein of 304 amino acid residues. According to PILEUP analysis, Elo2 clustered with other Elo-type sequences associated with the elongation of PUFA, including those with activity on Δ6 or Δ9 PUFA (Figure 6). Elo2 was on the same sub-branch as the Δ9 elongase isolated from *Isochrysis galbana* (AX571775). Elo2 displayed 31% identity to the *Isochrysis* gene across its entire coding region. The Elo2 ORF also displayed a conserved amino acid motif found in all other Elo-type elongases.

Example 8. Functional Characterization of Δ5 Fatty Acid Elongase in Yeast and Plant Cells

Yeast

The entire coding region of the *P. salina* Elo1 gene was ligated into pYES2, generating pYES2-psELO1, for characterisation in yeast. This genetic construct was introduced into yeast strains and tested for activity by growth in media containing exogenous fatty acids as listed in the Table 8. Yeast cells containing pYES2-psELO1 were able to convert 20:5ω3 into 22:5ω3, confirming Δ5 elongase activity on C20 substrate. The conversion ratio of 7% indicated high activity for this substrate. The same yeast cells converted 18:4ω3 (Δ6,9,12,15) to 20:4ω3 and 18:3ω6 (Δ6,9,12) to 20:3ω6, demonstrating that the elongase also had Δ6 elongase activity in yeast cells, but at approximately 10-fold lower conversion rates (Table 8). This indicated that the Elo1 gene encodes a specific or selective Δ5 elongase in yeast cells. This represents the first report of a specific Δ5 elongase, namely an enzyme that has a greater Δ5 elongase activity when compared to Δ6 elongase activity. This molecule is also the first Δ5 elongase isolated from an algael source. This enzyme is critical in the conversion of EPA to DPA (Figure 1).

Plants

The Δ5 elongase, Elo1 isolated from *Pavlova* is expressed in plants to confirm its ability to function in plants. Firstly, a plant expression construct is made for constitutive expression of Elo1. For this purpose, the Elo1 sequence is placed under the control of the 35S promoter in the plant binary vector pBI121 (Clontech). This construct is introduced into *Arabidopsis* using the floral dip method described above. Analysis of leaf lipids is used to determine the specificity of fatty acids elongated by the Elo1 sequence. In another approach, co expression of the Elo1 construct with the zebra fish Δ5/Δ6 desaturase/*C. elegans elongase* construct and the Δ4 desaturase isolated from *Pavlova*, results in DHA synthesis from ALA in *Arabidopsis* seed, demonstrating the use of the Δ5 elongase in producing DHA in cells. In a further approach, the Elo1 gene may be co-expressed with Δ6-desaturase and Δ5 desaturase genes, or a Δ6/Δ5 bifunctional desaturase gene, to produce DPA from ALA in cells, particularly plant cells. In an alternative approach, the Δ5 elongase and Δ4 elongase genes are used in combination with the PKS genes of *Shewanella* which produce EPA (Takeyama et al., 1997), in plants, for the synthesis of DHA.

TABLE 8. Conversion of fatty acids in yeast cells transformed with genetic constructs expressing Elo1 or Elo2.

Clone	Fatty acid precursor/ (% of total FA)	Fatty acid formed/ (% of total FA)	Conversion ratio (%)
pYES2-psELO1	20:5n-3 / 3%	22:5n-3 / 0.21%	7%
pYES2-psELO1	18:4n-3 / 16.9%	20:4n-3 / 0.15%	0.89%
pYES2-psELO1	18:3n-6 / 19.8%	20:3n-6 / 0.14%	0.71%
pYES2-psELO2	20:5n-3 / 2.3%	22:5n-3 / tr	-
pYES2-psELO2	18:4n-3 / 32.5%	20:4n-3 / 0.38%	1.2%
pYES2-psELO2	18:3n-6 / 12.9%	20:3n-6 / 0.08%	0.62%
pYES2-psELO2	18:2n-6 / 30.3%	20:2n-6 / 0.12%	0.40%
pYES2-psELO2	18:3n-3 / 42.9%	18:3n-3 / 0.20%	0.47%

tr: trace amounts (<0.02%) detected.

Example 9. Functional Characterization of Δ9 Fatty Acid Elongase in Yeast and Plant Cells

Expression in yeast cells

The entire coding region of the *P. salina* Elo2 gene encoding a protein of 304 amino acids (SEQ ID NO:3) was ligated into pYES2, generating pYES2-psELO2, for characterisation in yeast. This genetic construct was introduced into yeast strains and tested for activity by growth in media containing exogenous fatty acids. Yeast cells containing pYES2-psELO2 were able to convert 18:2ω6 into 20:2ω6 (0.12% of total fatty acids) and 18:3ω3 into 20:3ω3 (0.20%), confirming Δ9 elongase activity on C18 substrates (Table 8). These cells were also able to convert 18:3ω6 into 20:3ω6 and 18:4ω3 into 20:4ω3, confirming Δ6 elongase activity on C18 substrates in yeast. However, since the 18:3ω6 and 18:4ω3 substrates also have a desaturation in the Δ9

position, it could be that the Elo2 enzyme is specific for Δ9-desaturated fatty acids, irrespective of whether they have a Δ6 desaturation as well. The cells were able to convert 20:5ω3 into the 22:5 product DPA. This is the first report of a Δ9 elongase that also has Δ6 elongase activity from a non-vertebrate source, in particular from a fungal or algal source.

As the coding region contained three possible ATG start codons corresponding to methionine (Met) amino acids at positions 1, 11 (SEQ ID NO:85) and 29 (SEQ ID NO:86) of SEQ ID NO:3, the possibility that polypeptides beginning at amino acid positions 11 or 29 would also be active was tested. Using 5' oligonucleotide (sense) primers corresponding to the nucleotide sequences of these regions, PCR amplification of the coding regions was performed, and the resultant products digested with *Eco*RI. The fragments are cloned into pYES2 to form pYES2-psELO2-11 and pYES2-psELO2-29. Both plasmids are shown to encode active Δ9-elongase enzymes in yeast. The three polypeptides may also be expressed in *Synechococcus* or other cells such as plant cells to demonstrate activity.

Expression in plant cells

The Δ9 elongase gene, Elo2, isolated from *Pavlova* was expressed in plants to confirm its ability to function in plants. Firstly, a plant expression construct is made for constitutive expression of Elo2. For this purpose, the Elo2 coding sequence from amino acid position 1 of SEQ ID NO:3 was placed under the control of the 35S promoter in the plant binary vector pBI121 (Clontech). This construct is introduced into *Arabidopsis* using the floral dip method described above. Analysis of leaf lipids indicates the specificity of fatty acids that are elongated by the Elo2 sequence.

Co-expression of Δ9 elongase and Δ8-desaturase genes in transformed cells

The *P. salina* Δ8-desaturase and Δ9-elongase were cloned into a single binary vector, each under the control of the constitutive 35S promoter and *nos* terminator. In this gene construct, pBI121 containing the Δ8-desaturase sequence was cut with *Hin*dIII and *Cla*I (blunt-ended) to release a fragment containing the 35S promoter and the Δ8-desaturase gene, which was then ligated to the *Hin*dIII + *Sac*I (blunt ended) cut pXZP143/Δ9-elongase vector to result in the intermediate pJRP013. This intermediate was then opened with *Hin*dIII and ligated to a pWvec8/Δ9-elongase binary vector (also *Hin*dIII-opened) to result in the construct pJRP014, which contains both genes between the left and right T-DNA borders, together with a hygromycin selectable marker gene suitable for plant transformation.

This double-gene construct was then used to transform tobacco using a standard *Agrobacterium*-mediated transformation technique. Following introduction of the construct into *Agrobacterium* strain AGL1, a single transformed colony was used to inoculate 20 mL of LB media and incubated with shaking for 48 hours at 28°C. The cells were pelleted (1000 *g* for 10 minutes), the supernatant discarded, and the pellet resuspended in 20 mL of sterile MS media. This was step was then repeated before 10 ml of this Agrobacterial solution was added to freshly cut (1 cm squares) tobacco leaves from cultivar W38. After gentle mixing, the tobacco leaf pieces and *Agrobacterium* solution were allowed to stand at room temperature for 10 min. The leaf pieces were transferred to MS plates, sealed, and incubated (co-cultivation) for 2 days at 24°C. Transformed cells were selected on medium containing hygromycin, and shoots regenerated. These shoots were then cut off and transferred to MS-rooting media pots for root growth, and eventually transferred to soil. Both leaf and seed lipids from these plants are analysed for the presence of 20:2ω6, 20:3ω6, 20:3ω3 and 20:4ω3 fatty acids, demonstrating the co-expression of the two genes.

Discussion

Biochemical evidence suggests that the fatty acid elongation consists of 4 steps: condensation, reduction, dehydration and a second reduction, and the reaction is catalysed by a complex of four proteins, the first of which catalyses the condensation step and is commonly called the elongase. There are 2 groups of condensing enzymes identified so far. The first are involved in the synthesis of saturated and monounsaturated fatty acids (C18-22). These are the FAE-like enzymes and do not play any role in LC-PUFA biosynthesis. The other class of elongases identified belong to the ELO family of elongases named after the ELO gene family whose activities are required for the synthesis of very LC fatty acids of sphingolipids in yeast. Apparent paralogs of the ELO-type elongases isolated from LC-PUFA synthesizing organisms like algae, mosses, fungi and nematodes have been shown to be involved in the elongation and synthesis of LC-PUFA. It has been shown that only the expression of the condensing component of the elongase is required for the elongation of the respective acyl chain. Thus the introduced condensing component of the elongase is able to successfully recruit the reduction and dehydration activities from the transgenic host to carry out successful acyl elongations. This was also true for the *P. salina* Δ9-elongase.

Example 10. Isolation of a Gene Encoding a Δ4-Desaturase from *P. salina*

The final step in the aerobic pathway of DHA synthesis in organisms other than vertebrates, such as microorganisms, lower plants including algae, mosses, fungi, and possibly lower animals, is catalysed by a Δ4-desaturase that introduces a double bond into the carbon chain of the fatty acid at the Δ4 position. Genes encoding such an enzyme have been isolated from the algae *Euglena* and *Pavlova* and from *Thraustochytrium*, using different approaches. For example, Δ4-desaturase genes from *Pavlova lutheri* and *Euglena gracilis* were isolated by random sequencing of cloned ESTs (EST approach, Meyer et al., 2003; Tonon et al., 2003), and a Δ4-desaturase gene from *Thraustochytrium* sp. ATCC21685 was isolated by RT-PCR using primers corresponding to a cytochrome b_5 HPGG domain and histidine box III region (Qiu et al., 2001). The cloned Δ4-desaturase genes encoded front-end desaturases whose members are characterised by the presence of an N-terminal cytochrome b_5-like domain (Napier et al., 1999; Sayanova and Napier, 2004).

Isolation of a gene fragment from a Δ4-desaturase gene from *P. salina*

Comparison of known moss and microalgae Δ4-desaturases revealed several conserved motifs including a HPGG (SEQ ID NO:52) motif within a cytochrome b_5- like domain and three histidine box motifs that are presumed to be required for activity. Novel degenerate PCR primers PavD4Des-F3 (5'-AGCACGACGSSARCCACGGCG-3') (SEQ ID NO:53) and PavD4Des-R3 (5'-GTGGTGCAYCABCACGTGCT-3') (SEQ ID NO:54) corresponding to the conserved amino acid sequence of histidine box I and complementary to a nucleotide sequence encoding the amino acid sequence of histidine box II, respectively, were designed as to amplify the corresponding region of *P. salina* desaturase genes, particularly a Δ4-desaturase gene. The use of degenerate PCR primers corresponding to histidine box I and histidine box II regions of Δ4-desaturase has not been reported previously.

PCR amplification reactions using these primers were carried out using *P. salina* first strand cDNA as template with cycling of 95°C, 5min for 1 cycle, 94°C 30 sec, 57°C 30 sec, 72°C 30 sec for 35 cycles, and 72°C 5 min for 1 cycles. The PCR products were cloned into pGEM-T-easy (Promega) vectors, and nucleotide sequences were determined with an ABI3730 automatic sequencer using a reverse primer from the pGEM-Teasy vector. Among 14 clones sequenced, three clones showed homology to Δ4-desaturase genes. Two of these three clones are truncated at one primer end. The nucleotide sequence of the cDNA insert of the third, clone 1803, is provided as SEQ ID NO:11.

The amino acid sequence encoded by SEQ ID NO:11 was used to search the NCBI protein sequence database using the BLASTX software. The results indicated that this sequence was homologous to known Δ4-desaturases. The amino acid sequence of the *P. salina* gene fragment showed 65%, 49%, 46% and 46% identity to that of Δ4-desaturases of *P. lutheri*, *Thraustochytrium* sp. ATCC21685, *Thraustochytrium aureum* and *Euglena gracilis* respectively.

Isolation of a full-length Δ4-desaturase gene

The insert from clone 1803 was excised, and used as probe to isolate full-length cDNAs corresponding to the putative Δ4-desaturase gene fragment. About 750,000 pfu of the *P. salina* cDNA library were screened at high stringency. The hybridization was performed at 60°C overnight and washing was done with 2xSSC/0.1%SDS 30min at 65°C then with 0.2xSSC/0.1%SDS 30min at 65°C. Eighteen hybridising clones were isolated and secondary screening with six clones was performed under the same hybridization conditions. Single plaques from secondary screening of these six clones were isolated. Plasmids from five single plaques were excised and the nucleotide sequences of the inserts determined with an ABI 3730 automatic sequencer with reverse and forward primers from the vector. Sequencing results showed that four clones each contained Δ4-desaturase cDNA of approximately 1.7kb in length, each with the same coding sequence and each apparently full-length. They differed slightly in the length of the 5' and 3' UTRs even though they contained identical protein coding regions. The cDNA sequence of the longest *P. salina* Δ4-desaturase cDNA is provided as SEQ ID NO:13, and the encoded protein as SEQ ID NO:4.

The full-length cDNA was 1687 nucleotides long and had a coding region encoding 447 amino acids. The *Pavlova salina* Δ4-desaturase showed all the conserved motifs typical of 'front-end desaturases' including the N-terminal cytochrome b_5-like domain and three conserved histidine-rich motifs. Comparison of the nucleotide and amino acid sequences with other Δ4-desaturase genes showed that the greatest extent of homology was for the *P. lutheri* Δ4-desaturase (Accession No. AY332747), which was 69.4% identical in nucleotide sequence over the protein coding region, and 67.2% identical in amino acid sequence.

Demonstration of enzyme activity of *Pavlova salina* Δ4-desaturase gene

A DNA fragment including the *Pavlova salina* Δ4-desaturase cDNA coding region was excised as an *Eco*RI-*Sal*I cDNA fragment and inserted into the pYES2 yeast expression vector using the *Eco*RI and *Xho*I sites. The resulted plasmid was transformed

into yeast cells. The transformants were grown in YMM medium and the gene induced by the addition of galactose, in the presence of added (exogenous) ω6 and ω3 fatty acids in order to demonstrate enzyme activity and the range of substrates that could be acted upon by the expressed gene. The fatty acids 22:5ω3 (DPA, 1.0mM), 20:4n-3 (ETA, 1.0mM), 22:4ω6 (DTAG, 1.0 mM) and 20:4ω6 (ARA, 1.0mM) were each added separately to the medium. After 72 hours incubation, the cells were harvested and fatty acid analysis carried out by capillary gas-liquid chromatography (GC) as described in Example 1. The data obtained are shown in Table 9.

TABLE 9. Yeast PUFA feeding showing activity of delta-4 desaturase gene.

	Exogenous fatty acid added to growth medium	
Fatty acid composition (% of total fatty acid)	**22:4ω6**	**22:5ω3**
14:0	0.63	0.35
15:0	0.06	0.06
16:1ω7c	43.45	40.52
16:1ω5	0.20	0.13
16:0	18.06	15.42
17:1ω8	0.08	0.09
17:0	0.08	-
18:1ω9	26.73	30.07
18:1ω7 (major) & 18:3ω3	1.43	1.61
18:1ω5c	0.02	tr
18:0	7.25	8.87
20:5ω3	0.40	0.62
20:1ω9 / ω11	0.03	tr
20:0	0.08	0.09
22:5ω6	0.03	0.00
22:6ω3	-	0.04
22:4ω6	0.97	-
22:5ω3	0.00	1.66
22:0	0.06	0.06
24:1ω7	0.31	0.37
24:0	0.12	0.04
Sum	100.00%	100.00%

This showed that the cloned gene encoded a Δ4-desaturase which was able to desaturate both C22:4ω6 (3.0% conversion to 22:5ω6) and C22:5ω3 (2.4% conversion to 22:6ω3) at the Δ4 position. The enzyme did not show any Δ5 desaturation activity when the yeast transformants were fed C20:3ω6 or C20:4ω3.

Example 11. Expression of *P. salina* Δ4-desaturase Gene in Plant Cells and Production of DHA

To demonstrate activity of the Δ4-desaturase gene in plant cells, the coding region may be expressed either separately to allow the conversion of DPA to DHA, or in the context of other LC-PUFA synthesis genes such as, for example, a Δ5–elongase gene for the conversion of EPA to DHA. For expression as a separate gene, the Δ4-desaturase coding region may be excised as a *Bam*HI-*Sal*I fragment and inserted between a seed-specific promoter and a polyadenylation/transcription termination sequence, such as, for example, in vector pGNAP (Lee et al., 1998), so that it is expressed under the control of the seed specific promoter. The expression cassette may then be inserted into a binary vector and introduced into plant cells. The plant material used for the transformation may be either untransformed plants or transformed plants containing a construct which expressed the zebrafish Δ5/Δ6-dual desaturase gene and *C. elegans* elongase gene each under the control of a seed specific promoter (Example 5). Transgenic *Arabidopsis* containing the latter, dual-gene construct had successfully produced EPA and DPA in seeds, and the combination with the Δ4-desaturase gene would allow the conversion of the DPA to DHA in the plant cells, as demonstrated below.

To demonstrate co-expression of a Δ5–elongase gene with the Δ4-desaturase gene in recombinant cells, particularly plant cells, and allow the production of DHA, the Δ4-desaturase and the Δ5–elongase genes from *P. salina* (Example 8) were combined in a binary vector as follows. Both coding regions were placed under the control of seed-specific (napin) promoters and nos3' terminators, and the binary vector construct had a kanamycin resistance gene as a selectable marker for selection in plant cells. The coding region of the Δ5-elongase gene was excised from its cDNA clone as a *Pst*I-*Sac*II fragment and inserted into an intermediate plasmid (pXZP143) between the promoter and terminator, resulting in plasmid pXZP144. The coding region of the Δ4-desaturase gene was excised from its cDNA clone as a *Bam*HI-*Sal*I fragment and inserted into plasmid pXZP143 between the promoter and *nos* 3' transcription terminator, resulting in plasmid pXZP150. These two expression cassettes were combined in one vector by inserting the *Hind*III-*Apa*I fragment from pXZP144 (containing promoter-Elo1-nos3') between the *Stu*I and *Apa*I sites of pXZP150, resulting in plasmid pXZP191. The *Hind*III-*Stu*I

fragment from pXZP191 containing both expression cassettes was then cloned into the binary vector pXZP330, a derivative of pBI121, resulting in plant expression vector pXZP355. This vector is shown schematically in Figure 7.

Plant transformation

The Δ5–elongase and the Δ4-desaturase genes on pXZP355 were introduced by the *Agrobacterium*-mediated floral dip transformation method into the *Arabidopsis* plants designated DO11 (Example 5) which were already transgenic for the zebrafish Δ5/Δ6 bifunctional desaturase and the *C. elegans* Δ5/Δ6 bifunctional elongase genes. Since those transgenes were linked to a hygromycin resistance gene as a selectable marker gene, the secondary transformation with pXZP355 used a kanamycin resistance selection, thus distinguishing the two sets of transgenes. Five transgenic plants are obtained, designated "DW" plants. Since the DO11 plants were segregating for the zebrafish Δ5/Δ6 bifunctional desaturase and the *C. elegans* Δ5/Δ6 bifunctional elongase genes, some of the transformed plants were expected to be heterozygous for these genes, while others were expected to be homozygous. Seed (T2 seed) of the five transformed plants were analysed and shown to contain up to at least 0.1% DPA and up to at least 0.5% DHA in the seed oils. Data are presented for two lines in Table 10. Analysis, by mass spectrometry (GC-MS), of the fatty acids in the peaks identified as EPA and DHA from the GC analysis proved that they were indeed EPA and DHA (Figure 8).

The fatty acid analysis of the T2 seedoil demonstrated that significant conversion of EPA to DHA had occurred in the DW2 and DW5 lines, having 0.2% and 0.5% DHA, respectively. Examination of the enzyme efficiencies in plant DW5 containing the higher level of DHA showed that 17% of the EPA produced in its seed was elongated to DPA by the *P. salina* Δ5-elongase, and greater than 80% of this DPA was converted to DHA by the *P. salina* Δ4-desaturase. Since the Δ5-elongase and Δ4-desaturase genes were segregating in the T2 seed, the fatty acid composition data represented an average of pooled null, heterozygous and homozygous genotypes for these genes. It is expected that levels of DHA in progeny lines of DW5 will be greater in seed that is uniformly homozygous for these genes.

TABLE 10. Fatty acid composition (% of total fatty acids) of seed oils from *Arabidopsis thaliana* (ecotype Columbia) and derivatives carrying EPA and DHA gene constructs – EPA, DPA and DHA synthesis in transgenic seed.

Fatty acid	Wild type	DO11 + DHA construct			
	Columbia	DW2	DW5		
		Total	Total	TAG	PL
Usual fatty acids					
16:0	7.2	6.7	6.1	5.5	12.5
18:0	2.9	3.8	4.4	4.3	4.5
18:1Δ^{9}	20.0	20.6	16.6	18.9	13.7
18:2$\Delta^{9,12}$ (LA)	27.5	26.0	25.9	25.5	33.1
18:3$\Delta^{9,12,15}$ (ALA)	15.1	13.2	15.0	13.6	15.1
20.0	2.2	2.1	1.8	1.9	0.6
20:1Δ^{11}	19.8	14.8	10.5	10.5	3.2
20:1Δ^{13}	2.2	3.0	4.2	4.8	1.4
20:2$\Delta^{11,14}$	0.1	1.7	3.5	3.8	3.7
22:1Δ^{13}	1.5	1.4	1.0	0.3	0.4
Other minor	1.5	2.9	2.7	2.4	3.8
Total	100.0	96.0	91.7	91.5	92.0
New ω6-PUFA					
18:3$\Delta^{6,9,12}$ (GLA)	0	0.2	0.4	0.4	0.2
20:3$\Delta^{8,11,14}$	0	0.8	1.5	1.5	1.7
20:4$\Delta^{5,8,11,14}$ (ARA)	0	0.4	1.0	1.1	1.2
22:4$\Delta^{7,10,13,16}$	0	0	0	0	0.2
22:5$\Delta^{4,7,10,13,16}$	0	0	0.1	0.1	0.1
Total	0	1.4	3.0	3.1	3.4
New ω3-PUFA					
18:4$\Delta^{6,9,12,15}$ (SDA)	0	0.7	1.5	1.6	0.5
20:4$\Delta^{8,11,14,17}$	0	0.5	0.8	0.7	0.9
20:5$\Delta^{5,8,11,14,17}$ (EPA)	0	1.1	2.4	2.5	2.3
22:5$\Delta^{7,10,13,16,19}$ (DPA)	0	0.1	0.1	0.2	0.7
22:6$\Delta^{4,7,10,13,16,19}$ (DHA)	0	0.2	0.5	0.4	0.2
Total	0	2.6	5.3	5.4	4.6
Total fatty acids	100.0	100.0	100.0	100.0	100.0
Total MUFA[a]	41.3	36.8	28.1	29.7	17.3

Total C_{18}-PUFA[b]	42.6	39.2	40.9	39.1	48.2
Total new PUFA[c]	0	4.0	8.3	8.5	8.0

[a] Total of 18:1Δ^9 and derived LC-MUFA (=18:1Δ^9 + 20:1Δ^{11} + 22:1Δ^{13})
[b] 18:2 + 18:3
[c] Total of all new ω6 and ω3-PUFA

Germination of 50 T2 seed from each of DW2 and DW5 on hygromycin-containing medium showed that the DW5 T1 plant was homozygous (50/50) for the Δ5/Δ6 bifunctional desaturase and Δ5/Δ6 bifunctional elongase genes, while the DW2 seed segregating in a 3:1 ration (resistant: susceptible) for these genes and DW2 was therefore heterozygous. This was consistent with the higher levels of EPA observed in DW5 seed compared to DW2 seed, and explained the increased level of DHA produced in the seed homozygous for these transgenes. This further demonstrated the desirability of seed that are homozygous for the trait.

We also noted the consequences of LC-PUFA synthesis on the overall fatty acid profile in these seed. Although we observed accumulation of new ω6 and ω3 PUFA (i.e. products of Δ6-desaturation) at levels of greater than 8% in DW5 seed, these seed had levels of the precursor fatty acids LA and ALA that were almost the same as in the wild-type seed. Rather than depleting LA and ALA, the levels of monounsaturated fatty acid C18:1Δ^9 and its elongated derivatives (20:1Δ^{11} and 22:1Δ^{13}) were significantly reduced. Thus it appeared that conversion of C_{18}-PUFA to LC-PUFA resulted in increased conversion of 18:1 to LA and ALA, and a corresponding reduction in 18:1 available for elongation.

The plant expression vector pXZP355 containing the Δ4-desaturase and the Δ5–elongase genes was also used to introduce the genes into plants of the homozygous line DO11-5, and 20 transgenic T1 plants were obtained. The levels of DHA and DPA in T2 seed from these plants were similar to those observed in seed from DW5. Reductions in the levels of the monounsaturated fatty acids were also observed in these seed.

Fractionation of the total seed lipids of DW5 seed revealed them to be comprised of 89% TAG and 11% polar lipids (largely made up phospholipids). Furthermore, fatty acid analysis of the TAG fraction from DW5 seed showed that the newly synthesised EPA and DHA were being incorporated into the seed oil and that the proportion of EPA and DHA in the fatty acid composition of the total seed lipid essentially reflected that of the TAG fraction (Table 10).

Example 12. Isolation of Homologous Genes from Other Sources

Homologs of the desaturase and elongase genes such as the *P. salina* genes described herein may be readily detected in other microalgae or other sources by hybridization to labelled probes derived from the genes, particularly to parts or all of the coding regions, for example by Southern blot hybridization or dot-blot hybridisation methods. The homologous genes may be isolated from genomic or cDNA libraries of such organisms, or by PCR amplification using primers corresponding to conserved regions. Similarly, homologs of vertebrate desaturases with high affinity for Acyl-CoA and/or freshwater fish bifunctional desaturases can be isolated by similar means using probes to the zebrafish Δ5/Δ6 desaturase.

Dot Blot Hybridisations

Genomic DNA from six microalgae species was isolated using a DNAeasy kit (Qiagen) using the suppliers instructions, and used in dot blot hybridization analyses for identification of homologous genes involved in LC-PUFA synthesis in these species. This also allowed evaluation of the sequence divergence of such genes compared to those isolated from *Pavlova salina*. The species of microalga examined in this analysis were from the genera *Melosira*, *Rhodomonas*, *Heterosigma*, *Nannochloropsis*, *Heterocapsa* and *Tetraselmis*. They were identified according to Hasle, G. R. & Syvertsen, E. E. 1996 Dinoflagellates. In: Tomas, C. R.(ed.) Identifying Marine Phytoplankton. Academic Press, San Diego, CA. pp 531-532. These microalga were included in the analysis on the basis of the presence of EPA, DHA, or both when cultured *in vitro* (Example 2).

Genomic DNA (approximately 100μg) isolated from each of the microalga was spotted onto strips of Hybond N+ membrane (Amersham). After air drying, each membrane strip was placed on a layer of 3MM filter paper saturated with 0.4 M NaOH for 20 min, for denaturation of the DNA, and then rinsed briefly in 2x SSC solution. The membrane strips were air dried and the DNA cross linked to the membranes under UV light. Probes labeled with ^{32}P nucleotides and consisting of the coding regions without the untranslated regions of a number of *Pavlova*-derived genes, including the Δ8, Δ5 and Δ4 desaturases and Δ9 and Δ5 elongases, were prepared and hybridized to each membrane strip/DNA dot blot. The membranes were hybridized with each probe overnight in a buffer containing 50 mM Tris-HCl, pH7.5, 1M NaCl, 50% formamide, 10x Denhardt's solution, 10% dextran sulfate, 1%SDS, 0.1% sodium pyrophosphate, and 0.1 mg/ml herring sperm DNA, at 42°C, then washed three times in a solution containing 2x SSC, 0.5% SDS at 50°C for 15 min each (low stringency wash in this experiment) or for a high stringency wash in 0.2x SSC, 0.5% SDS at 65°C for 20 minutes each.

It is well understood that the stringency of the washing conditions employed in DNA blot/hybridizations can reveal useful information regarding the sequence relatedness of genes. Thus hybridizations maintained when subjected to a high stringency wash indicate a high level of sequence relatedness (e.g. 80% or greater nucleotide identity over at least 100-200 nucleotides), while hybridizations maintained only during low stringency washes indicate a relatively lower degree of DNA conservation between genes (e.g. 60% or greater nucleotide identity over at least 200 nucleotides).

The hybridized dot blots were exposed to BioMax X-ray film (Kodak), and the autoradiograms are shown in Figure 9. The autoradiograms reveal the presence of homologs to the *P. salina* LC-PUFA genes in these species, and moreover reveal a range of homologies based on the different levels of hybridization seen under the high and low stringency conditions. It appeared that some of the microalgal species examined have LC-PUFA genes that may differ substantially from the genes in *P. salina*, while others are more related in sequence. For example, genes from *Tetraselmis sp* appeared to be highly similar to the Δ4- and Δ5-desaturases and the Δ5 elongase from *Pavlova salina* on the basis of the strength of hybridizations. In contrast, all of the LC-PUFA genes identified in *Melosira sp* appeared to have lower degrees of similarity to the *P. salina* genes.

Isolation of an LC-PUFA elongase gene from *Heterocapsa* sp.

Heterocapsa spp. such as *Heterocapsa niei* in the CSIRO collection (Example 2) are dinoflagellates that were identified as producers of LC-PUFA including EPA and DHA. To exemplify the isolation of LC-PUFA synthesis genes from these dinoflagellates, DNA was purified from cells of a *Heterocapsa niei* strain originally isolated in Port Hacking, NSW, Australia in 1977. DNA was isolated using a DNAeasy kit (Qiagen) using the suppliers instructions. Based on published multiple amino acid sequence alignments for fatty acid elongases (Qi et al., 2002; Parker-Barnes et al., 2000), the consensus amino acid blocks FLHXYH (SEQ ID NO:48) and MYXYYF (SEQ ID NO:49) were identified and corresponding degenerate primers encoding these sequences 5'-CAGGATCCTTYYTNCATNNNTAYCA-3' (SEQ ID NO:50) (sense) or complementary to these sequences 5'-GATCTAGARAARTARTANNNRTACAT-3' (SEQ ID NO:51) (antisense) were synthesised. PCR amplification reactions were carried out in reaction volumes of 20μL with 20pmol of each primer, 200ng of *Heterocapsa* sp. genomic DNA and Hotstar Taq DNA polymerase (Qiagen) with buffer and nucleotide components as specified by the supplier. The reactions were cycled as follows: 1 cycle of

95°C for 15 minutes, 5 cycles of 95°C, 1min, 38°C, 1min, 72°C, 1min, 35 cycles of 95°C, 35 sec, 52°C, 30 sec, 72°C, 1min, 1 cycle of 72°C, 10min. Fragments of approximately 350bp were generated and ligated into pGEM-Teasy for sequence analysis.

Of eight clones isolated, two identical clones had nucleotide and encoded amino acid sequences with similarity to regions of known elongases. These were designated Het350Elo, and the nucleotide and amino acid sequences are provided as SEQ ID NO:79 and SEQ ID NO:80 respectively. BLAST analysis and the presence of an in-frame stop codon suggested the presence of an intron between approximate positions 33 and 211.

The best matches to the amino acid sequence were animal elongase sequences, see for example Meyer et al. (2004), indicating that the isolated *Heterocapsa* gene sequence was probably involved in elongation of C18 and C20 fatty acid substrates.

Full-length clones of the elongase can readily be isolated by screening a *Heterocapsa* cDNA library or by 5'- and 3' RACE techniques, well known in the art.

Construction of *Melosira* sp. cDNA library and EST sequencing

mRNA, for the construction of a cDNA library, was isolated from *Melosira* sp. cells using the following method. 2 g (wet weight) of *Melosira* sp. cells were powdered using a mortar and pestle in liquid nitrogen and sprinkled slowly into a beaker containing 22 ml of extraction buffer that was being stirred constantly. To this, 5% insoluble polyvinylpyrrolidone, 90mM 2-mercaptoethanol, and 10mM dithiotheitol were added and the mixture stirred for a further 10 minutes prior to being transferred to a Corex™ tube. 18.4 ml of 3M ammonium acetate was added and mixed well. The sample was then centrifuged at 6000xg for 20 minutes at 4°C. The supernatant was transferred to a new tube and nucleic acid precipitated by the addition of 0.1 volume of 3M NaAc (pH 5.2) and 0.5 volume of cold isopropanol. After 1 hour incubation at –20°C, the sample was centrifuged at 6000xg for 30 minutes in a swing-out rotor. The pellet was resuspended in 1 ml of water and extracted with phenol/chloroform. The aqueous layer was transferred to a new tube and nucleic acids were precipitated once again by the addition of 0.1 volume 3M NaAc (pH 5.2) and 2.5 volume of ice cold ethanol. The pellet was resuspended in water, the concentration of nucleic acid determined and then mRNA was isolated using the Oligotex mRNA system (Qiagen).

First strand cDNA was synthesised using an oligo(dT) linker-primer supplied with the ZAP-cDNA synthesis kit (Stratagene – cat # 200400) and the reverse transcriptase SuperscriptIII (Invitrogen). Double stranded cDNA was ligated to *Eco*RI adaptors and from this a library was constructed using the ZAP-cDNA synthesis kit as described in the accompanying instruction manual (Stratagene – cat # 200400). A primary library of 1.4 x

10^6 plaque forming units (pfu) was obtained. The average insert size of cDNA inserts in the library was 0.9 kilobases based on 47 random plaques and the percentage of recombinants in the library was 99%.

Single pass nucleotide sequencing of 8684 expressed sequence tags (ESTs) was performed with SK primer (5'-CGCTCTAGAACTAGTGGATC-3') (SEQ ID NO:87) using the ABI BigDye system. Sequences of 6750 ESTs were longer than 400 nucleotides, showing the inserts were at least this size. ESTs showing homology to several fatty acid desaturases and one PUFA elongase were identified by BlastX analysis.

The amino acid sequence (partial) (SEQ ID NO:88) encoded by the cDNA clone Mm301461 showed 75% identity to *Thalassiosira pseudonana* fatty acid elongase 1 (Accession No. AY591337). The nucleotide sequence of EST clone Mm301461 is provided as SEQ ID NO:89. The high degree of identity to a known elongase makes it highly likely that Mm301461 encodes a Melosira fatty acid elongase. RACE techniques can readily be utilized to isolate the full-length clone encoding the elongase.

Example 13. Isolation of FAE-like elongase gene fragment from *P. salina*

Random cDNA clones from the *P. salina* cDNA library were sequenced by an EST approach. In an initial round of sequencing, 73 clones were sequenced. One clone, designated 11.B1, was identified as encoding a protein (partial sequence) having sequence similarity with known beta keto-acyl synthase-like fatty acid elongases, based on BLASTX analysis. The nucleotide sequence of 11.B1 from the 3' end is provided as (SEQ ID NO:55).

These plant elongases are different to the ELO class elongase in that they are known to be involved in the elongation of C16 to C18 fatty acids and also the elongation of very-long-chain saturated and monounsaturated fatty acids. Clone 11.B1, represents the first non-higher plant gene in this class isolated.

Example 14. Isolation of a Gene Encoding a Δ5-Desaturase from *P. salina*

Isolation of a gene fragment from a Δ5-desaturase gene from *P. salina*

In order to isolate a Δ5-desaturase gene from *P. salina*, oligonucleotides were designed for a conserved region of desaturases. The oligonucleotides designated d5A and d5B shown below were made corresponding to a short DNA sequence from a Δ5-desaturase gene from *Pavlova lutheri*. Oligo d5A: 5'-TGGGTTGAGTACTCGGCCAACCACACGACCAACTGCGCGCCCTCGTGGTGGT GCGACTGGTGGATGTCTTACCTCAACTACCAGATCGAGCATCATCTGT-3' (nucleotides 115-214 of International patent application published as WO03078639-A2,

Figure 4a) (SEQ ID NO:56) and oligo d5B: 5'-ATAGTGCAGCCCGTGCTTCTCGAAGAGCGCCTTGACGCGCGGCGCGATCGTCGGGTGGCGGAATTGCGGCATGGACGGGAACAGATGATGCTCGATCTGG-3' (corresponding to the complement of nucleotides 195-294 of WO03078639-A2, Figure 4a) (SEQ ID NO:57). These oligonucleotides were annealed and extended in a PCR reaction. The PCR product from was inserted into pGEM-T Easy vector and the nucleotide sequence confirmed.

The cloned fragment was labelled and used as a hybridization probe for screening of a *Pavlova salina* cDNA library under moderately high stringency conditions, hybridizing at 55°C overnight with an SSC hybridization solution and washing the blots at 60°C with 2x SSC/0.1% SDS three times each for 10 minutes. From screening of about 500,000 plaques, 60 plaques were isolated which gave at least a weak hybridization signal. Among 13 clones that were sequenced, one clone designated p1918 contained a partial-length cDNA encoding an amino acid sequence with homology to known Δ5-desaturase genes. For example, the amino acid sequence was 53% identical to amino acid residues 210-430 from the C-terminal region of a *Thraustochytrium* Δ5-desaturase gene (Accession No. AF489588).

