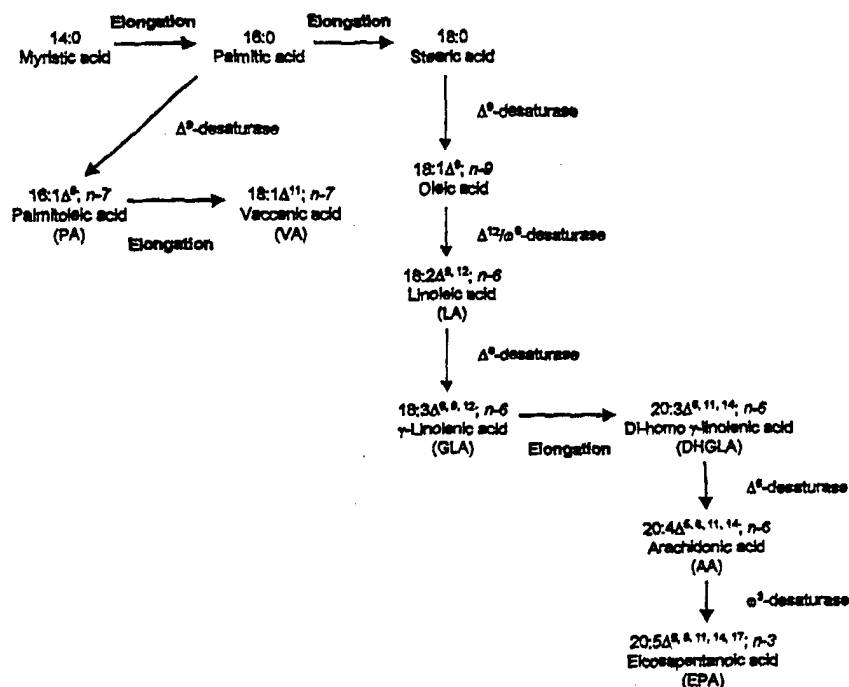




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <p>(51) International Patent Classification ⁷ : C12N 15/54, 9/10, 1/21, A01H 5/00, C07C 57/03, 57/12, A61K 38/45, 31/202, A23L 1/30</p> | A1 | <p>(11) International Publication Number: WO 00/55330</p> <p>(43) International Publication Date: 21 September 2000 (21.09.00)</p> |
| <p>(21) International Application Number: PCT/GB00/01035</p> <p>(22) International Filing Date: 20 March 2000 (20.03.00)</p> <p>(30) Priority Data: 9906307.5 18 March 1999 (18.03.99) GB 0003869.5 18 February 2000 (18.02.00) GB</p> <p>(71) Applicant (for all designated States except US): THE UNIVERSITY OF BRISTOL [GB/GB]; Senate House, Tyndall Avenue, Bristol BS8 1TH (GB).</p> <p>(72) Inventor; and (75) Inventor/Applicant (for US only): NAPIER, Johnathan, A. [GB/GB]; IACR - Long Ashton Research Station, Dept. of Agriculture Sciences, University of Bristol, Long Ashton, Bristol BS41 9AF (GB).</p> <p>(74) Agents: DEAN, John, Paul et al.; Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).</p> | | <p>(81) Designated States: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p> |

(54) Title: POLYSATURATED FATTY ACID (PUFA) ELONGASE FROM CAENORHABDITIS ELEGANS



(57) Abstract

A isolated polypeptide comprising a functional long chain polyunsaturated fatty acid (PUFA) elongase.

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POLYSATURATED FATTY ACID (PUFA) ELONGASE FROM CAENORHABDITIS ELEGANS

The present invention relates to polyunsaturated fatty acid (PUFA) elongases. More specifically, the invention relates to a DNA sequence from *C. elegans* encoding a PUFA elongase.

Unsaturated fatty acids are essential components required for normal cellular function, being involved in a diverse number of roles ranging from membrane fluidity to acting as signal molecules (Gill, I., Valivety, R. (1997). *Trends Biotechnol.* **15**, 401-409; Broun, P., *et al* (1999) *Ann. Rev. Nutr.* **19**, 197-216). In particular, the class of fatty acids known as the polyunsaturated fatty acids (PUFAs) has attracted considerable interest as pharmaceutical and nutraceutical compounds (Broun *supra*; Horrobin, D. F. (1990) *Reviews in Contemp Pharmacotherapy* **1**, 1-45).

The synthesis of PUFAs i.e. fatty acids of 18 carbons or more in length and containing two or more double bonds, is thought to be catalyzed in a variety of organisms by a specific fatty acid elongase enzyme. This elongase is responsible for the addition of 2 carbon units to an 18 carbon PUFA, resulting in a 20 carbon fatty acid. An example of this reaction is the elongation of γ -linolenic acid (GLA; 18:3 $\Delta^{6,9,12}$) to di-homo- γ -linolenic acid (DHGLA; 20:3 $\Delta^{8,11,14}$) in which the tri-unsaturated 18 carbon fatty acid is elongated by the addition of a two carbon unit to yield the tri-unsaturated 20 carbon fatty acid. Since there is considerable interest in the production of long chain PUFAs of more than 18 carbons in chain length, for example arachidonic acid and eicosapentanoic acid, the identification of this enzyme is of both academic and commercial interest.

At present, there are no examples of identified cloned genes encoding PUFA elongases, though a number of genes encoding enzymes likely to be involved in other aspects of lipid synthesis have been identified. For example, an *Arabidopsis* gene (FAE1) has been shown to be required for the synthesis of very long chain monounsaturated fatty acids (such as erucic acid; 20:1 Δ^{11}) (James, D. W. *et al*, (1995) *Plant Cell* **7**, 309-319). However, it is clear that this enzyme does not recognize di- and tri-unsaturated 18 carbon fatty acids, for example, linoleic acid, 18:2 $\Delta^{9,12}$ or α -linolenic acid, 18:3 $\Delta^{9,12,15}$ respectively, as substrates,

and is therefore not involved in the synthesis of long chain PUFAs (Millar & Kunst (1997), *Plant Journal* **12**, 121-131). This in itself is not surprising, since, of the plant kingdom, only a very few lower plant species, such as the moss *Physcomitrella patens* (Girke *et al.*, (1998), *Plant J*, **15**: 39-48); are capable of synthesising long chain PUFAs, and therefore *Arabidopsis* would not be expected to contain any such enzymes (Napier *et al.* (1997), *Biochem J*, **328**: 717-720; Napier *et al.*, (1999) *Trends in Plant Sci* **4**, 2-5).

A schematic diagram representing a generalized pathway for the product of PUFAs is shown in Figure 1. Biochemical characterisation of mammalian elongation systems (most notably from liver microsomes) has indicated that a mammalian elongase consists of four subunits, made up of a condensing enzyme, a β -ketoreductase, a dehydrase and an enoyl reductase (reviewed in Cinti, D. L., *et al* (1992) *Prog. Lipid Res.* **31**, 1-51). The *Arabidopsis FAE1* gene product encodes a polypeptide of 56kDa, which shows very limited homology to condensing enzymes such as chalcone synthase and stilbene synthase (James, D. W. *supra*). Although *FAE1* is normally only expressed in seed tissues, ectopic expression in non-seed tissue (or heterologously in yeast) revealed that *FAE1* could direct the synthesis of erucic acid (Millar, A. A., Kunst, L. (1997) *Plant J.* **12**, 121-131).