Isolation of a full-length Δ5-desaturase gene

The partial-length sequence in p1918 was used to design a pair of sequence specific primers, which were then used in PCR screening of the 60 isolated plaques mentioned above. Nineteen of the 60 were positive, having the same or similar cDNA sequence. One of the clones that showed a strong hybridization signal using the partial-length sequence as a probe was used to determine the full-length sequence provided as SEQ ID NO:58, and the amino acid sequence (425 amino acids in length) encoded thereby is provided as SEQ ID NO:60.

The amino acid sequence was used to search the NCBI protein sequence database using the BLASTX software. The results indicated that this sequence was homologous to known Δ5-desaturases. The amino acid sequence of the *P. salina* protein showed 81% identity to a *P. lutheri* sequence of undefined activity in WO03/078639-A2, and 50% identity to a Δ5-desaturase from *Thraustochytrium* (Accession No. AF489588). The *Pavlova salina* Δ5-desaturase showed all the conserved motifs typical of 'front-end desaturases' including the N-terminal cytochrome b_5-like domain and three conserved histidine-rich motifs.

Co-expression of Δ9 elongase, Δ8-desaturase and Δ5-desaturase genes in transformed cells

Co-expression of the Δ5-desaturase gene together with the Δ9 elongase gene (Elo2, Example 7) and the Δ8-desaturase gene (Example 6) was achieved in cells as follows. The plant expression vector pXZP354 containing the three genes, each from *P. salina*, and each expressed from the seed specific napin promoter was constructed. The *P. salina* Δ8-desaturase coding region from the cDNA clone (above) was first inserted as a *Bam*HI-*Nco*I fragment into pXZP143 between the seed specific napin promoter and *Nos* terminator, resulting in plasmid pXZP146. The *P. salina* Δ9-elongase gene was likewise inserted, as a *Pst*I-*Xho*I fragment from its cDNA clone, into pXZP143 resulting in plasmid pXZP143-Elo2. The *P. salina* Δ5-desaturase gene was also inserted, as a *Pst*I-*Bss*HII fragment from its cDNA clone, into pXZP143, resulting in plasmid pXZP147. Then, the *Hind*III-*Apa*I fragment containing the Δ9-elongase expression cassette from pXZP143-Elo2 was inserted into pXZP146 downstream of the Δ8-desaturase expression cassette, resulting in plasmid pXZP148. The *Hind*III-*Apa*I fragment containing the Δ5-desaturase expression cassette from pXZP147 was inserted into pXZP148 downstream of Δ8-desaturase and Δ9-elongase expression cassettes, resulting in plasmid pXZP149. Then, as a final step, the *Hind*III-*Apa*I fragment containing the three genes from pXZP149 was inserted into a derivative of the binary vector pART27, containing a hygromycin resistance gene selection marker, resulting in plant expression plasmid pXZP354.

Plasmid pXZP354 was introduced into *Arabidopsis* by the *Agrobacterium*-mediated floral dip method, either in the simultaneous presence or the absence of expression plasmid pXZP355 (Example 11) containing the *P. salina* Δ5-elongase and Δ4-desaturase genes. Co-transformation of the vectors could be achieved since they contained different selectable marker genes. In the latter case, the transgenic plants (designated "DR" plants) were selected using hygromycin as selective agent, while in the former case, the plants ("DU" plants) were selected with both hygromycin and kanamycin.

Twenty-one DR plants (T1 plants) were obtained. Fatty acid analysis of seedoil from T2 seed from ten of these plants showed the presence of low levels of 20:2ω (EDA), 20:3ω6 (DGLA) and 20:4ω6 (ARA), including up to 0.4% ARA. Fatty acid analysis of seedoil from T2 seed from seven DU plants showed similar levels of these fatty acids. From the relative ratios of these fatty acids, it was concluded that the Δ5-desaturase and Δ8-desaturase genes were functioning efficiently in seed transformed with pXZP354 but that the activity of the Δ9 elongase gene was suboptimal. It is likely that shortening of the

coding region at the N-terminal end, to initiate translation at amino acid position 11 or 29 of SEQ ID NO:3 (Example 9) (see SEQ ID NO's 85 and 86) will improve the level of activity of the Δ9 elongase gene. Expression of one or two of the genes from seed-specific promoters other than the napin promoter, so they are not all expressed from the napin promoter, is also expected to improve the expression level of the Δ9 elongase gene.

Example 15. Isolation of a Gene Encoding a Δ6-Desaturase from *Echium plantagineum*

Some plant species such as evening primrose (*Oenothera biennis*), common borage (*Borago officinalis*), blackcurrant (*Ribes nigrum*), and some *Echium* species belonging to the *Boragenacae* family contain the ω6- and ω3–desaturated C18 fatty acids, γ-linolenic acid (18:3ω6, GLA) and stearidonic acid (18:4ω3, SDA) in their leaf lipids and seed TAGs (Guil-Guerrero et al., 2000). GLA and SDA are recognized as beneficial fatty acids in human nutrition. The first step in the synthesis of LC-PUFA is a Δ6-desaturation. GLA is synthesized by a Δ6-desaturase that introduces a double bond into the Δ6-position of LA. The same enzyme is also able to introduce a double bond into Δ6-position of ALA, producing SDA. Δ6-Desaturase genes have been cloned from members of the *Boraginacae*, like borage (Sayanova et al., 1997) and two *Echium* species (Garcia-Maroto et al., 2002).

Echium plantagineum is a winter annual native to Mediterranean Europe and North Africa. Its seed oil is unusual in that it has a unique ratio of ω3 and ω6 fatty acids and contains high amounts of GLA (9.2%) and SDA (12.9%) (Guil-Guerrero et al., 2000), suggesting the presence of Δ6-desaturase activity involved in desaturation of both ω3 and ω6 fatty acids in seeds of this plant.

Cloning of *E. platangineum* EplD6Des gene

Degenerate primers with built-in *Xba*I or *Sac*I restriction sites corresponding to N- and C-termini amino acid sequences MANAIKKY (SEQ ID NO: 61) and EALNTHG (SEQ ID NO: 62) of known *Echium pitardii* and *Echium gentianoides* (Garcia-Maroto et al., 2002) Δ6-desaturases were used for RT-PCR amplification of Δ6-desaturase sequences from *E. platangineum* using a proofreading DNA polymerase Pfu Turbo® (Stratagene). The 1.35kb PCR amplification product was inserted into pBluescript SK(+) at the *Xba*I and *Sac*I sites to generate plasmid pXZP106. The nucleotide sequence of the insert was determined (SEQ ID NO:63). It comprised an open reading frame encoding a polypeptide of 438 amino acid residues (SEQ ID NO:64) which had a high degree of homology with other reported Δ6- and Δ8-desaturases from *E. gentianoides* (SEQ ID

NO:65), *E. pitardii* (SEQ ID NO:66), *Borago officinalis* (SEQ ID NO:67 and 68), *Helianthus annuus* (SEQ ID NO:69) and *Arabidopsis thaliana* (SEQ ID NO:70 and SEQ ID NO:71) (Figure 10). It has a cytochrome b_5 domain at the N-terminus, including the HPGG (SEQ ID NO:72) motif in the heme-binding region, as reported for other Δ6- and Δ8-desaturases (Sayanova *et al.* 1997; Napier *et al.* 1999). In addition, the *E. plantagineum* Δ6 desaturase contains three conserved histidine boxes, including the third histidine box containing the signature QXXHH (SEQ ID NO:73) motif present in majority of the 'front-end' desaturases (Figure 10) (Napier et al., 1999). Cluster analysis including representative members of Δ6 and Δ8 desaturases showed a clear grouping of the cloned gene with other Δ6 desaturases especially those from *Echium* species.

Heterologous expression of *E. plantagineum* Δ6-desaturase gene in yeast

Expression experiments in yeast were carried out to confirm that the cloned *E. platangineum* gene encoded a Δ6-desaturase enzyme. The gene fragment was inserted as an *Xba*I-*Sac*I fragment into the *Sma*I-*Sac*I sites of the yeast expression vector pSOS (Stratagene) containing the constitutive *ADH1* promoter, resulting in plasmid pXZP271. This was transformed into yeast strain S288Cα by a heat shock method and transformant colonies selected by plating on minimal media plates. For the analysis of enzyme activity, 2mL yeast clonal cultures were grown to an $O.D._{600}$ of 1.0 in yeast minimal medium in the presence of 0.1% NP-40 at 30°C with shaking. Precursor free-fatty acids, either linoleic or linolenic acid as 25mM stocks in ethanol, were added so that the final concentration of fatty acid was 0.5mM. The cultures were transferred to 20°C and grown for 2-3 days with shaking. Yeast cells were harvested by repeated centrifugation and washing first with 0.1% NP-40, then 0.05%NP-40 and finally with water. Fatty acids were extracted and analyzed. The peak identities of fatty acids were confirmed by GC-MS.

The transgenic yeast cells expressing the *Echium EplD6Des* were able to convert LA and ALA to GLA and SDA, respectively. Around 2.9% of LA was converted to GLA and 2.3% of ALA was converted to SDA, confirming the Δ6-desaturase activity encoded by the cloned gene.

Functional expression of *E. platangineum* Δ6-desaturase gene in transgenic tobacco

In order to demonstrate that the *EplD6Des* gene could confer the synthesis of Δ6 desaturated fatty acids in transgenic plants, the gene was expressed in tobacco plants. To do this, the gene fragment was excised from pXZP106 as an *Xba*I-*Sac*I fragment and cloned into the plant expression vector pBI121 (Clonetech) at the *Xba*I and *Sac*I sites

under the control of a constitutive 35S CaMV promoter, to generate plant expression plasmid pXZP341. This was introduced into *Agrobacterium tumefaciens* AGL1, and used for transformation of tobacco W38 plant tissue, by selection with kanamycin.

Northern blot hybridization analysis of transformed plants was carried out to detect expression of the introduced gene, and total fatty acids present in leaf lipids of wild-type tobacco W38 and transformed tobacco plants were analysed as described above. Untransformed plants contained appreciable amounts of LA (21 % of total fatty acids) and ALA (37% of total fatty acids) in leaf lipids. As expected, neither GLA nor SDA, products of Δ6-desaturation, were detected in the untransformed leaf. Furthermore, transgenic tobacco plants transformed with the pBI121 vector had similar leaf fatty acid composition to the untransformed W38 plants. In contrast, leaves of transgenic tobacco plants expressing the *EplD6Des* gene showed the presence of additional peaks with retention times corresponding to GLA and SDA. The identity of the GLA and SDA peaks were confirmed by GC-MS. Notably, leaf fatty acids of plants expressing the *EplD6Des* gene consistently contained approximately a two-fold higher concentration of GLA than SDA even when the total Δ6-desaturated fatty acids amounted up to 30% of total fatty acids in their leaf lipids (Table 11).

TABLE 11. Fatty acid composition in lipid from transgenic tobacco leaves (%).

Plant	16:0	18:0	18:1	18:2	GLA	18:3	SDA	Total Δ6-desaturate products
W38	21.78	5.50	2.44	21.21	-	37.62	-	-
ET27-1	20.33	1.98	1.25	10.23	10.22	41.10	6.35	16.57
ET27-2	18.03	1.79	1.58	14.42	1.47	53.85	0.48	1.95
ET27-4	19.87	1.90	1.35	7.60	20.68	29.38	9.38	30.07
ET27-5	15.43	2.38	3.24	11.00	0.84	49.60	0.51	1.35
ET27-6	19.85	2.05	1.35	11.12	4.54	50.45	2.19	6.73
ET27-8	19.87	2.86	2.55	11.71	17.02	27.76	7.76	24.78
ET27-11	17.78	3.40	2.24	12.62	1.11	51.56	0.21	1.32
ET27-12	16.84	2.16	1.75	13.49	2.71	50.80	1.15	3.86

Northern analysis of multiple independent transgenic tobacco lines showed variable levels of the *EplD6Des* transcript which generally correlated with the levels of Δ6-desaturated products synthesized in the plants. For example, transgenic plant ET27-2 which contained low levels of the *EplD6Des* transcript synthesised only 1.95% of its total leaf lipids as Δ6-desaturated fatty acids. On the other hand, transgenic plant ET27-4 contained significantly higher levels of *EplD6Des* transcript and also had a much higher proportion (30%) of Δ6-desaturated fatty acids in its leaf lipids.

Analysis of the individual tobacco plants showed that, without exception, GLA was present at a higher concentration than SDA even though a higher concentration of ALA than LA was present in untransformed plants. In contrast, expression of *EplD6Des* in yeast had resulted in approximately equivalent levels of conversion of LA into GLA and ALA into SDA. *Echium plantagineum* seeds, on the other hand, contain higher levels of SDA than GLA. EplD6Des probably carries out its desaturation *in vivo* in *Echium plantagineum* seeds on LA and ALA esterified to phosphatidyl choline (PC) (Jones and Harwood 1980). In the tobacco leaf assay, the enzyme is most likely desaturating LA and ALA esterified to the chloroplast lipid monogalactosyldiacylglyerol (MGDG) (Browse and Slack, 1981). In the yeast assay, free fatty acid precursors LA and ALA added to the medium most likely enter the acyl-CoA pool and are available to be acted upon by EplD6Des in this form.

Functional expression of *E. platangineum* Δ6-desaturase gene in transgenic seed

To show seed-specific expression of the *Echium* Δ6-desaturase gene, the coding region was inserted into the seed-specific expression cassette as follows. An *Nco*I-*Sac*I fragment including the Δ6-desaturase coding region was inserted into pXZP6, a pBluescriptSK derivative containing a *Nos* terminator, resulting in plasmid pXZP157. The *Sma*I-*Apa*I fragment containing the coding region and terminator EplD6Des-NosT was cloned into pWVec8-Fp1 downstream of the *Fp1* prompter, resulting in plasmid pXZP345. The plasmid pXZP345 was used for transforming wild type *Arabidopsis* plants, ecotype Columbia, and transgenic plants selected by hygromycin B selection. The transgenic plants transformed with this gene were designated "DP" plants.

Fatty acid composition analysis of the seed oil from T2 seed from eleven T1 plants transformed with the construct showed the presence of GLA and SDA in all of the lines, with levels of Δ6-desaturation products reaching to at least 11% (Table 12). This demonstrated the efficient Δ6-desaturation of LA and ALA in the seed.

TABLE 12. Fatty acid composition in transgenic *Arabidopsis* seeds expressing Δ6-desaturase from *Echium*.

Plant	Fatty acid (%)									Total Δ6-desaturation products (%)
	16:0	18:0	$18:1^{\Delta9}$	$18:2^{\Delta9,12}$ (LA)	$18:3^{\Delta6,9,12}$ (GLA)	$18:3^{\Delta9,12,15}$ (ALA)	$18:4^{\Delta6,9,12,15}$ (SDA)	20:0	20:1	
Columbia										
DP-2	8.0	2.8	22.9	27.3	2.5	11.3	0.7	1.6	15.8	3.2
DP-3	7.8	2.7	20.6	25.9	3.0	12.1	0.8	1.7	17.8	3.8
DP-4	7.8	2.8	20.4	28.5	1.2	13.7	0.4	1.7	16.1	1.5
DP-5	8.2	3.2	17.4	29.3	1.2	14.2	0.3	2.1	15.6	1.6
DP-7	8.2	2.9	18.4	26.7	5.0	12.7	1.4	1.7	15.2	6.4
DP-11	9.0	3.5	17.8	28.4	3.0	13.4	0.9	2.1	13.9	3.8
DP-12	8.6	3.0	18.9	27.8	3.3	12.6	1.0	1.8	15.4	4.3
DP-13	8.7	2.9	14.4	27.3	8.5	13.7	2.6	1.7	12.4	11.1
DP-14	9.3	2.9	14.2	32.3	2.1	15.4	0.7	1.8	12.8	2.8
DP-15	8.2	2.9	17.8	30.1	0.3	15.3	0.2	1.9	15.5	0.5
DP-16	8.0	2.8	19.5	29.2	2.7	13.1	0.8	1.7	14.2	3.5

Example 16. Mutagenesis of *E. platangineum* EplD6Des Gene

To determine whether variability could be introduced into the Δ6-desaturase gene and yet retain desaturase activity, the *E. platangineum* Δ6-desaturase cDNA was randomly mutated by PCR using Taq polymerase and EPD6DesF1 and EPD6DesR1 primers in the presence of dITP as described by Zhou and Christie (1997). The PCR products were cloned as *Xba*I-*Sac*I fragments in pBluescript SK(+) at *Xba*I and *Sac*I sites, and sequences of randomly selected clones determined. Random variants with amino acid residue changes were chosen to clone as *Xba*I-*Sac*I fragments into pBI121 and the enzyme activities of proteins expressed from these variants characterized in transgenic tobacco leaves as described above for the wild-type gene.

Figure 11A represents the activity of the *EplD6Des* sequence variants when expressed in tobacco plants. The variants could be divided into two broad classes in terms of their ability to carry out Δ6-desaturation. Mutations represented as empty diamonds showed substantial reductions in the Δ6-desaturation activity while mutations denoted as solid diamonds had little or no effect on the activity of the encoded Δ6-desaturase enzyme. Figure 11B represents the quantitative effect that a selection of mutations in the *EplD6Des* gene had on the Δ6-desaturase activity. An L14P mutation in the cytochrome b_5 domain and an S301P mutation between histidine box II and histidine box III of *EplD6Des* caused substantial reductions in their Δ6-desaturase activities, resulting in a 3- to 5-fold reduction in total Δ6-desaturated fatty acids when compared to the wild-type enzyme in W38 plants. Surprisingly, significant activity was retained for each. In contrast, most of the variants examined, as exemplified by the S205N mutation, had no effect on the Δ6-desaturation activity of *EplD6Des* gene.

Example 17. Comparison of acyl-CoA and acyl-PC substrate dependent desaturases for production of LC-PUFA in cells

As described above, the synthesis of LC-PUFA such as EPA and DHA in cells by the conventional Δ6 desaturation pathway requires the sequential action of PUFA desaturases and elongases, shown schematically in Figure 12 part A. This conventional pathway operates in algae, mosses, fungi, diatoms, nematodes and some freshwater fish (Sayanova and Napier, 2004). The PUFA desaturases from algae, fungi, mosses and worms are selective for desaturation of fatty acids esterified to the *sn*-2 position of phosphatidylcholine (PC) while the PUFA elongases act on fatty acids in the form of acyl-CoA substrates represented in the acyl-CoA pool in the endoplasmic reticulum (ER), which is physiologically separated from the PC component of the ER. Therefore, sequentially desaturation and elongation reactions on a fatty acid substrate requires that

the fatty acid is transferred between the acyl-PC and acyl-CoA pools in the ER. This requires acyltransferases that are able to accommodate LC-PUFA substrates. This "substrate switching" requirement may account for the low efficiency observed in earlier reported attempts to re-constitute LC-PUFA biosynthesis (Beaudoin et al., 2000, Domergue et al., 2003a). The alternative Δ8 desaturation pathway (Figure 12 part B) suffers from the same disadvantage of requiring "substrate switching".

As described in Example 5, the strategy of using a vertebrate desaturase which was able to desaturate acyl-CoA substrates, provided relatively efficient production of LC-PUFA in plant cells including in seed. In Example 5, the combination of a Δ5/Δ6 desaturase from zebra fish with a Δ6 elongase from *C. elegans* had the advantage that both the desaturase and the elongase enzymes had activity on acyl-CoA substrates in the acyl-CoA pool. This may explain why this strategy was more efficient in the synthesis of LC-PUFA. To provide a direct comparison of the relative efficiencies of using an acyl-CoA substrate-dependent desaturase compared to an acyl-PC substrate-dependent desaturase, we conducted the following experiment. This compared the use of the *Echium* Δ6 desaturase (Example 15) and the *P. salina* Δ5 desaturase (Example 14), both of which are thought to use acyl-PC substrates, with the zebrafish Δ6/Δ5 desaturase which uses an acyl-CoA substrate (Example 5).

A construct was prepared containing two acyl-PC dependent desaturases, namely the *Echium* Δ6 desaturase and *P. salina* Δ5 desaturase, in combination with the *C. elegans* Δ6 elongase. The *Echium* Δ6 desaturase gene on an *Nco*I-*Sac*I fragment was inserted into pXZP143 (Example 15) resulting in pXZP192. The *C. elegans* Δ6 elongase gene (Fp1-CeElo-NosT expression cassette) on the *Hind*III-*Apa*I fragment of pCeloPWVec8 (Example 5) was inserted into the *Stu*I-*Apa*I sites of pXZP147 (Example 14) to make pXZP193. The *Hind*III-*Apa*I fragment of pXZP193 containing both genes (Fp1-PsD5Des-NosT and Fp1-CeElo-NosT) was inserted into the *Apa*I-*Stu*I sites of pXZP192, resulting in plasmid pXZP194 containing the three expression cassettes. The *Xba*I-*Apa*I fragment from pXZP194 was inserted in a pWvec8 derivative, resulting in pXZP357.

The plasmid pXZP357 was used to transform plants of wild-type *Arabidopsis* ecotype Columbia by *Agrobacterium*-mediated floral dip method, and six transgenic plants were obtained after hygromycin B (20 mg/L) selection. The transgenic T1 plants were designated "DT" plants. The hygromycin resistant transformed plants were transferred into soil and self-fertilised. The T2 seed were harvested and the seed fatty acid composition of two lines, DT1 and DT2, was analysed. The seed fatty acids of DT1 and DT2 contained low levels of 18:3ω6 and 18:4ω4 (0.9 and 0.8 % of GLA,

0.3% and 0.1% of SDA, respectively, Table 13). In addition, both DT1 and DT2 seed also contained 0.3% and 0.1% of the 20:4ω6 (ARA). However, there was no apparent synthesis of the ω3 fatty acid EPA in either of the T2 seed lines, which probably reflected the greater desaturation ability of the *Echium* Δ6 desaturase on the ω6 substrate LA compared to the ω3 substrate ALA (Example 15).

TABLE 13. Fatty acid composition of seed-oil from T2 seed of DT1 and DT2. Fatty acid values are % of total fatty acids.

Fatty acid	Control	DT1	DT2
16:0	7.2	6.5	6.5
18:0	2.9	3.6	3.3
18:1ω9	20.0	23.2	22.3
18:2ω6	27.5	23.6	24.4
18:3ω3	15.1	15.4	16.1
20:0	2.2	2.0	1.9
20:1ω9/ω11	19.9	19.4	19.5
20:1ω7	2.2	3.4	3.0
20:2ω6	0.1	0.0	0.0
22:1ω7	0.0	0.0	0.0
Other minor	2.8	1.5	1.9
Total	100.0	98.6	98.9
New ω6-PUFA			
18:3ω6	0.0	0.9	0.8
20:3ω6	0.0	0.0	0.0
20:4ω6	0.0	0.3	0.1
Total	0.0	1.2	0.9
New ω3-PUFA			
18:4ω3	0.0	0.3	0.2
20:4ω3	0.0	0.0	0.0
20:5ω3	0.0	0.0	0.0
Total	0.0	0.3	0.2
Total fatty acids	100.0	100.0	100.0

This data was in clear contrast to Example 5, above, where expression of the acyl-CoA dependent desaturase from zebrafish in combination with a Δ6 elongase resulted in the production of at least 1.1% ARA and 2.3% EPA in T2 seed fatty acids. Thus it would appear that acyl-PC dependent desaturases were less effective than acyl-CoA dependent desaturases in driving the synthesis of LC-PUFA in plants cells.

Example 18. Expression of LC-PUFA genes in *Synechococcus*

Synechococcus spp. (Bacteria; Cyanobacteria; Chroococcales; *Synechococcus* species for example *Synechococcus elongatus*, also known as Synechocystis spp.) are unicellular, photosynthetic, marine or freshwater bacteria in the order cyanobacteria that utilize chlorophyll *a* in the light-harvesting apparatus. The species include important primary producers in the marine environment. One distinct biochemical feature of *Synechococcus* is the presence of phycoerythrin, an orange fluorescent compound that can be detected at an excitation wavelength of 540 nm, and which can be used to identify *Synechococcus*. Members of the marine *synechococccus* group are closely related at the level of 16s rRNA. They are obligately marine and have elevated growth requirements for Na^+, Cl^-, Mg^{2+}, and Ca^{2+}, but can be grown readily in both natural and artificial seawater liquid media as well as on plates (Waterbury et al. 1988). Since they have a rapid heterotrophic or autotrophic growth rate, contain fatty acid precursors such as LA and ALA, and are relatively simple to transform, they are suitable for functional studies involving LC-PUFA synthesis genes, or for production of LC-PUFA in fermenter type production systems. Strains such as *Synechococcus* sp. strain WH8102, PCC7002 (7002, marine), or PCC7942 (freshwater) can be grown easily and are amenable to biochemical and genetic manipulation (Carr, N.G., and N. H. Mann. 1994. The oceanic cyanobacterial picoplankton, p. 27-48. In D. A. Bryant (ed.), the Molecular biology of cyanobacteria. Kluwer Academic publishers, Boston). For example, *Synechococcus* has been used as a heterologous expression system for desaturases (Domergue 2003b).

Wildtype *Synechococcus* 7002 Fatty Acid Profile and Growth Rates

To show that cyanobacterium *Synechococcus* 7002 was a suitable host for the transformation of fatty acid synthesis genes and that this expression system could be used to rapidly test the functions and specificities of fatty acid synthesis genes, the growth of the wildtype strain 7002 was first analysed at 22°C, 25°C and 30°C and the resultant fatty acid profiles analysed by gas chromatography for growth at 22°C and 30°C (Table 14).

TABLE 14. *Synechococcus* 7002 wildtype fatty acid profiles at 22°C and 30°C growth temperatures (% of total fatty acid).

Temp	Myristic	Palmitic	Palmit-oleic	Stearic	Oleic	18:1iso	Linoleic	GLA	Lino-lenic
22°C	0.79	42.5	10.6	0.92	8.4	1.5	7.5	0.54	27.1
30°C	0.76	47.1	10.9	0.67	17.0	0.34	20.4		2.9

Growth at 30°C was much more rapid than at 22°C, with intermediate rates at 25°C (Figure 13). The cells were found to contain both linoleic (LA, 18:2 ω6) and linolenic (ALA, 18:3 ω3) acids which could be used as precursors for LC-PUFA synthesis. Although some of the preferred precursor ALA was produced at the 30°C, higher levels were obtained at 22°C. Tests were also carried out to determine whether cells could be grown at 30°C, followed by reducing the incubation temperature to 22°C after sufficient biomass had been achieved, to see if this would result in a shift to higher production of linolenic acid (Figure 14). In this experiment, levels of ALA obtained were greater than 5%. In further experiments, 25°C was used as the preferred temperature for strain 7002, providing adequate growth rates and suitable precursor fatty acid profile.

Transformation Strategy

Both replicative plasmid vectors and non-replicative homologous recombination vectors have been used previously to transform various cyanobacterial species, including *Synechococcus* 7002 (Williams and Szalay, 1983; Ikeda et al., 2002; Akiyama et al., 1998a). The recombination vectors may be preferred in certain applications, and have been used to inactivate a gene, rather than create an expression strain.

A recombination vector was constructed that was suitable for introduction of one or more fatty acid synthesis genes into the chromosome of *Synechococcus* strains such as strain 7002. This vector contained the *Synechococcus* 7002 sul2 gene in a pBluescript plasmid backbone, which provided an ampicillin gene as a selectable marker and allowed bacterial replication in species such as *E. coli*. The vector was engineered to contain a plac promoter from *E. coli* fused to a downstream multiple cloning site, with the two elements inserted approximately in the centre of the sul2 gene. The sul2 gene in *Synechococcus* encodes a low affinity sulfate which is not essential under normal growth conditions. Any gene other than sul2, preferably a non-essential gene, could have been chosen for incorporation in the recombination vector.

The sul2 gene was amplified from *Synechococcus* 7002 genomic DNA using gene-specific primers, based on the near-identical sequence in strain PCC6803 (Genbank Accession No. NC_000911, nucleotides 2902831 to 2904501) and inserted into the vector pGEM-T. The plac promoter from pBluescript was amplified using the primers 5'-gctacgcccggggatcctcgaggctggcgcaacgcaattaatgtga-3' (SEQ ID NO:81) (sense) and 5'-cacaggaaacagcttgacatcgattaccggcaattgtacggcggccgctacggatatcctcgctcgagctcgcccgggg tagct-3' (SEQ ID NO:82) (antisense), which also introduced a number of restriction sites at the ends of the promoter sequence. The amplified fragment was then digested with *Sma*I and ligated to the large *Pvu*II fragment of pBluescript including the beta-lactamase gene. This intermediate vector was then digested with *Eco*RV and *Sac*I and ligated to the *Hpa*I to *Sac*I fragment (designated sul2b) of the sul2 gene. The resultant plasmid was digested with *Bam*HI, treated with DNA polymerase I (Klenow fragment) to fill in the ends, and ligated to the *Sma*I to *Hpa*I fragment (designated sul2a) of the sul2 gene. Excess restriction sites were then removed from this vector by digestion with *Sac*I and *Spe*I, blunting the ends with T4 DNA polymerase, and religation. Finally, a multiple cloning site was introduced downstream of the plac promoter by digesting the vector with *Cla*I and *Not*I, and ligating in a *Cla*I to *Not*I fragment from pBluescript, generating the recombination vector which was designated pJRP3.2.

Various genes related to LC-PUFA synthesis were adapted by PCR methods to include flanking restriction sites as well as ribosome binding site (RBS) sequences that were suitable for expression in the prokaryote, *Synechococcus*. For example, the *Echium plantagineum* Δ6-desaturase (Example 15) was amplified with the primers 5'-AGCACATCGATGAAGGAGATATACCCatggctaatgcaatcaagaa-3' (SEQ ID NO:83) (sense) and 5'-ACGATGCGGCCGCTCAACCATGAGTATTAAGAGCTT-3' (SEQ ID NO:84) (antisense).

The amplified product was digested with *Cla*I and *Not*I and cloned into the *Cla*I to *Not*I sites of pJRP3.2. A selectable marker gene comprising a chloramphenicol acetyl transferase coding region (CAT) (catB3 gene, Accession No. AAC53634) downstream of a pbsA promoter (psbA-CAT) was inserted into the *Xho*I site of pJRP3.2, producing the vector pJRP3.3. The selectable marker gene was inserted within the sulB gene to enable easy selection for homologous recombination events after introduction of the recombination vector into *Synechococcus*.

Transformation of *Synechococcus* 7002 was achieved by mixing vector DNA with cells during the exponential phase of growth, during which DNA uptake occurred, as follows. Approximately 1μg of the recombination vector DNA resuspended in

100µL of 10 mM Tris-HCl was added to 900µL of mid-log phase cells growing in BG-11 broth. The cells were incubated for 90 min at 30°C and light intensity of 20µmol photons.$m^{-2}.s^{-1}$. 250µL aliquots were then added to 2mL BG-11 broth, mixed with 2mL molten agar (1.5%) and poured onto BG-11 agar plates containing 50µg/mL chloramphenicol (Cm) for selection of recombinant cells. The plates were incubated for 10-14 days at the same temperature/light conditions before the Cm-resistant colonies were clearly visible. These colonies were then re-streaked several times onto fresh BG-11/Cm50 plates. After several rounds of restreaking on selective plates, liquid medium was inoculated with individual colonies and the cultures incubated at 25°C

Synechococcus 7002 cells containing the *Echium* Δ6-desaturase gene inserted into the sulB gene via the recombination vector and expressed from the plac promoter are shown to produce GLA (18:3 Δ6,9,12) and SDA (18:4, Δ6,9,12,15) from endogenous linoleic acid (LA) and linolenic acid (ALA), respectively, as substrates.

Episomal vectors can also be used in *Synechococcus* rather than the integrative/recombinational vectors described above. *Synechococcus* species have native plasmids that have been adapted for use in transformation, for example pAQ-EX1, where a fragment of the native plasmid pAQ1 (Accession No. NC_005025) was fused with an *E. coli* plasmid to form a shuttle vector with both *E. coli* and *Synechococcus* origins of replication (Ikeda et al., 2002; Akiyama et al., 1998b).

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 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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Sequence Listing in Electronic Form

In accordance with Section 111(1) of the Patent Rules, this description contains a sequence listing in electronic form in ASCII text format (file: 79314-44D2 Seq 25-10-18 v1.txt).