Three fatty acid elongase activities have been characterised from the yeast *S. cerevisiae*. Again, this organism does not synthesis PUFAs, and therefore does not contain genes encoding a PUFA elongase. One gene ELO1, was identified on the basis of a screen to isolate mutants defective in elongation of 14 carbon (i.e. medium) chain saturated fatty acids (Toke & Martin (1996) *J Biol Chem* **271**, 18413-18422). Complementation of *elol* mutants restored viability, and the ELO1 gene product was shown to encode a polypeptide which was responsible for the specific elongation of 14:0 fatty acids to 16:0 fatty acids.

Two related genes were also detected in the genome of *S. cerevisiae*, and their function determined by disruption. These two genes, subsequently named ELO2 and ELO3, were shown to be involved in the elongation of the very long chain saturated fatty acids found in sphingolipid molecules (Oh *et al* (1997), *J. Biol Chem* **272**, 17376-17384). In particular, ELO2 was required for elongation of fatty acids up to 24 carbons, and ELO3 was required for elongation of the 24 carbon fatty acid to 26 carbons. However, neither gene was

essential for viability. Examination of these three fatty acid elongases revealed the presence of a conserved "histidine box" motif (Shanklin *et al.*, (1994), *Biochemistry*, **33**, 12787-12794) (His-X-X-His-His, where X is any amino acid) towards the centre of the polypeptide sequences. Importantly, there was no detectable homology between the yeast elongases (ELO1,2,3) and the plant very long chain mono-unsaturated fatty acid elongase (FAE1) (Oh *et al.*, *supra*).

In order to identify genes encoding PUFA elongases, it is necessary to study systems in which the synthesis of PUFAs is well documented; a good example of this is the model animal system *C. elegans*, a small free-living worm (Tanaka *et al.*, (1996), *Lipids* **31**, 1173-1178). *C. elegans*, like most other animals, and in contrast to higher plants, synthesises PUFAs such as arachidonic acid (AA; 20:4 $\Delta^{5,8,11,14}$) as precursors to a class of molecules known as the eicosanoids, which in turn serve as precursors for compounds such as prostaglandins and leucotrienes (Horrobin, (1990), *Reviews in Contemp Pharmacotherapy*, **1**:1-45). The presence of AA and other long chain polyunsaturated fatty acids in *C. elegans* is well documented (Tanaka *et al.*, (1996), *Lipids* **31**, 1173-1178). The complete sequence of the nematode's genome is now publicly available (*The C. elegans consortium*, 1998, *Science* **282**, 2012-2018: Database at http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml).

An object of the invention is to provide an isolated PUFA elongase.

Using the above-mentioned *C. elegans* genomic sequence, together with suitable search strings, the inventors identified eight related putative open reading frames (ORFs) encoding for PUFA elongases. A number of different search criteria were applied to identify a number of (ORFs) which were likely to encode polypeptides with fatty acid elongase activities. These ORFs were then subject to functional characterisation by heterologous expression in yeast, allowing the identification of a PUFA elongase.

Accordingly, a first aspect of the invention provides an isolated polypeptide comprising a functional long chain polyunsaturated fatty acid (PUFA) elongase i.e. the polypeptide has the function of extending the chain length of an 18 carbon PUFA to 20 carbons in length.

This polypeptide can be used to elevate PUFA levels in animals, thereby providing a ready source of PUFAs.

The polypeptide may be from a eukaryote.

The polypeptide may comprise at least a portion of the amino acid shown in SEQ ID. 15, or variants thereof.

For the purposes of the present application, the term "variant" in relation to a certain sequence means a protein or polypeptide which is derived from the sequence through the insertion or deletion of one or more amino acid residues or the substitution of one or more amino acid residues with amino acid residues having similar properties, e.g. the replacement of a polar amino acid residue with another polar amino acid residue, or the replacement of a non-polar amino acid residue with another non-polar amino acid residue. In all cases, variants must have an elongase function as defined herein.

A second aspect of the invention provides a polypeptide having at least 60 % homology to a polypeptide according to a first aspect of the invention. The polypeptide may have at least 80%, or as much as 90% or more homology to a polypeptide according to a first aspect of the invention.

The polypeptide according to either aspect of the invention may include a sequence motif responsible for Endoplasmic Reticulum (ER) - retention. This allows the polypeptide to be specifically located or targeted to the ER of a cell.

The polypeptide may also be able to elongate palmitoleic acid (PA; 16:1 Δ^9) to vacceric acid (VA; 18:1 Δ^{11}). Thus, the polypeptide is also capable of elongation of a Δ^9 - monounsaturated 16C fatty acid.

Preferably, the polypeptide is from an animal, more preferably, the animal is an invertebrate such as a worm. Where the animal is a worm, it is preferably *C. elegans*. Alternatively, the animal is a vertebrate, preferably a mammal such as a human, rat or mouse.

A third aspect of the invention provides an isolated DNA sequence, preferably a cDNA sequence, encoding a polypeptide according to a first or second aspect of the invention. This DNA sequence may be used to engineer transgenic organisms.

Preferably, the DNA sequence comprises the sequence shown in SEQ ID NO: 7 or variants of that sequence due, for example, to base substitutions, deletions, and/or additions.

A fourth aspect of the invention provides an engineered organism, such as a transgenic animal, engineered to express a polypeptide according to a first or second aspect of the invention. The engineered organism may be engineered to express elevated levels of the polypeptide, thereby providing a supply of polypeptide at a reduced cost as a reduced number of organisms need be used.

Preferably, the engineered organism is a mammal such as a rat, mouse or monkey.

A fifth aspect of the invention provides an engineered organism containing a synthetic pathway for the production of a polypeptide according to a first or second aspect of the invention. This has the advantage of allowing greater control over the production of PUFAs by the pathway by an organism.

The pathway may include Δ^5 -fatty acid desaturase, and/or Δ^6 -fatty acid desaturase.

The engineered organism according to a fourth or fifth aspect of the invention may be a lower eukaryote, such as yeast. Alternatively, the transgenic organism may be a fish.

A sixth aspect of the invention provides a transgenic plant engineered to express a polypeptide according to a first aspect of the invention.

A seventh aspect of the invention provides a transgenic plant containing a DNA sequence according to a third aspect of the invention.

An eighth aspect of the invention provides a method of producing a PUFA comprising carrying out an elongase reaction catalysed by a polypeptide according to a first or second aspect of the invention.

The PUFA may be di-homo-gamma-linoleic acid ($20:3\Delta^{8,11,14}$), arachidonic acid ($20:4\Delta^{5,8,11,14}$), eicosapentanoic acid ($20:5\Delta^{5,8,11,14,17}$), docosatrienoic acid ($22:3\Delta^{3,16,19}$), docosatetraenoic acid ($22:4\Delta^{7,10,13,16}$), docosapentaenoic acid ($22:5\Delta^{7,10,13,16,19}$) or docosahexaenoic acid ($22:6\Delta^{4,7,10,13,16,19}$).

The PUFA may be a 24 carbon fatty acid with at least 4 double bonds.

A ninth aspect of the invention provides a PUFA produced by a method according to an eighth aspect of the invention.

The PUFA may be used in foodstuffs, dietary supplements or pharmaceutical compositions.

A tenth aspect of the invention provides a foodstuff comprising a PUFA according to a fifth aspect of the invention. The foodstuff can be fed to an animal.

An eleventh aspect of the invention provides a dietary supplement comprising a PUFA according to a fifth aspect of the invention. The dietary supplement can be supplied to an animal to augment its PUFA levels.

An twelfth aspect of the invention provides a pharmaceutical composition comprising a polypeptide according to a first or second aspect of the invention or a PUFA according to a ninth aspect of the invention.

Preferably, the pharmaceutical composition comprises a pharmaceutically-acceptable diluent, carrier, excipient or extender. This allows the composition to be supplied in a form which best suits the pharmaceutical application in question. For example, a topical application would preferably be a cream or lotion, whereas if the composition was to be ingested a different form would be more suitable.

A thirteenth aspect of the invention provides a method of treatment of an animal, such as a mammal, or a plant, comprising supplying to the animal or plant a DNA sequence according to a third aspect of the invention, a foodstuff according to a tenth aspect of the invention, a dietary supplement according to an eleventh aspect of the invention, a pharmaceutical composition according to a twelfth aspect of the invention or a PUFA according to a ninth aspect of the invention.

Preferably, the mammal is a human.

The invention will now be further described, by way of example only, with reference to SEQ ID1 to 16, and Figures 2 to 11, in which;

SEQ ID1 to 8 show the putative ORFs encoding PUFA elongases A to H respectively; and

SEQ ID9 to 16 show the deduced amino acid sequences of the putative ORFs of SEQ ID NO: 1 to 8 respectively; and

Figures 2 to 9 show hydrophobicity plots for each of PUFA elongases A to H respectively.

Figure 10 shows an amino acid sequence line-up comparing the *C. elegans* ORF F56H11.4 (Z68749) with related sequences.

Figure 11 shows chromatograms of fatty acid methyl esters from transformed yeast.

Introduction to general strategy

Initially the *C. elegans* databases were searched for any sequences which showed low levels of homology to yeast ELO genes (*ELO2* and *ELO3*) using the TBLASTN programme. A similar search was carried out using short (20 to 50 amino acid) stretches of ELO genes which were conserved amongst the three ELO polypeptide sequences. *C. elegans* sequences which were identified by this method were then used themselves as search probes, to identify any related *C. elegans* genes which the initial search with the yeast sequences failed to identify. This was necessary because the level of homology between the yeast ELO genes

and any worm genes is always low (see BLAST scores later). To allow for a more sensitive search of worm sequences, a novel approach was adopted to circumvent the major drawback with searches using the BLAST programmes, namely that the search string (i.e. the input search motif) must be longer than 15 characters for the algorithm to work. Thus, if it was desired to search for a short motif (like a histidine box), then the BLAST programme would not be capable of doing this. A complete list of all the predicted ORFs present in the *C. elegans* genome exists as a database called Wormpep, which is freely available from the Sanger WWW site (http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml). The latest version of Wormpep was downloaded to the hard disc of a Pentium PC, and re-formatted as a Microsoft Word6 document, resulting in a document of about 3,500 pages. This was then searched using the "Search & Replace" function of Word6, which also allows for the introduction of "wildcard" characters into the search motif. So, for example, it is possible to search both for the short text string HPGG, which would identify any predicted worm ORF present in the Wormpep 3,500 page document containing this motif, or alternatively search with HPGX (where X is a wild card character). Clearly, such (manual) searches of a 3,500 page document are extremely time-consuming and demanding, also requiring visual inspection of each and every identified ORF. For example, searching with a motif such as HXXHH identifies in excess of 300 different ORFs. However, by using a number of different short search strings (as outlined below), and combining these with other methods for identifying putative elongase enzymes, a number of candidate ORFs have been identified.

Database search using the FAE1 polypeptide sequence

As a negative control, to demonstrate that the FAE1 gene sequence was unlikely to provide a useful search sequence in the identification of *C.elegans* sequences encoding for PUFA elongases, the GenBank databases (<http://www.ncbi.nlm.nih.gov/Web/Search/index.html>) were searched using the *Arabidopsis* FAE1 polypeptide sequence to identify related genes or expressed sequence transcripts (ESTs). GenBank is the NIH genetic sequence database, an annotated collection of all publicly available DNA sequences (*Nucleic Acid Research* (1998) **26**, 1-7). There are approximately 2,162,000,000 bases in 3,044,000 sequence records as of December 1998. The search was carried out using the BLAST2 (Basic Local Alignment Search Tool) algorithm (Altschul *et al.*, (1990) *J Mol Biol* **215**,403,410) Although a number

of plant ORFs and ESTs were reported as being related, no animal sequences were identified by this search, confirming the observation that FAE1 was unlikely to be a suitable candidate as a search template for PUFA elongases.

Database search using yeast ELO sequences

Using the three yeast fatty acid elongase sequences (ELO 1, 2, 3) as probes, a number of putative ORFs in the DNA of *C. elegans*-derived cosmid sequences which form the *C. elegans* genomic sequence database were identified. Moreover, an extensive and time-consuming search of a downloaded copy of the WormPep database (<ftp://ftp.sanger.ac.uk/pub/databases/wormpep>) using manual search strings in MSWord 6, identified a number of *C. elegans* ORFs which contained presumptive histidine boxes. Wormpep contains predicted proteins from the *Caenorhabditis elegans* genome sequence project, which is carried out jointly by the Sanger Centre in Cambridge, UK and Genome Sequencing Center in St. Louis, USA. The current Wormpep database, Wormpep 16, contains 16,332 protein sequences (7,120,115 residues). Search strings used included [HXXHH], [HXXXHH], [QXXHH] and [YHH]. Comparison of the data from the two different searches indicated a small (<10) number of putative ORFs as candidate elongases. The histidine box motifs are shown in bold in SEQ ID 9 to 16.

Hydrophobicity plot analysis

Since the fatty acid elongase reaction is predicted to be carried out on the cytosolic face of the endomembrane system (Toke & Martin (1996), *supra*; Oh *et al* (1997), *supra*), the putative *C. elegans* ORFs were examined for potential membrane spanning domains, via Kyte & Doolittle hydrophobicity plots (*J. Mol Biol*, (1982), **157**, 105-132). This revealed a number of ORFs with possible membrane-spanning domains, and also indicated a degree of similarity in the secondary-structure of a number of identified ORFs.