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

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<212> DNA
<213> Pavlova salina

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<212> DNA
<213> Pavlova salina

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<212> DNA
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<212> PRT
<213> Caenorhabditis elegans

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35 40 45
Gln Ala Ser Ile Leu Tyr Met Val Val Val Phe Gly Thr Lys Trp Phe
50 55 60
Met Arg Asn Arg Gln Pro Phe Gln Leu Thr Ile Pro Leu Asn Ile Trp
65 70 75 80
Asn Phe Ile Leu Ala Ala Phe Ser Ile Ala Gly Ala Val Lys Met Thr
85 90 95
Pro Glu Phe Phe Gly Thr Ile Ala Asn Lys Gly Ile Val Ala Ser Tyr
100 105 110
Cys Lys Val Phe Asp Phe Thr Lys Gly Glu Asn Gly Tyr Trp Val Trp
115 120 125
Leu Phe Met Ala Ser Lys Leu Phe Glu Leu Val Asp Thr Ile Phe Leu
130 135 140
Val Leu Arg Lys Arg Pro Leu Met Phe Leu His Trp Tyr His His Ile
145 150 155 160
Leu Thr Met Ile Tyr Ala Trp Tyr Ser His Pro Leu Thr Pro Gly Phe
165 170 175
Asn Arg Tyr Gly Ile Tyr Leu Asn Phe Val Val His Ala Phe Met Tyr
180 185 190
Ser Tyr Tyr Phe Leu Arg Ser Met Lys Ile Arg Val Pro Gly Phe Ile
195 200 205
Ala Gln Ala Ile Thr Ser Leu Gln Ile Val Gln Phe Ile Ile Ser Cys
210 215 220
Ala Val Leu Ala His Leu Gly Tyr Leu Met His Phe Thr Asn Ala Asn
225 230 235 240
Cys Asp Phe Glu Pro Ser Val Phe Lys Leu Ala Val Phe Met Asp Thr
245 250 255
Thr Tyr Leu Ala Leu Phe Val Asn Phe Phe Leu Gln Ser Tyr Val Leu
260 265 270
Arg Gly Gly Lys Asp Lys Tyr Lys Ala Val Pro Lys Lys Lys Asn Asn
275 280 285

<210> 15
<211> 444
<212> PRT
<213> Danio rerio

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Gly Asp Gln Trp Val Val Val Glu Arg Lys Val Tyr Asn Val Ser Gln
35 40 45
Trp Val Lys Arg His Pro Gly Gly Leu Arg Ile Leu Gly His Tyr Ala
50 55 60
Gly Glu Asp Ala Thr Glu Ala Phe Thr Ala Phe His Pro Asn Leu Gln
65 70 75 80

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Phe Arg Ala Leu Arg Glu Arg Leu Glu Ala Glu Gly Cys Phe Lys Thr
        115                 120                 125
Gln Pro Leu Phe Phe Ala Leu His Leu Gly His Ile Leu Leu Leu Glu
    130                 135                 140
Ala Ile Ala Phe Met Met Val Trp Tyr Phe Gly Thr Gly Trp Ile Asn
145                 150                 155                 160
Thr Leu Ile Val Ala Val Ile Leu Ala Thr Ala Gln Ser Gln Ala Gly
                165                 170                 175
Trp Leu Gln His Asp Phe Gly His Leu Ser Val Phe Lys Thr Ser Gly
            180                 185                 190
Met Asn His Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly Ala
        195                 200                 205
Ser Ala Gly Trp Trp Asn His Arg His Phe Gln His His Ala Lys Pro
    210                 215                 220
Asn Ile Phe Lys Lys Asp Pro Asp Val Asn Met Leu Asn Ala Phe Val
225                 230                 235                 240
Val Gly Asn Val Gln Pro Val Glu Tyr Gly Val Lys Lys Ile Lys His
                245                 250                 255
Leu Pro Tyr Asn His Gln His Lys Tyr Phe Phe Phe Ile Gly Pro Pro
            260                 265                 270
Leu Leu Ile Pro Val Tyr Phe Gln Phe Gln Ile Phe His Asn Met Ile
        275                 280                 285
Ser His Gly Met Trp Val Asp Leu Leu Trp Cys Ile Ser Tyr Tyr Val
    290                 295                 300
Arg Tyr Phe Leu Cys Tyr Thr Gln Phe Tyr Gly Val Phe Trp Ala Ile
305                 310                 315                 320
Ile Leu Phe Asn Phe Val Arg Phe Met Glu Ser His Trp Phe Val Trp
                325                 330                 335
Val Thr Gln Met Ser His Ile Pro Met Asn Ile Asp Tyr Glu Lys Asn
            340                 345                 350
Gln Asp Trp Leu Ser Met Gln Leu Val Ala Thr Cys Asn Ile Glu Gln
        355                 360                 365
Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
    370                 375                 380
His His Leu Phe Pro Thr Val Pro Arg His Asn Tyr Trp Arg Ala Ala
385                 390                 395                 400
Pro Arg Val Arg Ala Leu Cys Glu Lys Tyr Gly Val Lys Tyr Gln Glu
                405                 410                 415
Lys Thr Leu Tyr Gly Ala Phe Ala Asp Ile Ile Arg Ser Leu Glu Lys
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Ser Gly Glu Leu Trp Leu Asp Ala Tyr Leu Asn Lys
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<210> 16
<211> 444
<212> PRT
<213> Homo sapiens

<400> 16

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Glu Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Ser Glu Phe
35 40 45
Thr Arg Arg His Pro Gly Gly Ser Arg Val Ile Ser His Tyr Ala Gly
50 55 60
Gln Asp Ala Thr Asp Pro Phe Val Ala Phe His Ile Asn Lys Gly Leu
65 70 75 80
Val Lys Lys Tyr Met Asn Ser Leu Leu Ile Gly Glu Leu Ser Pro Glu
85 90 95
Gln Pro Ser Phe Glu Pro Thr Lys Asn Lys Glu Leu Thr Asp Glu Phe
100 105 110
Arg Glu Leu Arg Ala Thr Val Glu Arg Met Gly Leu Met Lys Ala Asn
115 120 125
His Val Phe Phe Leu Leu Tyr Leu Leu His Ile Leu Leu Leu Asp Gly
130 135 140
Ala Ala Trp Leu Thr Leu Trp Val Phe Gly Thr Ser Phe Leu Pro Phe
145 150 155 160
Leu Leu Cys Ala Val Leu Leu Ser Ala Val Gln Ala Gln Ala Gly Trp
165 170 175
Leu Gln His Asp Phe Gly His Leu Ser Val Phe Ser Thr Ser Lys Trp
180 185 190
Asn His Leu Leu His His Phe Val Ile Gly His Leu Lys Gly Ala Pro
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Ala Ser Trp Trp Asn His Met His Phe Gln His His Ala Lys Pro Asn
210 215 220
Cys Phe Arg Lys Asp Pro Asp Ile Asn Met His Pro Phe Phe Phe Ala
225 230 235 240
Leu Gly Lys Ile Leu Ser Val Glu Leu Gly Lys Gln Lys Lys Asn Tyr
245 250 255
Met Pro Tyr Asn His Gln His Lys Tyr Phe Phe Leu Ile Gly Pro Pro
260 265 270
Ala Leu Leu Pro Leu Tyr Phe Gln Trp Tyr Ile Phe Tyr Phe Val Ile
275 280 285
Gln Arg Lys Lys Trp Val Asp Leu Ala Trp Met Ile Thr Phe Tyr Val
290 295 300
Arg Phe Phe Leu Thr Tyr Val Pro Leu Leu Gly Leu Lys Ala Phe Leu
305 310 315 320
Gly Leu Phe Phe Ile Val Arg Phe Leu Glu Ser Asn Trp Phe Val Trp
325 330 335
Val Thr Gln Met Asn His Ile Pro Met His Ile Asp His Asp Arg Asn
340 345 350
Met Asp Trp Val Ser Thr Gln Leu Gln Ala Thr Cys Asn Val His Lys
355 360 365
Ser Ala Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
370 375 380
His His Leu Phe Pro Thr Met Pro Arg His Asn Tyr His Lys Val Ala
385 390 395 400
Pro Leu Val Gln Ser Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Ser
405 410 415
Lys Pro Leu Leu Ser Ala Phe Ala Asp Ile Ile His Ser Leu Lys Glu
420 425 430
Ser Gly Gln Leu Trp Leu Asp Ala Tyr Leu His Gln
435 440

<210> 17
<211> 456
<212> PRT
<213> Pythium irregulare

<400> 17

Met	Gly	Thr	Asp	Gln	Gly	Lys	Thr	Phe	Thr	Trp	Gln	Glu	Val	Ala	Lys
1				5				10					15		
His	Asn	Thr	Ala	Lys	Ser	Ala	Trp	Val	Ile	Ile	Arg	Gly	Glu	Val	Tyr
			20					25					30		
Asp	Val	Thr	Glu	Trp	Ala	Asp	Lys	His	Pro	Gly	Gly	Ser	Glu	Leu	Ile
		35					40					45			
Val	Leu	His	Ser	Gly	Arg	Glu	Cys	Thr	Asp	Thr	Phe	Tyr	Ser	Tyr	His
	50					55					60				
Pro	Phe	Ser	Asn	Arg	Ala	Asp	Lys	Ile	Leu	Ala	Lys	Tyr	Lys	Ile	Gly
65					70					75					80
Lys	Leu	Val	Gly	Gly	Tyr	Glu	Phe	Pro	Val	Phe	Lys	Pro	Asp	Ser	Gly
				85					90					95	
Phe	Tyr	Lys	Glu	Cys	Ser	Glu	Arg	Val	Ala	Glu	Tyr	Phe	Lys	Thr	Asn
			100					105					110		
Asn	Leu	Asp	Pro	Lys	Ala	Ala	Phe	Ala	Gly	Leu	Trp	Arg	Met	Val	Phe
		115					120					125			
Val	Phe	Ala	Val	Ala	Ala	Leu	Ala	Tyr	Met	Gly	Met	Asn	Glu	Leu	Ile
	130					135					140				
Pro	Gly	Asn	Val	Tyr	Ala	Gln	Tyr	Ala	Trp	Gly	Val	Val	Phe	Gly	Val
145					150					155					160
Phe	Gln	Ala	Leu	Pro	Leu	Leu	His	Val	Met	His	Asp	Ser	Ser	His	Ala
				165					170					175	
Ala	Cys	Ser	Ser	Ser	Pro	Ala	Met	Trp	Gln	Ile	Ile	Gly	Arg	Gly	Val
			180					185					190		
Met	Asp	Trp	Phe	Ala	Gly	Ala	Ser	Met	Val	Ser	Trp	Leu	Asn	Gln	His
		195					200					205			
Val	Val	Gly	His	His	Ile	Tyr	Thr	Asn	Val	Ala	Gly	Ala	Asp	Pro	Asp
	210					215					220				
Leu	Pro	Val	Asp	Phe	Glu	Ser	Asp	Val	Arg	Arg	Ile	Val	His	Arg	Gln
225					230					235					240
Val	Leu	Leu	Pro	Ile	Tyr	Lys	Phe	Gln	His	Ile	Tyr	Leu	Pro	Pro	Leu
				245					250					255	
Tyr	Gly	Val	Leu	Gly	Leu	Lys	Phe	Arg	Ile	Gln	Asp	Val	Phe	Glu	Thr
			260					265					270		
Phe	Val	Ser	Leu	Thr	Asn	Gly	Pro	Val	Arg	Val	Asn	Pro	His	Pro	Val
		275					280					285			
Ser	Asp	Trp	Val	Gln	Met	Ile	Phe	Ala	Lys	Ala	Phe	Trp	Thr	Phe	Tyr
	290					295					300				
Arg	Ile	Tyr	Ile	Pro	Leu	Val	Trp	Leu	Lys	Ile	Thr	Pro	Ser	Thr	Phe
305					310					315					320
Trp	Gly	Val	Phe	Phe	Leu	Ala	Glu	Phe	Thr	Thr	Gly	Trp	Tyr	Leu	Ala
				325					330					335	
Phe	Asn	Phe	Gln	Val	Ser	His	Val	Ser	Thr	Glu	Cys	Glu	Tyr	Pro	Cys
			340					345					350		
Gly	Asp	Ala	Pro	Ser	Ala	Glu	Val	Gly	Asp	Glu	Trp	Ala	Ile	Ser	Gln
		355					360					365			
Val	Lys	Ser	Ser	Val	Asp	Tyr	Ala	His	Gly	Ser	Pro	Leu	Ala	Ala	Phe
	370					375					380				
Leu	Cys	Gly	Ala	Leu	Asn	Tyr	Gln	Val	Thr	His	His	Leu	Tyr	Pro	Gly
385					390					395					400

Ile Ser Gln Tyr His Tyr Pro Ala Ile Ala Pro Ile Ile Ile Asp Val
405 410 415
Cys Lys Lys Tyr Asn Ile Lys Tyr Thr Val Leu Pro Thr Phe Thr Glu
420 425 430
Ala Leu Leu Ala His Phe Lys His Leu Lys Asn Met Gly Glu Leu Gly
435 440 445
Lys Pro Val Glu Ile His Met Gly
450 455

<210> 18
<211> 439
<212> PRT
<213> Thraustochytrium sp.

<400> 18
Met Gly Lys Gly Ser Glu Gly Arg Ser Ala Ala Arg Glu Met Thr Ala
1 5 10 15
Glu Ala Asn Gly Asp Lys Arg Lys Thr Ile Leu Ile Glu Gly Val Leu
20 25 30
Tyr Asp Ala Thr Asn Phe Lys His Pro Gly Gly Ser Ile Ile Asn Phe
35 40 45
Leu Thr Glu Gly Glu Ala Gly Val Asp Ala Thr Gln Ala Tyr Arg Glu
50 55 60
Phe His Gln Arg Ser Gly Lys Ala Asp Lys Tyr Leu Lys Ser Leu Pro
65 70 75 80
Lys Leu Asp Ala Ser Lys Val Glu Ser Arg Phe Ser Ala Lys Glu Gln
85 90 95
Ala Arg Arg Asp Ala Met Thr Arg Asp Tyr Ala Ala Phe Arg Glu Glu
100 105 110
Leu Val Ala Glu Gly Tyr Phe Asp Pro Ser Ile Pro His Met Ile Tyr
115 120 125
Arg Val Val Glu Ile Val Ala Leu Phe Ala Leu Ser Phe Trp Leu Met
130 135 140
Ser Lys Ala Ser Pro Thr Ser Leu Val Leu Gly Val Val Met Asn Gly
145 150 155 160
Ile Ala Gln Gly Arg Cys Gly Trp Val Met His Glu Met Gly His Gly
165 170 175
Ser Phe Thr Gly Val Ile Trp Leu Asp Asp Arg Met Cys Glu Phe Phe
180 185 190
Tyr Gly Val Gly Cys Gly Met Ser Gly His Tyr Trp Lys Asn Gln His
195 200 205
Ser Lys His His Ala Ala Pro Asn Arg Leu Glu His Asp Val Asp Leu
210 215 220
Asn Thr Leu Pro Leu Val Ala Phe Asn Glu Arg Val Val Arg Lys Val
225 230 235 240
Lys Pro Gly Ser Leu Leu Ala Leu Trp Leu Arg Val Gln Ala Tyr Leu
245 250 255
Phe Ala Pro Val Ser Cys Leu Leu Ile Gly Leu Gly Trp Thr Leu Tyr
260 265 270
Leu His Pro Arg Tyr Met Leu Arg Thr Lys Arg His Met Glu Phe Val
275 280 285
Trp Ile Phe Ala Arg Tyr Ile Gly Trp Phe Ser Leu Met Gly Ala Leu
290 295 300
Gly Tyr Ser Pro Gly Thr Ser Val Gly Met Tyr Leu Cys Ser Phe Gly
305 310 315 320

Leu Gly Cys Ile Tyr Ile Phe Leu Gln Phe Ala Val Ser His Thr His
325 330 335
Leu Pro Val Thr Asn Pro Glu Asp Gln Leu His Trp Leu Glu Tyr Ala
340 345 350
Ala Asp His Thr Val Asn Ile Ser Thr Lys Ser Trp Leu Val Thr Trp
355 360 365
Trp Met Ser Asn Leu Asn Phe Gln Ile Glu His His Leu Phe Pro Thr
370 375 380
Ala Pro Gln Phe Arg Phe Lys Glu Ile Ser Pro Arg Val Glu Ala Leu
385 390 395 400
Phe Lys Arg His Asn Leu Pro Tyr Tyr Asp Leu Pro Tyr Thr Ser Ala
405 410 415
Val Ser Thr Thr Phe Ala Asn Leu Tyr Ser Val Gly His Ser Val Gly
420 425 430
Ala Asp Thr Lys Lys Gln Asp
435

<210> 19
<211> 446
<212> PRT
<213> Mortierella alpina

<400> 19
Met Gly Thr Asp Gln Gly Lys Thr Phe Thr Trp Glu Glu Leu Ala Ala
1 5 10 15
His Asn Thr Lys Gly Asp Leu Phe Leu Ala Ile Arg Gly Arg Val Tyr
20 25 30
Asp Val Thr Lys Phe Leu Ser Arg His Pro Gly Gly Val Asp Thr Leu
35 40 45
Leu Leu Gly Ala Gly Arg Asp Val Thr Pro Val Phe Glu Met Tyr His
50 55 60
Ala Phe Gly Ala Ala Asp Ala Ile Met Lys Lys Tyr Tyr Val Gly Thr
65 70 75 80
Leu Val Ser Asn Glu Leu Pro Val Phe Pro Glu Pro Thr Val Phe His
85 90 95
Lys Thr Ile Lys Thr Arg Val Glu Gly Tyr Phe Thr Asp Arg Asp Ile
100 105 110
Asp Pro Lys Asn Arg Pro Glu Ile Trp Gly Arg Tyr Ala Leu Ile Phe
115 120 125
Gly Ser Leu Ile Ala Ser Tyr Tyr Ala Gln Leu Phe Val Pro Phe Val
130 135 140
Val Glu Arg Thr Trp Leu Gln Val Val Phe Ala Ile Ile Met Gly Phe
145 150 155 160
Ala Cys Ala Gln Val Gly Leu Asn Pro Leu His Asp Ala Ser His Phe
165 170 175
Ser Val Thr His Asn Pro Thr Val Trp Lys Ile Leu Gly Ala Thr His
180 185 190
Asp Phe Phe Asn Gly Ala Ser Tyr Leu Val Trp Met Tyr Gln His Met
195 200 205
Leu Gly His His Pro Tyr Thr Asn Ile Ala Gly Ala Asp Pro Asp Val
210 215 220
Ser Thr Phe Glu Pro Asp Val Arg Arg Ile Lys Pro Asn Gln Lys Trp
225 230 235 240
Phe Val Asn His Ile Asn Gln Asp Met Phe Val Pro Phe Leu Tyr Gly
245 250 255

Leu Leu Ala Phe Lys Val Arg Ile Gln Asp Ile Asn Ile Leu Tyr Phe
260 265 270
Val Lys Thr Asn Asp Ala Ile Arg Val Asn Pro Ile Ser Thr Trp His
275 280 285
Thr Val Met Phe Trp Gly Gly Lys Ala Phe Phe Val Trp Tyr Arg Leu
290 295 300
Ile Val Pro Leu Gln Tyr Leu Pro Leu Gly Lys Val Leu Leu Leu Phe
305 310 315 320
Thr Val Ala Asp Met Val Ser Ser Tyr Trp Leu Ala Leu Thr Phe Gln
325 330 335
Ala Asn His Val Val Glu Glu Val Gln Trp Pro Leu Pro Asp Glu Asn
340 345 350
Gly Ile Ile Gln Lys Asp Trp Ala Ala Met Gln Val Glu Thr Thr Gln
355 360 365
Asp Tyr Ala His Asp Ser His Leu Trp Thr Ser Ile Thr Gly Ser Leu
370 375 380
Asn Tyr Gln Ala Val His His Leu Phe Pro Asn Val Ser Gln His His
385 390 395 400
Tyr Pro Asp Ile Leu Ala Ile Ile Lys Asn Thr Cys Ser Glu Tyr Lys
405 410 415
Val Pro Tyr Leu Val Lys Asp Thr Phe Trp Gln Ala Phe Ala Ser His
420 425 430
Leu Glu His Leu Arg Val Leu Gly Leu Arg Pro Lys Glu Glu
435 440 445

<210> 20
<211> 447
<212> PRT
<213> Caenorhabditis elegans

<400> 20
Met Val Leu Arg Glu Gln Glu His Glu Pro Phe Phe Ile Lys Ile Asp
1 5 10 15
Gly Lys Trp Cys Gln Ile Asp Asp Ala Val Leu Arg Ser His Pro Gly
20 25 30
Gly Ser Ala Ile Thr Thr Tyr Lys Asn Met Asp Ala Thr Thr Val Phe
35 40 45
His Thr Phe His Thr Gly Ser Lys Glu Ala Tyr Gln Trp Leu Thr Glu
50 55 60
Leu Lys Lys Glu Cys Pro Thr Gln Glu Pro Glu Ile Pro Asp Ile Lys
65 70 75 80
Asp Asp Pro Ile Lys Gly Ile Asp Asp Val Asn Met Gly Thr Phe Asn
85 90 95
Ile Ser Glu Lys Arg Ser Ala Gln Ile Asn Lys Ser Phe Thr Asp Leu
100 105 110
Arg Met Arg Val Arg Ala Glu Gly Leu Met Asp Gly Ser Pro Leu Phe
115 120 125
Tyr Ile Arg Lys Ile Leu Glu Thr Ile Phe Thr Ile Leu Phe Ala Phe
130 135 140
Tyr Leu Gln Tyr His Thr Tyr Tyr Leu Pro Ser Ala Ile Leu Met Gly
145 150 155 160
Val Ala Trp Gln Gln Leu Gly Trp Leu Ile His Glu Phe Ala His His
165 170 175
Gln Leu Phe Lys Asn Arg Tyr Tyr Asn Asp Leu Ala Ser Tyr Phe Val
180 185 190

```
Gly Asn Phe Leu Gln Gly Phe Ser Ser Gly Gly Trp Lys Glu Gln His
        195                 200                 205
Asn Val His His Ala Ala Thr Asn Val Val Gly Arg Asp Gly Asp Leu
    210                 215                 220
Asp Leu Val Pro Phe Tyr Ala Thr Val Ala Glu His Leu Asn Asn Tyr
225                 230                 235                 240
Ser Gln Asp Ser Trp Val Met Thr Leu Phe Arg Trp Gln His Val His
                245                 250                 255
Trp Thr Phe Met Leu Pro Phe Leu Arg Leu Ser Trp Leu Leu Gln Ser
            260                 265                 270
Ile Ile Phe Val Ser Gln Met Pro Thr His Tyr Tyr Asp Tyr Tyr Arg
        275                 280                 285
Asn Thr Ala Ile Tyr Glu Gln Val Gly Leu Ser Leu His Trp Ala Trp
    290                 295                 300
Ser Leu Gly Gln Leu Tyr Phe Leu Pro Asp Trp Ser Thr Arg Ile Met
305                 310                 315                 320
Phe Phe Leu Val Ser His Leu Val Gly Gly Phe Leu Leu Ser His Val
                325                 330                 335
Val Thr Phe Asn His Tyr Ser Val Glu Lys Phe Ala Leu Ser Ser Asn
            340                 345                 350
Ile Met Ser Asn Tyr Ala Cys Leu Gln Ile Met Thr Thr Arg Asn Met
        355                 360                 365
Arg Pro Gly Arg Phe Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln
    370                 375                 380
Ile Glu His His Leu Phe Pro Thr Met Pro Arg His Asn Leu Asn Thr
385                 390                 395                 400
Val Met Pro Leu Val Lys Glu Phe Ala Ala Ala Asn Gly Leu Pro Tyr
                405                 410                 415
Met Val Asp Asp Tyr Phe Thr Gly Phe Trp Leu Glu Ile Glu Gln Phe
            420                 425                 430
Arg Asn Ile Ala Asn Val Ala Ala Lys Leu Thr Lys Lys Ile Ala
        435                 440                 445
```

<210> 21
<211> 444
<212> PRT
<213> Homo sapiens

<400> 21

```
Met Gly Lys Gly Gly Asn Gln Gly Glu Gly Ala Ala Glu Arg Glu Val
1               5                   10                  15
Ser Val Pro Thr Phe Ser Trp Glu Glu Ile Gln Lys His Asn Leu Arg
            20                  25                  30
Thr Asp Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Ile Thr Lys
        35                  40                  45
Trp Ser Ile Gln His Pro Gly Gly Gln Arg Val Ile Gly His Tyr Ala
    50                  55                  60
Gly Glu Asp Ala Thr Asp Ala Phe Arg Ala Phe His Pro Asp Leu Glu
65                  70                  75                  80
Phe Val Gly Lys Phe Leu Lys Pro Leu Leu Ile Gly Glu Leu Ala Pro
                85                  90                  95
Glu Glu Pro Ser Gln Asp His Gly Lys Asn Ser Lys Ile Thr Glu Asp
            100                 105                 110
Phe Arg Ala Leu Arg Lys Thr Ala Glu Asp Met Asn Leu Phe Lys Thr
        115                 120                 125
```

```
Asn His Val Phe Phe Leu Leu Leu Leu Ala His Ile Ile Ala Leu Glu
    130                 135                 140
Ser Ile Ala Trp Phe Thr Val Phe Tyr Phe Gly Asn Gly Trp Ile Pro
145                 150                 155                 160
Thr Leu Ile Thr Ala Phe Val Leu Ala Thr Ser Gln Ala Gln Ala Gly
                165                 170                 175
Trp Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Arg Lys Pro Lys
            180                 185                 190
Trp Asn His Leu Val His Lys Phe Val Ile Gly His Leu Lys Gly Ala
        195                 200                 205
Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His Ala Lys Pro
    210                 215                 220
Asn Ile Phe His Lys Asp Pro Asp Val Asn Met Leu His Val Phe Val
225                 230                 235                 240
Leu Gly Glu Trp Gln Pro Ile Glu Tyr Gly Lys Lys Lys Leu Lys Tyr
                245                 250                 255
Leu Pro Tyr Asn His Gln His Glu Tyr Phe Phe Leu Ile Gly Pro Pro
            260                 265                 270
Leu Leu Ile Pro Met Tyr Phe Gln Tyr Gln Ile Ile Met Thr Met Ile
        275                 280                 285
Val His Lys Asn Trp Val Asp Leu Ala Trp Ala Val Ser Tyr Tyr Ile
    290                 295                 300
Arg Phe Phe Ile Thr Tyr Ile Pro Phe Tyr Gly Ile Leu Gly Ala Leu
305                 310                 315                 320
Leu Phe Leu Asn Phe Ile Arg Phe Leu Glu Ser His Trp Phe Val Trp
                325                 330                 335
Val Thr Gln Met Asn His Ile Val Met Glu Ile Asp Gln Glu Ala Tyr
            340                 345                 350
Arg Asp Trp Phe Ser Ser Gln Leu Thr Ala Thr Cys Asn Val Glu Gln
        355                 360                 365
Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
    370                 375                 380
His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His Lys Ile Ala
385                 390                 395                 400
Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Glu
                405                 410                 415
Lys Pro Leu Leu Arg Ala Leu Leu Asp Ile Ile Arg Ser Leu Lys Lys
            420                 425                 430
Ser Gly Lys Leu Trp Leu Asp Ala Tyr Leu His Lys
        435                 440
```

<210> 22
<211> 444
<212> PRT
<213> Mus musculus

<400> 22

```
Met Gly Lys Gly Gly Asn Gln Gly Glu Gly Ser Thr Glu Arg Gln Ala
1               5                   10                  15
Pro Met Pro Thr Phe Arg Trp Glu Glu Ile Gln Lys His Asn Leu Arg
            20                  25                  30
Thr Asp Arg Trp Leu Val Ile Asp Arg Lys Val Tyr Asn Val Thr Lys
        35                  40                  45
Trp Ser Gln Arg His Pro Gly Gly His Arg Val Ile Gly His Tyr Ser
    50                  55                  60
```

```
Gly Glu Asp Ala Thr Asp Ala Phe Arg Ala Phe His Leu Asp Leu Asp
65                  70                  75                  80
Phe Val Gly Lys Phe Leu Lys Pro Leu Leu Ile Gly Glu Leu Ala Pro
                85                  90                  95
Glu Glu Pro Ser Leu Asp Arg Gly Lys Ser Ser Gln Ile Thr Glu Asp
            100                 105                 110
Phe Arg Ala Leu Lys Lys Thr Ala Glu Asp Met Asn Leu Phe Lys Thr
        115                 120                 125
Asn His Leu Phe Phe Phe Leu Leu Leu Ser His Ile Ile Val Met Glu
    130                 135                 140
Ser Leu Ala Trp Phe Ile Leu Ser Tyr Phe Gly Thr Gly Trp Ile Pro
145                 150                 155                 160
Thr Leu Val Thr Ala Phe Val Leu Ala Thr Ser Gln Ala Gln Ala Gly
                165                 170                 175
Trp Leu Gln His Asp Tyr Gly His Leu Ser Val Tyr Lys Lys Ser Ile
            180                 185                 190
Trp Asn His Val Val His Lys Phe Val Ile Gly His Leu Lys Gly Ala
        195                 200                 205
Ser Ala Asn Trp Trp Asn His Arg His Phe Gln His His Ala Lys Pro
    210                 215                 220
Asn Ile Phe His Lys Asp Pro Asp Ile Lys Ser Leu His Val Phe Val
225                 230                 235                 240
Leu Gly Glu Trp Gln Pro Leu Glu Tyr Gly Lys Lys Lys Leu Lys Tyr
                245                 250                 255
Leu Pro Tyr Asn His Gln His Glu Tyr Phe Phe Leu Ile Gly Pro Pro
            260                 265                 270
Leu Leu Ile Pro Met Tyr Phe Gln Tyr Gln Ile Ile Met Thr Met Ile
        275                 280                 285
Ser Arg Arg Asp Trp Val Asp Leu Ala Trp Ala Ile Ser Tyr Tyr Met
    290                 295                 300
Arg Phe Phe Tyr Thr Tyr Ile Pro Phe Tyr Gly Ile Leu Gly Ala Leu
305                 310                 315                 320
Val Phe Leu Asn Phe Ile Arg Phe Leu Glu Ser His Trp Phe Val Trp
                325                 330                 335
Val Thr Gln Met Asn His Leu Val Met Glu Ile Asp Leu Asp His Tyr
            340                 345                 350
Arg Asp Trp Phe Ser Ser Gln Leu Ala Ala Thr Cys Asn Val Glu Gln
        355                 360                 365
Ser Phe Phe Asn Asp Trp Phe Ser Gly His Leu Asn Phe Gln Ile Glu
    370                 375                 380
His His Leu Phe Pro Thr Met Pro Arg His Asn Leu His Lys Ile Ala
385                 390                 395                 400
Pro Leu Val Lys Ser Leu Cys Ala Lys His Gly Ile Glu Tyr Gln Glu
                405                 410                 415
Lys Pro Leu Leu Arg Ala Leu Ile Asp Ile Val Ser Ser Leu Lys Lys
            420                 425                 430
Ser Gly Glu Leu Trp Leu Asp Ala Tyr Leu His Lys
        435                 440
```

<210> 23
<211> 459
<212> PRT
<213> Pythium irregulare

<400> 23

Met Val Asp Leu Lys Pro Gly Val Lys Arg Leu Val Ser Trp Lys Glu
1 5 10 15
Ile Arg Glu His Ala Thr Pro Ala Thr Ala Trp Ile Val Ile His His
20 25 30
Lys Val Tyr Asp Ile Ser Lys Trp Asp Ser His Pro Gly Gly Ser Val
35 40 45
Met Leu Thr Gln Ala Gly Glu Asp Ala Thr Asp Ala Phe Ala Val Phe
50 55 60
His Pro Ser Ser Ala Leu Lys Leu Leu Glu Gln Phe Tyr Val Gly Asp
65 70 75 80
Val Asp Glu Thr Ser Lys Ala Glu Ile Glu Gly Glu Pro Ala Ser Asp
85 90 95
Glu Glu Arg Ala Arg Arg Glu Arg Ile Asn Glu Phe Ile Ala Ser Tyr
100 105 110
Arg Arg Leu Arg Val Lys Val Lys Gly Met Gly Leu Tyr Asp Ala Ser
115 120 125
Ala Leu Tyr Tyr Ala Trp Lys Leu Val Ser Thr Phe Gly Ile Ala Val
130 135 140
Leu Ser Met Ala Ile Cys Phe Phe Phe Asn Ser Phe Ala Met Tyr Met
145 150 155 160
Val Ala Gly Val Ile Met Gly Leu Phe Tyr Gln Gln Ser Gly Trp Leu
165 170 175
Ala His Asp Phe Leu His Asn Gln Val Cys Glu Asn Arg Thr Leu Gly
180 185 190
Asn Leu Ile Gly Cys Leu Val Gly Asn Ala Trp Gln Gly Phe Ser Val
195 200 205
Gln Trp Trp Lys Asn Lys His Asn Leu His His Ala Val Pro Asn Leu
210 215 220
His Ser Ala Lys Asp Glu Gly Phe Ile Gly Asp Pro Asp Ile Asp Thr
225 230 235 240
Met Pro Leu Leu Ala Trp Ser Lys Glu Met Ala Arg Lys Ala Phe Glu
245 250 255
Ser Ala His Gly Pro Phe Phe Ile Arg Asn Gln Ala Phe Leu Tyr Phe
260 265 270
Pro Leu Leu Leu Leu Ala Arg Leu Ser Trp Leu Ala Gln Ser Phe Phe
275 280 285
Tyr Val Phe Thr Glu Phe Ser Phe Gly Ile Phe Asp Lys Val Glu Phe
290 295 300
Asp Gly Pro Glu Lys Ala Gly Leu Ile Val His Tyr Ile Trp Gln Leu
305 310 315 320
Ala Ile Pro Tyr Phe Cys Asn Met Ser Leu Phe Glu Gly Val Ala Tyr
325 330 335
Phe Leu Met Gly Gln Ala Ser Cys Gly Leu Leu Leu Ala Leu Val Phe
340 345 350
Ser Ile Gly His Asn Gly Met Ser Val Tyr Glu Arg Glu Thr Lys Pro
355 360 365
Asp Phe Trp Gln Leu Gln Val Thr Thr Thr Arg Asn Ile Arg Ala Ser
370 375 380
Val Phe Met Asp Trp Phe Thr Gly Gly Leu Asn Tyr Gln Ile Asp His
385 390 395 400
His Leu Phe Pro Leu Val Pro Arg His Asn Leu Pro Lys Val Asn Val
405 410 415
Leu Ile Lys Ser Leu Cys Lys Glu Phe Asp Ile Pro Phe His Glu Thr
420 425 430

```
Gly Phe Trp Glu Gly Ile Tyr Glu Val Val Asp His Leu Ala Asp Ile
        435                 440                 445
Ser Lys Glu Phe Ile Thr Glu Phe Pro Ala Met
    450                 455
```

<210> 24
<211> 448
<212> PRT
<213> Borago officinalis

<400> 24

```
Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
1               5                   10                  15
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
            20                  25                  30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
        35                  40                  45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
    50                  55                  60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
65                  70                  75                  80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
                85                  90                  95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
            100                 105                 110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
        115                 120                 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
    130                 135                 140
Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145                 150                 155                 160
Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
                165                 170                 175
Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
            180                 185                 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
        195                 200                 205
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
    210                 215                 220
Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
225                 230                 235                 240
Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
                245                 250                 255
Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
            260                 265                 270
Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala Gln Glu Leu Leu Gly
        275                 280                 285
Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
                325                 330                 335
Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
            340                 345                 350
```

```
Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
    370                 375                 380
Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
        435                 440                 445
```

<210> 25
<211> 446
<212> PRT
<213> Anemone leveillei

<400> 25

```
Met Ala Glu Lys Arg Arg Ser Ile Ser Ser Asp Asp Leu Arg Ser His
1               5                  10                  15
Asn Lys Pro Gly Asp Val Trp Ile Ser Ile Gln Gly Lys Ile Tyr Asp
            20                  25                  30
Val Thr Glu Trp Gly Lys Asp His Pro Gly Gly Glu Gly Pro Leu Leu
        35                  40                  45
Asn Leu Ala Gly Gln Asp Val Thr Asp Ala Phe Val Ala Phe His Pro
    50                  55                  60
Gly Ser Ala Trp Lys Asn Leu Asp Lys Phe His Ile Gly Tyr Leu Gln
65                  70                  75                  80
Asp Tyr Val Val Ser Asp Val Ser Lys Asp Tyr Arg Lys Leu Val Ser
                85                  90                  95
Glu Phe Ser Lys Ala Gly Leu Tyr Glu Lys Lys Gly His Gly His Leu
            100                 105                 110
Ile Arg Leu Leu Val Met Ser Leu Val Phe Ile Ala Ser Val Ser Gly
        115                 120                 125
Val Val Leu Ser Asp Lys Thr Ser Val His Val Gly Ser Ala Val Leu
    130                 135                 140
Leu Ala Val Ile Trp Met Gln Phe Gly Phe Ile Gly His Asp Ser Gly
145                 150                 155                 160
His Tyr Asn Ile Met Thr Ser Pro Glu Leu Asn Arg Tyr Met Gln Ile
                165                 170                 175
Phe Ser Val Asn Val Val Ser Gly Val Ser Val Gly Trp Trp Lys Arg
            180                 185                 190
Tyr His Asn Ala His His Ile Ala Val Asn Ser Leu Glu Tyr Asp Pro
        195                 200                 205
Asp Leu Gln Tyr Val Pro Phe Leu Val Val Ser Thr Ala Ile Phe Asp
    210                 215                 220
Ser Leu Thr Ser His Phe Tyr Arg Lys Lys Met Thr Phe Asp Ala Val
225                 230                 235                 240
Ala Arg Phe Leu Val Ser Phe Gln His Trp Thr Phe Tyr Pro Leu Met
                245                 250                 255
Ala Ile Gly Arg Val Ser Phe Leu Ala Gln Ser Ile Gly Val Leu Leu
            260                 265                 270
Ser Lys Lys Pro Leu Pro Asp Arg His Leu Glu Trp Phe Gly Leu Val
        275                 280                 285
```

```
Val Phe Trp Ala Trp Tyr Ser Leu Leu Ile Ser Cys Leu Pro Asn Trp
    290                 295                 300
Trp Glu Arg Val Ile Phe Ile Ala Val Asn Phe Ala Val Thr Gly Ile
305                 310                 315                 320
Gln His Val Gln Phe Cys Leu Asn His Tyr Ser Ala Gln Thr Tyr Ile
                325                 330                 335
Gly Ala Pro Cys Ala Asn Asp Trp Phe Glu Lys Gln Thr Lys Gly Ser
            340                 345                 350
Ile Asp Ile Ser Cys Ser Pro Trp Thr Asp Trp Phe His Gly Gly Leu
        355                 360                 365
Gln Phe Gln Ile Glu His His Leu Phe Pro Arg Met Pro Arg Cys Asn
    370                 375                 380
Leu Arg Lys Ile Ser Pro Phe Val Lys Glu Leu Cys Arg Lys His Asn
385                 390                 395                 400
Leu Val Tyr Thr Ser Val Ser Phe Phe Glu Gly Asn Arg Arg Thr Leu
                405                 410                 415
Ala Thr Leu Lys Asn Ala Ala Leu Lys Ala Arg Asp Leu Thr Ser Pro
            420                 425                 430
Ile Pro Lys Asn Leu Val Trp Glu Ala Val His Thr His Gly
        435                 440                 445
```

<210> 26
<211> 520
<212> PRT
<213> Ceratodon purpureus

<400> 26

```
Met Val Ser Gln Gly Gly Gly Leu Ser Gln Gly Ser Ile Glu Glu Asn
1               5                   10                  15
Ile Asp Val Glu His Leu Ala Thr Met Pro Leu Val Ser Asp Phe Leu
            20                  25                  30
Asn Val Leu Gly Thr Thr Leu Gly Gln Trp Ser Leu Ser Thr Thr Phe
        35                  40                  45
Ala Phe Lys Arg Leu Thr Thr Lys Lys His Ser Ser Asp Ile Ser Val
    50                  55                  60
Glu Ala Gln Lys Glu Ser Val Ala Arg Gly Pro Val Glu Asn Ile Ser
65                  70                  75                  80
Gln Ser Val Ala Gln Pro Ile Arg Arg Arg Trp Val Gln Asp Lys Lys
                85                  90                  95
Pro Val Thr Tyr Ser Leu Lys Asp Val Ala Ser His Asp Met Pro Gln
            100                 105                 110
Asp Cys Trp Ile Ile Ile Lys Glu Lys Val Tyr Asp Val Ser Thr Phe
        115                 120                 125
Ala Glu Gln His Pro Gly Gly Thr Val Ile Asn Thr Tyr Phe Gly Arg
    130                 135                 140
Asp Ala Thr Asp Val Phe Ser Thr Phe His Ala Ser Thr Ser Trp Lys
145                 150                 155                 160
Ile Leu Gln Asn Phe Tyr Ile Gly Asn Leu Val Arg Glu Glu Pro Thr
                165                 170                 175
Leu Glu Leu Leu Lys Glu Tyr Arg Glu Leu Arg Ala Leu Phe Leu Arg
            180                 185                 190
Glu Gln Leu Phe Lys Ser Ser Lys Ser Tyr Tyr Leu Phe Lys Thr Leu
        195                 200                 205
Ile Asn Val Ser Ile Val Ala Thr Ser Ile Ala Ile Ile Ser Leu Tyr
    210                 215                 220
```

Lys Ser Tyr Arg Ala Val Leu Leu Ser Ala Ser Leu Met Gly Leu Phe
225 230 235 240
Ile Gln Gln Cys Gly Trp Leu Ser His Asp Phe Leu His His Gln Val
245 250 255
Phe Glu Thr Arg Trp Leu Asn Asp Val Val Gly Tyr Val Val Gly Asn
260 265 270
Val Val Leu Gly Phe Ser Val Ser Trp Trp Lys Thr Lys His Asn Leu
275 280 285
His His Ala Ala Pro Asn Glu Cys Asp Gln Lys Tyr Thr Pro Ile Asp
290 295 300
Glu Asp Ile Asp Thr Leu Pro Ile Ile Ala Trp Ser Lys Asp Leu Leu
305 310 315 320
Ala Thr Val Glu Ser Lys Thr Met Leu Arg Val Leu Gln Tyr Gln His
325 330 335
Leu Phe Phe Leu Val Leu Leu Thr Phe Ala Arg Ala Ser Trp Leu Phe
340 345 350
Trp Ser Ala Ala Phe Thr Leu Arg Pro Glu Leu Thr Leu Gly Glu Lys
355 360 365
Leu Leu Glu Arg Gly Thr Met Ala Leu His Tyr Ile Trp Phe Asn Ser
370 375 380
Val Ala Phe Tyr Leu Leu Pro Gly Trp Lys Pro Val Val Trp Met Val
385 390 395 400
Val Ser Glu Leu Met Ser Gly Phe Leu Leu Gly Tyr Val Phe Val Leu
405 410 415
Ser His Asn Gly Met Glu Val Tyr Asn Thr Ser Lys Asp Phe Val Asn
420 425 430
Ala Gln Ile Ala Ser Thr Arg Asp Ile Lys Ala Gly Val Phe Asn Asp
435 440 445
Trp Phe Thr Gly Gly Leu Asn Arg Gln Ile Glu His His Leu Phe Pro
450 455 460
Thr Met Pro Arg His Asn Leu Asn Lys Ile Ser Pro His Val Glu Thr
465 470 475 480
Leu Cys Lys Lys His Gly Leu Val Tyr Glu Asp Val Ser Met Ala Ser
485 490 495
Gly Thr Tyr Arg Val Leu Lys Thr Leu Lys Asp Val Ala Asp Ala Ala
500 505 510
Ser His Gln Gln Leu Ala Ala Ser
515 520