Screening for ER-retention signal sequences

The inventors postulated that since fatty acid elongases are expected to be endoplasmic reticulum (ER) membrane proteins, they might be expected to have peptide signals which are responsible for "ER-retention". In the case of ER membrane proteins, this signal often takes the form of a C-terminal motif [K-K-X_{2,3}-Stop], or similar variants thereof (Jackson *et*

al., (1990), *EMBO J.*, **9**, 3153-3162). Further sequence analysis of the *C. elegans* putative elongases revealed that 4 ORFs (F41H10.7, F41H10.8, F56H11.4, Y53F4B.c) had C-terminal motifs that exactly matched this search pattern, and that a further 2 ORFs (F11E6.5, C40H1.4) had related sequences. These sequence motifs are underlined in SEQ ID 9 to 13, 15 and 16.

Chromosome mapping

Since the inventors had previously observed that *C.elegans* genes involved in the synthesis of PUFA may exist in tandem (for example the $\Delta 5$ and $\Delta 6$ desaturases required for AA and GLA synthesis, respectively, are < 1 kB apart on chromosome IV (Michaelson *et al.*, (1998), *FEBS Letts* **439**, 215-218), the positions of the putative *C. elegans* elongase ORFs were determined using the Sanger Centre's WebAce *C. elegans* server (http://www.sanger.ac.uk/Projects/C_elegans/webace_front_end.shtml). This indicated that two pairs of putative elongases were in close proximity to each other on the *C. elegans* chromosome IV.

F41H10.7 and F41H10.8 were identified as being approximately 10 Kb apart on chromosome IV, and F56H11.3 and F56H11.4 were identified as being approximately 2 Kb apart on chromosome IV.

Putative *C. elegans* fatty acid elongases

The positions of the putative ORFs in the *C. elegans* genome are shown below i.e. chromosome number, and map position in centiMorgans, together with the GenBank database accession numbers.

The designations used employ the same method as used on the Sanger Centre's *C. elegans* database, i.e. ORF C40H1.4 is predicted coding sequence 4 on cosmid C40H1.

| <u>Elongase</u> | <u>Cosmid Sanger ID</u> <u>Code</u> | <u>GenBank Acc</u> | <u>Chromosome</u> |
|-----------------|--|--------------------|-------------------|
| A | C40H1.4 | Z19154 | III |

| | | | |
|---|-----------------------|--------|----------|
| B | D2024.3 | U41011 | IV, 7.68 |
| C | F11E6.5 | Z81058 | IV, 18.8 |
| D | F41H10.7* | U61954 | IV, 29.8 |
| E | F41H10.8* | U61954 | IV, 29.8 |
| F | F56H11.3 [#] | Z68749 | IV, 2.5 |
| G | F56H11.4 [#] | Z68749 | IV, 2.5 |
| H | Y53F4B.c | Z92860 | II |

* or [#] indicates genes in tandem

Comparison of *C. elegans* putative elongase ORFs with yeast genes:

Each of the three yeast ELO polypeptides were compared against all of the worm putative elongase translated ORF sequences, and then ranked in order of similarity (as measured by the BLAST score) (Altschul *et al* (1990), *supra*)

The results are shown below, with the ORF sequences ranked from most similar to least similar, and the BLAST scores are shown in brackets:

Yeast ELO1 (14 to 16 carbon fatty acid elongase)

G (262) > E (241) > D (225) > C (219) > A (216) > F (215) > H (197) > B (172)

Yeast ELO2 (24 carbon sphingolipid elongase)

E (231) > C (226) > G (189) > A (181) > F (166) > D (150) > H (141) > B (140)

Yeast ELO3 (24 to 26 sphingolipid elongase)

D (171) > G (163) > F (154) > A (152) > E (150) > C (131) > B (132) > H (128)

It is clear from the numeric values of the BLAST scores that the sequences are related, but the levels of homology are low. For comparison, the BLAST score for homology between two related worm proteins, the $\Delta 5$ and the $\Delta 6$ desaturase is in excess of 500.

Analysis of potential sphingolipid ancestry

Previously, the inventors had noted the similarities between the fatty acid $\Delta 6$ desaturase and sphingolipid desaturases in plants, and that the two distinct enzymes could have arisen from one ancestral gene. Moreover, it was considered likely that the sphingolipid desaturase predated the fatty acid desaturase, and may in fact have been the ancestral progenitor. Therefore it is plausible that the next step in the arachidonic acid biosynthetic pathway has also evolved from the sphingolipid metabolic pathway. It is therefore considered highly significant that some of the *C. elegans* ORF putative elongases have similarity to sphingolipid enzymes. For this reason, these ORFs are considered to be very clear candidates for PUFA elongases. It has previously been considered that the *C. elegans* $\Delta 5$ and $\Delta 6$ fatty acid desaturases have evolved from 1 ancestral gene (Michaelson *et al.*, (1998), *FEBS Letts* **439**, 215-218). It is also significant that one pair of *C. elegans* putative elongase ORFs (F & G) genetically maps close to the $\Delta 5/\Delta 6$ fatty acid desaturase genes, with both gene pairs being located at the top end of chromosome IV.

| <u>Cosmid Sanger ID Code</u> | <u>GenBank Acc</u> | <u>Chromosome</u> | <u>Encoded Peptide</u> |
|------------------------------|--------------------|-------------------|----------------------------------|
| W08D2.4 | Z70271 | IV, 3.06 | $\Delta 6$ fatty acid desaturase |
| T13F2.1 | Z81122 | IV, 3.06 | $\Delta 5$ fatty acid desaturase |

Cloning of Desaturase and Elongase Genes in Yeast Expression Vectors

Putative elongases sequences F56H11.4 and F41H10.8 were cloned by PCR into the pYES2 vector (Invitrogen). A *C. elegans* mixed stage cDNA library was used as a PCR template. F56H11.4 was amplified using primers:

56h114.for 5'-GCGGGTACCATGGCTCAGCATCCGCTC-3' and;

56h114.rev 5'-GCGGGATCCCTTAGTTGTTCTTCTTCTT-3'.

F41H10.8 was amplified using primers:

41h108.for 5'-GCGGGTACCATGCCACAGGGAGAAGTC-3' and;

41h108.rev 5'-GCGGGATCCCTTATTCAATTTTTCTTTT-3'.

Amplified sequences were then restricted using *KpnI* and *BamHI* (underlined in the forward and reverse primers, respectively), purified using the Qiagen PCR purification kit, and ligated into a *KpnI/BamHI* cut pYes2 vector.

An ORF encoding the *Mortierella alpina* Δ^5 -fatty acid desaturase (Michaelson, L. V., *et al* (1998) *J. Biol. Chem.* **273**, 19055-19059) was amplified using primers:

Mad5.for 5'-GCGAATTCACCATGGGTACGGACCAAGGA-3' and;

Mad5.rev 5'-GCGGAGCTCCTACTCTTCCTTGGGACG-3',

and restricted using *EcoRI* and *SacI*, gel purified as described and ligated into a *EcoRI/SacI* cut pESC-TRP vector (Stratagene) to generate pESC/ Δ^5 .

An ORF encoding the borage Δ^6 -fatty acid desaturase (Sayanova, O., *et al* (1997) *Proc. Natl. Acad. Sci USA* **94**, 4211-4216) was restricted from pGEM3 using *BamHI* and *XhoI* and ligated into a *BamHI/XhoI* cut pESC-TRP vector to generate pESC/ Δ^6 .

A double construct was also generated by ligating the *Bam*HI/*Xho*I borage Δ^6 insert into the pESC/ Δ^5 construct described previously, generating pESC/(Δ^5, Δ^6).

Functional Characterisation in Yeast

Elongases and desaturase constructs were introduced in *Saccharomyces cerevisiae* W303-1A using a lithium acetate based method (Elble, R. (1992) *Biotechniques* **13**, 18-20) and expression of the transgenes was induced by addition of galactose to 2% (w/v) as described in Napier *et al* (Napier, J. A., *et al* (1998) *Biochem J* **330**, 611-614; Michaelson L. V., *supra*; Michaelson, L. V., (1998) *FEBS Letts* **439**, 215-218). Yeast transformants containing pYES2-derived constructs were grown on synthetic minimal media (SD, the composition of which is defined in Sherman, F (1991) *Methods in Enzymology* **194**, 3-21); synthetic minimal medium minus uracil; pESC-derived constructs were grown on SD minimal medium minus tryptophan. Co-transformed yeast (containing both pYES2 and pESC derivatives) were grown on SD minimal medium minus uracil and tryptophan. Prior to induction, cultures were grown in the presence of 2% raffinose and supplemented with 0.5 mM of the appropriate fatty acid substrate in the presence of 1% tergitol-(NP40) (Sigma). All cultures were then grown for a further 48-h unless indicated.

Fatty Acid Analysis

To identify the elongation reaction responsible for the synthesis of di-homo- γ -linolenic acid (DHGLA; 20:3 $\Delta^{8,11,14}$) from GLA, this latter fatty acid was supplied as the (exogenous) substrate.

Lipids were extracted from transformed and control yeast by homogenisation in MeOH-CHCl₃ using a modification of the method of Bligh and Dyer (Dickenson & Lester (1999) *Biochim Biophys Acta* **1426**, 347-357). The resulting CHCl₃ phase was evaporated to dryness under nitrogen gas and the samples were transmethylated with 1M HCl in methanol at 80 °C for 1 hour. Fatty acid methyl esters (FAMES) were extracted in hexane and purified using a small column packed with Florisil. Analysis of FAMES was conducted using a Hewlett Packard 5880A Series Gas Chromatograph equipped with a 25M x 0.32mm RSL-500BP bonded capillary column and a flame ionisation detector. Fatty acids were identified by comparison of retention times with FAME standards (Sigma)

separated on the same GC. Quantitation was carried out using peak height area integrals expressed as a total of all integrals (Bligh, E.G. & Dyer, W.J. (1959) *Can. J. Biochem. Physiol.* **37**, 911-917).

Total fatty acids extracted from yeast cultures were analysed by gas chromatography (GC) of methyl ester derivatives. Lipids were extracted, transmethylated and the fatty acid methyl esters (FAMES) analysed as described by Sayanova *et al.*

Figure 11 shows chromatograms of fatty acid methyl esters from yeast transformed with the control (empty) plasmid pYES2 (Fig. 11A) or with ORF F56H11.4 in pYES2 (Fig. 11B). Exogenous substrate in the form of GLA was supplied to the cultures. Two novel peaks are observed in (B); these peaks (annotated as 20:3 and 18:1*) were identified (against known standards) as DHGLA and vaccenic acid, respectively. Detection was by flame ionisation.

One cDNA ORF tested in this manner displayed a high level of elongase activity on the GLA substrate, converting 44% to DHGLA. The identity of this elongation product was confirmed as DHGLA by comparison with a known standard (the standards used were known standards for either DHGLA, AA, EPA or VA from Sigma Chemicals, Ltd.), using GCMS analysis using a Kratos MS80RFA (Napier, J. A., *supra*; Michaelson, L. V., *supra*; Michaelson, L. V., *supra*). The deduced amino acid sequence of the functional elongase clone identified it as being encoded by the *C. elegans* gene F56H11.4, and comparison with the yeast *ELO* genes showed low homology confined to a few short amino acid motifs (see Fig. 10). Some similarity with a mouse gene Cig30 (Tvrđik, P., (1997) *J. Biol. Chem.* **272**, 31738-31746), which has been implicated in the recruitment of brown adipose tissue in liver tissue, was also observed, as well as a potential human homologue encoded by a gene located on chromosome 4q25, BAC 207d4. The most closely related *C. elegans* ORFs, F41H10.8 (U61954) and F56H11.3 (Z68749) are also shown, as is part of a related human gene present on chromosome IV (present on BAC clone B207d4; AC004050). The GenBank accession numbers are given for all sequences.

The range of fatty acids synthesised by *C. elegans* can potentially require a number of different elongation reactions (Tanaka, T., (1996) *Lipids* **31**, 1173-1178). The substrate-specificity of the F56H11.4 PUFA elongase was therefore determined using a

range of exogenously supplied fatty acids. This revealed that GLA is the major substrate, with a number of other fatty acids being elongated at a lower efficiency (see Table 1). Although most of these substrates are polyunsaturated fatty acids, it was unexpectedly observed that palmitoleic acid (PA; 16:1 Δ^9) was also elongated by F56H11.4 to yield vaccenic acid (VA; 18:1 Δ^{11}). The biosynthetic pathway for VA is unclear, but the data indicate that it may be synthesised by elongation of Δ^9 -monounsaturated 16C fatty acid.

The *C. elegans* PUFA elongase ORF F56H11.4 maps to the top of chromosome IV (at 4.32 cM) with a related sequence (F56H11.3; 51 % similarity) located 1,824bp downstream. Another *C. elegans* gene (F41H10.8) was also observed, which is present on chromosome IV, and which shows a slightly higher level (53%) of similarity to the PUFA elongase than F56H11.3 (see Fig. 10). However, when a PCR product encoding ORF F41H10.8 was expressed in yeast in a manner identical to that used for F56H11.4, the former failed to direct the elongation of any fatty acids, despite the provision of a range of substrates (see Table II).

In order to reconstitute the PUFA biosynthetic pathway in a heterologous system, the PUFA elongase F56H11.4 was expressed in yeast in conjunction with either the Δ^6 - or Δ^5 -fatty acid desaturases previously isolated and characterised by the inventor (Napier, J. A., *supra*; Michaelson, L. V., *supra*). Expression of the Δ^6 -fatty acid desaturase and F56H11.4 was carried out in the presence of two different substrates (LA or ALA) while the Δ^5 -fatty acid desaturase and the elongase were expressed in the presence of GLA only. This demonstrated that was possible to combine a desaturase and an elongase in yeast to generate significant amounts of a final "product" (see Table III). In the case of the elongase and the Δ^6 -fatty acid desaturase, the reactions proved highly efficient with the production of 4.5% of DHGLA from the LA substrate. This resulted from 25% desaturation of the LA substrate to GLA, which was then elongated to DHGLA at a similar level of efficiency (18%). This is lower than the % conversion observed for GLA when supplied exogenously (see Table I), indicating that the *in vivo* production of substrates for elongation may be rate-limiting.