<210> 27
<211> 525
<212> PRT
<213> Physcomitrella patens

<400> 27
Met Val Phe Ala Gly Gly Gly Leu Gln Gln Gly Ser Leu Glu Glu Asn
1 5 10 15
Ile Asp Val Glu His Ile Ala Ser Met Ser Leu Phe Ser Asp Phe Phe
20 25 30
Ser Tyr Val Ser Ser Thr Val Gly Ser Trp Ser Val His Ser Ile Gln
35 40 45
Pro Leu Lys Arg Leu Thr Ser Lys Lys Arg Val Ser Glu Ser Ala Ala
50 55 60
Val Gln Cys Ile Ser Ala Glu Val Gln Arg Asn Ser Ser Thr Gln Gly
65 70 75 80

Thr Ala Glu Ala Leu Ala Glu Ser Val Val Lys Pro Thr Arg Arg Arg
85 90 95
Ser Ser Gln Trp Lys Lys Ser Thr His Pro Leu Ser Glu Val Ala Val
100 105 110
His Asn Lys Pro Ser Asp Cys Trp Ile Val Val Lys Asn Lys Val Tyr
115 120 125
Asp Val Ser Asn Phe Ala Asp Glu His Pro Gly Gly Ser Val Ile Ser
130 135 140
Thr Tyr Phe Gly Arg Asp Gly Thr Asp Val Phe Ser Ser Phe His Ala
145 150 155 160
Ala Ser Thr Trp Lys Ile Leu Gln Asp Phe Tyr Ile Gly Asp Val Glu
165 170 175
Arg Val Glu Pro Thr Pro Glu Leu Leu Lys Asp Phe Arg Glu Met Arg
180 185 190
Ala Leu Phe Leu Arg Glu Gln Leu Phe Lys Ser Ser Lys Leu Tyr Tyr
195 200 205
Val Met Lys Leu Leu Thr Asn Val Ala Ile Phe Ala Ala Ser Ile Ala
210 215 220
Ile Ile Cys Trp Ser Lys Thr Ile Ser Ala Val Leu Ala Ser Ala Cys
225 230 235 240
Met Met Ala Leu Cys Phe Gln Gln Cys Gly Trp Leu Ser His Asp Phe
245 250 255
Leu His Asn Gln Val Phe Glu Thr Arg Trp Leu Asn Glu Val Val Gly
260 265 270
Tyr Val Ile Gly Asn Ala Val Leu Gly Phe Ser Thr Gly Trp Trp Lys
275 280 285
Glu Lys His Asn Leu His His Ala Ala Pro Asn Glu Cys Asp Gln Thr
290 295 300
Tyr Gln Pro Ile Asp Glu Asp Ile Asp Thr Leu Pro Leu Ile Ala Trp
305 310 315 320
Ser Lys Asp Ile Leu Ala Thr Val Glu Asn Lys Thr Phe Leu Arg Ile
325 330 335
Leu Gln Tyr Gln His Leu Phe Phe Met Gly Leu Leu Phe Phe Ala Arg
340 345 350
Gly Ser Trp Leu Phe Trp Ser Trp Arg Tyr Thr Ser Thr Ala Val Leu
355 360 365
Ser Pro Val Asp Arg Leu Leu Glu Lys Gly Thr Val Leu Phe His Tyr
370 375 380
Phe Trp Phe Val Gly Thr Ala Cys Tyr Leu Leu Pro Gly Trp Lys Pro
385 390 395 400
Leu Val Trp Met Ala Val Thr Glu Leu Met Ser Gly Met Leu Leu Gly
405 410 415
Phe Val Phe Val Leu Ser His Asn Gly Met Glu Val Tyr Asn Ser Ser
420 425 430
Lys Glu Phe Val Ser Ala Gln Ile Val Ser Thr Arg Asp Ile Lys Gly
435 440 445
Asn Ile Phe Asn Asp Trp Phe Thr Gly Gly Leu Asn Arg Gln Ile Glu
450 455 460
His His Leu Phe Pro Thr Met Pro Arg His Asn Leu Asn Lys Ile Ala
465 470 475 480
Pro Arg Val Glu Val Phe Cys Lys Lys His Gly Leu Val Tyr Glu Asp
485 490 495
Val Ser Ile Ala Thr Gly Thr Cys Lys Val Leu Lys Ala Leu Lys Glu
500 505 510
Val Ala Glu Ala Ala Ala Glu Gln His Ala Thr Thr Ser
515 520 525

<210> 28
<211> 457
<212> PRT
<213> Mortierella alpina

<400> 28

Met	Ala	Ala	Ala	Pro	Ser	Val	Arg	Thr	Phe	Thr	Arg	Ala	Glu	Ile	Leu
1				5				10					15		
Asn	Ala	Glu	Ala	Leu	Asn	Glu	Gly	Lys	Lys	Asp	Ala	Glu	Ala	Pro	Phe
		20					25					30			
Leu	Met	Ile	Ile	Asp	Asn	Lys	Val	Tyr	Asp	Val	Arg	Glu	Phe	Val	Pro
	35					40					45				
Asp	His	Pro	Gly	Gly	Ser	Val	Ile	Leu	Thr	His	Val	Gly	Lys	Asp	Gly
50					55					60					
Thr	Asp	Val	Phe	Asp	Thr	Phe	His	Pro	Glu	Ala	Ala	Trp	Glu	Thr	Leu
65				70					75					80	
Ala	Asn	Phe	Tyr	Val	Gly	Asp	Ile	Asp	Glu	Ser	Asp	Arg	Ala	Ile	Lys
				85					90					95	
Asn	Asp	Asp	Phe	Ala	Ala	Glu	Val	Arg	Lys	Leu	Arg	Thr	Leu	Phe	Gln
			100					105					110		
Ser	Leu	Gly	Tyr	Tyr	Asp	Ser	Ser	Lys	Ala	Tyr	Tyr	Ala	Phe	Lys	Val
		115					120					125			
Ser	Phe	Asn	Leu	Cys	Ile	Trp	Gly	Leu	Ser	Thr	Phe	Ile	Val	Ala	Lys
	130					135					140				
Trp	Gly	Gln	Thr	Ser	Thr	Leu	Ala	Asn	Val	Leu	Ser	Ala	Ala	Leu	Leu
145					150					155					160
Gly	Leu	Phe	Trp	Gln	Gln	Cys	Gly	Trp	Leu	Ala	His	Asp	Phe	Leu	His
				165					170					175	
His	Gln	Val	Phe	Gln	Asp	Arg	Phe	Trp	Gly	Asp	Leu	Phe	Gly	Ala	Phe
			180					185					190		
Leu	Gly	Gly	Val	Cys	Gln	Gly	Phe	Ser	Ser	Ser	Trp	Trp	Lys	Asp	Lys
		195					200					205			
His	Asn	Thr	His	His	Ala	Ala	Pro	Asn	Val	His	Gly	Glu	Asp	Pro	Asp
	210					215					220				
Ile	Asp	Thr	His	Pro	Leu	Leu	Thr	Trp	Ser	Glu	His	Ala	Leu	Glu	Met
225					230					235					240
Phe	Ser	Asp	Val	Pro	Asp	Glu	Glu	Leu	Thr	Arg	Met	Trp	Ser	Arg	Phe
				245					250					255	
Met	Val	Leu	Asn	Gln	Thr	Trp	Phe	Tyr	Phe	Pro	Ile	Leu	Ser	Phe	Ala
			260					265					270		
Arg	Leu	Ser	Trp	Cys	Leu	Gln	Ser	Ile	Met	Phe	Val	Leu	Pro	Asn	Gly
		275					280					285			
Gln	Ala	His	Lys	Pro	Ser	Gly	Ala	Arg	Val	Pro	Ile	Ser	Leu	Val	Glu
	290					295					300				
Gln	Leu	Ser	Leu	Ala	Met	His	Trp	Thr	Trp	Tyr	Leu	Ala	Thr	Met	Phe
305					310					315					320
Leu	Phe	Ile	Lys	Asp	Pro	Val	Asn	Met	Ile	Val	Tyr	Phe	Leu	Val	Ser
				325					330					335	
Gln	Ala	Val	Cys	Gly	Asn	Leu	Leu	Ala	Ile	Val	Phe	Ser	Leu	Asn	His
			340					345					350		
Asn	Gly	Met	Pro	Val	Ile	Ser	Lys	Glu	Glu	Ala	Val	Asp	Met	Asp	Phe
		355					360					365			
Phe	Thr	Lys	Gln	Ile	Ile	Thr	Gly	Arg	Asp	Val	His	Pro	Gly	Leu	Phe
	370					375					380				
Ala	Asn	Trp	Phe	Thr	Gly	Gly	Leu	Asn	Tyr	Gln	Ile	Glu	His	His	Leu
385					390					395					400

```
Phe Pro Ser Met Pro Arg His Asn Phe Ser Lys Ile Gln Pro Ala Val
                405                 410                 415
Glu Thr Leu Cys Lys Lys Tyr Gly Val Arg Tyr His Thr Thr Gly Met
            420                 425                 430
Ile Glu Gly Thr Ala Glu Val Phe Ser Arg Leu Asn Glu Val Ser Lys
        435                 440                 445
Ala Ala Ser Lys Met Gly Lys Ala Gln
    450                 455
```

<210> 29
<211> 443
<212> PRT
<213> Caenorhabditis elegans

<400> 29

```
Met Val Val Asp Lys Asn Ala Ser Gly Leu Arg Met Lys Val Asp Gly
1               5                  10                  15
Lys Trp Leu Tyr Leu Ser Glu Glu Leu Val Lys Lys His Pro Gly Gly
            20                  25                  30
Ala Val Ile Glu Gln Tyr Arg Asn Ser Asp Ala Thr His Ile Phe His
        35                  40                  45
Ala Phe His Glu Gly Ser Ser Gln Ala Tyr Lys Gln Leu Asp Leu Leu
    50                  55                  60
Lys Lys His Gly Glu His Asp Glu Phe Leu Glu Lys Gln Leu Glu Lys
65                  70                  75                  80
Arg Leu Asp Lys Val Asp Ile Asn Val Ser Ala Tyr Asp Val Ser Val
                85                  90                  95
Ala Gln Glu Lys Lys Met Val Glu Ser Phe Glu Lys Leu Arg Gln Lys
            100                 105                 110
Leu His Asp Asp Gly Leu Met Lys Ala Asn Glu Thr Tyr Phe Leu Phe
        115                 120                 125
Lys Ala Ile Ser Thr Leu Ser Ile Met Ala Phe Ala Phe Tyr Leu Gln
    130                 135                 140
Tyr Leu Gly Trp Tyr Ile Thr Ser Ala Cys Leu Leu Ala Leu Ala Trp
145                 150                 155                 160
Gln Gln Phe Gly Trp Leu Thr His Glu Phe Cys His Gln Gln Pro Thr
                165                 170                 175
Lys Asn Arg Pro Leu Asn Asp Thr Ile Ser Leu Phe Phe Gly Asn Phe
            180                 185                 190
Leu Gln Gly Phe Ser Arg Asp Trp Trp Lys Asp Lys His Asn Thr His
        195                 200                 205
His Ala Ala Thr Asn Val Ile Asp His Asp Gly Asp Ile Asp Leu Ala
    210                 215                 220
Pro Leu Phe Ala Phe Ile Pro Gly Asp Leu Cys Lys Tyr Lys Ala Ser
225                 230                 235                 240
Phe Glu Lys Ala Ile Leu Lys Ile Val Pro Tyr Gln His Leu Tyr Phe
                245                 250                 255
Thr Ala Met Leu Pro Met Leu Arg Phe Ser Trp Thr Gly Gln Ser Val
            260                 265                 270
Gln Trp Val Phe Lys Glu Asn Gln Met Glu Tyr Lys Val Tyr Gln Arg
        275                 280                 285
Asn Ala Phe Trp Glu Gln Ala Thr Ile Val Gly His Trp Ala Trp Val
    290                 295                 300
Phe Tyr Gln Leu Phe Leu Leu Pro Thr Trp Pro Leu Arg Val Ala Tyr
305                 310                 315                 320
```

```
Phe Ile Ile Ser Gln Met Gly Gly Gly Leu Leu Ile Ala His Val Val
                325                 330                 335
Thr Phe Asn His Asn Ser Val Asp Lys Tyr Pro Ala Asn Ser Arg Ile
            340                 345                 350
Leu Asn Asn Phe Ala Ala Leu Gln Ile Leu Thr Thr Arg Asn Met Thr
        355                 360                 365
Pro Ser Pro Phe Ile Asp Trp Leu Trp Gly Gly Leu Asn Tyr Gln Ile
    370                 375                 380
Glu His His Leu Phe Pro Thr Met Pro Arg Cys Asn Leu Asn Ala Cys
385                 390                 395                 400
Val Lys Tyr Val Lys Glu Trp Cys Lys Glu Asn Asn Leu Pro Tyr Leu
                405                 410                 415
Val Asp Asp Tyr Phe Asp Gly Tyr Ala Met Asn Leu Gln Gln Leu Lys
            420                 425                 430
Asn Met Ala Glu His Ile Gln Ala Lys Ala Ala
        435                 440
```

<210> 30
<211> 299
<212> PRT
<213> Homo sapiens

<400> 30

```
Met Glu His Phe Asp Ala Ser Leu Ser Thr Tyr Phe Lys Ala Leu Leu
1               5                   10                  15
Gly Pro Arg Asp Thr Arg Val Lys Gly Trp Phe Leu Leu Asp Asn Tyr
            20                  25                  30
Ile Pro Thr Phe Ile Cys Ser Val Ile Tyr Leu Leu Ile Val Trp Leu
        35                  40                  45
Gly Pro Lys Tyr Met Arg Asn Lys Gln Pro Phe Ser Cys Arg Gly Ile
    50                  55                  60
Leu Val Val Tyr Asn Leu Gly Leu Thr Leu Leu Ser Leu Tyr Met Phe
65                  70                  75                  80
Cys Glu Leu Val Thr Gly Val Trp Glu Gly Lys Tyr Asn Phe Phe Cys
                85                  90                  95
Gln Gly Thr Arg Thr Ala Gly Glu Ser Asp Met Lys Ile Ile Arg Val
            100                 105                 110
Leu Trp Trp Tyr Tyr Phe Ser Lys Leu Ile Glu Phe Met Asp Thr Phe
        115                 120                 125
Phe Phe Ile Leu Arg Lys Asn Asn His Gln Ile Thr Val Leu His Val
    130                 135                 140
Tyr His His Ala Ser Met Leu Asn Ile Trp Trp Phe Val Met Asn Trp
145                 150                 155                 160
Val Pro Cys Gly His Ser Tyr Phe Gly Ala Thr Leu Asn Ser Phe Ile
                165                 170                 175
His Val Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Ser Val Pro Ser Met
            180                 185                 190
Arg Pro Tyr Leu Trp Trp Lys Lys Tyr Ile Thr Gln Gly Gln Leu Leu
        195                 200                 205
Gln Phe Val Leu Thr Ile Ile Gln Thr Ser Cys Gly Val Ile Trp Pro
    210                 215                 220
Cys Thr Phe Pro Leu Gly Trp Leu Tyr Phe Gln Ile Gly Tyr Met Ile
225                 230                 235                 240
Ser Leu Ile Ala Leu Phe Thr Asn Phe Tyr Ile Gln Thr Tyr Asn Lys
                245                 250                 255
```

Lys Gly Ala Ser Arg Arg Lys Asp His Leu Lys Asp His Gln Asn Gly
260 265 270
Ser Met Ala Ala Val Asn Gly His Thr Asn Ser Phe Ser Pro Leu Glu
275 280 285
Asn Asn Val Lys Pro Arg Lys Leu Arg Lys Asp
290 295

<210> 31
<211> 290
<212> PRT
<213> Physcomitrella patens

<400> 31
Met Glu Val Val Glu Arg Phe Tyr Gly Glu Leu Asp Gly Lys Val Ser
1 5 10 15
Gln Gly Val Asn Ala Leu Leu Gly Ser Phe Gly Val Glu Leu Thr Asp
20 25 30
Thr Pro Thr Thr Lys Gly Leu Pro Leu Val Asp Ser Pro Thr Pro Ile
35 40 45
Val Leu Gly Val Ser Val Tyr Leu Thr Ile Val Ile Gly Gly Leu Leu
50 55 60
Trp Ile Lys Ala Arg Asp Leu Lys Pro Arg Ala Ser Glu Pro Phe Leu
65 70 75 80
Leu Gln Ala Leu Val Leu Val His Asn Leu Phe Cys Phe Ala Leu Ser
85 90 95
Leu Tyr Met Cys Val Gly Ile Ala Tyr Gln Ala Ile Thr Trp Arg Tyr
100 105 110
Ser Leu Trp Gly Asn Ala Tyr Asn Pro Lys His Lys Glu Met Ala Ile
115 120 125
Leu Val Tyr Leu Phe Tyr Met Ser Lys Tyr Val Glu Phe Met Asp Thr
130 135 140
Val Ile Met Ile Leu Lys Arg Ser Thr Arg Gln Ile Ser Phe Leu His
145 150 155 160
Val Tyr His His Ser Ser Ile Ser Leu Ile Trp Trp Ala Ile Ala His
165 170 175
His Ala Pro Gly Gly Glu Ala Tyr Trp Ser Ala Ala Leu Asn Ser Gly
180 185 190
Val His Val Leu Met Tyr Ala Tyr Tyr Phe Leu Ala Ala Cys Leu Arg
195 200 205
Ser Ser Pro Lys Leu Lys Asn Lys Tyr Leu Phe Trp Gly Arg Tyr Leu
210 215 220
Thr Gln Phe Gln Met Phe Gln Phe Met Leu Asn Leu Val Gln Ala Tyr
225 230 235 240
Tyr Asp Met Lys Thr Asn Ala Pro Tyr Pro Gln Trp Leu Ile Lys Ile
245 250 255
Leu Phe Tyr Tyr Met Ile Ser Leu Leu Phe Leu Phe Gly Asn Phe Tyr
260 265 270
Val Gln Lys Tyr Ile Lys Pro Ser Asp Gly Lys Gln Lys Gly Ala Lys
275 280 285
Thr Glu
290

<210> 32
<211> 318

<212> PRT
<213> Mortierella alpina

<400> 32
Met Glu Ser Ile Ala Pro Phe Leu Pro Ser Lys Met Pro Gln Asp Leu
1 5 10 15
Phe Met Asp Leu Ala Thr Ala Ile Gly Val Arg Ala Ala Pro Tyr Val
20 25 30
Asp Pro Leu Glu Ala Ala Leu Val Ala Gln Ala Glu Lys Tyr Ile Pro
35 40 45
Thr Ile Val His His Thr Arg Gly Phe Leu Val Ala Val Glu Ser Pro
50 55 60
Leu Ala Arg Glu Leu Pro Leu Met Asn Pro Phe His Val Leu Leu Ile
65 70 75 80
Val Leu Ala Tyr Leu Val Thr Val Phe Val Gly Met Gln Ile Met Lys
85 90 95
Asn Phe Glu Arg Phe Glu Val Lys Thr Phe Ser Leu Leu His Asn Phe
100 105 110
Cys Leu Val Ser Ile Ser Ala Tyr Met Cys Gly Gly Ile Leu Tyr Glu
115 120 125
Ala Tyr Gln Ala Asn Tyr Gly Leu Phe Glu Asn Ala Ala Asp His Thr
130 135 140
Phe Lys Gly Leu Pro Met Ala Lys Met Ile Trp Leu Phe Tyr Phe Ser
145 150 155 160
Lys Ile Met Glu Phe Val Asp Thr Met Ile Met Val Leu Lys Lys Asn
165 170 175
Asn Arg Gln Ile Ser Phe Leu His Val Tyr His His Ser Ser Ile Phe
180 185 190
Thr Ile Trp Trp Leu Val Thr Phe Val Ala Pro Asn Gly Glu Ala Tyr
195 200 205
Phe Ser Ala Ala Leu Asn Ser Phe Ile His Val Ile Met Tyr Gly Tyr
210 215 220
Tyr Phe Leu Ser Ala Leu Gly Phe Lys Gln Val Ser Phe Ile Lys Phe
225 230 235 240
Tyr Ile Thr Arg Ser Gln Met Thr Gln Phe Cys Met Met Ser Val Gln
245 250 255
Ser Ser Trp Asp Met Tyr Ala Met Lys Val Leu Gly Arg Pro Gly Tyr
260 265 270
Pro Phe Phe Ile Thr Ala Leu Leu Trp Phe Tyr Met Trp Thr Met Leu
275 280 285
Gly Leu Phe Tyr Asn Phe Tyr Arg Lys Asn Ala Lys Leu Ala Lys Gln
290 295 300
Ala Lys Ala Asp Ala Ala Lys Glu Lys Ala Arg Lys Leu Gln
305 310 315

<210> 33
<211> 519
<212> PRT
<213> Thraustochytrium sp.

<400> 33
Met Thr Val Gly Tyr Asp Glu Glu Ile Pro Phe Glu Gln Val Arg Ala
1 5 10 15
His Asn Lys Pro Asp Asp Ala Trp Cys Ala Ile His Gly His Val Tyr
20 25 30

Asp Val Thr Lys Phe Ala Ser Val His Pro Gly Gly Asp Ile Ile Leu
35 40 45
Leu Ala Ala Gly Lys Glu Ala Thr Val Leu Tyr Glu Thr Tyr His Val
50 55 60
Arg Gly Val Ser Asp Ala Val Leu Arg Lys Tyr Arg Ile Gly Lys Leu
65 70 75 80
Pro Asp Gly Gln Gly Gly Ala Asn Glu Lys Glu Lys Arg Thr Leu Ser
85 90 95
Gly Leu Ser Ser Ala Ser Tyr Tyr Thr Trp Asn Ser Asp Phe Tyr Arg
100 105 110
Val Met Arg Glu Arg Val Val Ala Arg Leu Lys Glu Arg Gly Lys Ala
115 120 125
Arg Arg Gly Gly Tyr Glu Leu Trp Ile Lys Ala Phe Leu Leu Leu Val
130 135 140
Gly Phe Trp Ser Ser Leu Tyr Trp Met Cys Thr Leu Asp Pro Ser Phe
145 150 155 160
Gly Ala Ile Leu Ala Ala Met Ser Leu Gly Val Phe Ala Ala Phe Val
165 170 175
Gly Thr Cys Ile Gln His Asp Gly Asn His Gly Ala Phe Ala Gln Ser
180 185 190
Arg Trp Val Asn Lys Val Ala Gly Trp Thr Leu Asp Met Ile Gly Ala
195 200 205
Ser Gly Met Thr Trp Glu Phe Gln His Val Leu Gly His His Pro Tyr
210 215 220
Thr Asn Leu Ile Glu Glu Glu Asn Gly Leu Gln Lys Val Ser Gly Lys
225 230 235 240
Lys Met Asp Thr Lys Leu Ala Asp Gln Glu Ser Asp Pro Asp Val Phe
245 250 255
Ser Thr Tyr Pro Met Met Arg Leu His Pro Trp His Gln Lys Arg Trp
260 265 270
Tyr His Arg Phe Gln His Ile Tyr Gly Pro Phe Ile Phe Gly Phe Met
275 280 285
Thr Ile Asn Lys Val Val Thr Gln Asp Val Gly Val Val Leu Arg Lys
290 295 300
Arg Leu Phe Gln Ile Asp Ala Glu Cys Arg Tyr Ala Ser Pro Met Tyr
305 310 315 320
Val Ala Arg Phe Trp Ile Met Lys Ala Leu Thr Val Leu Tyr Met Val
325 330 335
Ala Leu Pro Cys Tyr Met Gln Gly Pro Trp His Gly Leu Lys Leu Phe
340 345 350
Ala Ile Ala His Phe Thr Cys Gly Glu Val Leu Ala Thr Met Phe Ile
355 360 365
Val Asn His Ile Ile Glu Gly Val Ser Tyr Ala Ser Lys Asp Ala Val
370 375 380
Lys Gly Thr Met Ala Pro Pro Lys Thr Met His Gly Val Thr Pro Met
385 390 395 400
Asn Asn Thr Arg Lys Glu Val Glu Ala Glu Ala Ser Lys Ser Gly Ala
405 410 415
Val Val Lys Ser Val Pro Leu Asp Asp Trp Ala Val Val Gln Cys Gln
420 425 430
Thr Ser Val Asn Trp Ser Val Gly Ser Trp Phe Trp Asn His Phe Ser
435 440 445
Gly Gly Leu Asn His Gln Ile Glu His His Leu Phe Pro Gly Leu Ser
450 455 460
His Glu Thr Tyr Tyr His Ile Gln Asp Val Phe Gln Ser Thr Cys Ala
465 470 475 480

Glu Tyr Gly Val Pro Tyr Gln His Glu Pro Ser Leu Trp Thr Ala Tyr
485 490 495
Trp Lys Met Leu Glu His Leu Arg Gln Leu Gly Asn Glu Glu Thr His
500 505 510
Glu Ser Trp Gln Arg Ala Ala
515

<210> 34
<211> 541
<212> PRT
<213> Euglena gracilis

<400> 34
Met Leu Val Leu Phe Gly Asn Phe Tyr Val Lys Gln Tyr Ser Gln Lys
1 5 10 15
Asn Gly Lys Pro Glu Asn Gly Ala Thr Pro Glu Asn Gly Ala Lys Pro
20 25 30
Gln Pro Cys Glu Asn Gly Thr Val Glu Lys Arg Glu Asn Asp Thr Ala
35 40 45
Asn Val Arg Pro Thr Arg Pro Ala Gly Pro Pro Pro Ala Thr Tyr Tyr
50 55 60
Asp Ser Leu Ala Val Ser Gly Gln Gly Lys Glu Arg Leu Phe Thr Thr
65 70 75 80
Asp Glu Val Arg Arg His Ile Leu Pro Thr Asp Gly Trp Leu Thr Cys
85 90 95
His Glu Gly Val Tyr Asp Val Thr Asp Phe Leu Ala Lys His Pro Gly
100 105 110
Gly Gly Val Ile Thr Leu Gly Leu Gly Arg Asp Cys Thr Ile Leu Ile
115 120 125
Glu Ser Tyr His Pro Ala Gly Arg Pro Asp Lys Val Met Glu Lys Tyr
130 135 140
Arg Ile Gly Thr Leu Gln Asp Pro Lys Thr Phe Tyr Ala Trp Gly Glu
145 150 155 160
Ser Asp Phe Tyr Pro Glu Leu Lys Arg Arg Ala Leu Ala Arg Leu Lys
165 170 175
Glu Ala Gly Gln Ala Arg Arg Gly Gly Leu Gly Val Lys Ala Leu Leu
180 185 190
Val Leu Thr Leu Phe Phe Val Ser Trp Tyr Met Trp Val Ala His Lys
195 200 205
Ser Phe Leu Trp Ala Ala Val Trp Gly Phe Ala Gly Ser His Val Gly
210 215 220
Leu Ser Ile Gln His Asp Gly Asn His Gly Ala Phe Ser Arg Asn Thr
225 230 235 240
Leu Val Asn Arg Leu Ala Gly Trp Gly Met Asp Leu Ile Gly Ala Ser
245 250 255
Ser Thr Val Trp Glu Tyr Gln His Val Ile Gly His His Gln Tyr Thr
260 265 270
Asn Leu Val Ser Asp Thr Leu Phe Ser Leu Pro Glu Asn Asp Pro Asp
275 280 285
Val Phe Ser Ser Tyr Pro Leu Met Arg Met His Pro Asp Thr Ala Trp
290 295 300
Gln Pro His His Arg Phe Gln His Leu Phe Ala Phe Pro Leu Phe Ala
305 310 315 320
Leu Met Thr Ile Ser Lys Val Leu Thr Ser Asp Phe Ala Val Cys Leu
325 330 335

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Ser Met Lys Lys Gly Ser Ile Asp Cys Ser Ser Arg Leu Val Pro Leu
            340                 345                 350
Glu Gly Gln Leu Leu Phe Trp Gly Ala Lys Leu Ala Asn Phe Leu Leu
        355                 360                 365
Gln Ile Val Leu Pro Cys Tyr Leu His Gly Thr Ala Met Gly Leu Ala
    370                 375                 380
Leu Phe Ser Val Ala His Leu Val Ser Gly Glu Tyr Leu Ala Ile Cys
385                 390                 395                 400
Phe Ile Ile Asn His Ile Ser Glu Ser Cys Glu Phe Met Asn Thr Ser
                405                 410                 415
Phe Gln Thr Ala Ala Arg Arg Thr Glu Met Leu Gln Ala Ala His Gln
            420                 425                 430
Ala Ala Glu Ala Lys Lys Val Lys Pro Thr Pro Pro Pro Asn Asp Trp
        435                 440                 445
Ala Val Thr Gln Val Gln Cys Cys Val Asn Trp Arg Ser Gly Gly Val
    450                 455                 460
Leu Ala Asn His Leu Ser Gly Gly Leu Asn His Gln Ile Glu His His
465                 470                 475                 480
Leu Phe Pro Ser Ile Ser His Ala Asn Tyr Pro Thr Ile Ala Pro Val
                485                 490                 495
Val Lys Glu Val Cys Glu Glu Tyr Gly Leu Pro Tyr Lys Asn Tyr Val
            500                 505                 510
Thr Phe Trp Asp Ala Val Cys Gly Met Val Gln His Leu Arg Leu Met
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Gly Ala Pro Pro Val Pro Thr Asn Gly Asp Lys Lys Ser
    530                 535                 540
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<210> 35
<211> 263
<212> PRT
<213> Isochrysis galbana

<400> 35

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Asp Pro Glu Ile Leu Ile Gly Thr Phe Ser Tyr Leu Leu Leu Lys Pro
            20                  25                  30
Leu Leu Arg Asn Ser Gly Leu Val Asp Glu Lys Lys Gly Ala Tyr Arg
        35                  40                  45
Thr Ser Met Ile Trp Tyr Asn Val Leu Leu Ala Leu Phe Ser Ala Leu
    50                  55                  60
Ser Phe Tyr Val Thr Ala Thr Ala Leu Gly Trp Asp Tyr Gly Thr Gly
65                  70                  75                  80
Ala Trp Leu Arg Arg Gln Thr Gly Asp Thr Pro Gln Pro Leu Phe Gln
                85                  90                  95
Cys Pro Ser Pro Val Trp Asp Ser Lys Leu Phe Thr Trp Thr Ala Lys
            100                 105                 110
Ala Phe Tyr Tyr Ser Lys Tyr Val Glu Tyr Leu Asp Thr Ala Trp Leu
        115                 120                 125
Val Leu Lys Gly Lys Arg Val Ser Phe Leu Gln Ala Phe His His Phe
    130                 135                 140
Gly Ala Pro Trp Asp Val Tyr Leu Gly Ile Arg Leu His Asn Glu Gly
145                 150                 155                 160
Val Trp Ile Phe Met Phe Phe Asn Ser Phe Ile His Thr Ile Met Tyr
                165                 170                 175
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Thr Tyr Tyr Gly Leu Thr Ala Ala Gly Tyr Lys Phe Lys Ala Lys Pro
            180                 185                 190
Leu Ile Thr Ala Met Gln Ile Cys Gln Phe Val Gly Gly Phe Leu Leu
        195                 200                 205
Val Trp Asp Tyr Ile Asn Val Pro Cys Phe Asn Ser Asp Lys Gly Lys
    210                 215                 220
Leu Phe Ser Trp Ala Phe Asn Tyr Ala Tyr Val Gly Ser Val Phe Leu
225                 230                 235                 240
Leu Phe Cys His Phe Phe Tyr Gln Asp Asn Leu Ala Thr Lys Lys Ser
                245                 250                 255
Ala Lys Ala Gly Lys Gln Leu
            260
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<210> 36
<211> 419
<212> PRT
<213> Euglena gracilis

<400> 36

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Thr Tyr Asp Val Val Asn Phe His Pro Gly Gly Ala Glu Ile Ile Glu
            20                  25                  30
Asn Tyr Gln Gly Arg Asp Ala Thr Asp Ala Phe Met Val Met His Phe
        35                  40                  45
Gln Glu Ala Phe Asp Lys Leu Lys Arg Met Pro Lys Ile Asn Pro Ser
    50                  55                  60
Phe Glu Leu Pro Pro Gln Ala Ala Val Asn Glu Ala Gln Glu Asp Phe
65                  70                  75                  80
Arg Lys Leu Arg Glu Glu Leu Ile Ala Thr Gly Met Phe Asp Ala Ser
                85                  90                  95
Pro Leu Trp Tyr Ser Tyr Lys Ile Ser Thr Thr Leu Gly Leu Gly Val
            100                 105                 110
Leu Gly Tyr Phe Leu Met Val Gln Tyr Gln Met Tyr Phe Ile Gly Ala
        115                 120                 125
Val Leu Leu Gly Met His Tyr Gln Gln Met Gly Trp Leu Ser His Asp
    130                 135                 140
Ile Cys His His Gln Thr Phe Lys Asn Arg Asn Trp Asn Asn Leu Val
145                 150                 155                 160
Gly Leu Val Phe Gly Asn Gly Leu Gln Gly Phe Ser Val Thr Cys Trp
                165                 170                 175
Lys Asp Arg His Asn Ala His His Ser Ala Thr Asn Val Gln Gly His
            180                 185                 190
Asp Pro Asp Ile Asp Asn Leu Pro Pro Leu Ala Trp Ser Glu Asp Asp
        195                 200                 205
Val Thr Arg Ala Ser Pro Ile Ser Arg Lys Leu Ile Gln Phe Gln Gln
    210                 215                 220
Tyr Tyr Phe Leu Val Ile Cys Ile Leu Leu Arg Phe Ile Trp Cys Phe
225                 230                 235                 240
Gln Cys Val Leu Thr Val Arg Ser Leu Lys Asp Arg Asp Asn Gln Phe
                245                 250                 255
Tyr Arg Ser Gln Tyr Lys Lys Glu Ala Ile Gly Leu Ala Leu His Trp
            260                 265                 270
Thr Leu Lys Ala Leu Phe His Leu Phe Phe Met Pro Ser Ile Leu Thr
        275                 280                 285
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Ser Leu Leu Val Phe Phe Val Ser Glu Leu Val Gly Gly Phe Gly Ile
290 295 300
Ala Ile Val Val Phe Met Asn His Tyr Pro Leu Glu Lys Ile Gly Asp
305 310 315 320
Pro Val Trp Asp Gly His Gly Phe Ser Val Gly Gln Ile His Glu Thr
325 330 335
Met Asn Ile Arg Arg Gly Ile Ile Thr Asp Trp Phe Phe Gly Gly Leu
340 345 350
Asn Tyr Gln Ile Glu His His Leu Trp Pro Thr Leu Pro Arg His Asn
355 360 365
Leu Thr Ala Val Ser Tyr Gln Val Glu Gln Leu Cys Gln Lys His Asn
370 375 380
Leu Pro Tyr Arg Asn Pro Leu Pro His Glu Gly Leu Val Ile Leu Leu
385 390 395 400
Arg Tyr Leu Ala Val Phe Ala Arg Met Ala Glu Lys Gln Pro Ala Gly
405 410 415
Lys Ala Leu

<210> 37
<211> 867
<212> DNA
<213> Caenorhabditis elegans

<400> 37
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gtggctattg ctactcatgg gccaaagaat ttccctgacg cagaaggtcg caagttcttt 120
gctgatcact ttgatgttac tattcaggct tcaatcctgt acatggtcgt tgtgttcgga 180
acaaaatggt tcatgcgtaa tcgtcaacca ttccaattga ctattccact caacatctgg 240
aatttcatcc tcgccgcatt ttccatcgca ggagctgtca aaatgacccc agagttcttt 300
ggaaccattg ccaacaaagg aattgtcgca tcctactgca aagtgtttga tttcacgaaa 360
ggagagaatg gatactgggt gtggctcttc atggcttcca aacttttcga acttgttgac 420
accatcttct tggttctccg taaacgtcca ctcatgttcc ttcactggta tcaccatatt 480
ctcaccatga tctacgcctg gtactctcat ccattgaccc caggattcaa cagatacgga 540
atttatctta actttgtcgt ccacgccttc atgtactctt actacttcct tcgctcgatg 600
aagattcgcg tgccaggatt catcgcccaa gctatcacat ctcttcaaat cgttcaattc 660
atcatctctt gcgccgttct tgctcatctt ggttatctca tgcacttcac caatgccaac 720
tgtgatttcg agccatcagt attcaagctc gcagttttca tggacacaac atacttggct 780
cttttcgtca acttcttcct ccaatcatat gttctccgcg gaggaaaaga caagtacaag 840
gcagtgccaa agaagaagaa caactaa 867