If ALA was used as a substrate, 27% of this was initially Δ^6 -desaturated to yield octadecatetraenoic acid (OTA; 18:4 $\Delta^{6,9,12,15}$) but only 8% of was subsequently elongated to yield eicosatetraenoic acid (20:4 $\Delta^{8,11,14,17}$). Thus, the conversion efficiency of ALA to the final 20-carbon tetraenoic PUFA was only about 2.2%.

Since DHGLA is an *n-6* fatty acid, whilst the OTA-derived eicostetraenoic acid is an *n-3* type, this demonstrates that the elongase is capable of accepting both forms of essential fatty acid, albeit with different efficiencies. Verification was also provided that the 20C PUFAs synthesised in the yeast expression system were generated by the Δ^6 -desaturation of 18C substrates which were subsequently elongated, as the Δ^6 -desaturase showed no activity on 20:2 or 20:3 substrates (see Table III).

The combination of the Δ^5 -desaturase and the elongase also demonstrated that these two enzymes could work in tandem, although the efficiency of this overall conversion was lower (3.3% AA from GLA) which was due to the previously observed low activity of the Δ^5 -desaturase enzyme itself (Michaelson, L. V., *supra*; Michaelson, L. V., *supra*). Thus, although nearly 45% of the GLA substrate was elongated to DHGLA, only 7.5% of this was then desaturated to AA (see Table III).

Finally, the production of either AA or eicosapentanoic acid (EPA; 20:5 $\Delta^{5,8,11,14,17}$) in yeast from dienoic or trienoic 18 carbon substrates was achieved via expression of all three enzymes (the two desaturases and the F56H11.4 PUFA elongase) simultaneously. As shown in Table IV, small but significant amounts of AA were produced when the yeast was supplied with the 18C dienoic fatty acid LA.

GC-Mass Spectroscopy (MS) Analysis

Peak identification and confirmation were carried out by GC-MS using a Kratos MS80RFA using known standards (Sigma). The identity of this 20C PUFA was verified by GCMS, indicating that the conversion efficiency from LA was 0.65%. When ALA was used as a substrate, 12.5% of the (Δ^6 -desaturated and elongated) eicosatetraenoic *n-3* fatty acid was Δ^5 -desaturated, resulting in a total conversion of 0.3% of the ALA substrate to EPA (the identity of EPA was confirmed by GCMS).

Expression of *C. elegans* elongase in plants

In order to express *C. elegans* elongase in plants, the following protocol is an example of a process which can be used to create the transgenic plants. *C. elegans* ORF sequence can be subcloned into a plant expression vector pJD330, which comprises a viral 35S promoter, and a Nos terminator. The resulting cassette or promoter/coding sequence/terminator can then be subcloned into the plant binary transformation vector pBin 19, and the resulting plasmid introduced into *Agrobacterium tumefaciens*. This *Agrobacterium* strain can then be used to transform Arabidopsis by the vacuum-infiltration of inflorescences, and the seeds harvested and plated onto selective media containing kanamycin. Since pBin 19 confers resistance to this antibiotic, only transformed plant material will grow. Resistant lines can therefore be identified and self-fertilized to produce homozygous material. Leaf material can then be analyzed for expression of *C. elegans* elongase.

Fatty acid methyl ester analysis can be carried out as previously described.

Table I
ORF F56E11.4
mole% Fatty Acids
(Control)

| Substrate | Induction | GLA | LA | ALA | EPA |
|-------------|------------|------------|------------|------------|------------|
| 16:0 | 17.5 ± 3.3 | 27.7 ± 1.4 | 22.9 ± 1.5 | 19.1 ± 0.7 | 23.4 ± 0.2 |
| 16:1 | 53.2 ± 7.2 | 32.5 ± 4.4 | 21.2 ± 2.2 | 18.1 ± 1.5 | 26.9 ± 0.7 |
| 18:0 | 4.5 ± 0.7 | 5.6 ± 0.5 | 5.1 ± 0.3 | 5.0 ± 0.3 | 5.3 ± 0.2 |
| 18:1 | 24.8 ± 3.9 | 16.9 ± 0.9 | 11.2 ± 2.4 | 10.1 ± 1.1 | 15.4 ± 0.4 |
| 18:1* | 9.6 ± 0.6 | 3.9 ± 0.6 | 3.2 ± 0.6 | 3.1 ± 0.4 | 6.2 ± 0.3 |
| LA | - | - | 34.4 ± 4.2 | - | - |
| ALA | - | - | - | 43.1 ± 3.9 | 45.8 ± 4.8 |
| GLA | - | 7.5 ± 1.2 | - | - | - |
| 20:2 | - | 14.0 ± 0.3 | 2.0 ± 0.9 | - | - |
| DEGLA | - | 5.8 ± 0.9 | - | - | - |
| 20:3 | - | - | - | 1.5 ± 0.1 | - |
| EPA | - | - | - | - | 22.8 ± 0.7 |
| % Elongated | - | - | - | - | 20.1 ± 2.4 |
| GLA | 44 | - | - | - | - |
| LA | - | 5.5 | - | - | - |
| ALA | - | - | 3.4 | - | - |
| EPA | - | - | - | 0 | - |

Table III

| Construct | mole% Fatty Acids | | | | | | | | | |
|-------------|-------------------|------------|-----------------------|------------|------------|-----------------------|------------|------------|-----|--|
| | Δ^6 | | F56H11.4 + Δ^6 | | | F56H11.4 + Δ^5 | | | GLA | |
| | 20:2 | 20:3 | LA | ALA | ALA | ALA | ALA | + | - | |
| Substrate | 20:2 | 20:3 | LA | ALA | ALA | ALA | ALA | + | - | |
| Induction | + | - | + | - | + | - | + | - | - | |
| 16:0 | 24.7 ± 1.3 | 25.2 ± 1.5 | 18.7 ± 0.6 | 23.7 ± 0.5 | 17.4 ± 0.7 | 21.0 ± 1.3 | 27.9 ± 4.2 | 29.8 ± 3.8 | | |
| 16:1 | 46.0 ± 2.8 | 43.7 ± 3.7 | 18.9 ± 1.2 | 24.6 ± 0.7 | 5.3 ± 0.6 | 9.1 ± 0.9 | 24.6 ± 3.4 | 25.1 ± 3.2 | | |
| 16:2 | 5.2 ± 1.2 | 4.1 ± 1.4 | 0.6 ± 0.1 | - | 0.4 ± 0.1 | - | - | - | | |
| 18:0 | 4.8 ± 0.4 | 5.1 ± 0.4 | 4.0 ± 0.3 | 5.1 ± 0.1 | 6.2 ± 0.7 | 5.4 ± 0.2 | 5.6 ± 0.8 | 5.4 ± 0.7 | | |
| 18:1 | 15.3 ± 1.1 | 16.1 ± 1.2 | 12.2 ± 1.4 | 11.2 ± 0.4 | 5.7 ± 0.8 | 6.0 ± 0.4 | 12.7 ± 2.9 | 13.0 ± 2.5 | | |
| 18:1* | - | - | 7.7 ± 0.7 | - | 2.6 ± 0.3 | - | 2.9 ± 0.9 | - | | |
| LA | - | - | 25.0 ± 3.2 | 35.4 ± 2.1 | - | - | - | - | | |
| ALA | - | - | - | - | 42.3 ± 3.3 | 58.5 ± 4.7 | - | - | | |
| GLA | - | - | 7.9 ± 2.2 | - | - | - | 13.2 ± 3.6 | 19.2 ± 3.5 | | |
| OTA | - | - | - | - | 15.3 ± 1.8 | - | - | - | | |
| 20:2 | 4.0 ± 0.3 | - | 3.3 ± 0.5 | - | - | - | - | - | | |
| DHGLA | - | - | 1.7 ± 0.2 | - | - | - | 9.8 ± 1.8 | - | | |
| 20:3 | - | 5.8 ± 0.5 | - | - | 3.4 ± 0.4 | - | - | - | | |
| AA | - | - | - | - | - | - | 0.8 ± 0.2 | - | | |
| 20:4 | - | - | - | - | 1.4 ± 0.2 | - | - | - | | |
| EPA | - | - | - | - | - | - | - | - | | |
| % Elongated | | | | | | | | | | |
| GLA | - | - | 17.7 | - | - | - | 44.5 | - | | |
| OTA | - | - | - | - | 8.4 | - | - | - | | |
| LA | - | - | 8.7 | - | - | - | - | - | | |
| ALA | - | - | - | - | 5.4 | - | - | - | | |

SEQ ID1

C40H1.4

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SEQ ID2

D2024.3

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SEQ ID3

F11E6.5

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SEQ ID4

F41H10.7

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SEQ ID5

F41H10.8 (ce477)

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| tctggatctc | aatggcatat | gttgtcgtta | tttttgggct | caaggctgct |
| atgacaaacc | gaaaaccatt | tgatctcacg | ggaccactga | atctctggaa |
| tgccgggtctt | gctattttct | caactctcgg | atcacttgcc | actacatttg |
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SEQ ID6

F56H11.3

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SEQ ID7

F56H11.4 (Ce 166)

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SEQ ID8

Y53F4B.c

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ttttttggca gtgttcagta ttatgggtac atggagattt ggaatcgaat
tctacgatgc tgttttcaga agaggcttca tgcattcgat ctgcttggt
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cggttatatt ccttcaactg tcatcacg ctggtgttct gatcctttct
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gaactatttg gtgcattcaa taatgtatac atactacgca ataacatcaa
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gcttcggaat ctacgcctca ttctggtgc tattctccag tttcttcaac
aatgcatatt tggtaaaaaa ggacaagaaa cccgatgtga agaaggatta
a

SEQ ID9**A**

1 MELAEFWNDL NTFIIYGNPH TDMTTKYKYS YHFPGEQVAD PQYWTILFQK
51 YWYHSITISV LYFILIKVIQ KFMENRKPFT LKYPLILWNG ALAAFSIIAT
101 LRFSIDPLRS LYAEGFYKTL CYSCNPTDVA AFWSFAFALS KIVELGDTMF
151 IILRKRPLIF **LHYYHHA**AVL IYTVHSGAEH TAAGRFYILM NYFAHSLMYT
201 YYTVSAMGYR LPKWVSMVT TVQTTQMLAG VGITWMVYKV KTEYKLPCQQ
251 SVANLYLAFV IYVTFAILFI QFFVKAYIIK SSKKSKSVKN E*

SEQ ID10**B**

1 MAKYDYNPKY GLENYSIFLP FETSFDAFRS TTWMQNHVYQ SITASVVYVA
51 VIFTGKKVVL IYKSRVITF ESSLQNAIKN RNRKSLNSSQ MFQIMEKYKP
101 FQLDTPLFVW NSFLAIFSIL GFLRMTPEFV WWSAEGNSF KYSICHSSYA
151 QGVTGFWTEQ FAMSKLFELI DTIFIVLRKR PLIFL**HWYHH** VTVMITYWHA
201 YKDHTASGRW FIWMNYGVHA LMYSYYALRS LKFRLLPKQMA MVVTTLQLAQ

251 MVMGVIIIGVT VYRIKSSGEY CQQTWDNLGL CFGVYFTYFL LFANFFYHAY
 301 VKKNNRTVNY ENNSKNFPDL VLIYLRKKVS RKSKNRQCSE NNYKIQFSSN
 351 FVNVDGKHKH KTYELILPRR KMTTILTFLE GKNRIFSKYQ KNRKNISIPV
 401 DFEILEPKED INANIAEPSI TTRSAAARRK VQKAD*

SEQ ID11**C**

1 MAAAQTSPAA TLVDVLTCPW SLDQTDSEMS TFFVPLSYKIM IGYLVTIYFG
 51 QKLMHRKPF DLQNTLALWN FGFSLEFSGIA AYKLIPELFG VFMKDGFFVAS
 101 YCQENENYTD ASTGFWGWAFF VMSKAPELGD TMFLVLRKKP VIFMHWYHHA
 151 LTFVYAVVTY SEHQAWARWS LALNLAVHTV MYFYFAVRAL NIQTPRPVAK
 201 FITTIQIVQF VISCYIFGHL VFIKSADSVF GCAVSWNVLS IGGLMYISYL
 251 FLFAKFFYKA YIQKRSPTKT SKQE*

SEQ ID12**D**

1 MSSDDRGRTR FKMMQILGT NFTYEGAKEV ARGLEGFSAK LAVGYIATIF
 51 GLKYMKDRK AFDLSTPLNI WNGILSTFSL LGFLFTFPTL LSVIRKDGFS
 101 HTYSHVSELY TDSTSGYWIF LWVISKIPEL LDTVFIVLRK RPLIFMHWYH
 151 HALTGYYALV CYHEDAVHNV WVVWMNYIIH AFMYGYLLK SLKVPIPPSV
 201 AQAITTSQMV QFAVAIFAQV HVSYKHYVEG VEGLAYSFRG TAIGFFMLTT
 251 YFYLWIIQFYK EHYLKNNGGK YNLAKDQAKT QTKKAN*

SEQ ID13**E**

MPQGEVSFFE VLTTAPFSHE LSKKHIAQTQ YAAFWISMAY VVVFGLKAV
 MTNRKPFDLT GPLNLWNAGL AIFSTLGLSL TTFGLLHEFF SRGFFESYIH
 IGDFYNGLSG MFTWLFVLSK VAEFGDTLFI ILRKKPLMFL **HWYHH**VLTMN
 YAFMSFEANL GFNTWITWMN FSVHSIMYGY YMLRSFGVKV PAWIAKNITT
 MQILQFVITH FILFHVGYLE VTGQSVDSTP GYYWFCLLME ISYVVLFGNF
 YYQSYIKGGG KKFNAEKKTE KKIE*