<210> 38
<211> 1335
<212> DNA
<213> Danio rerio

<400> 38
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tacacctggg aggaggtgca gaaacacacc aaacatggag atcagtgggt ggtggtggag 120
aggaaggttt ataacgtcag ccagtgggtg aagagacacc ccggaggact gaggatcctc 180
ggacactatg ctggagaaga cgccacggag gcgttcactg cgtttcatcc aaaccttcag 240
ctggtgagga aatacctgaa gccgctgcta atcggagagc tggaggcgtc tgaacccagt 300
caggaccggc agaaaaacgc tgctctcgtg gaggatttcc gagccctgcg tgagcgtctg 360
gaggctgaag gctgttttaa aacgcagccg ctgtttttcg ctctgcattt gggccacatt 420
ctgctcctgg aggccatcgc tttcatgatg gtgtggtatt tcggcaccgg ttggatcaac 480

acgctcatcg tcgctgttat tctggctact gcacagtcac aagctggatg gttgcagcat 540
gacttcggtc atctgtccgt gtttaaaacc tctggaatga atcatttggt gcacaaattt 600
gtcatcggac acctgaaggg agcgtctgcg ggctggtgga accatcggca cttccagcat 660
cacgctaaac ccaacatctt caagaaggac ccggacgtca acatgctgaa cgcctttgtg 720
gtgggaaacg tgcagcccgt ggagtatggc gttaagaaga tcaagcatct gccctacaac 780
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ttccagatcg agcatcatct ctttcccaca gtgcctcggc acaactactg gcgcgccgct 1200
ccacgggtgc gagcgttgtg tgagaaatac ggagtcaaat accaagagaa gaccttgtac 1260
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tatctcaaca aataa 1335

<210> 39
<211> 31
<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 39
cccaagctta ctatgggtgg cggaggacag c 31

<210> 40
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 40
ccgctggagt tatttgttga gatacgc 27

<210> 41
<211> 27
<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 41
gcgggtacca tggctcagca tccgctc 27

<210> 42
<211> 27

<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 42
gcgggatcct tagttgttct tcttctt 27

<210> 43
<211> 6
<212> PRT
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<220>
<223> Conserved region

<400> 43
Asp His Pro Gly Gly Ser
1 5

<210> 44
<211> 7
<212> PRT
<213> Artificial sequence

<220>
<223> Conserved region

<400> 44
Trp Trp Lys Asp Lys His Asn
1 5

<210> 45
<211> 7
<212> PRT
<213> Artificial sequence

<220>
<223> Conserved region

<400> 45
Gln Ile Glu His His Leu Phe
1 5

<210> 46
<211> 21
<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 46
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<210> 47
<211> 30
<212> DNA
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<220>
<223> Oligonucleotide primer

<220>
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<223> n = any nucleotide

<400> 47
gcgagggatc caaggraana rrtgrtgytc 30

<210> 48
<211> 6
<212> PRT
<213> Artificial sequence

<220>
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<220>
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<223> X = any amino acid

<400> 48
Phe Leu His Xaa Tyr His
1 5

<210> 49
<211> 6
<212> PRT
<213> Artificial sequence

<220>
<223> Conserved region

<220>
<221> MISC_FEATURE
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<223> X = any amino acid

<400> 49
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1 5

<210> 50
<211> 25
<212> DNA
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<220>
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<222> (19)..(19)
<223> n = any nucleotide

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<222> (20)..(20)
<223> n = any nucleotide

<400> 50
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<210> 51
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<222> (20)..(20)
<223> n = any nucleotide

<400> 51
gatctagara artartannn rtacat 26

<210> 52
<211> 4
<212> PRT
<213> Artificial sequence

<220>
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<400> 52
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<210> 53
<211> 21
<212> DNA
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<220>
<223> Oligonucleotide primer

<400> 53
agcacgacgs sarccacggc g 21

<210> 54
<211> 20
<212> DNA
<213> Artificial sequence

<220>
<223> Oligonucleotide primer

<400> 54
gtggtgcayc abcacgtgct 20

<210> 55
<211> 642
<212> DNA
<213> Pavlova salina

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<223> n = unknown

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cgttgtaaaa cgacggccag tgaattgtaa tacgactcac tatagggcga attgggtacc 180
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tntgagctcg actgacgagc tcggagctgc ggtacagaca ctgtcggcgg ctcgagaggg 360
ctgcgacttc agacgtgatc gggagattgt gcattggtgc gccgccgggc gcggcctgcc 420
gcccgggcgc tgcacgtcat cgtcagtagt cacggtcggc atcagcgccc ggcccgtggt 480
tggtacgtgg tagcgcaggc tgcgcagctg ccaacagccg ccgcccgagg tgggtggtgg 540
gactccgggt gtcagtcaca ctcagtggcg gccgccggca gtaggccgtg actctgccgt 600
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<210> 56
<211> 100
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 56
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tggatgtctt acctcaacta ccagatcgag catcatctgt 100

<210> 57
<211> 100
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 57
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gaattgcggc atggacggga acagatgatg ctcgatctgg 100

<210> 58
<211> 1612
<212> DNA
<213> Pavlova salina

<400> 58
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gaccgcgcgt acgacgtgac caactttgtg aagcgccacc cgggtggcaa gatcatcgca 180
taccaggttg gcacagatgc gacggacgcg tacaagcagt tccatgtgcg gtctgccaag 240
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gccggcgccg cgctcatctg gcacgggtac accttcgcgg gcattgccat gctcggcgtt 480
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tgcggcagcc gccaagtggt aatccatgac acgctgctcc acgacgcgca cgccttccat 1560
cttgacaatc agcatggacg tagcatcatc agttcagtga ctaattcctt tc 1612

<210> 59
<211> 1278
<212> DNA
<213> Pavlova salina

<400> 59
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accaactttg tgaagcgcca cccgggtggc aagatcatcg cataccaggt tggcacagat 180
gcgacggacg cgtacaagca gttccatgtg cggtctgcca aggcggacaa gatgctcaag 240
tcgctgcctt cgcgcccggt gcacaagggc tactcgcccc gccgcgctga cctcattgcc 300
gacttccagg agttcaccaa gcagctggag gcggagggca tgtttgagcc gtcgctgccg 360
cacgtggcat accgcctggc ggaggtgatc gcgatgcacg tggccggcgc cgcgctcatc 420
tggcacgggt acaccttcgc gggcattgcc atgctcggcg ttgtgcaggg ccgctgcggc 480
tggctcatgc acgagggcgg ccactactcg ctcacgggca acattgcttt tgaccgtgcc 540
atccaagtcg cgtgctacgg ccttggctgc ggcatgtcgg gcgcgtggtg gcgcaaccag 600

cacaacaagc accacgcgac gccgcagaag ttgcagcacg acgtcgacct cgacaccctc 660
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tggcttagta tgcaggcgaa gctcttcgcg ccagtgacca cgctgctggt cgcgctgggc 780
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<210> 60
<211> 425
<212> PRT
<213> Pavlova salina

<400> 60

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Ile	Gly	Asp	Arg	Ala	Tyr	Asp	Val	Thr	Asn	Phe	Val	Lys	Arg	His	Pro
		35					40					45			
Gly	Gly	Lys	Ile	Ile	Ala	Tyr	Gln	Val	Gly	Thr	Asp	Ala	Thr	Asp	Ala
	50					55					60				
Tyr	Lys	Gln	Phe	His	Val	Arg	Ser	Ala	Lys	Ala	Asp	Lys	Met	Leu	Lys
65				70					75						80
Ser	Leu	Pro	Ser	Arg	Pro	Val	His	Lys	Gly	Tyr	Ser	Pro	Arg	Arg	Ala
				85					90					95	
Asp	Leu	Ile	Ala	Asp	Phe	Gln	Glu	Phe	Thr	Lys	Gln	Leu	Glu	Ala	Glu
			100					105					110		
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		115					120					125			
Val	Ile	Ala	Met	His	Val	Ala	Gly	Ala	Ala	Leu	Ile	Trp	His	Gly	Tyr
	130					135					140				
Thr	Phe	Ala	Gly	Ile	Ala	Met	Leu	Gly	Val	Val	Gln	Gly	Arg	Cys	Gly
145				150					155						160
Trp	Leu	Met	His	Glu	Gly	Gly	His	Tyr	Ser	Leu	Thr	Gly	Asn	Ile	Ala
				165					170					175	
Phe	Asp	Arg	Ala	Ile	Gln	Val	Ala	Cys	Tyr	Gly	Leu	Gly	Cys	Gly	Met
			180					185					190		
Ser	Gly	Ala	Trp	Trp	Arg	Asn	Gln	His	Asn	Lys	His	His	Ala	Thr	Pro
		195					200					205			
Gln	Lys	Leu	Gln	His	Asp	Val	Asp	Leu	Asp	Thr	Leu	Pro	Leu	Val	Ala
	210					215					220				
Phe	His	Glu	Arg	Ile	Ala	Ala	Lys	Val	Lys	Ser	Pro	Ala	Met	Lys	Ala
225				230					235						240
Trp	Leu	Ser	Met	Gln	Ala	Lys	Leu	Phe	Ala	Pro	Val	Thr	Thr	Leu	Leu
				245					250					255	
Val	Ala	Leu	Gly	Trp	Gln	Leu	Tyr	Leu	His	Pro	Arg	His	Met	Leu	Arg
			260					265					270		
Thr	Lys	His	Tyr	Asp	Glu	Leu	Ala	Met	Leu	Gly	Ile	Arg	Tyr	Gly	Leu
		275					280					285			

```
Val Gly Tyr Leu Ala Ala Asn Tyr Gly Ala Gly Tyr Val Leu Ala Cys
    290                 295                 300
Tyr Leu Leu Tyr Val Gln Leu Gly Ala Met Tyr Ile Phe Cys Asn Phe
305                 310                 315                 320
Ala Val Ser His Thr His Leu Pro Val Val Glu Pro Asn Glu His Ala
                325                 330                 335
Thr Trp Val Glu Tyr Ala Ala Asn His Thr Thr Asn Cys Ser Pro Ser
            340                 345                 350
Trp Trp Cys Asp Trp Trp Met Ser Tyr Leu Asn Tyr Gln Ile Glu His
        355                 360                 365
His Leu Tyr Pro Ser Met Pro Gln Phe Arg His Pro Lys Ile Ala Pro
    370                 375                 380
Arg Val Lys Gln Leu Phe Glu Lys His Gly Leu His Tyr Asp Val Arg
385                 390                 395                 400
Gly Tyr Phe Glu Ala Met Ala Asp Thr Phe Ala Asn Leu Asp Asn Val
                405                 410                 415
Ala His Ala Pro Glu Lys Lys Met Gln
            420                 425
```

<210> 61
<211> 8
<212> PRT
<213> Echium pitardii

<400> 61
```
Met Ala Asn Ala Ile Lys Lys Tyr
1               5
```

<210> 62
<211> 7
<212> PRT
<213> Echium pitardii

<400> 62
```
Glu Ala Leu Asn Thr His Gly
1               5
```

<210> 63
<211> 1347
<212> DNA
<213> Echium plantagineum

<400> 63
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atggctaatg caatcaagaa gtacattact gcagaggagc tgaagaagca tgataaagca     60
ggggatctct ggatctccat tcaaggaaaa atctatgatg tttcagattg gttgaaggac    120
catccaggtg ggaacttccc cttgctgagc cttgctggcc aagaggtaac tgatgcattt    180
gttgcatttc attctggtac aacttggaag cttcttgaaa aattcttcac tggttattac    240
cttaaagatt actctgtttc tgaggtgtcc aaagattaca ggaagcttgt gtttgagttt    300
aataaaatgg gcttgtttga caaaaagggt catattgttc ttgtgactgt cttgtttata    360
gctatgttgt ttggtatgag tgtttatggg gttttgtttt gtgagggtgt tttggtacat    420
ttgcttgctg gggggttgat gggttttgtc tggattcaga gtggttggat tggtcatgat    480
gctgggcatt atattgttat gcctgatgct aggcttaata agcttatggg tattgttgct    540
gccaattgtt tatctggaat aagcattggt tggtggaaat ggaaccataa tgcacatcac    600
```

attgcctgta atagcctcga ttacgacccg gatttgcagt acattccgtt tcttgttgtg 660
tcgtccaagt tgtttagctc gctcacctct catttctatg aaaagaaact gacatttgac 720
tctttatcga gattctttgt aagccatcag cattggacgt tttacccggt tatgtgtatg 780
gctagggtta atatgtttgt gcagtctctg ataatgttgt tgactaagcg aaatgtgttc 840
tatagaagtc aagaactgtt gggattggtg gtgttttgga tttggtaccc gttgcttgtt 900
tcttgcttgc ctaattgggg agaacgagta atgttcgttg ttgctagtct ctcggtgact 960
ggaatgcaac aagtgcagtt ctctttgaac catttctcgt cgagtgttta tgttggtcag 1020
cctaaaggga acgattggtt cgagaaacaa acatgtggga cgctcgacat ttcttgccct 1080
tcgtggatgg attggtttca tggtggattg caattccaag ttgagcatca tttgttccct 1140
aagctgccca gatgccacct tcggaaaatc tccccgttcg tgatggagtt atgcaagaag 1200
cataatttgt cttacaattg tgcatctttc tccgaggcca acaatatgac actcagaaca 1260
ttaagggaca cagcattgca agctcgcgat ttaaccaagc cgctccccaa gaatttggta 1320
tgggaagctc ttaatactca tggttga 1347

<210> 64
<211> 448
<212> PRT
<213> Echium plantagineum

<400> 64
Met Ala Asn Ala Ile Lys Lys Tyr Ile Thr Ala Glu Glu Leu Lys Lys
1 5 10 15
His Asp Lys Ala Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ile Tyr
20 25 30
Asp Val Ser Asp Trp Leu Lys Asp His Pro Gly Gly Asn Phe Pro Leu
35 40 45
Leu Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
50 55 60
Ser Gly Thr Thr Trp Lys Leu Leu Glu Lys Phe Phe Thr Gly Tyr Tyr
65 70 75 80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
85 90 95
Val Phe Glu Phe Asn Lys Met Gly Leu Phe Asp Lys Lys Gly His Ile
100 105 110
Val Leu Val Thr Val Leu Phe Ile Ala Met Leu Phe Gly Met Ser Val
115 120 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Leu Ala Gly
130 135 140
Gly Leu Met Gly Phe Val Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145 150 155 160
Ala Gly His Tyr Ile Val Met Pro Asp Ala Arg Leu Asn Lys Leu Met
165 170 175
Gly Ile Val Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
180 185 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Asp Tyr
195 200 205
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Leu
210 215 220
Phe Ser Ser Leu Thr Ser His Phe Tyr Glu Lys Lys Leu Thr Phe Asp
225 230 235 240
Ser Leu Ser Arg Phe Phe Val Ser His Gln His Trp Thr Phe Tyr Pro
245 250 255
Val Met Cys Met Ala Arg Val Asn Met Phe Val Gln Ser Leu Ile Met
260 265 270

```
Leu Leu Thr Lys Arg Asn Val Phe Tyr Arg Ser Gln Glu Leu Leu Gly
        275                 280                 285
Leu Val Val Phe Trp Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Val Met Phe Val Val Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
                325                 330                 335
Tyr Val Gly Gln Pro Lys Gly Asn Asp Trp Phe Glu Lys Gln Thr Cys
            340                 345                 350
Gly Thr Leu Asp Ile Ser Cys Pro Ser Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Val Glu His His Leu Phe Pro Lys Leu Pro Arg
    370                 375                 380
Cys His Leu Arg Lys Ile Ser Pro Phe Val Met Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Ser Tyr Asn Cys Ala Ser Phe Ser Glu Ala Asn Asn Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asp Thr Ala Leu Gln Ala Arg Asp Leu Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu Asn Thr His Gly
        435                 440                 445
```

<210> 65
<211> 448
<212> PRT
<213> Echium gentianoides

<400> 65

```
Met Ala Asn Ala Ile Lys Lys Tyr Ile Thr Ala Glu Glu Leu Lys Lys
1               5                   10                  15
His Asp Lys Glu Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Val Tyr
            20                  25                  30
Asp Val Ser Asp Trp Leu Lys Asp His Pro Gly Gly Lys Phe Pro Leu
        35                  40                  45
Leu Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
    50                  55                  60
Ser Gly Ser Thr Trp Lys Phe Leu Asp Ser Phe Phe Thr Gly Tyr Tyr
65                  70                  75                  80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
                85                  90                  95
Val Phe Glu Phe Asn Lys Met Gly Leu Phe Asp Lys Lys Gly His Ile
            100                 105                 110
Val Leu Val Thr Val Leu Phe Ile Ala Met Met Phe Ala Met Ser Val
        115                 120                 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Leu Ala Gly
    130                 135                 140
Gly Leu Met Gly Phe Val Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145                 150                 155                 160
Ala Gly His Tyr Ile Val Met Pro Asn Pro Arg Leu Asn Lys Leu Met
                165                 170                 175
Gly Ile Val Ala Gly Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
            180                 185                 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Asp Tyr
        195                 200                 205
```

```
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Leu
    210                 215                 220
Phe Ser Ser Leu Thr Ser His Phe Tyr Glu Lys Lys Leu Thr Phe Asp
225                 230                 235                 240
Ser Leu Ser Arg Phe Phe Val Ser His Gln His Trp Thr Phe Tyr Pro
                245                 250                 255
Val Met Cys Ser Ala Arg Val Asn Met Phe Val Gln Ser Leu Ile Met
            260                 265                 270
Leu Leu Thr Lys Arg Asn Val Phe Tyr Arg Ser Gln Glu Leu Leu Gly
        275                 280                 285
Leu Val Val Phe Trp Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Ile Met Phe Val Val Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ala Ser Val
                325                 330                 335
Tyr Val Gly Gln Pro Lys Gly Asn Asp Trp Phe Glu Lys Gln Thr Cys
            340                 345                 350
Gly Thr Leu Asp Ile Ser Cys Pro Ser Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Val Glu His His Leu Phe Pro Lys Leu Pro Arg
    370                 375                 380
Cys His Leu Arg Lys Ile Ser Pro Phe Val Met Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Ser Tyr Asn Cys Ala Ser Phe Ser Glu Ala Asn Glu Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asp Thr Ala Leu Gln Ala Arg Asp Leu Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu Asn Thr His Gly
        435                 440                 445
```

<210> 66
<211> 448
<212> PRT
<213> Echium pitardii

<400> 66

```
Met Ala Asn Ala Ile Lys Lys Tyr Ile Thr Ala Glu Glu Leu Lys Lys
1               5                   10                  15
His Asp Lys Glu Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Val Tyr
            20                  25                  30
Asp Val Ser Asp Trp Leu Lys Asp His Pro Gly Gly Lys Phe Pro Leu
        35                  40                  45
Leu Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
    50                  55                  60
Ser Gly Ser Thr Trp Lys Leu Leu Asp Ser Phe Phe Thr Gly Tyr Tyr
65                  70                  75                  80
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
                85                  90                  95
Val Phe Glu Phe Asn Lys Met Gly Leu Phe Asp Lys Lys Gly His Ile
            100                 105                 110
Val Leu Val Thr Val Phe Phe Ile Ala Met Met Phe Ala Met Ser Val
        115                 120                 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Leu Ala Gly
    130                 135                 140
```

```
Gly Leu Met Gly Phe Val Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145                 150                 155                 160
Ala Gly His Tyr Ile Val Met Pro Asn Pro Lys Leu Asn Lys Leu Met
                165                 170                 175
Gly Ile Val Ala Ser Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
            180                 185                 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Asp Tyr
        195                 200                 205
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Leu
    210                 215                 220
Phe Ser Ser Leu Thr Ser His Phe Tyr Glu Lys Lys Leu Thr Phe Asp
225                 230                 235                 240
Ser Leu Ser Arg Phe Phe Val Ser His Gln His Trp Thr Phe Tyr Pro
                245                 250                 255
Val Met Cys Ser Ala Arg Val Asn Met Phe Val Gln Ser Leu Ile Met
            260                 265                 270
Leu Leu Thr Lys Arg Asn Val Phe Tyr Arg Ser Gln Glu Leu Leu Gly
        275                 280                 285
Leu Val Val Phe Trp Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Ile Met Phe Val Val Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Leu Gln Gln Val Gln Phe Ser Leu Asn His Phe Ala Ala Ser Val
                325                 330                 335
Tyr Val Gly Gln Pro Lys Gly Ile Asp Trp Phe Glu Lys Gln Thr Cys
            340                 345                 350
Gly Thr Leu Asp Ile Ser Cys Pro Ser Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Val Glu His His Leu Phe Pro Lys Leu Pro Arg
    370                 375                 380
Cys His Leu Arg Lys Ile Ser Pro Phe Val Met Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Ser Tyr Asn Cys Ala Ser Phe Ser Gln Ala Asn Glu Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asp Thr Ala Leu Gln Ala Arg Asp Leu Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu Asn Thr His Gly
        435                 440                 445
```

<210> 67
<211> 448
<212> PRT
<213> Borago officinalis

<400> 67

```
Met Ala Ala Gln Ile Lys Lys Tyr Ile Thr Ser Asp Glu Leu Lys Asn
1               5                   10                  15
His Asp Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Ala Tyr
            20                  25                  30
Asp Val Ser Asp Trp Val Lys Asp His Pro Gly Gly Ser Phe Pro Leu
        35                  40                  45
Lys Ser Leu Ala Gly Gln Glu Val Thr Asp Ala Phe Val Ala Phe His
    50                  55                  60
Pro Ala Ser Thr Trp Lys Asn Leu Asp Lys Phe Phe Thr Gly Tyr Tyr
65                  70                  75                  80
```

```
Leu Lys Asp Tyr Ser Val Ser Glu Val Ser Lys Asp Tyr Arg Lys Leu
                85                  90                  95
Val Phe Glu Phe Ser Lys Met Gly Leu Tyr Asp Lys Lys Gly His Ile
            100                 105                 110
Met Phe Ala Thr Leu Cys Phe Ile Ala Met Leu Phe Ala Met Ser Val
        115                 120                 125
Tyr Gly Val Leu Phe Cys Glu Gly Val Leu Val His Leu Phe Ser Gly
    130                 135                 140
Cys Leu Met Gly Phe Leu Trp Ile Gln Ser Gly Trp Ile Gly His Asp
145                 150                 155                 160
Ala Gly His Tyr Met Val Val Ser Asp Ser Arg Leu Asn Lys Phe Met
                165                 170                 175
Gly Ile Phe Ala Ala Asn Cys Leu Ser Gly Ile Ser Ile Gly Trp Trp
            180                 185                 190
Lys Trp Asn His Asn Ala His His Ile Ala Cys Asn Ser Leu Glu Tyr
        195                 200                 205
Asp Pro Asp Leu Gln Tyr Ile Pro Phe Leu Val Val Ser Ser Lys Phe
    210                 215                 220
Phe Gly Ser Leu Thr Ser His Phe Tyr Glu Lys Arg Leu Thr Phe Asp
225                 230                 235                 240
Ser Leu Ser Arg Phe Phe Val Ser Tyr Gln His Trp Thr Phe Tyr Pro
                245                 250                 255
Ile Met Cys Ala Ala Arg Leu Asn Met Tyr Val Gln Ser Leu Ile Met
            260                 265                 270
Leu Leu Thr Lys Arg Asn Val Ser Tyr Arg Ala His Glu Leu Leu Gly
        275                 280                 285
Cys Leu Val Phe Ser Ile Trp Tyr Pro Leu Leu Val Ser Cys Leu Pro
    290                 295                 300
Asn Trp Gly Glu Arg Ile Met Phe Val Ile Ala Ser Leu Ser Val Thr
305                 310                 315                 320
Gly Met Gln Gln Val Gln Phe Ser Leu Asn His Phe Ser Ser Ser Val
                325                 330                 335
Tyr Val Gly Lys Pro Lys Gly Asn Asn Trp Phe Glu Lys Gln Thr Asp
            340                 345                 350
Gly Thr Leu Asp Ile Ser Cys Pro Pro Trp Met Asp Trp Phe His Gly
        355                 360                 365
Gly Leu Gln Phe Gln Ile Glu His His Leu Phe Pro Lys Met Pro Arg
    370                 375                 380
Cys Asn Leu Arg Lys Ile Ser Pro Tyr Val Ile Glu Leu Cys Lys Lys
385                 390                 395                 400
His Asn Leu Pro Tyr Asn Tyr Ala Ser Phe Ser Lys Ala Asn Glu Met
                405                 410                 415
Thr Leu Arg Thr Leu Arg Asn Thr Ala Leu Gln Ala Arg Asp Ile Thr
            420                 425                 430
Lys Pro Leu Pro Lys Asn Leu Val Trp Glu Ala Leu His Thr His Gly
        435                 440                 445
```

<210> 68
<211> 446
<212> PRT
<213> Borago officinalis

<400> 68

```
Met Glu Gly Thr Lys Lys Tyr Ile Ser Val Gly Glu Leu Glu Lys His
1               5                  10                  15
```

Asn Gln Leu Gly Asp Val Trp Ile Ser Ile Gln Gly Lys Val Tyr Asn
20 25 30
Val Thr Asp Trp Ile Lys Lys His Pro Gly Gly Asp Val Pro Ile Met
35 40 45
Asn Leu Ala Gly Gln Asp Ala Thr Asp Ala Phe Ile Ala Tyr His Pro
50 55 60
Gly Thr Ala Trp Lys Asn Leu Glu Asn Leu Phe Thr Gly Tyr His Leu
65 70 75 80
Glu Asp Tyr Leu Val Ser Glu Ile Ser Lys Asp Tyr Arg Lys Leu Ala
85 90 95
Ser Glu Phe Ser Lys Ala Gly Leu Phe Glu Lys Lys Gly His Thr Val
100 105 110
Ile Tyr Cys Leu Ser Phe Ile Ala Leu Leu Leu Cys Gly Cys Val Tyr
115 120 125
Gly Val Leu Cys Ser Asn Ser Leu Trp Val His Met Leu Ser Gly Ala
130 135 140
Met Leu Gly Met Cys Phe Ile Gln Ala Ala Tyr Leu Gly His Asp Ser
145 150 155 160
Gly His Tyr Thr Met Met Ser Ser Lys Gly Tyr Asn Lys Phe Ala Gln
165 170 175
Val Leu Asn Gly Asn Cys Leu Thr Gly Ile Ser Ile Ala Trp Trp Lys
180 185 190
Trp Thr His Asn Ala His His Ile Ala Cys Asn Ser Leu Asp Tyr Asp
195 200 205
Pro Asp Leu Gln His Leu Pro Val Phe Ala Val Pro Ser Ser Phe Phe
210 215 220
Lys Ser Leu Thr Ser Arg Phe Tyr Gly Arg Glu Leu Thr Phe Asp Gly
225 230 235 240
Leu Ser Arg Phe Leu Val Ser Tyr Gln His Phe Thr Ile Tyr Leu Val
245 250 255
Met Ile Phe Gly Arg Ile Asn Leu Tyr Val Gln Thr Phe Leu Leu Leu
260 265 270
Phe Ser Thr Arg Lys Val Pro Asp Arg Ala Leu Asn Ile Ile Gly Ile
275 280 285
Leu Val Tyr Trp Thr Trp Phe Pro Tyr Leu Val Ser Cys Leu Pro Asn
290 295 300
Trp Asn Glu Arg Val Leu Phe Val Leu Thr Cys Phe Ser Val Thr Ala
305 310 315 320
Leu Gln His Ile Gln Phe Thr Leu Asn His Phe Ala Ala Asp Val Tyr
325 330 335
Val Gly Pro Pro Thr Gly Thr Asn Trp Phe Glu Lys Gln Ala Ala Gly
340 345 350
Thr Ile Asp Ile Ser Cys Ser Ser Trp Met Asp Trp Phe Phe Gly Gly
355 360 365
Leu Gln Phe Gln Leu Glu His His Leu Phe Pro Arg Met Pro Arg Cys
370 375 380
Gln Leu Arg Asn Ile Ser Pro Ile Val Gln Asp Tyr Cys Lys Lys His
385 390 395 400
Asn Leu Pro Tyr Arg Ser Leu Ser Phe Phe Asp Ala Asn Val Ala Thr
405 410 415
Ile Lys Thr Leu Arg Thr Ala Ala Leu Gln Ala Arg Asp Leu Thr Val
420 425 430
Val Pro Gln Asn Leu Leu Trp Glu Ala Phe Asn Thr His Gly
435 440 445

<210> 69
<211> 458
<212> PRT
<213> Helianthus annus

<400> 69

```
Met Val Ser Pro Ser Ile Glu Val Leu Asn Ser Ile Ala Asp Gly Lys
1               5                  10                  15
Lys Tyr Ile Thr Ser Lys Glu Leu Lys Lys His Asn Asn Pro Asn Asp
            20                  25                  30
Leu Trp Ile Ser Ile Leu Gly Lys Val Tyr Asn Val Thr Glu Trp Ala
        35                  40                  45
Lys Glu His Pro Gly Gly Asp Ala Pro Leu Ile Asn Leu Ala Gly Gln
    50                  55                  60
Asp Val Thr Asp Ala Phe Ile Ala Phe His Pro Gly Thr Ala Trp Lys
65                  70                  75                  80
His Leu Asp Lys Leu Phe Thr Gly Tyr His Leu Lys Asp Tyr Gln Val
                85                  90                  95
Ser Asp Ile Ser Arg Asp Tyr Arg Lys Leu Ala Ser Glu Phe Ala Lys
            100                 105                 110
Ala Gly Met Phe Glu Lys Lys Gly His Gly Val Ile Tyr Ser Leu Cys
        115                 120                 125
Phe Val Ser Leu Leu Leu Ser Ala Cys Val Tyr Gly Val Leu Tyr Ser
    130                 135                 140
Gly Ser Phe Trp Ile His Met Leu Ser Gly Ala Ile Leu Gly Leu Ala
145                 150                 155                 160
Trp Met Gln Ile Ala Tyr Leu Gly His Asp Ala Gly His Tyr Gln Met
                165                 170                 175
Met Ala Thr Arg Gly Trp Asn Lys Phe Ala Gly Ile Phe Ile Gly Asn
            180                 185                 190
Cys Ile Thr Gly Ile Ser Ile Ala Trp Trp Lys Trp Thr His Asn Ala
        195                 200                 205
His His Ile Ala Cys Asn Ser Leu Asp Tyr Asp Pro Asp Leu Gln His
    210                 215                 220
Leu Pro Met Leu Ala Val Ser Ser Lys Leu Phe Asn Ser Ile Thr Ser
225                 230                 235                 240
Val Phe Tyr Gly Arg Gln Leu Thr Phe Asp Pro Leu Ala Arg Phe Phe
                245                 250                 255
Val Ser Tyr Gln His Tyr Leu Tyr Tyr Pro Ile Met Cys Val Ala Arg
            260                 265                 270
Val Asn Leu Tyr Leu Gln Thr Ile Leu Leu Leu Ile Ser Lys Arg Lys
        275                 280                 285
Ile Pro Asp Arg Gly Leu Asn Ile Leu Gly Thr Leu Ile Phe Trp Thr
    290                 295                 300
Trp Phe Pro Leu Leu Val Ser Arg Leu Pro Asn Trp Pro Glu Arg Val
305                 310                 315                 320
Ala Phe Val Leu Val Ser Phe Cys Val Thr Gly Ile Gln His Ile Gln
                325                 330                 335
Phe Thr Leu Asn His Phe Ser Gly Asp Val Tyr Val Gly Pro Pro Lys
            340                 345                 350
Gly Asp Asn Trp Phe Glu Lys Gln Thr Arg Gly Thr Ile Asp Ile Ala
        355                 360                 365
Cys Ser Ser Trp Met Asp Trp Phe Phe Gly Gly Leu Gln Phe Gln Leu
    370                 375                 380
Glu His His Leu Phe Pro Arg Leu Pro Arg Cys His Leu Arg Ser Ile
385                 390                 395                 400
```

```
Ser Pro Ile Cys Arg Glu Leu Cys Lys Lys Tyr Asn Leu Pro Tyr Val
                405                 410                 415
Ser Leu Ser Phe Tyr Asp Ala Asn Val Thr Thr Leu Lys Thr Leu Arg
            420                 425                 430
Thr Ala Ala Leu Gln Ala Arg Asp Leu Thr Asn Pro Ala Pro Gln Asn
        435                 440                 445
Leu Ala Trp Glu Ala Phe Asn Thr His Gly
    450                 455
```

<210> 70
<211> 449
<212> PRT
<213> Arabidopsis thaliana

<400> 70

```
Met Ala Asp Gln Thr Lys Lys Arg Tyr Val Thr Ser Glu Asp Leu Lys
1               5                  10                  15
Lys His Asn Lys Pro Gly Asp Leu Trp Ile Ser Ile Gln Gly Lys Val
            20                  25                  30
Tyr Asp Val Ser Asp Trp Val Lys Ser His Pro Gly Gly Glu Ala Ala
        35                  40                  45
Ile Leu Asn Leu Ala Gly Gln Asp Val Thr Asp Ala Phe Ile Ala Tyr
    50                  55                  60
His Pro Gly Thr Ala Trp His His Leu Glu Lys Leu His Asn Gly Tyr
65                  70                  75                  80
His Val Arg Asp His His Val Ser Asp Val Ser Arg Asp Tyr Arg Arg
                85                  90                  95
Leu Ala Ala Glu Phe Ser Lys Arg Gly Leu Phe Asp Lys Lys Gly His
            100                 105                 110
Val Thr Leu Tyr Thr Leu Thr Cys Val Gly Val Met Leu Ala Ala Val
        115                 120                 125
Leu Tyr Gly Val Leu Ala Cys Thr Ser Ile Trp Ala His Leu Ile Ser
    130                 135                 140
Ala Val Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Val Gly His
145                 150                 155                 160
Asp Ser Gly His Tyr Thr Val Thr Ser Thr Lys Pro Cys Asn Lys Leu
                165                 170                 175
Ile Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ser Ile Ala Trp
            180                 185                 190
Trp Lys Trp Thr His Asn Ala His His Ile Ala Cys Asn Ser Leu Asp
        195                 200                 205
His Asp Pro Asp Leu Gln His Ile Pro Ile Phe Ala Val Ser Thr Lys
    210                 215                 220
Phe Phe Asn Ser Met Thr Ser Arg Phe Tyr Gly Arg Lys Leu Thr Phe
225                 230                 235                 240
Asp Pro Leu Ala Arg Phe Leu Ile Ser Tyr Gln His Trp Thr Phe Tyr
                245                 250                 255
Pro Val Met Cys Val Gly Arg Ile Asn Leu Phe Ile Gln Thr Phe Leu
            260                 265                 270
Leu Leu Phe Ser Lys Arg His Val Pro Asp Arg Ala Leu Asn Ile Ala
        275                 280                 285
Gly Ile Leu Val Phe Trp Thr Trp Phe Pro Leu Leu Val Ser Phe Leu
    290                 295                 300
Pro Asn Trp Gln Glu Arg Phe Ile Phe Val Phe Val Ser Phe Ala Val
305                 310                 315                 320
```

Thr Ala Ile Gln His Val Gln Phe Cys Leu Asn His Phe Ala Ala Asp
325 330 335
Val Tyr Thr Gly Pro Pro Asn Gly Asn Asp Trp Phe Glu Lys Gln Thr
340 345 350
Ala Gly Thr Leu Asp Ile Ser Cys Arg Ser Phe Met Asp Trp Phe Phe
355 360 365
Gly Gly Leu Gln Phe Gln Leu Glu His His Leu Phe Pro Arg Leu Pro
370 375 380
Arg Cys His Leu Arg Thr Val Ser Pro Val Val Lys Glu Leu Cys Lys
385 390 395 400
Lys His Asn Leu Pro Tyr Arg Ser Leu Ser Trp Trp Glu Ala Asn Val
405 410 415
Trp Thr Ile Arg Thr Leu Lys Asn Ala Ala Ile Gln Ala Arg Asp Ala
420 425 430
Thr Asn Pro Val Leu Lys Asn Leu Leu Trp Glu Ala Val Asn Thr His
435 440 445
Gly

<210> 71
<211> 449
<212> PRT
<213> Arabidopsis thaliana

<400> 71
Met Ala Glu Glu Thr Glu Lys Lys Tyr Ile Thr Asn Glu Asp Leu Lys
1 5 10 15
Lys His Asn Lys Ser Gly Asp Leu Trp Ile Ala Ile Gln Gly Lys Val
20 25 30
Tyr Asn Val Ser Asp Trp Ile Lys Thr His Pro Gly Gly Asp Thr Val
35 40 45
Ile Leu Asn Leu Val Gly Gln Asp Val Thr Asp Ala Phe Ile Ala Phe
50 55 60
His Pro Gly Thr Ala Trp His His Leu Asp His Leu Phe Thr Gly Tyr
65 70 75 80
His Ile Arg Asp Phe Gln Val Ser Glu Val Ser Arg Asp Tyr Arg Arg
85 90 95
Met Ala Ala Glu Phe Arg Lys Leu Gly Leu Phe Glu Asn Lys Gly His
100 105 110
Val Thr Leu Tyr Thr Leu Ala Phe Val Ala Ala Met Phe Leu Gly Val
115 120 125
Leu Tyr Gly Val Leu Ala Cys Thr Ser Val Phe Ala His Gln Ile Ala
130 135 140
Ala Ala Leu Leu Gly Leu Leu Trp Ile Gln Ser Ala Tyr Ile Gly His
145 150 155 160
Asp Ser Gly His Tyr Val Ile Met Ser Asn Lys Ser Tyr Asn Arg Phe
165 170 175
Ala Gln Leu Leu Ser Gly Asn Cys Leu Thr Gly Ile Ser Ile Ala Trp
180 185 190
Trp Lys Trp Thr His Asn Ala His His Leu Ala Cys Asn Ser Leu Asp
195 200 205
Tyr Asp Pro Asp Leu Gln His Ile Pro Val Phe Ala Val Ser Thr Lys
210 215 220
Phe Phe Ser Ser Leu Thr Ser Arg Phe Tyr Asp Arg Lys Leu Thr Phe
225 230 235 240

Asp Pro Val Ala Arg Phe Leu Val Ser Tyr Gln His Phe Thr Tyr Tyr
245 250 255
Pro Val Met Cys Phe Gly Arg Ile Asn Leu Phe Ile Gln Thr Phe Leu
260 265 270
Leu Leu Phe Ser Lys Arg Glu Val Pro Asp Arg Ala Leu Asn Phe Ala
275 280 285
Gly Ile Leu Val Phe Trp Thr Trp Phe Pro Leu Leu Val Ser Cys Leu
290 295 300
Pro Asn Trp Pro Glu Arg Phe Phe Phe Val Phe Thr Ser Phe Thr Val
305 310 315 320
Thr Ala Leu Gln His Ile Gln Phe Thr Leu Asn His Phe Ala Ala Asp
325 330 335
Val Tyr Val Gly Pro Pro Thr Gly Ser Asp Trp Phe Glu Lys Gln Ala
340 345 350
Ala Gly Thr Ile Asp Ile Ser Cys Arg Ser Tyr Met Asp Trp Phe Phe
355 360 365
Gly Gly Leu Gln Phe Gln Leu Glu His His Leu Phe Pro Arg Leu Pro
370 375 380
Arg Cys His Leu Arg Lys Val Ser Pro Val Val Gln Glu Leu Cys Lys
385 390 395 400
Lys His Asn Leu Pro Tyr Arg Ser Met Ser Trp Phe Glu Ala Asn Val
405 410 415
Leu Thr Ile Asn Thr Leu Lys Thr Ala Ala Tyr Gln Ala Arg Asp Val
420 425 430
Ala Asn Pro Val Val Lys Asn Leu Val Trp Glu Ala Leu Asn Thr His
435 440 445
Gly

<210> 72
<211> 4
<212> PRT
<213> Artificial Sequence

<220>
<223> Conserved motif

<400> 72
His Pro Gly Gly
1

<210> 73
<211> 5
<212> PRT
<213> Artificial Sequence

<220>
<223> Conserved motif

<220>
<221> MISC_FEATURE
<222> (2)..(2)
<223> X = any amino acid

<220>
<221> MISC_FEATURE
<222> (3)..(3)
<223> X = any amino acid

<400> 73
Gln Xaa Xaa His His
1 5

<210> 74
<211> 271
<212> PRT
<213> Thraustochytrium sp.

<400> 74
Met Met Glu Pro Leu Asp Arg Tyr Arg Ala Leu Ala Glu Leu Ala Ala
1 5 10 15
Arg Tyr Ala Ser Ser Ala Ala Phe Lys Trp Gln Val Thr Tyr Asp Ala
20 25 30
Lys Asp Ser Phe Val Gly Pro Leu Gly Ile Arg Glu Pro Leu Gly Leu
35 40 45
Leu Val Gly Ser Val Val Leu Tyr Leu Ser Leu Leu Ala Val Val Tyr
50 55 60
Ala Leu Arg Asn Tyr Leu Gly Gly Leu Met Ala Leu Arg Ser Val His
65 70 75 80
Asn Leu Gly Leu Cys Leu Phe Ser Gly Ala Val Trp Ile Tyr Thr Ser
85 90 95
Tyr Leu Met Ile Gln Asp Gly His Phe Arg Ser Leu Glu Ala Ala Thr
100 105 110
Cys Glu Pro Leu Lys His Pro His Phe Gln Leu Ile Ser Leu Leu Phe
115 120 125
Ala Leu Ser Lys Ile Trp Glu Trp Phe Asp Thr Val Leu Leu Ile Val
130 135 140
Lys Gly Asn Lys Leu Arg Phe Leu His Val Leu His His Ala Thr Thr
145 150 155 160
Phe Trp Leu Tyr Ala Ile Asp His Ile Phe Leu Ser Ser Ile Lys Tyr
165 170 175
Gly Val Ala Val Asn Ala Phe Ile His Thr Val Met Tyr Ala His Tyr
180 185 190
Phe Arg Pro Phe Pro Lys Gly Leu Arg Pro Leu Ile Thr Gln Leu Gln
195 200 205
Ile Val Gln Phe Ile Phe Ser Ile Gly Ile His Thr Ala Ile Tyr Trp
210 215 220
His Tyr Asp Cys Glu Pro Leu Val His Thr His Phe Trp Glu Tyr Val
225 230 235 240
Thr Pro Tyr Leu Phe Val Val Pro Phe Leu Ile Leu Phe Phe Asn Phe
245 250 255
Tyr Leu Gln Gln Tyr Val Leu Ala Pro Ala Lys Thr Lys Lys Ala
260 265 270

<210> 75
<211> 266
<212> PRT
<213> Dabio rerio

<400> 75
Met Ser Val Leu Ala Leu Gln Glu Tyr Glu Phe Glu Arg Gln Phe Asn
1 5 10 15
Glu Asp Glu Ala Ile Arg Trp Met Gln Glu Asn Trp Lys Lys Ser Phe
20 25 30
Leu Phe Ser Ala Leu Tyr Ala Ala Cys Ile Leu Gly Gly Arg His Val
35 40 45
Met Lys Gln Arg Glu Lys Phe Glu Leu Arg Lys Pro Leu Val Leu Trp
50 55 60
Ser Leu Thr Leu Ala Ala Phe Ser Ile Phe Gly Ala Ile Arg Thr Gly
65 70 75 80
Gly Tyr Met Val Asn Ile Leu Met Thr Lys Gly Leu Lys Gln Ser Val
85 90 95
Cys Asp Gln Ser Phe Tyr Asn Gly Pro Val Ser Lys Phe Trp Ala Tyr
100 105 110
Ala Phe Val Leu Ser Lys Ala Pro Glu Leu Gly Asp Thr Leu Phe Ile
115 120 125
Val Leu Arg Lys Gln Lys Leu Ile Phe Leu His Trp Tyr His His Ile
130 135 140
Thr Val Leu Leu Tyr Ser Trp Tyr Ser Tyr Lys Asp Met Val Ala Gly
145 150 155 160
Gly Gly Trp Phe Met Thr Met Asn Tyr Leu Val His Ala Val Met Tyr
165 170 175
Ser Tyr Tyr Ala Leu Arg Ala Ala Gly Phe Lys Ile Ser Arg Lys Phe
180 185 190
Ala Met Phe Ile Thr Leu Thr Gln Ile Thr Gln Met Val Met Gly Cys
195 200 205
Val Val Asn Tyr Leu Val Tyr Leu Trp Met Gln Gln Gly Gln Glu Cys
210 215 220
Pro Ser His Val Gln Asn Ile Val Trp Ser Ser Leu Met Tyr Leu Ser
225 230 235 240
Tyr Phe Val Leu Phe Cys Gln Phe Phe Phe Glu Ala Tyr Ile Thr Lys
245 250 255
Arg Lys Ser Asn Ala Ala Lys Lys Ser Gln
260 265

<210> 76
<211> 320
<212> PRT
<213> Pavlova lutheri

<400> 76
His Glu Ala Ser Cys Arg Ile Arg His Glu Ala Ala Leu Trp Ser Trp
1 5 10 15
Leu Pro Thr Tyr Asp Glu Phe Val Asp Gly Leu Ser Phe Val Asp Arg
20 25 30
Glu Lys Ile Gly Val His Met Val Asp Gln Gly Val Ile Thr Ser Ala
35 40 45
Glu Trp Ala Ala Ile Ser Val Asp Lys His Met Ser Phe Phe Ser Asp
50 55 60
Ala Ala Glu Phe Thr Gly Asp His Trp Ile Ile Pro Leu Val Ala Val
65 70 75 80
Ala Leu Tyr Leu Val Met Ile Val Val Gly Pro Met Ile Met Ala Asn
85 90 95

```
Arg Pro Pro Leu Pro Val Asn Gly Leu Ala Cys Ala Trp Asn Trp Phe
            100                 105                 110
Leu Ala Ala Phe Ser Thr Phe Gly Val Ala Cys Thr Trp His Cys Ile
        115                 120                 125
Phe Thr Arg Leu Arg Ser Arg Gly Phe Glu Ser Thr Thr Cys Gly Ser
    130                 135                 140
Ala Met Phe Met Ser Gln Gly Tyr Val Gly Leu Ala Met Leu Leu Phe
145                 150                 155                 160
Ile Tyr Ser Lys Leu Phe Glu Leu Ile Asp Thr Phe Phe Leu Ile Ala
                165                 170                 175
Lys Lys Ala Asp Val Ile Phe Leu His Trp Tyr His His Val Thr Val
            180                 185                 190
Leu Leu Tyr Cys Trp His Ser His Ser Val Arg Ile Pro Ser Gly Ile
        195                 200                 205
Trp Phe Ala Ala Met Asn Tyr Phe Val His Ala Ile Met Tyr Ser Tyr
    210                 215                 220
Phe Ala Met Thr Gln Met Gly Pro Arg Tyr Arg Lys Leu Val Arg Pro
225                 230                 235                 240
Tyr Ala Arg Leu Ile Thr Thr Leu Gln Ile Ser Gln Met Phe Val Gly
                245                 250                 255
Leu Ile Val Asn Gly Ser Ile Ile Tyr Phe Thr Ser Leu Gly His Ala
            260                 265                 270
Cys Lys Ser Ser Lys Thr Asn Thr Ile Leu Ser Trp Leu Met Tyr Leu
        275                 280                 285
Ser Tyr Phe Val Leu Phe Gly Leu Leu Tyr Leu Arg Asn Tyr Ile Leu
    290                 295                 300
Gly Thr His Gly Lys Pro Ala Gly Lys Arg Ala Lys Gly Lys Ala Glu
305                 310                 315                 320
```

<210> 77
<211> 282
<212> PRT
<213> Danio rerio

<400> 77

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Met Glu Thr Phe Ser His Arg Val Asn Ser Tyr Ile Asp Ser Trp Met
1               5                   10                  15
Gly Pro Arg Asp Leu Arg Val Thr Gly Trp Phe Leu Leu Asp Asp Tyr
            20                  25                  30
Ile Pro Thr Phe Ile Phe Thr Val Met Tyr Leu Leu Ile Val Trp Met
        35                  40                  45
Gly Pro Lys Tyr Met Lys Asn Arg Gln Ala Tyr Ser Cys Arg Ala Leu
    50                  55                  60
Leu Val Pro Tyr Asn Leu Cys Leu Thr Leu Leu Ser Leu Tyr Met Phe
65                  70                  75                  80
Tyr Glu Leu Val Met Ser Val Tyr Gln Gly Gly Tyr Asn Phe Phe Cys
                85                  90                  95
Gln Asn Thr His Ser Gly Gly Asp Ala Asp Asn Arg Met Met Asn Val
            100                 105                 110
Leu Trp Trp Tyr Tyr Phe Ser Lys Leu Ile Glu Phe Met Asp Thr Phe
        115                 120                 125
Phe Phe Ile Leu Arg Lys Asn Asn His Gln Ile Thr Phe Leu His Val
    130                 135                 140
Tyr His His Ala Thr Met Leu Asn Ile Trp Trp Phe Val Met Asn Trp
145                 150                 155                 160
```

```
Val Pro Cys Gly His Ser Tyr Phe Gly Ala Thr Phe Asn Ser Phe Ile
                165                 170                 175
His Val Leu Met Tyr Ser Tyr Tyr Gly Leu Ser Ala Val Pro Ala Leu
            180                 185                 190
Arg Pro Tyr Leu Trp Trp Lys Lys Tyr Ile Thr Gln Gly Gln Leu Val
        195                 200                 205
Gln Phe Val Leu Thr Met Phe Gln Thr Ser Cys Ala Val Val Trp Pro
    210                 215                 220
Cys Gly Phe Pro Met Gly Trp Leu Tyr Phe Gln Ile Ser Tyr Met Val
225                 230                 235                 240
Thr Leu Ile Leu Leu Phe Ser Asn Phe Tyr Ile Gln Thr Tyr Lys Lys
                245                 250                 255
Arg Ser Gly Ser Val Asn Gly His Thr Asn Gly Val Met Ser Ser Glu
            260                 265                 270
Lys Ile Lys His Arg Lys Ala Arg Ala Asp
        275                 280
```

<210> 78
<211> 396
<212> PRT
<213> Pavlova lutheri

<400> 78

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Arg Gly Leu Val Pro Asn Ser Ala Arg Gly Leu Arg Asp Asp Lys Asp
1               5                  10                  15
Asp Gly Ser Leu Ser Ala Thr Ser Asp Phe Phe Arg Ser Thr Ile Thr
            20                  25                  30
Asp Cys Gly Asn Phe Cys Asp Glu Ser Val Asp Phe Gln Met Lys Leu
        35                  40                  45
Phe Glu Arg Asn Gln Ile Ser Glu Arg Cys Tyr Phe Pro Pro Gly Ile
    50                  55                  60
Arg Ala Tyr Arg Lys Gly Glu Arg Asp Phe Asp Phe Ser Met Ala Ala
65                  70                  75                  80
Ala Arg Lys Glu Phe Glu Thr Val Val Phe Thr Thr Val Asp Glu Leu
                85                  90                  95
Leu Ala Lys Thr Gly Val Lys Pro Arg Asp Ile Asp Ile Leu Val Val
            100                 105                 110
Asn Cys Ser Leu Phe Asn Pro Thr Pro Ser Leu Ala Ala Ile Val Ile
        115                 120                 125
Asn His Tyr Gln Met Lys Asp Ser Val Gln Ser Tyr Ser Leu Gly Gly
    130                 135                 140
Met Gly Cys Ser Ala Gly Leu Ile Ser Ile His Leu Ala Lys Asp Leu
145                 150                 155                 160
Leu Gln Val Tyr Pro Arg Lys Arg Ala Leu Val Ile Ser Thr Glu Asn
                165                 170                 175
Ile Thr Gln Asn Phe Tyr Gln Gly Asn Glu Lys Ser Met Leu Ile Ser
            180                 185                 190
Asn Thr Leu Phe Arg Met Gly Gly Ala Ala Val Leu Leu Ser Gly Arg
        195                 200                 205
His Ala Asp Arg Arg Val Ala Lys Tyr Gln Leu Leu His Thr Val Arg
    210                 215                 220
Thr His Lys Gly Ala Asp Pro Asp Ala Tyr Arg Cys Val Phe Gln Glu
225                 230                 235                 240
Glu Asp Lys Ala Gly His Val Gly Val Arg Leu Ser Lys Asp Val Met
                245                 250                 255
```

```
Glu Cys Ala Gly Ala Ala Met Lys Thr Asn Ile Ser Val Leu Ala Pro
            260                 265                 270
Leu Ile Leu Pro Val Ser Glu Gln Val Arg Phe Leu Ala Asn Tyr Val
        275                 280                 285
Ala Arg Lys Trp Leu Arg Met Lys Gly Val Lys Gly Tyr Val Pro Asp
    290                 295                 300
Phe Thr Thr Ala Val Gln His Phe Cys Ile His Thr Gly Gly Arg Ala
305                 310                 315                 320
Val Leu Asp Ala Leu Gln Ala Asn Leu Ser Leu Ser Asp Tyr Tyr Leu
                325                 330                 335
Glu Pro Ser Arg Tyr Ser Leu Trp Arg Trp Gly Asn Val Ser Ser Ala
            340                 345                 350
Ser Val Trp Tyr Glu Leu Asp Trp Leu Glu Lys Ser Gly Arg Ile Arg
        355                 360                 365
Arg Gly Asp Lys Val Trp Gln Ile Gly Phe Gly Ser Gly Phe Lys Cys
    370                 375                 380
Asn Ser Ala Val Trp Arg Ala Cys Arg Ala Met Pro
385                 390                 395
```

<210> 79
<211> 315
<212> DNA
<213> Heterocapsa niei

<400> 79
gattcaggat ccttttgca taggtaccac tccaccatgt ttccgatctg gtagggttgt 60
gtgcgtggtc cacgcctctt cacttggaca agtgccgtcg ggccaagtgc cgtcgggcca 120
agtgccgtcg ggccaaggaa agcactccag cgctcacaac cacctcaccc ccccctcccg 180
ccccccgctt cgttttcgct tgctttcagg tggatggggg cccgctgggt gcctggaggc 240
cagtcgtatt tttgtgcgac catcaattcc accgtgcatg ttgtcatgta cgcctattac 300
ttttctagat caatc 315

<210> 80
<211> 100
<212> PRT
<213> Artificial Sequence

<220>
<223> Protein encoded by SEQ ID NO:79 which comprises a stop codon between residues 17 and 18 suggesting the presence of an intron

<400> 80
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Asp Ser Gly Ser Phe Leu His Arg Tyr His Ser Thr Met Phe Pro Ile
1               5                   10                  15
Trp Gly Cys Val Arg Gly Pro Arg Leu Phe Thr Trp Thr Ser Ala Val
            20                  25                  30
Gly Pro Ser Ala Val Gly Pro Ser Ala Val Gly Pro Arg Lys Ala Leu
        35                  40                  45
Gln Arg Ser Gln Pro Pro His Pro Pro Leu Pro Pro Pro Ala Ser Phe
    50                  55                  60
Ser Leu Ala Phe Arg Trp Met Gly Ala Arg Trp Val Pro Gly Gly Gln
65                  70                  75                  80
```

Ser Tyr Phe Cys Ala Thr Ile Asn Ser Thr Val His Val Val Met Tyr
85 90 95
Ala Tyr Tyr Phe
100

<210> 81
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 81
gctacgcccg gggatcctcg aggctggcgc aacgcaatta atgtga 46

<210> 82
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 82
cacaggaaac agcttgacat cgattaccgg caattgtacg gcggccgcta cggatatcct 60
cgctcgagct cgcccggggt agct 84

<210> 83
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 83
agcacatcga tgaaggagat atacccatgg ctaatgcaat caagaa 46

<210> 84
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 84
acgatgcggc cgctcaacca tgagtattaa gagctt 36

<210> 85
<211> 294
<212> PRT
<213> Pavlova salina

<400> 85
Met Pro Thr Trp Gly Glu Phe Val Ala Gly Leu Thr Tyr Val Glu Arg
1 5 10 15
Gln Gln Met Ser Glu Glu Leu Val Arg Ala Asn Lys Leu Pro Leu Ser
20 25 30
Leu Ile Pro Glu Val Asp Phe Phe Thr Ile Ala Ser Val Tyr Val Gly
35 40 45
Asp His Trp Arg Ile Pro Phe Thr Ala Ile Ser Ala Tyr Leu Val Leu
50 55 60
Ile Thr Leu Gly Pro Gln Leu Met Ala Arg Arg Pro Pro Leu Pro Ile
65 70 75 80
Asn Thr Leu Ala Cys Leu Trp Asn Phe Ala Leu Ser Leu Phe Ser Phe
85 90 95
Val Gly Met Ile Val Thr Trp Thr Thr Ile Gly Glu Arg Leu Trp Lys
100 105 110
Asn Gly Ile Glu Asp Thr Val Cys Gly His Pro Ile Phe Met Gly Tyr
115 120 125
Gly Trp Ile Gly Tyr Val Met Leu Ala Phe Ile Trp Ser Lys Leu Phe
130 135 140
Glu Leu Ile Asp Thr Val Phe Leu Val Ala Lys Lys Ala Asp Val Ile
145 150 155 160
Phe Leu His Trp Tyr His His Val Thr Val Leu Leu Tyr Cys Trp His
165 170 175
Ser Tyr Ala Val Arg Ile Pro Ser Gly Ile Trp Phe Ala Ala Met Asn
180 185 190
Tyr Phe Val His Ala Ile Met Tyr Ala Tyr Phe Gly Met Thr Gln Ile
195 200 205
Gly Pro Arg Gln Arg Lys Leu Val Arg Pro Tyr Ala Arg Leu Ile Thr
210 215 220
Thr Phe Gln Leu Ser Gln Met Gly Val Gly Leu Ala Val Asn Gly Leu
225 230 235 240
Ile Ile Arg Tyr Pro Ser Ile Gly His His Cys His Ser Asn Lys Thr
245 250 255
Asn Thr Ile Leu Ser Trp Ile Met Tyr Ala Ser Tyr Phe Val Leu Phe
260 265 270
Ala Ala Leu Tyr Val Lys Asn Tyr Ile Phe Ser Lys Leu Lys Ser Pro
275 280 285
Lys Arg Lys Lys Val Glu
290

<210> 86
<211> 276
<212> PRT
<213> Pavlova salina

<400> 86
Met Ser Glu Glu Leu Val Arg Ala Asn Lys Leu Pro Leu Ser Leu Ile
1 5 10 15
Pro Glu Val Asp Phe Phe Thr Ile Ala Ser Val Tyr Val Gly Asp His
20 25 30

```
Trp Arg Ile Pro Phe Thr Ala Ile Ser Ala Tyr Leu Val Leu Ile Thr
        35                  40                  45
Leu Gly Pro Gln Leu Met Ala Arg Arg Pro Pro Leu Pro Ile Asn Thr
    50                  55                  60
Leu Ala Cys Leu Trp Asn Phe Ala Leu Ser Leu Phe Ser Phe Val Gly
65                  70                  75                  80
Met Ile Val Thr Trp Thr Thr Ile Gly Glu Arg Leu Trp Lys Asn Gly
                85                  90                  95
Ile Glu Asp Thr Val Cys Gly His Pro Ile Phe Met Gly Tyr Gly Trp
            100                 105                 110
Ile Gly Tyr Val Met Leu Ala Phe Ile Trp Ser Lys Leu Phe Glu Leu
        115                 120                 125
Ile Asp Thr Val Phe Leu Val Ala Lys Lys Ala Asp Val Ile Phe Leu
    130                 135                 140
His Trp Tyr His His Val Thr Val Leu Leu Tyr Cys Trp His Ser Tyr
145                 150                 155                 160
Ala Val Arg Ile Pro Ser Gly Ile Trp Phe Ala Ala Met Asn Tyr Phe
                165                 170                 175
Val His Ala Ile Met Tyr Ala Tyr Phe Gly Met Thr Gln Ile Gly Pro
            180                 185                 190
Arg Gln Arg Lys Leu Val Arg Pro Tyr Ala Arg Leu Ile Thr Thr Phe
        195                 200                 205
Gln Leu Ser Gln Met Gly Val Gly Leu Ala Val Asn Gly Leu Ile Ile
    210                 215                 220
Arg Tyr Pro Ser Ile Gly His His Cys His Ser Asn Lys Thr Asn Thr
225                 230                 235                 240
Ile Leu Ser Trp Ile Met Tyr Ala Ser Tyr Phe Val Leu Phe Ala Ala
                245                 250                 255
Leu Tyr Val Lys Asn Tyr Ile Phe Ser Lys Leu Lys Ser Pro Lys Arg
            260                 265                 270
Lys Lys Val Glu
        275
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<210> 87
<211> 20
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 87
cgctctagaa ctagtggatc 20

<210> 88
<211> 223
<212> PRT
<213> Melosira sp.

<400> 88

```
Thr Ile Phe Lys Ser Asn Ala Val Pro Ala Leu Asp Pro Tyr Pro Ile
1               5                   10                  15
Lys Phe Val Tyr Asn Val Ser Gln Ile Met Met Cys Ala Tyr Met Thr
            20                  25                  30
```

```
Ile Glu Ala Gly Leu Val Ala Tyr Arg Ser Gly Tyr Thr Val Met Pro
        35                  40                  45
Cys Asn Asp Tyr Asn Thr Asn Asn Pro Pro Val Gly Asn Leu Leu Trp
    50                  55                  60
Leu Phe Tyr Ile Ser Lys Val Trp Asp Phe Trp Asp Thr Ile Phe Ile
65                  70                  75                  80
Val Ile Gly Lys Lys Trp Lys Gln Leu Ser Phe Leu His Val Tyr His
                85                  90                  95
His Thr Thr Ile Phe Leu Phe Tyr Trp Leu Asn Ser His Val Asn Tyr
            100                 105                 110
Asp Gly Asp Ile Tyr Leu Thr Ile Leu Leu Asn Gly Phe Ile His Thr
        115                 120                 125
Val Met Tyr Thr Tyr Tyr Phe Val Cys Met His Thr Lys Val Pro Glu
    130                 135                 140
Thr Gly Lys Ser Leu Pro Ile Trp Trp Lys Ser Ser Leu Thr Met Met
145                 150                 155                 160
Gln Met Ile Gln Phe Val Thr Met Met Ser Gln Ala Ser Tyr Leu Leu
                165                 170                 175
Val Thr Asn Cys Glu Lys Thr Ser Arg Gly Val Val Ile Ala Tyr Phe
            180                 185                 190
Val Tyr Ile Phe Thr Leu Leu Val Leu Phe Ala Gln Phe Phe Arg Ala
        195                 200                 205
Ser Tyr Met Lys Pro Lys Gly Lys Lys Ala Lys Met Lys Lys Val
    210                 215                 220
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<210> 89
<211> 683
<212> DNA
<213> Melosira sp.

<400> 89
acgatcttca agtcaaacgc cgtccctgcc ctggatccat accccatcaa attcgtttac 60
aatgtgtccc agatcatgat gtgcgcgtac atgacgatcg aggcaggcct ggtggcctac 120
cgcagtggct atactgtcat gccatgcaac gactacaaca ccaacaaccc ccctgtcggg 180
aacctgctgt ggctgtttta catttccaaa gtttgggact tttgggacac catctttatc 240
gtgattggca aaaagtggaa gcagctgagc ttcttgcacg tgtaccacca caccaccatc 300
tttttgttct actggctcaa ctcgcatgtc aactacgacg gagatattta tctgacgatt 360
ctgttgaacg gcttcatcca caccgtcatg tacacttatt acttcgtttg catgcacacg 420
aaggtgcccg agactggaaa gtcgttgccc atttggtgga aatccagtct caccatgatg 480
caaatgatcc aattcgtcac catgatgagc caggcttcgt acttgctcgt gacgaactgc 540
gaaaagacca gtcgggggt cgttattgcg tactttgtgt acattttcac tctactcgtc 600
ttatttgctc agttcttccg agcatcttac atgaagccca agggaaagaa ggcgaaaatg 660
aagaaggtat aagctgctgg cat 683

CLAIMS:

1. A recombinant plant cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode:

a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or

b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes.

2. The cell of claim 1 which is a plant cell from an angiosperm or an oilseed plant cell.

3. The cell of claim 1, wherein the plant cell is a *Brassica napus* cell.

4. The cell according to any one of claims 1 to 3, wherein at least one promoter is a seed specific promoter.

5. The cell according to any one of claims 1 to 4, wherein said cell is capable of synthesising DPA.

6. The cell according to any one of claims 1 to 5, wherein said cell is capable of synthesising docosahexaenoic acid (DHA).

7. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the cell comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase.

8. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the cell comprises heterologous polynucleotides which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase.

9. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the total fatty acid of the cell comprises 1.5% EPA.

10. The cell of claim 1 or claim 9 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the total fatty acid of the cell comprises 0.13% DPA.

11. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the total fatty acid of the cell comprises 4.1% ω3 C20 long chain polyunsaturated fatty acid (LC-PUFA).

12. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the efficiency of conversion of α-linolenic acid (ALA) to EPA in the cell is at least 14.6%.

13. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of α-linolenic acid (ALA), wherein the efficiency of conversion of ALA to said products in the cell is at least 22%.

14. The cell of claim 8 or claim 10 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the efficiency of conversion of EPA to DPA in the cell is at least 7%.

15. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell, wherein the total fatty acid of the cell comprises 2.5% ω3 C20 long chain polyunsaturated fatty acid (LC-PUFA), wherein the cell comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

16. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising ALA and EPA, wherein the efficiency of conversion of α-linolenic acid (ALA) to EPA in the cell is at least 14.6%, wherein the cell comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

17. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of α-linolenic acid (ALA) and/or the products of Δ9 elongation of ALA, which products are synthesized in the cell, wherein the efficiency of conversion of ALA to said products in the cell is at least 22%, wherein the cell comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that

are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

18. The cell of claim 10 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising DPA which is synthesized from EPA in the cell, wherein the efficiency of conversion of EPA to DPA in the cell is at least 7%, wherein the cell comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said in *Brassica napus* cells or *Arabidopsis thaliana* cells.

19. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising at least one ω3 C20 long chain polyunsaturated fatty acid (LC-PUFA) which is synthesized in the cell, wherein the total fatty acid of the cell comprises 2.5% ω3 C20 LC-PUFA, wherein the heterologous polynucleotides encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

20. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising α-linolenic acid (ALA) and EPA, wherein the efficiency of conversion of ALA to EPA in the cell is at least 14.6%, wherein the cell comprises heterologous polynucleotides which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

21. The cell of claim 1 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of α-linolenic acid (ALA) and/or the products of Δ9 elongation of ALA, which products are synthesized in the cell, wherein the efficiency of conversion of ALA to said products in the cell is at least 22%, wherein the cell comprises heterologous polynucleotides which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

22. The cell of claim 8 or claim 10 which is a *Brassica napus* cell or *Arabidopsis thaliana* cell comprising DPA which is synthesized from EPA in the cell, wherein the efficiency of conversion of EPA to DPA in the cell is at least 7%, wherein the heterologous polynucleotides encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* cells or *Arabidopsis thaliana* cells.

23. The cell according to any one of claims 1 to 22 which is homozygous for the heterologous polynucleotides.

24. The cell according to any one of claims 6 to 23, wherein at least 50% (w/w) of the EPA, DHA and DPA of the cell is incorporated into triacylglycerols in the cell.

25. A recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode:

a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or

b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

wherein the cell comprises oil having a total fatty acid content which comprises at least 2.1% EPA and less than 0.1% eicosatrienoic acid (w/w), and wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes.

26. A method of producing a recombinant plant cell which synthesises one or more long-chain polyunsaturated fatty acids (LC-PUFA(s)), the method comprising introducing into the cell more than one heterologous polynucleotide, wherein said polynucleotides encode enzymes which comprise:

a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or

b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein said one or more LC-PUFAs comprise EPA, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes.

27. The method of claim 26, wherein the plant cell is a *Brassica napus* cell or *Arabidopsis thaliana* cell, and wherein the heterologous polynucleotides encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase.

28. The method of claim 26, wherein the plant cell is a *Brassica napus* cell or *Arabidopsis thaliana* cell, and wherein the heterologous polynucleotides encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase.

29. A method of producing a long-chain polyunsaturated fatty acid (LC-PUFA) in a cell or a plant, the method comprising:

i) culturing, under suitable conditions, a recombinant cell according to any one of claims 1 to 24, and/or

ii) cultivating, under suitable conditions, a transgenic plant comprising at least one cell according to any one of claims 1 to 24, wherein the cell is a transgenic plant cell,

wherein the cell or plant produces LC-PUFA which comprises EPA.

30. The method of claim 29, wherein the LC-PUFA comprises DPA.

31. The method of claim 29 or claim 30, wherein the LC-PUFA comprises DHA.

32. The method of claim 29, wherein the LC-PUFA comprises EPA, DPA and DHA.

33. The method according to any one of claims 29 to 32, wherein the recombinant cell is a seed cell.

34. The method according to any one of claims 29 to 33, wherein the recombinant cell is a *Brassica napus* cell.

35. The method of claim 29, wherein the recombinant cell is a *Brassica napus* seed cell in a seed and the LC-PUFA comprises EPA, DPA and DHA.

36. A method of producing oil comprising long chain polyunsaturated fatty acid (LC-PUFA), wherein said LC-PUFA comprises eicosapentaenoic acid (EPA), the method comprising

i) obtaining seed comprising a recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell which synthesises eicosapentaenoic acid (EPA), comprising more than one heterologous polynucleotide, wherein said polynucleotides encode:

a) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or

b) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

wherein the seed comprises oil having a total fatty acid content which comprises at least 1.5% EPA, and wherein the more than one polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in the cell, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate, and wherein the synthesis of EPA requires the sequential action of said enzymes, and

ii) extracting the oil from said seed.

37. A method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell according to any one of claims 1 to 24, and extracting oil from said seed.

38. The method of claim 37, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed, wherein the total fatty acid of the seed comprises 0.13% DPA.

39. The method of claim 37, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed, wherein the total fatty acid of the seed comprises 4.1% ω3 C20 LC-PUFA.

40. The method of claim 37, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed, wherein the efficiency of conversion of ALA to EPA in the seed is at least 14.6%.

41. The method of claim 37, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, wherein the efficiency of conversion of ALA to said products in the seed is at least 22%.

42. The method of claim 38, wherein the efficiency of conversion of EPA to DPA in the seed is at least 7%.

43. A method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell of claim 1, and extracting oil from said seed, and wherein the seed comprises more than one heterologous polynucleotide which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase.

44. The method of claim 43, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed, wherein the total fatty acid of the seed comprises 2.5% ω3 C20 LC-PUFA, wherein the seed comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

45. The method of claim 43, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising ALA and EPA, wherein the efficiency of conversion of ALA to EPA in the seed is at least 14.6%, wherein the seed comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

46. The method of claim 43, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, which products are synthesized in the seed, wherein the efficiency of conversion of ALA to said products in the seed is at least 22%, wherein the seed comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

47. The method of claim 43, wherein the efficiency of conversion of EPA to DPA in the seed is at least 7%, wherein the seed comprises heterologous polynucleotides which encode a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

48. A method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining seed comprising a cell of claim 1, and extracting oil from said seed, and wherein the seed comprises more than one heterologous polynucleotide which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase.

49. The method of claim 48, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising at least one ω3 C20 LC-PUFA which is synthesized in the seed, wherein the total fatty acid of the seed comprises 2.5% ω3 C20 LC-PUFA, wherein the heterologous polynucleotides encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

50. The method of claim 48, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising ALA and EPA, wherein the efficiency of conversion of ALA to EPA in the cell is at least 14.6%, wherein the seed comprises heterologous polynucleotides which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

51. The method of claim 48, wherein the seed is a *Brassica napus* seed or *Arabidopsis thaliana* seed comprising ω3 polyunsaturated fatty acids that are the products of Δ6-desaturation of ALA and/or the products of Δ9 elongation of ALA, which products are synthesized in the seed, wherein the efficiency of conversion of ALA to said products in the seed is at least 22%, wherein the seed comprises heterologous polynucleotides which encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

52. The method of claim 48, wherein the efficiency of conversion of EPA to DPA in the seed is at least 7%, wherein the heterologous polynucleotides encode a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase, and wherein the polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seeds or *Arabidopsis thaliana* seeds.

53. The method according to any one of claims 37 to 52, wherein said oil is further purified.

54. The method according to any one of claims 37 to 53, wherein said oil is further treated by hydrolysis with a strong base to release the free fatty acids.

55. A method of producing oil comprising LC-PUFA, wherein said LC-PUFA comprises EPA, comprising obtaining *Brassica napus* or *Arabidopsis thaliana* seed comprising a cell of claim 25, and extracting oil from said seed.

56. A feedstuff comprising the recombinant cell according to any one of claims 1 to 24.

57. A feedstuff comprising the recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell of claim 25.

58. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 1 to 24 with a suitable carrier.

59. A method of preparing a feedstuff, the method comprising admixing a recombinant *Brassica napus* cell or *Arabidopsis thaliana* cell of claim 25 with a suitable carrier.

60. A method of preparing a feedstuff, the method comprising admixing oil produced using the method according to any one of claims 37 to 55 with a suitable carrier.

61. A recombinant nucleic acid molecule comprising:

a polynucleotide which encodes a Δ5/Δ6 bifunctional desaturase which catalyses desaturation of α-linolenic acid (ALA) to stearidonic acid (SDA) and desaturation of eicosatetraenoic acid (ETA) to eicosapentaenoic acid (EPA); and

a polynucleotide which encodes a Δ6 elongase which catalyses elongation of SDA to ETA;

wherein each polynucleotide is operably linked to a promoter which directs expression of the polynucleotide in a plant seed.

62. The recombinant nucleic acid molecule of claim 61, wherein the Δ5/Δ6 bifunctional desaturase catalyses desaturation of an acyl-CoA substrate.

63. The recombinant nucleic acid molecule of claim 61 or claim 62, wherein the Δ6 elongase is a Δ5/Δ6 bifunctional elongase.

64. The recombinant nucleic acid molecule according to any one of claims 61 to 63, wherein the plant seed is a *Brassica napus* or *Arabidopsis thaliana* seed.

65. A cell comprising the recombinant nucleic acid molecule according to any one of claims 61 to 64.

66. The cell of claim 65 which is a plant cell.

67. The cell of claim 65 or claim 66, wherein the cell comprises an additional exogenous polynucleotide which encodes a Δ4 desaturase which catalyses desaturation of DPA to docosahexaenoic acid (DHA), wherein the additional exogenous polynucleotide is operably linked to a promoter which directs expression of the polynucleotide in the plant seed.

68. The cell of claim 67, wherein the Δ4 desaturase is a *Pavlova* Δ4 desaturase.

69. The cell of claim 68, wherein the Δ4 desaturase has the amino acid sequence encoded by the nucleotide sequence set forth in Genbank Accession No. AY332747.

70. The cell according to any one of claims 65 to 69 which comprises EPA.

71. A process of producing oil, comprising:

i) obtaining plant seed comprising a cell according to any one of claims 66 to 70, and

ii) extracting oil from the plant seed.

72. The process of claim 71, comprising a step of purifying the extracted oil.

73. A process for feeding fish comprising:

a) obtaining feedstuff which comprises oil extracted from seed comprising a cell according to any one of claims 1 to 25 or 65 to 70, or oil produced using a method according to any one of claims 37 to 55, 71 or 72; and

b) providing the feedstuff to fish,

thereby feeding fish.

74. A process for feeding fish comprising:

a) obtaining feedstuff which comprises oil extracted from transgenic *Brassica* seeds which comprise eicosapentaenoic acid (EPA) and heterologous polynucleotides which encode enzymes that comprise:

i) a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase; or

ii) a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase;

wherein the heterologous polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica* seed cells, wherein the enzymes encoded by said polynucleotides comprise at least one desaturase which is able to act on an acyl-CoA substrate; and

b) providing the feedstuff to fish,

thereby feeding fish.

75. The process of claim 74, wherein at least one promoter is a seed specific promoter.

76. The process of claim 74 or claim 75, wherein the *Brassica* seeds comprise docosapentaenoic (DPA) which is produced from LA in the seed.

77. The process according to any one of claims 74 to 76, wherein the *Brassica* seeds comprise docosahexaenoic acid (DHA) which is produced from LA in the seed.

78. The process according to any one of claims 74 to 77, wherein the *Brassica* seeds have a germination rate which is substantially the same as that of isogenic non-transgenic seed.

79. The process according to any one of claims 74 to 78, wherein the *Brassica* seeds are homozygous for the heterologous polynucleotides.

80. The process according to any one of claims 74 to 79, wherein the heterologous polynucleotides encode enzymes which comprise a Δ6 desaturase, a Δ6 elongase and a Δ5 desaturase.

81. The process according to any one of claims 74 to 79, wherein the heterologous polynucleotides encode enzymes which comprise a Δ5/Δ6 bifunctional desaturase and a Δ5/Δ6 bifunctional elongase.

82. A process for feeding fish comprising:

a) obtaining feedstuff which comprises oil extracted from transgenic *Brassica napus* seeds which comprise docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols in the seeds, and heterologous polynucleotides which encode enzymes that comprise:

i) a Δ5 elongase;

ii) a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31;

iii) a Δ4 desaturase whose amino acid sequence is at least 99% identical to the sequence set forth as SEQ ID NO:33;

iv) a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18; and

v) a Δ6 desaturase;

wherein the heterologous polynucleotides are operably linked to one or more promoters that are capable of directing expression of said polynucleotides in *Brassica napus* seed cells; and

b) providing the feedstuff to fish,

thereby feeding fish.

83. A *Brassica* plant cell comprising:

a polynucleotide which encodes a Δ4 desaturase whose amino acid sequence is at least 99.5% identical to the sequence set forth as SEQ ID NO:33, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ5 elongase operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ6 desaturase operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and

an exogenous desaturase which desaturates an acyl-CoA substrate.

84. The *Brassica* plant cell of claim 83, wherein the Δ6 desaturase is a Δ5/Δ6 bifunctional desaturase.

85. The *Brassica* plant cell of claim 83 or claim 84 which is a *Brassica napus* plant cell.

86. A process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising:

i) obtaining *Brassica* seed comprising a cell according to any one of claims 83 to 85, and

ii) extracting triacylglycerols from the seed.

87. The process of claim 86 which further comprises purifying the extracted triacylglycerols.

88. A *Brassica* plant cell for producing docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols in the plant cell comprising:

a polynucleotide which encodes a Δ4 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ6 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and

a polynucleotide which encodes a Δ5/Δ6 bifunctional elongase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell.

89. The *Brassica* plant cell of claim 88 which is a *Brassica napus* plant cell.

90. The *Brassica* plant cell of claim 88 or claim 89, further comprising an exogenous desaturase which desaturates an acyl-CoA substrate.

91. A *Brassica* plant cell comprising:

a polynucleotide which encodes a Δ4 desaturase which has the amino acid sequence encoded by a Δ4 desaturase gene from *Pavlova lutheri*, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO: 18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ6 desaturase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and

a polynucleotide which encodes a Δ5/Δ6 bifunctional elongase, operably linked to a promoter which directs expression of the polynucleotide in the plant cell.

92. The *Brassica* plant cell of claim 91 which is a *Brassica napus* plant cell.

93. The *Brassica* plant cell of claim 92, comprising docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols.

94. The *Brassica* plant cell according to any one of claims 91 to 93, further comprising an exogenous desaturase which desaturates an acyl-CoA substrate.

95. The *Brassica* plant cell according to any one of claims 91 to 94, wherein the Δ6 desaturase is a Δ5/Δ6 bifunctional desaturase.

96. A *Brassica* plant cell comprising:

a polynucleotide which encodes a Δ4 desaturase which has the amino acid sequence encoded by a Δ4 desaturase gene from *Pavlova lutheri*, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ5 desaturase which has the amino acid sequence set forth as SEQ ID NO:18, operably linked to a promoter which directs expression of the polynucleotide in the plant cell;

a polynucleotide which encodes a Δ6 desaturase operably linked to a promoter which directs expression of the polynucleotide in the plant cell; and

a polynucleotide which encodes a Δ6 elongase which has the amino acid sequence set forth as SEQ ID NO:31, operably linked to a promoter which directs expression of the polynucleotide in the plant cell.

97. The *Brassica* plant cell of claim 96 which is a *Brassica napus* plant cell.

98. The *Brassica* plant cell of claim 96 or claim 97 comprising docasapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in an esterified form as part of triacylglycerols.

99. The *Brassica* plant cell according to any one of claims 96 to 98, further comprising an exogenous desaturase which desaturates an acyl-CoA substrate.

100. The *Brassica* plant cell according to any one of claims 96 to 99, wherein the Δ6 desaturase is a Δ5/Δ6 bifunctional desaturase.

101. The *Brassica* plant cell according to any one of claims 96 to 100, wherein the Δ6 desaturase desaturates an acyl-CoA substrate.

102. A process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising:

i) obtaining *Brassica napus* seed comprising a cell according to any one of claims 88 to 90, and

ii) extracting triacylglycerols from the seed.

103. The process of claim 102 which further comprises purifying the extracted triacylglycerols.

104. A process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising:

i) obtaining *Brassica napus* seed comprising a cell according to any one of claims 91 to 95, and

ii) extracting triacylglycerols from the seed.

105. The process of claim 104 which further comprises purifying the extracted triacylglycerols.

106. A process for producing DPA and DHA in an esterified form as part of triacylglycerols comprising:

i) obtaining *Brassica napus* seed comprising a cell according to any one of claims 96 to 101, and

ii) extracting triacylglycerols from the seed.

107. The process of claim 106 which further comprises purifying the extracted triacylglycerols.

108. An extract from a cell according to any one of claims 1 to 25, 65 to 70, 83 to 85 or 88 to 101, wherein said extract comprises an increased level of EPA in its fatty acid relative to a corresponding extract from an isogenic non-transformed cell, and further comprises the exogenous polynucleotide(s) defined in any one of claims 1 to 25, 65 to 70, 83 to 85 or 88 to 101.

109. A method of producing DHA in a cell or a plant, the method comprising:

i) culturing, under suitable conditions, a recombinant cell according to any one of claims 83 to 85 or 88 to 101, and/or

ii) cultivating, under suitable conditions, a transgenic plant comprising at least one cell according to any one of claims 83 to 85 or 88 to 101, wherein the cell is a transgenic plant cell,

wherein the cell or plant produces DHA.

110. A composition comprising a cell according to any one of claims 1 to 25, 65 to 70, 83 to 85 or 88 to 101, and a suitable carrier.

111. A feedstuff comprising a cell according to any one of claims 65 to 70.

112. A feedstuff comprising a cell according to any one of claims 83 to 85.

113. A feedstuff comprising a cell according to any one of claims 88 to 90.

114. A feedstuff comprising a cell according to any one of claims 91 to 95.

115. A feedstuff comprising a cell according to any one of claims 96 to 101.

116. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 65 to 70 with a suitable carrier.

117. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 83 to 85 with a suitable carrier.

118. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 88 to 90 with a suitable carrier.

119. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 91 to 95 with a suitable carrier.

120. A method of preparing a feedstuff, the method comprising admixing a cell according to any one of claims 96 to 101 with a suitable carrier.

121. A method of preparing a feedstuff, the method comprising admixing oil produced using the method of claim 71 or claim 72 with a suitable carrier.

122. A method of preparing a feedstuff, the method comprising admixing triacylglycerols produced using the method of claim 86 or claim 87 with a suitable carrier.

123. A method of preparing a feedstuff, the method comprising admixing triacylglycerols produced using the method of claim 102 or claim 103 with a suitable carrier.

124. A method of preparing a feedstuff, the method comprising admixing triacylglycerols produced using the method of claim 104 or claim 105 with a suitable carrier.

125. A method of preparing a feedstuff, the method comprising admixing triacylglycerols produced using the method of claim 106 or claim 107 with a suitable carrier.

126. Use of a cell according to any one of claims 1 to 25, 65 to 70, 83 to 85 or 88 to 101, or oil produced using the method according to any one of claims 37 to 55, 71, 72, 86, 87 or 102 to 107, for the manufacture of a medicament for treating or preventing a condition which would benefit from at least one or more or all of EPA, DPA and DHA, wherein the cell comprises EPA.

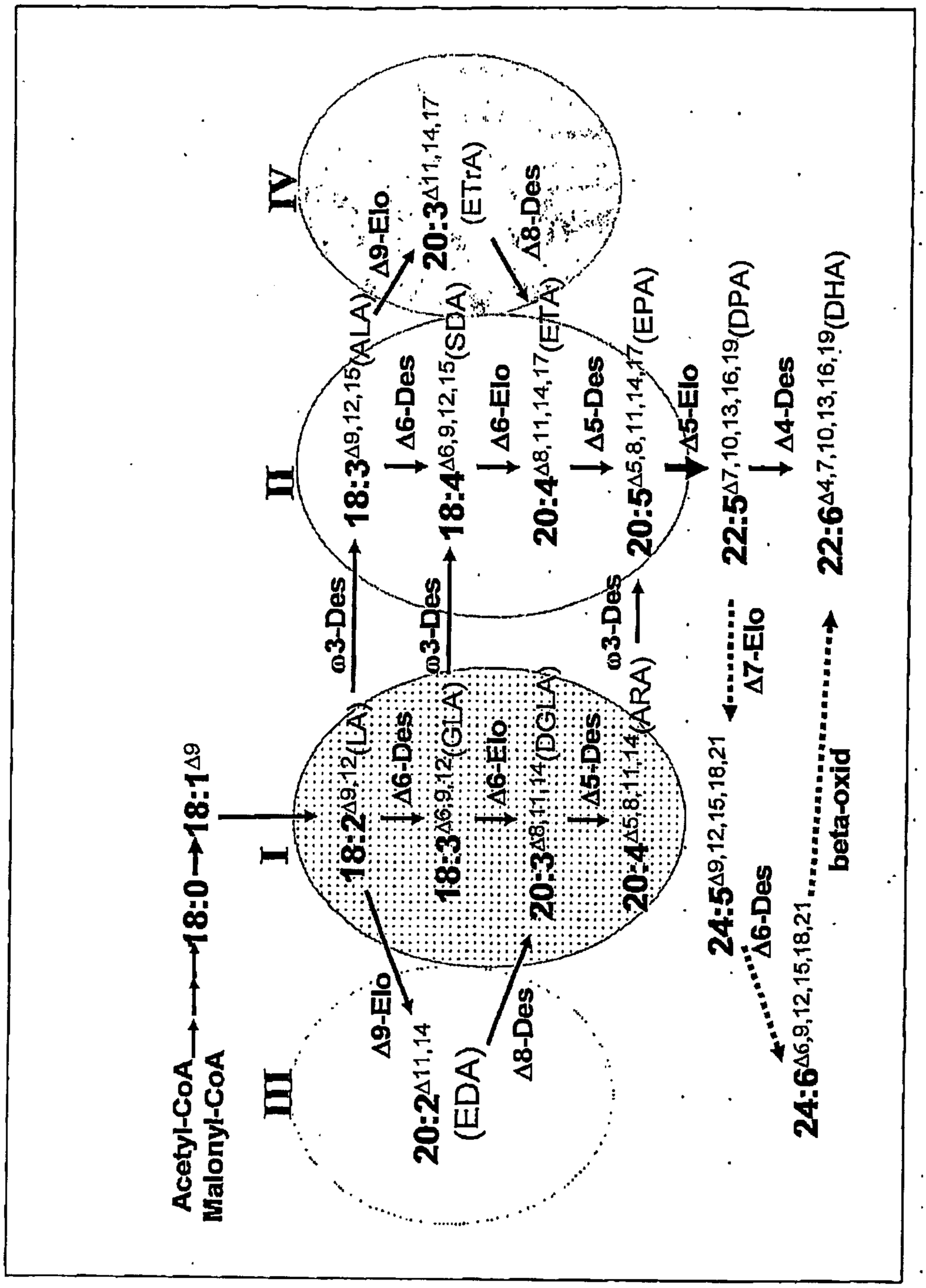

Acetyl-CoA
Malonyl-CoA
18:0
18:1Δ9
I
18:2Δ9,12(LA)
Δ6-Des
18:3Δ6,9,12(GLA)
Δ6-Elo
20:3Δ8,11,14(DGLA)
Δ5-Des
20:4Δ5,8,11,14(ARA)
ω3-Des
III
Δ9-Elo
20:2Δ11,14
(EDA)
Δ8-Des
II
18:3Δ9,12,15(ALA)
Δ6-Des
18:4Δ6,9,12,15(SDA)
Δ6-Elo
20:4Δ8,11,14,17(ETA)
Δ5-Des
20:5Δ5,8,11,14,17(EPA)
Δ5-Elo
22:5Δ7,10,13,16,19(DPA)
Δ4-Des
22:6Δ4,7,10,13,16,19(DHA)
ω3-Des
IV
Δ9-Elo
20:3Δ11,14,17
(ETrA)
Δ8-Des
24:5Δ9,12,15,18,21
Δ7-Elo
Δ6-Des
24:6Δ6,9,12,15,18,21
beta-oxid

Figure 1

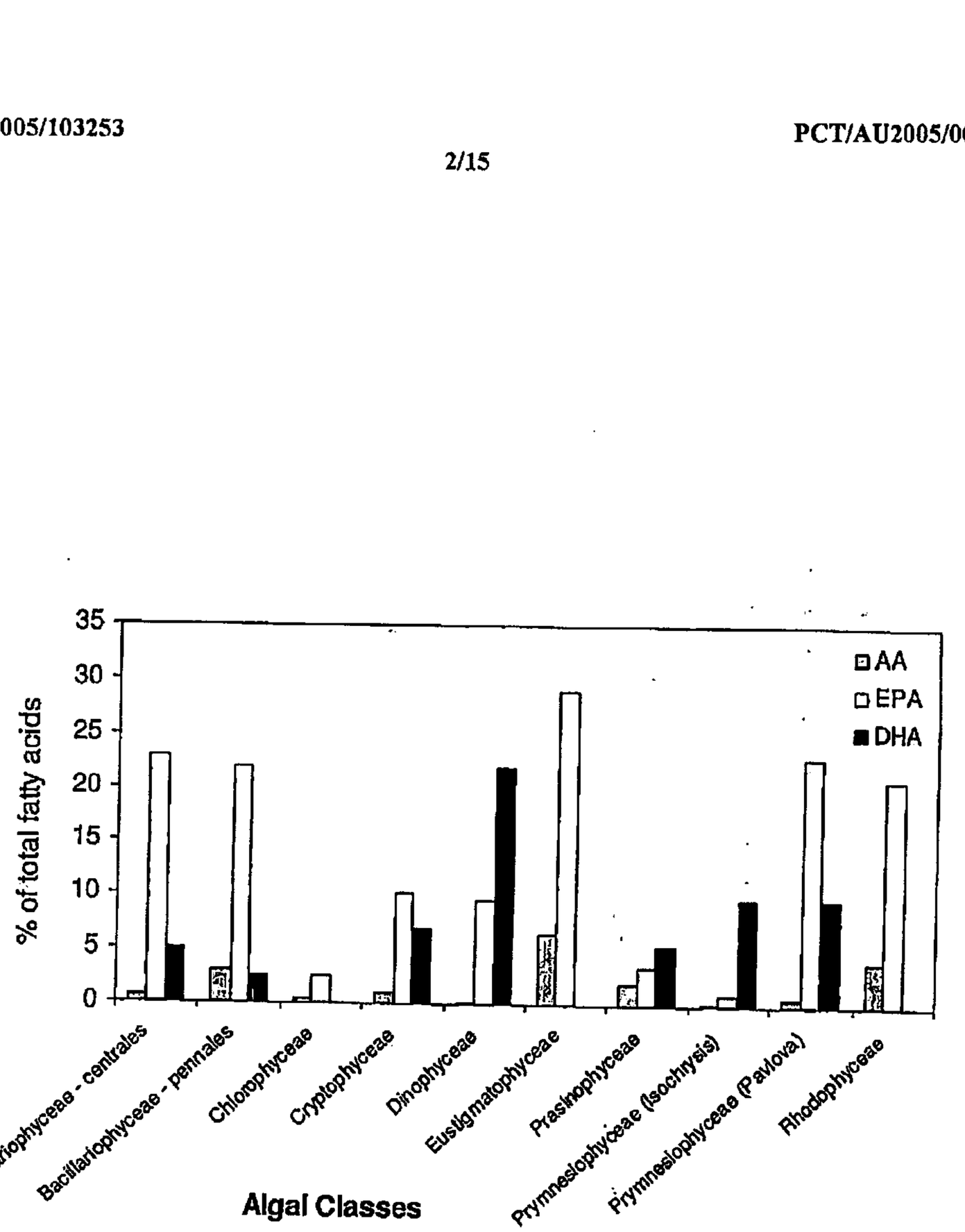

35
30
25
20
15
10
5
0
% of total fatty acids
AA
EPA
DHA
Bacillariophyceae - centrales
Bacillariophyceae - pennales
Chlorophyceae
Cryptophyceae
Dinophyceae
Eustigmatophyceae
Prasinophyceae
Prymnesiophyceae (Isochrysis)
Prymnesiophyceae (Pavlova)
Rhodophyceae
Algal Classes

Figure 2

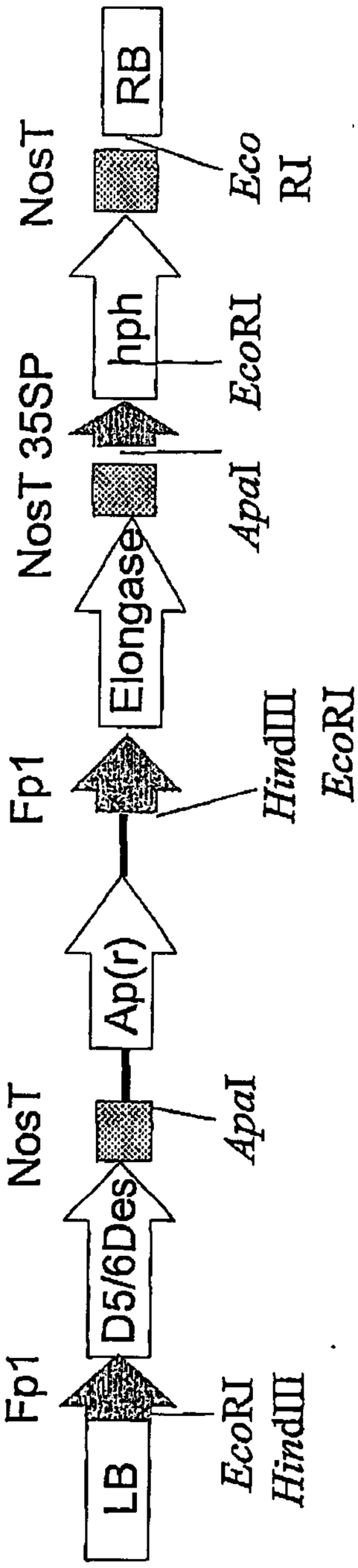

RB
NosT
Eco
RI
hph
EcoRI
NosT 35SP
ApaI
Elongase
Fp1
HindIII
EcoRI
Ap(r)
NosT
ApaI
D5/6Des
Fp1
EcoRI
HindIII
LB

Figure 3

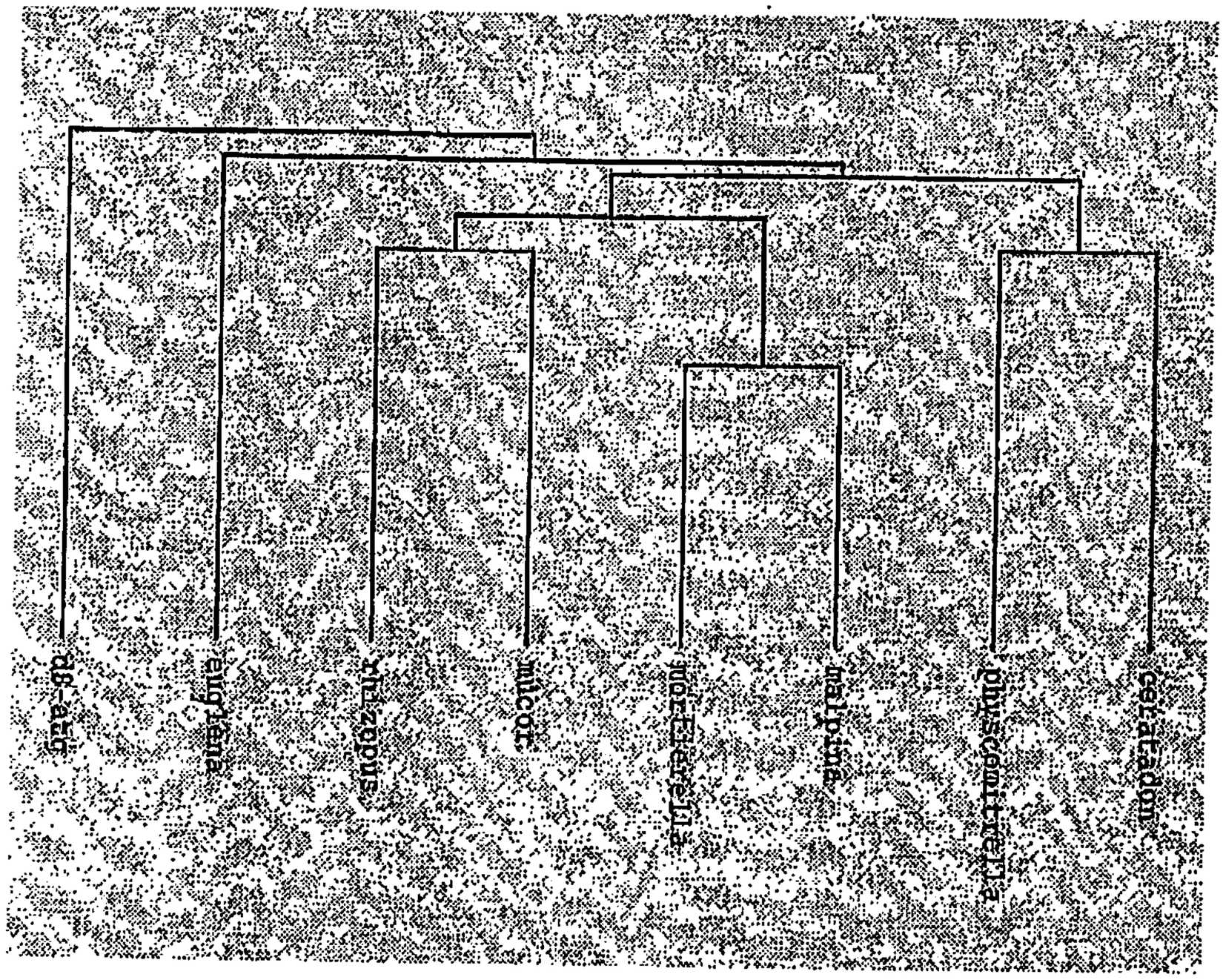

Cellacdom
Physcomitrella
Malpighia
Morella
Mucor
Rhizopus
Euglena
dg-arg

Figure 4

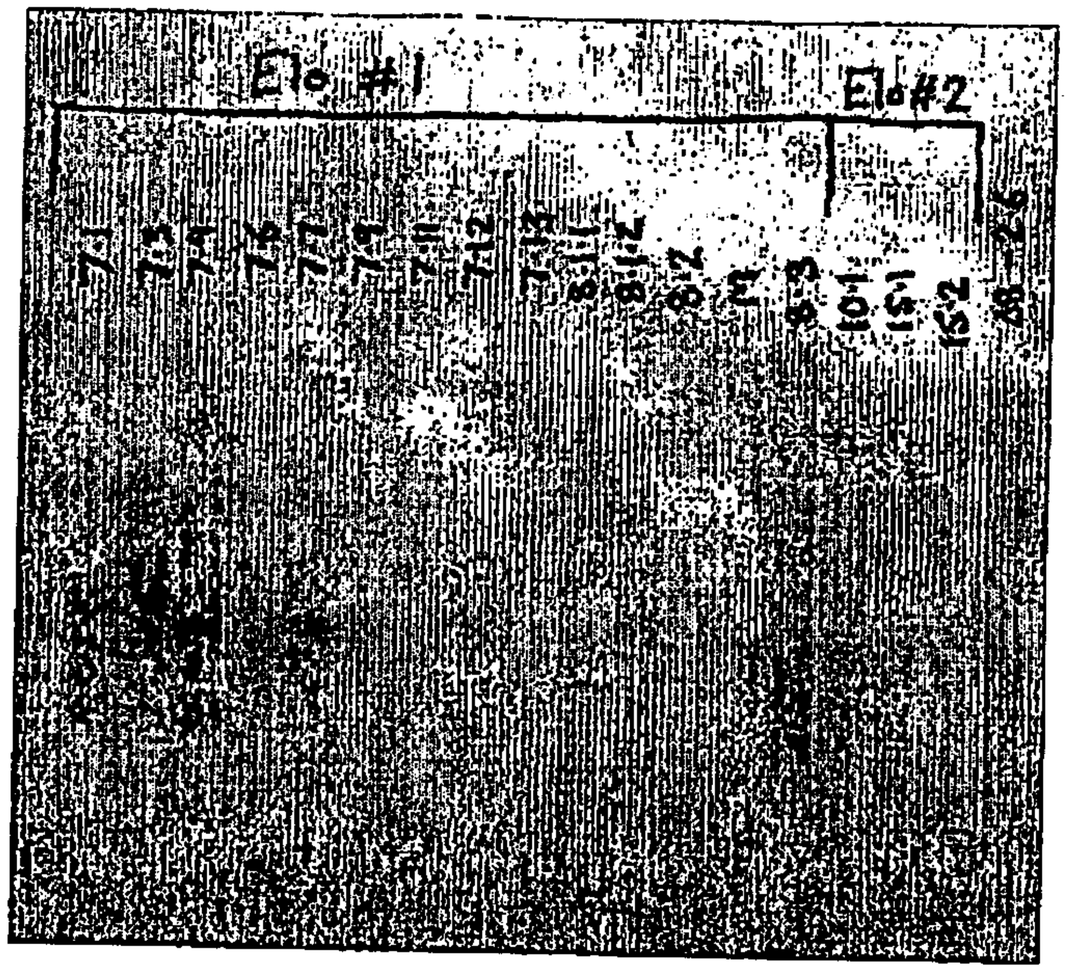


Figure 5

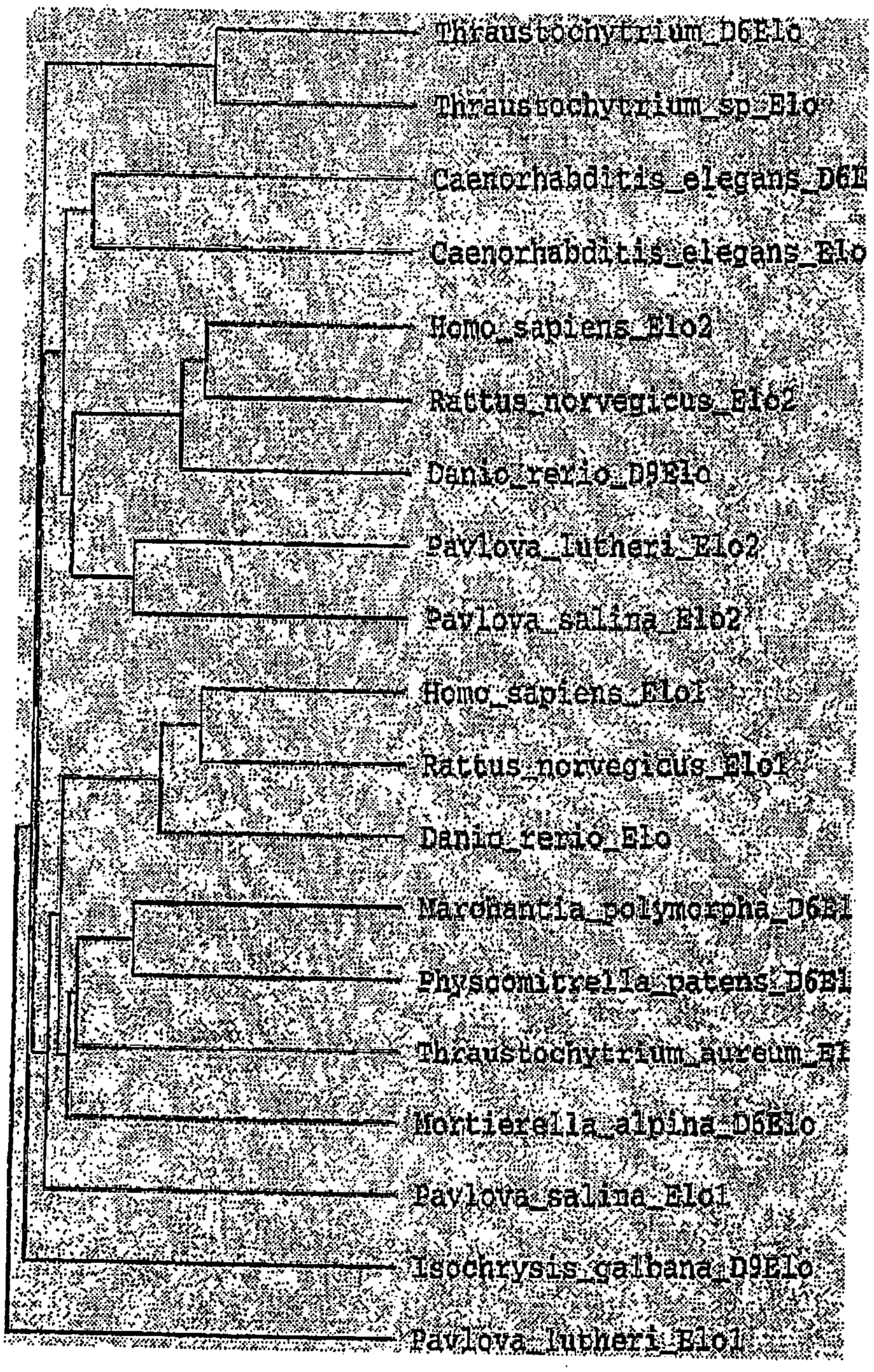

Thraustochytrium_D6Elo
Thraustochytrium_sp_Elo
Caenorhabditis_elegans_D6E
Caenorhabditis_elegans_Elo
Homo_sapiens_Elo2
Rattus_norvegicus_Elo2
Danio_rerio_D9Elo
Pavlova_lutheri_Elo2
Pavlova_salina_Elo2
Homo_sapiens_Elo1
Rattus_norvegicus_Elo1
Danio_rerio_Elo
Marchantia_polymorpha_D6El
Physcomitrella_patens_D6El
Thraustochytrium_aureum_El
Mortierella_alpina_D6Elo
Pavlova_salina_Elo1
Isochrysis_galbana_D9Elo
Pavlova_lutheri_Elo1

Figure 6

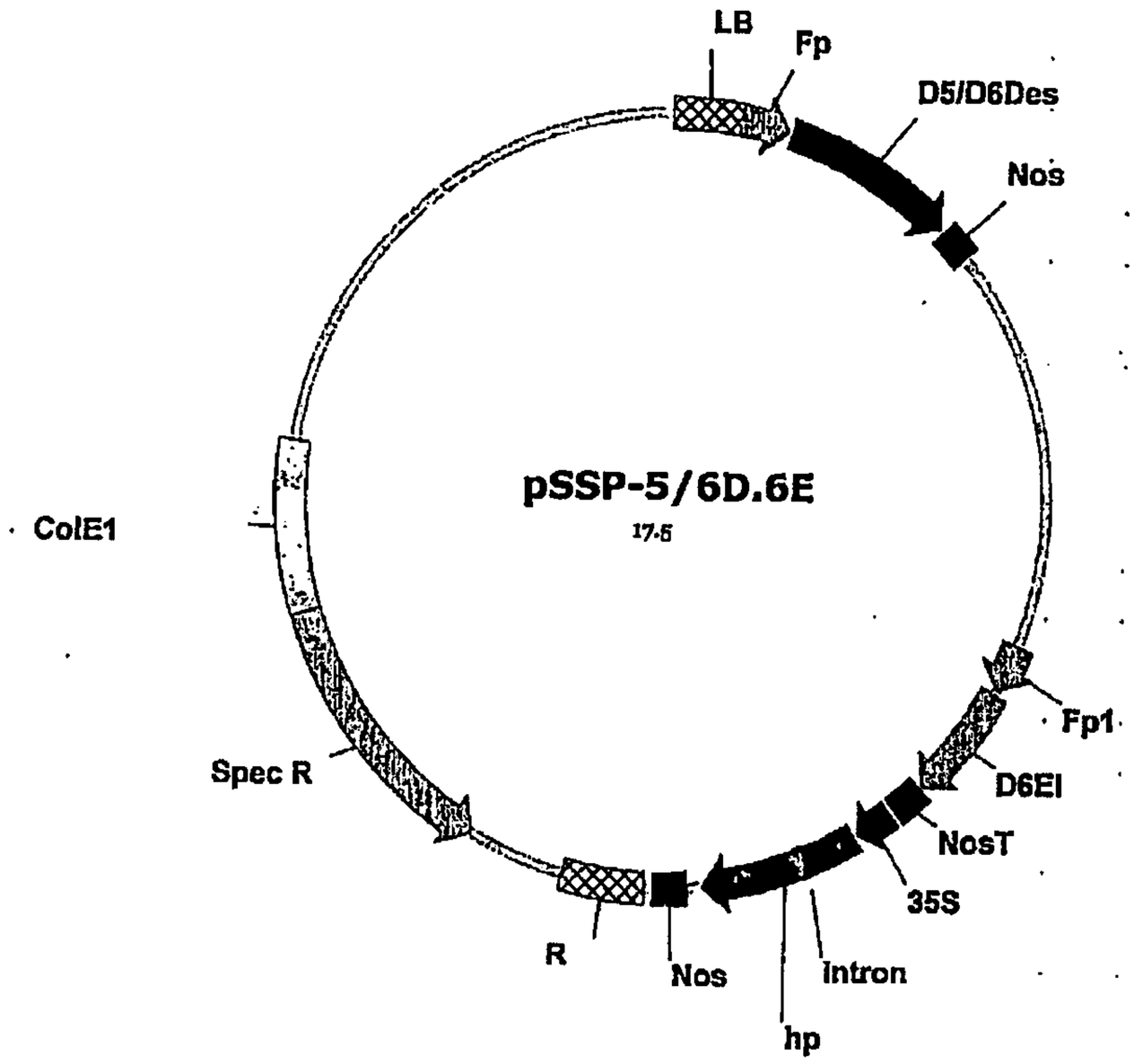

LB
Fp
D5/D6Des
Nos
pSSP-5/6D.6E
17.6
ColE1
Fp1
D6El
NosT
35S
Spec R
R
Nos
Intron
hp

Figure 7A

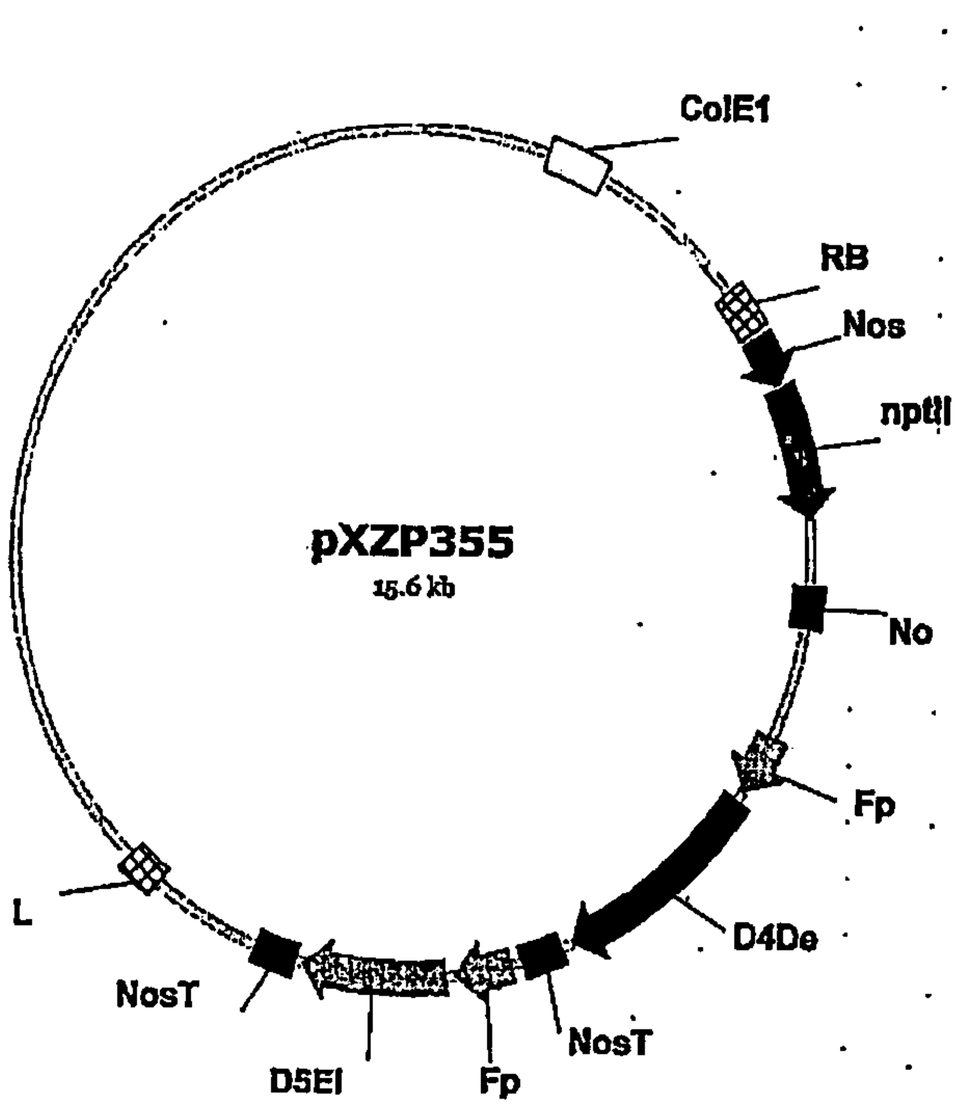
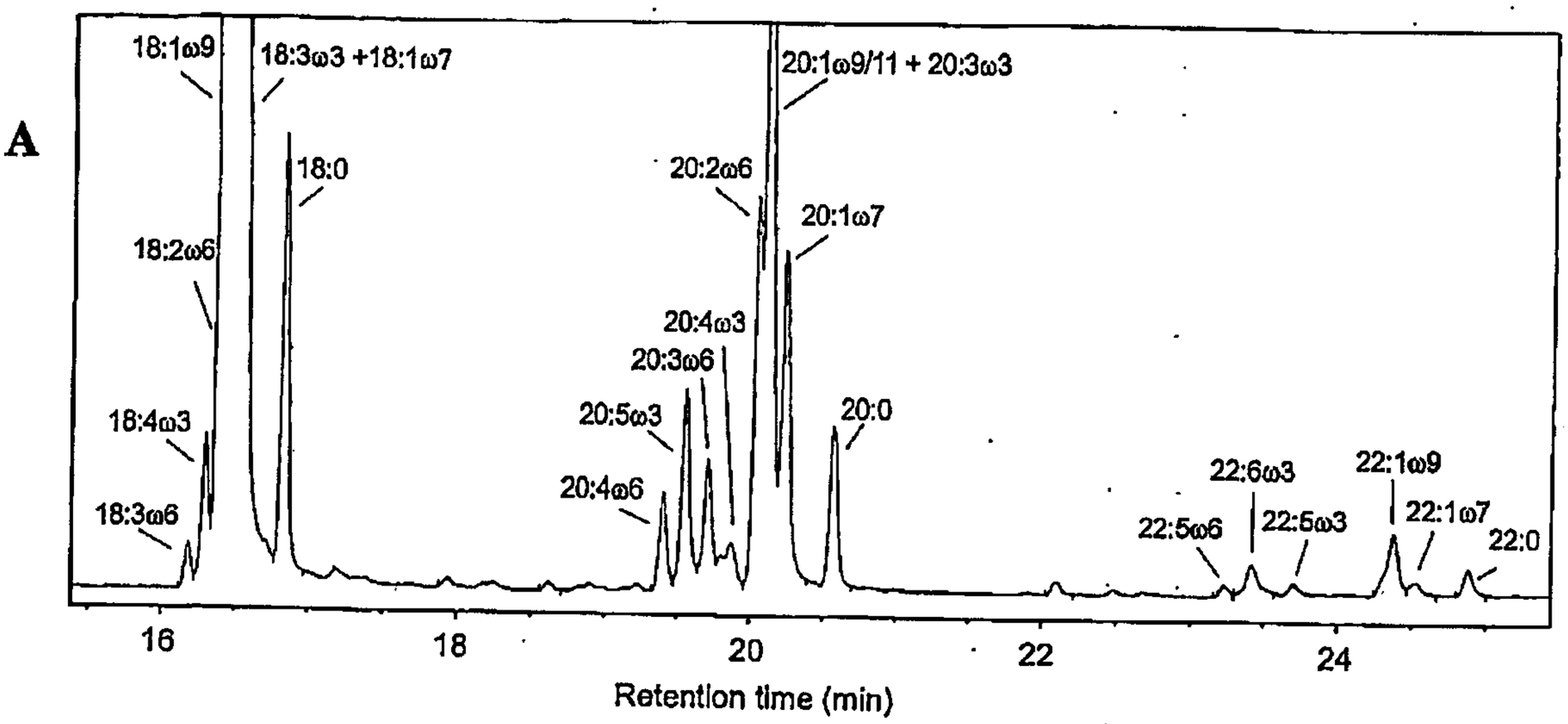
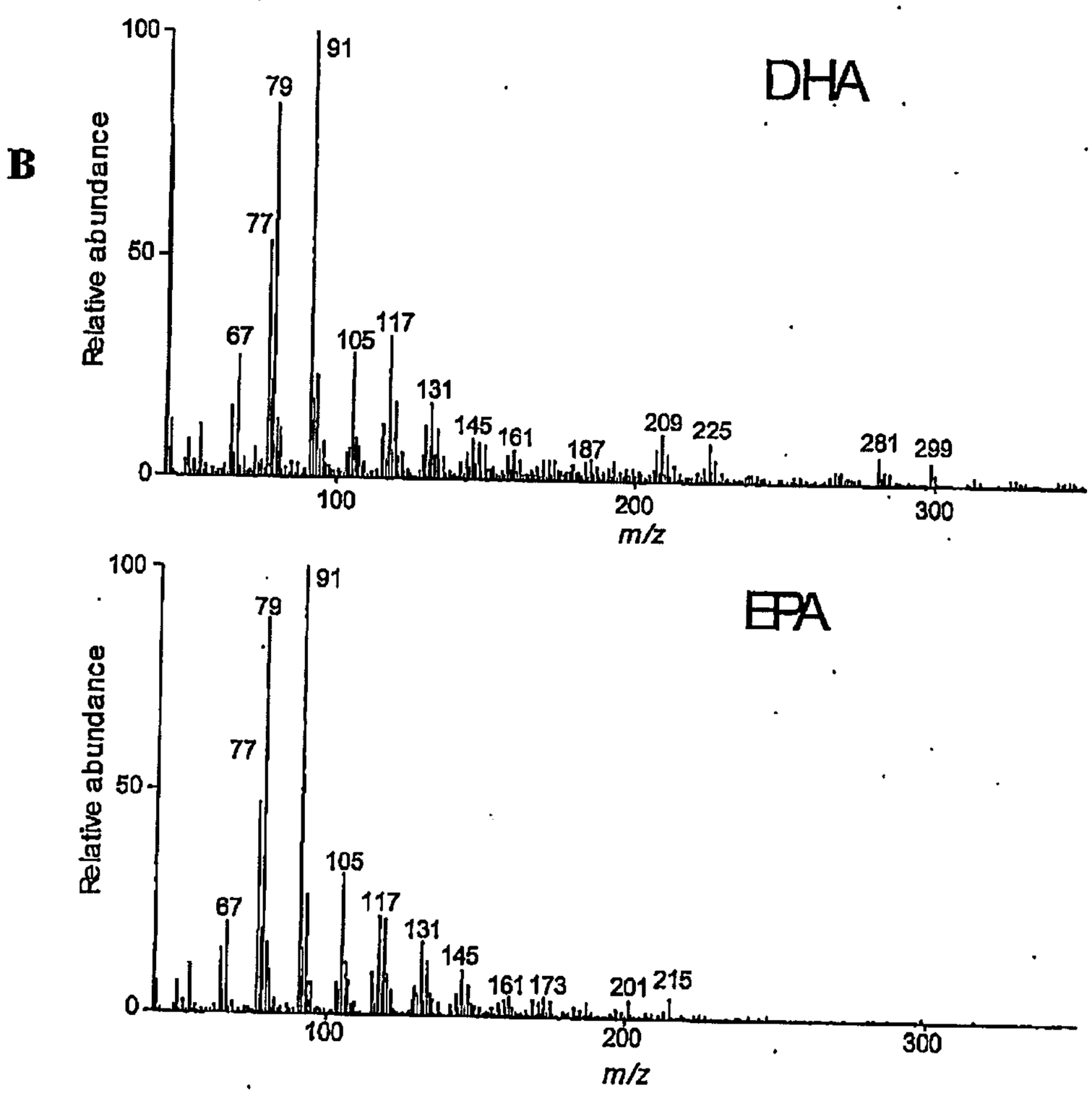

ColE1
RB
Nos
nptII
pXZP355
15.6 kb
No
Fp
D4De
NosT
D5El
Fp
NosT
L

Figure 7B


A
18:1ω9
18:3ω3 +18:1ω7
20:1ω9/11 + 20:3ω3
18:0
20:2ω6
18:2ω6
20:1ω7
20:4ω3
20:3ω6
18:4ω3
20:5ω3
20:0
20:4ω6
22:6ω3
22:1ω9
22:5ω6
22:5ω3
22:1ω7
22:0
18:3ω6
16
18
20
22
24
Retention time (min)


B
DHA
Relative abundance
100
91
79
77
50
67
105
117
131
145
161
187
209
225
281
299
0
100
200
300
m/z

EPA
Relative abundance
100
91
79
77
50
67
105
117
131
145
161
173
201
215
0
100
200
300
m/z

Figure 8

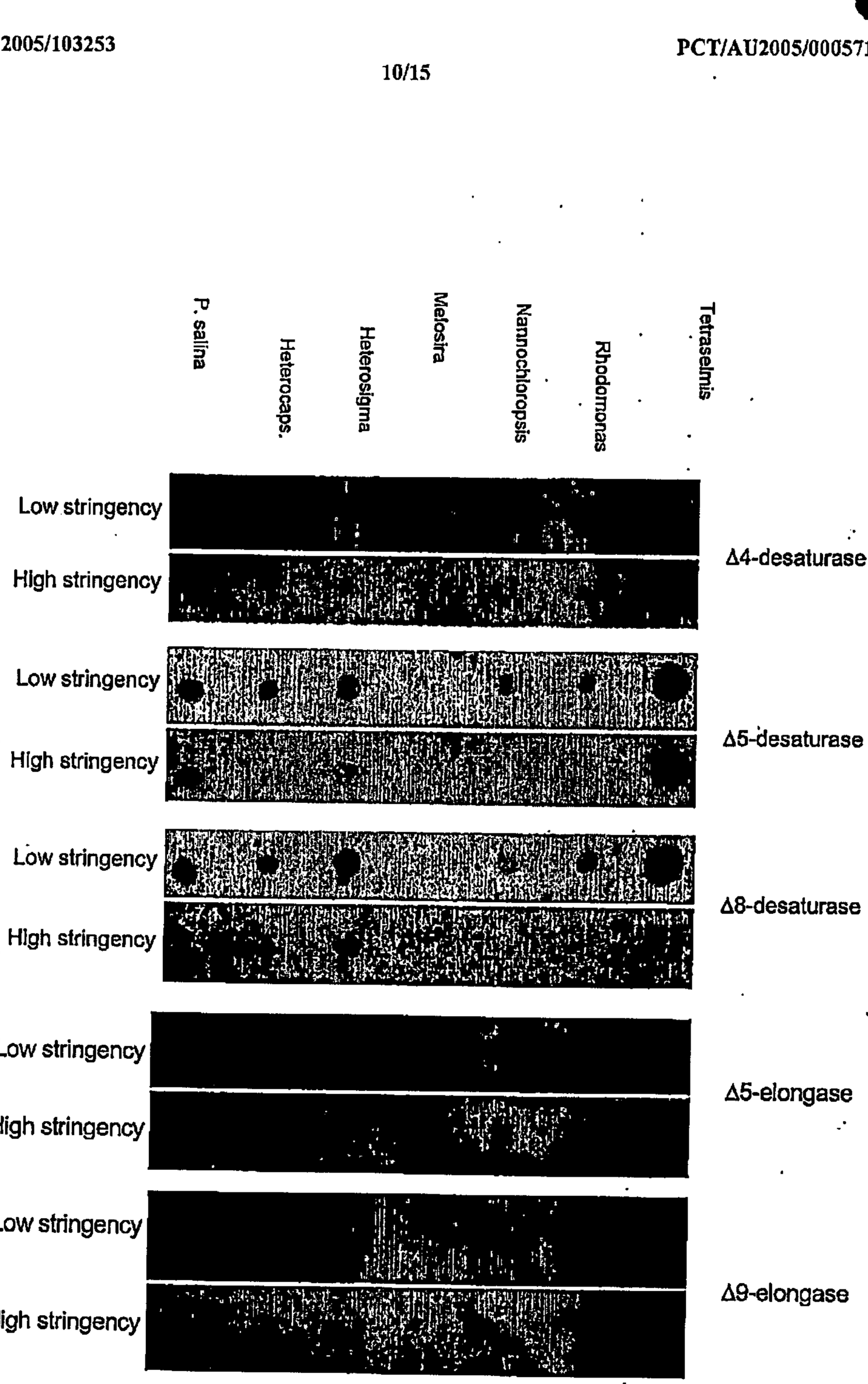

Tetraselmis
Rhodomonas
Nannochloropsis
Melosira
Heterosigma
Heterocaps.
P. salina
Low stringency
High stringency
Δ4-desaturase
Low stringency
High stringency
Δ5-desaturase
Low stringency
High stringency
Δ8-desaturase
Low stringency
High stringency
Δ5-elongase
Low stringency
High stringency
Δ9-elongase

Figure 9

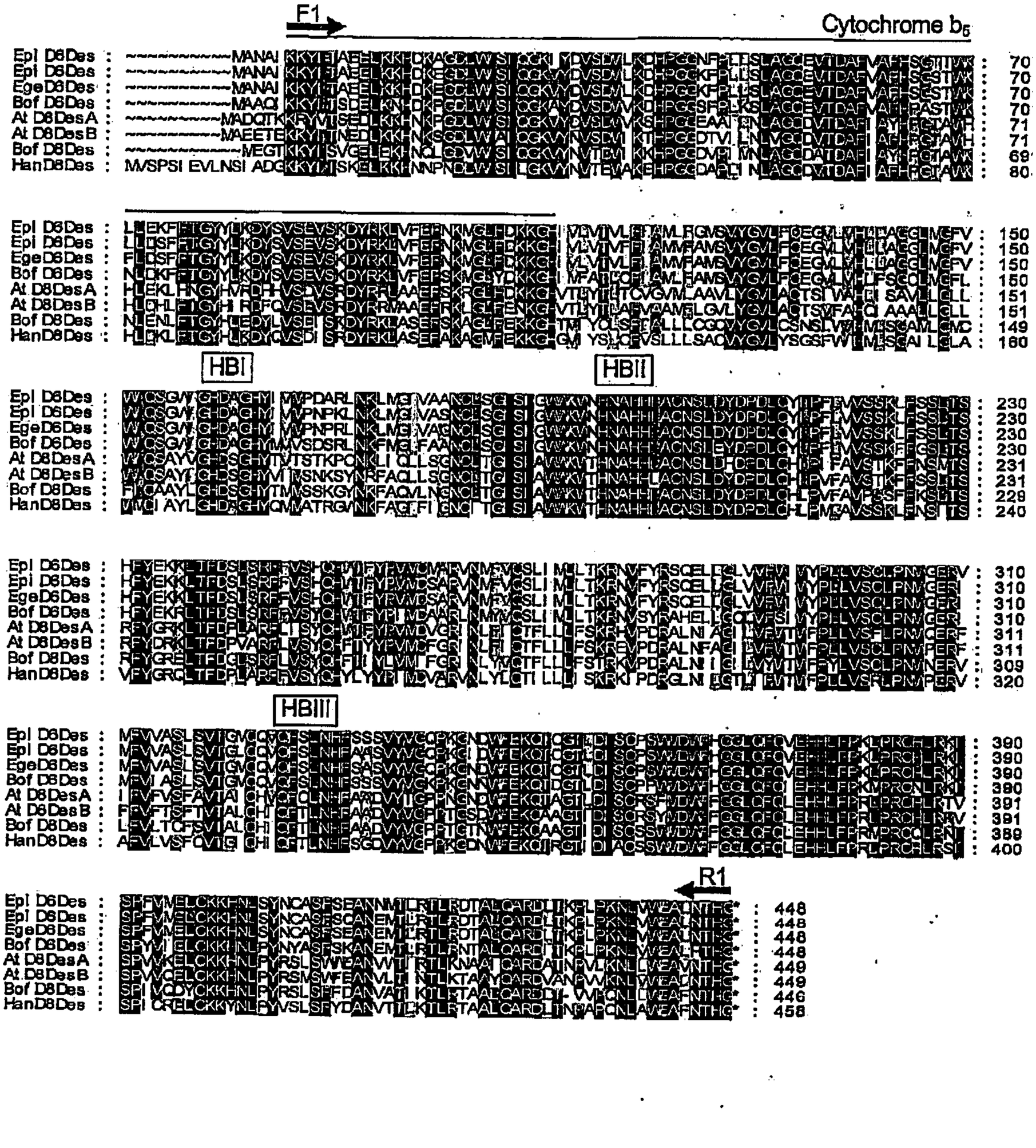


Figure 10

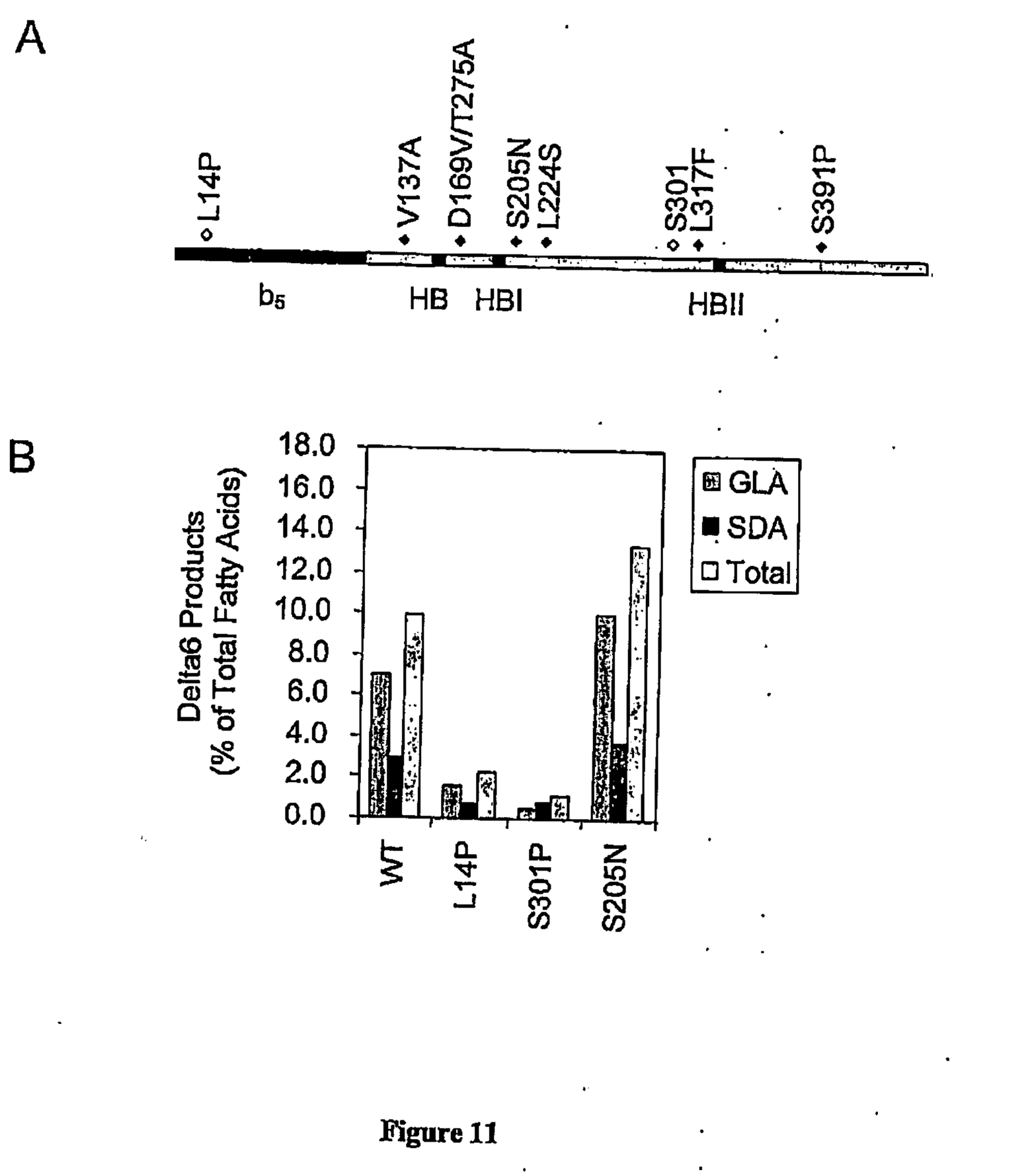

A
L14P
V137A
D169V/T275A
S205N
L224S
S301
L317F
S391P
b5
HB
HBI
HBII
B
Delta6 Products
(% of Total Fatty Acids)
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
GLA
SDA
Total
WT
L14P
S301P
S205N

Figure 11

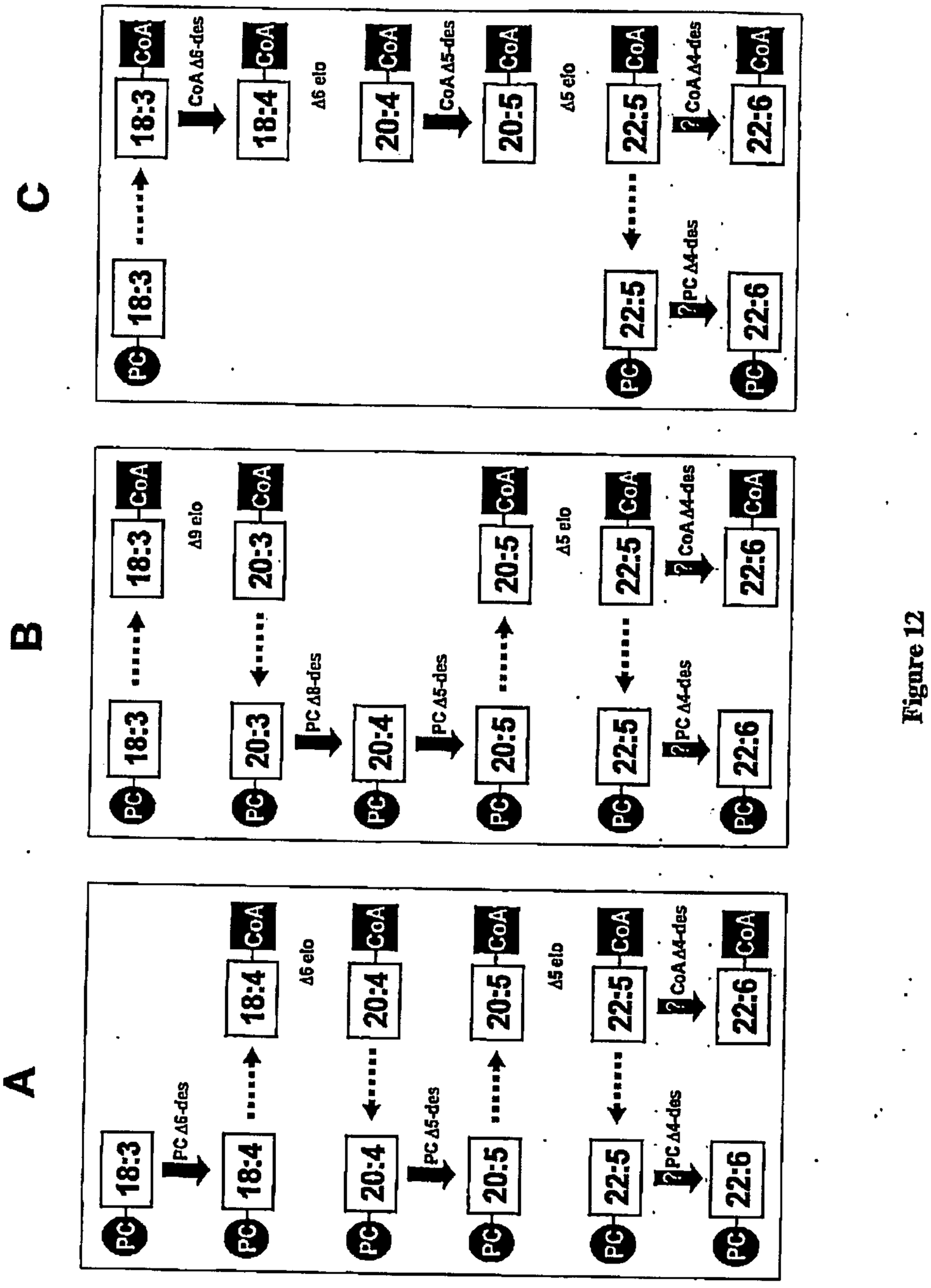

A
18:3
CoA
Δ6 elo
18:4
20:4
20:5
Δ5 elo
22:5
22:6
PC
B
Δ9 elo
20:3
C
PC Δ6-des
PC Δ8-des
PC Δ5-des
PC Δ4-des
CoA Δ6-des
CoA Δ5-des
CoA Δ4-des

Figure 12

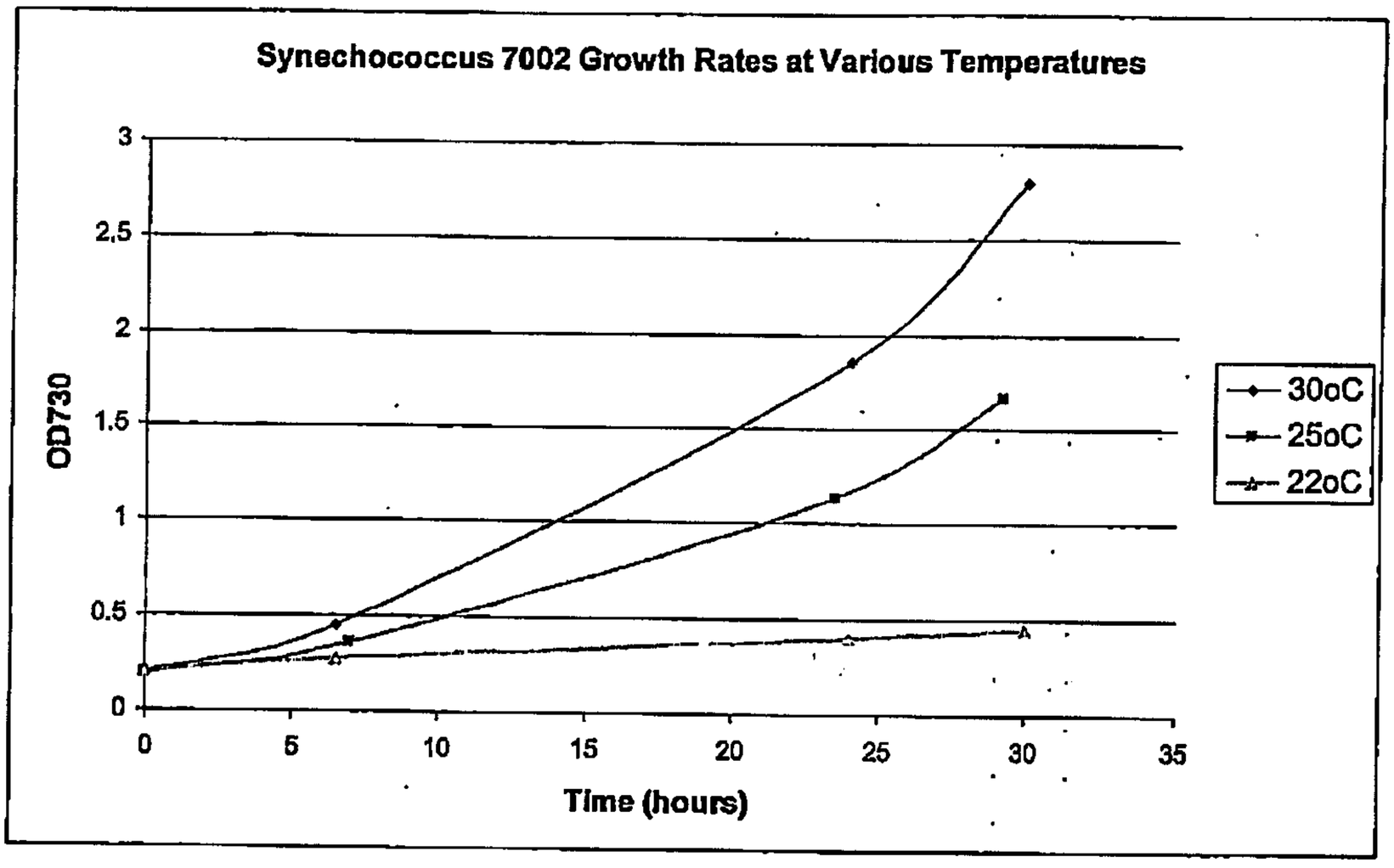

Synechococcus 7002 Growth Rates at Various Temperatures
OD730
3
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
Time (hours)
30oC
25oC
22oC

Figure 13

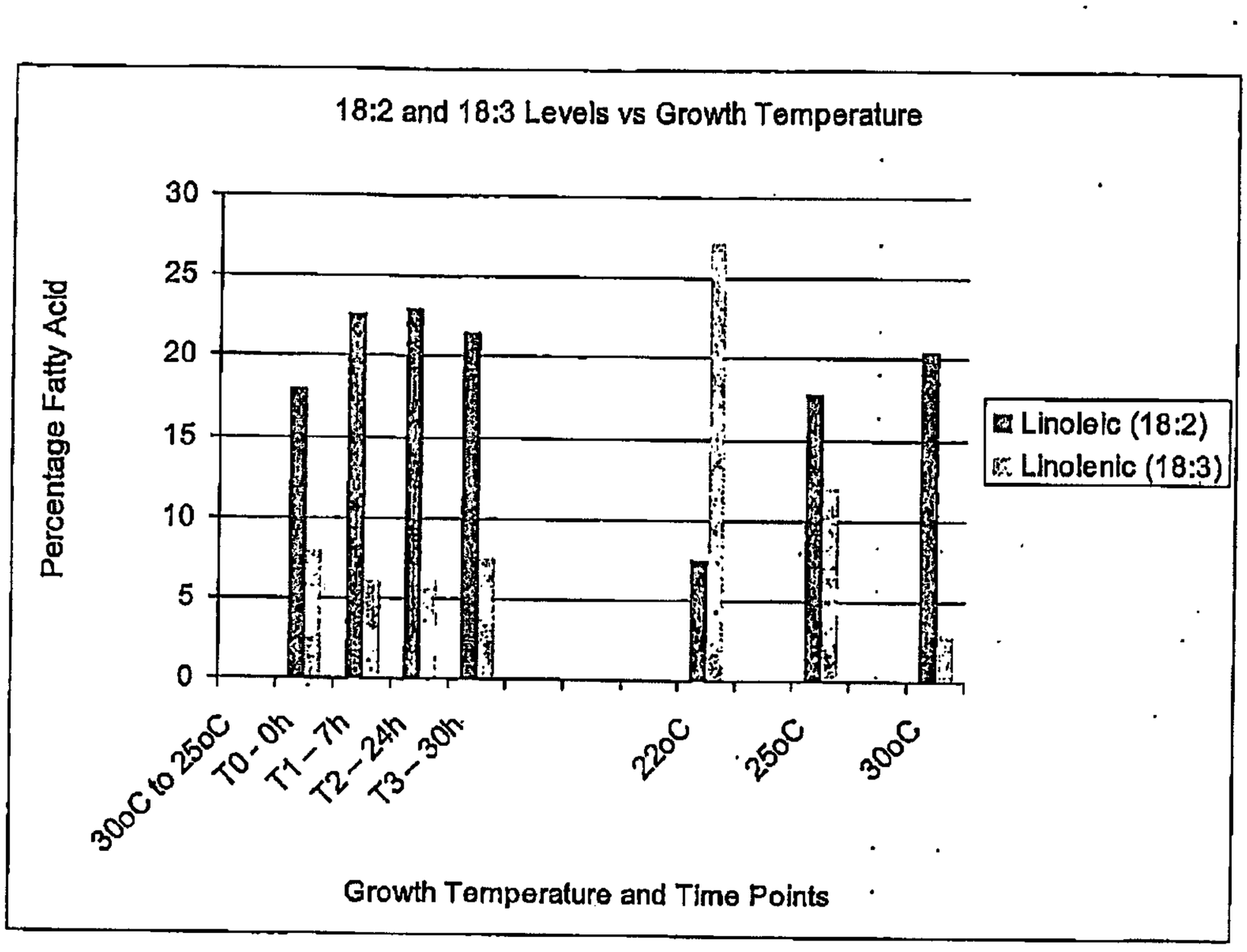
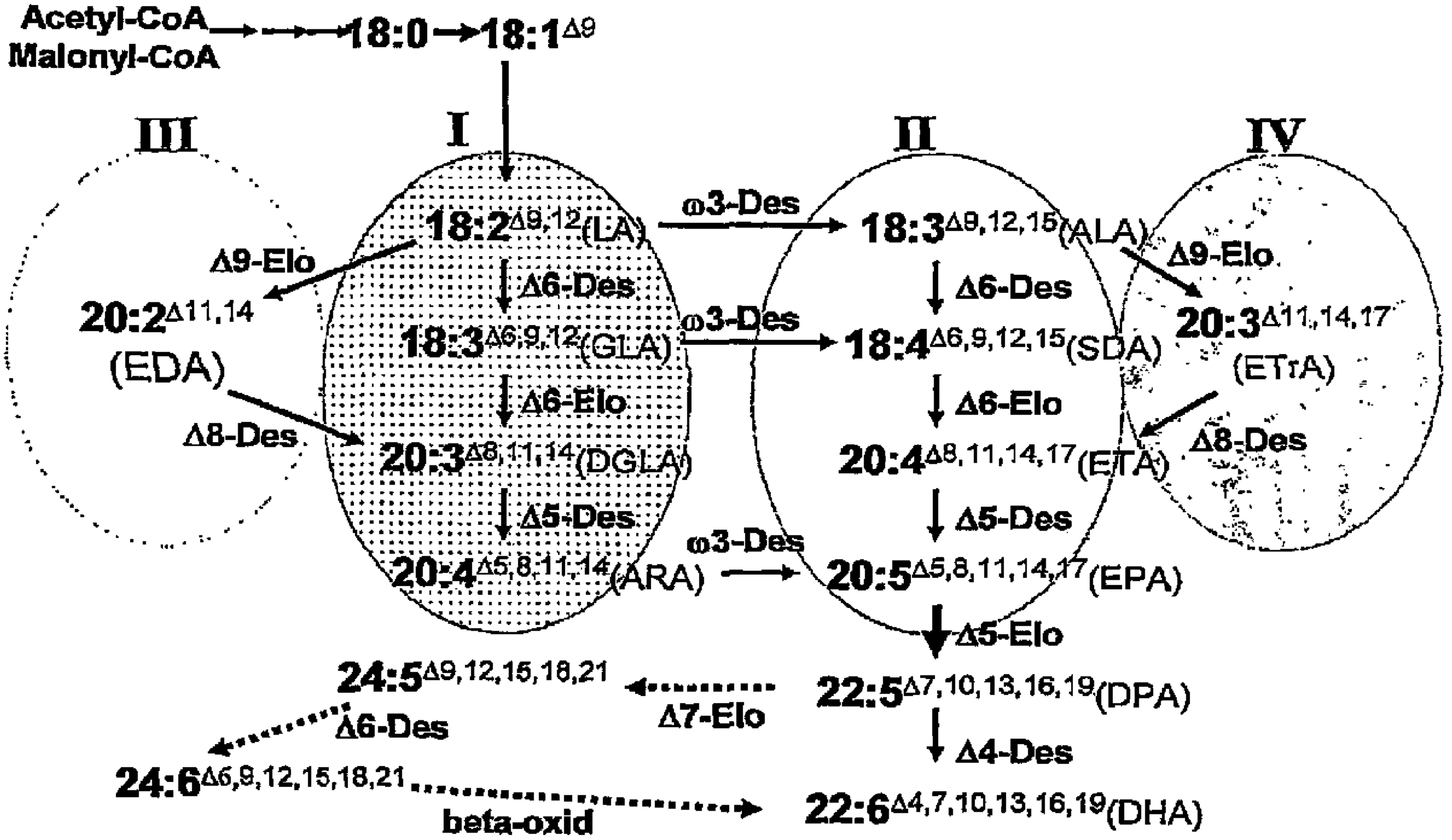

18:2 and 18:3 Levels vs Growth Temperature
Percentage Fatty Acid
30
25
20
15
10
5
0
Linoleic (18:2)
Linolenic (18:3)
30oC to 25oC
T0 - 0h
T1 - 7h
T2 - 24h
T3 - 30h
22oC
25oC
30oC
Growth Temperature and Time Points

Figure 14


Acetyl-CoA
Malonyl-CoA
18:0
18:1Δ9
III
I
II
IV
18:2Δ9,12(LA)
ω3-Des
18:3Δ9,12,15(ALA)
Δ9-Elo
Δ9-Elo
20:2Δ11,14
(EDA)
Δ6-Des
Δ6-Des
ω3-Des
18:3Δ6,9,12(GLA)
18:4Δ6,9,12,15(SDA)
20:3Δ11,14,17
(ETrA)
Δ6-Elo
Δ6-Elo
Δ8-Des
20:3Δ8,11,14(DGLA)
20:4Δ8,11,14,17(ETA)
Δ8-Des
Δ5-Des
Δ5-Des
ω3-Des
20:4Δ5,8,11,14(ARA)
20:5Δ5,8,11,14,17(EPA)
Δ5-Elo
24:5Δ9,12,15,18,21
Δ7-Elo
22:5Δ7,10,13,16,19(DPA)
Δ6-Des
Δ4-Des
24:6Δ6,9,12,15,18,21
beta-oxid
22:6Δ4,7,10,13,16,19(DHA)