SEQ ID14**F**

1 MYLNYFATEI FHRSVACETE ACRSSKIMIA DVFKWKFDAN ELWSLLTNQD
 51 EVFPHIRARR FIQEHFGLFV QMAIAYVILV FSIKRFMRDR EPFQLTTALR
 101 LWNFFLSVFS IYGSWTMFPF MVQQIRLYGL YGCGCEALSN LPSQAEYWLF

151 LTILSKAVEF VDTFFLVLRK KPLIFL**HWYH** HMAFVFFCS NYPTSSQSR
201 VGVIVNLFVH AFMYPYYFTR SMNIKVPAKI SMAVTVLQLT QFMCFIYGCT
251 LMYYSLATNQ ARYPSNTPAT LQCLSYTLHL L*

SEQ ID15**G**

MAQHPLVQRL LDVKFDTKRF VAIATHGPKN FPDAEGRKFF ADHFDVTIQA
SILYVVVFG TKWFMRNRQP FQLTIPLNIW NFILAAFSIA GAVKMTPEFF
GTIANKGIVA SYCKVDFDFTK GENGYWVWLF MASKLFELVD TIFLVLRKRP
LMFL**HWYHHI** LTMIYAWYSH PLTPGFNRYG IYLNFFVHAF MYSYYFLRSM
KIRVPGFIAQ AITSLQIVQF IISCAVLAHL GYLMHFTNAN CDFEPSVFKL
AVFMDTTYLA LFNFFLQSY VLRGGKDKYK AVPKKKNN*

SEQ ID16**H**

MSAEVSERFKVWTGNNETIIYSPFEYDSTLLIESCRCTYQLLILLRQI
YYRDIWSHGKACDXLLAWNGFLAVFSIMGTWRFGIEFYDAVFRXG
FIXSICLAVNPRSPSAFWACMFALSKIAEFGDTMFLVLRKRPVIFL**HWYHH**
AVVLILSWHAAIELTAPGRWFI FMNYLVHSIMYTYAITSIGYRXPKIVSMT
VTFLQTLQMLIGVSI SCIVLYLKLNGEMCQOSYDNLALSFGIYASFLVLSSFF
NNAYLVKKDKKPDVKKD*

Claims

1. An isolated polypeptide comprising a functional long chain polyunsaturated fatty acid (PUFA) elongase as herein defined.
2. A polypeptide according to claim 1 wherein the polypeptide is from a eukaryote.
3. A polypeptide according to claim 1 or claim 2 wherein the polypeptide has at least a portion of the amino acid sequence shown in SEQ ID 15, or variants thereof.
4. A polypeptide having at least 60% homology to a polypeptide according to claim 3 and having a PUFA elongase function.
5. A polypeptide according to claim 4 having at least 80% homology.
6. A polypeptide according to claim 5 having at least 90% homology.
7. A polypeptide according to any preceding claim wherein the polypeptide sequence includes a sequence motif responsible for Endoplasmic Reticulum (ER) - retention.
8. A polypeptide according to any preceding claim wherein the polypeptide is capable of elongating palmitoleic acid (PA; 16:1 Δ^9) to vacceric acid (VA; 18:1 Δ^{11}).
9. A polypeptide according to any preceding claim wherein the polypeptide is from an animal.
10. A polypeptide according to claim 9 wherein the animal is an invertebrate.
11. A polypeptide according to claim 10 wherein the invertebrate is a worm.
12. A polypeptide according to claim 11 wherein the worm is *C. elegans*.

13. A polypeptide according to claim 9 wherein the animal is a vertebrate.
14. A polypeptide according to claim 13 wherein the vertebrate is a mammal.
15. A polypeptide according to claim 14 wherein the mammal is a human, rat or mouse.
16. A DNA sequence encoding a polypeptide according to any preceding claim.
17. A DNA sequence according to claim 16 wherein the DNA comprises the sequence shown in SEQ ID 7 or variants of that sequence due to base substitutions, deletions and/or additions.
18. An engineered organism engineered to express a polypeptide according to any one of claims 1 to 15.
19. An engineered organism according to claim 18 wherein the animal is a mammal.
20. An engineered organism according to claim 19 wherein the mammal is a rat, mouse or monkey.
21. An engineered organism containing a synthetic pathway for the production of a polypeptide according to any one of claims 1 to 15.
22. An engineered organism according to claim 21 wherein the pathway includes Δ^5 -fatty acid desaturase.
23. An engineered organism according to claim 21 or 22 wherein the pathway includes Δ^6 -fatty acid desaturase.
24. An engineered organism according to any one of claims 21 to 23 wherein the animal is a lower eukaryote.

25. An engineered organism according to claim 24 wherein the lower eukaryote is a yeast.
26. An engineered organism according to claim 18 wherein the animal is a fish.
27. A transgenic plant engineered to express a polypeptide according to any one of claims 1 to 15.
28. A transgenic plant containing a DNA sequence according to claim 16 or 17.
29. A method of producing a PUFA comprising carrying out an elongase reaction catalysed by a polypeptide according to any one of claims 1 to 15.
30. A method according to claim 29 wherein the PUFA is di-homo-gamma-linoleic acid ($20:3\Delta^{8,11,14}$), arachidonic acid ($20:4\Delta^{5,8,11,14}$), eicosapentanoic acid ($20:5\Delta^{5,8,11,14,17}$), docosatrienoic acid ($22:3\Delta^{3,16,19}$), docosatetraenoic acid ($22:4\Delta^{7,10,13,16}$), docosapentaenoic acid ($22:5\Delta^{7,10,13,16,19}$) or docosahexaenoic acid ($22:6\Delta^{4,7,10,13,16,19}$).
31. A method according to claim 29 wherein the PUFA is a 24 carbon fatty acid with at least 4 double bonds.
32. A PUFA produced by a method according to any one of claims 29 to 31.
33. A foodstuff comprising a PUFA according to claim 32.
34. A dietary supplement comprising a PUFA according to claim 32.
35. A pharmaceutical composition comprising a polypeptide according to any one of claims 1 to 15.
36. A pharmaceutical composition comprising a PUFA according to claim 32.

37. A pharmaceutical composition according to claim 35 or claim 36 wherein the composition comprises a pharmaceutically-acceptable diluent, carrier, excipient or extender.
38. A method of elevating the PUFA levels of an animal or a plant by supplying to the animal or plant a polypeptide according to any of claims 1 to 15, a DNA sequence according to claim 16 or claim 17, a foodstuff according to claim 33, a dietary supplement according to claim 34, a pharmaceutical composition according to any of claims 35 to 37 or a PUFA according to claim 32.
39. A method of treatment according to claim 38 wherein the animal is a mammal.
40. A method of treatment according to claim 39 wherein the mammal is a human.

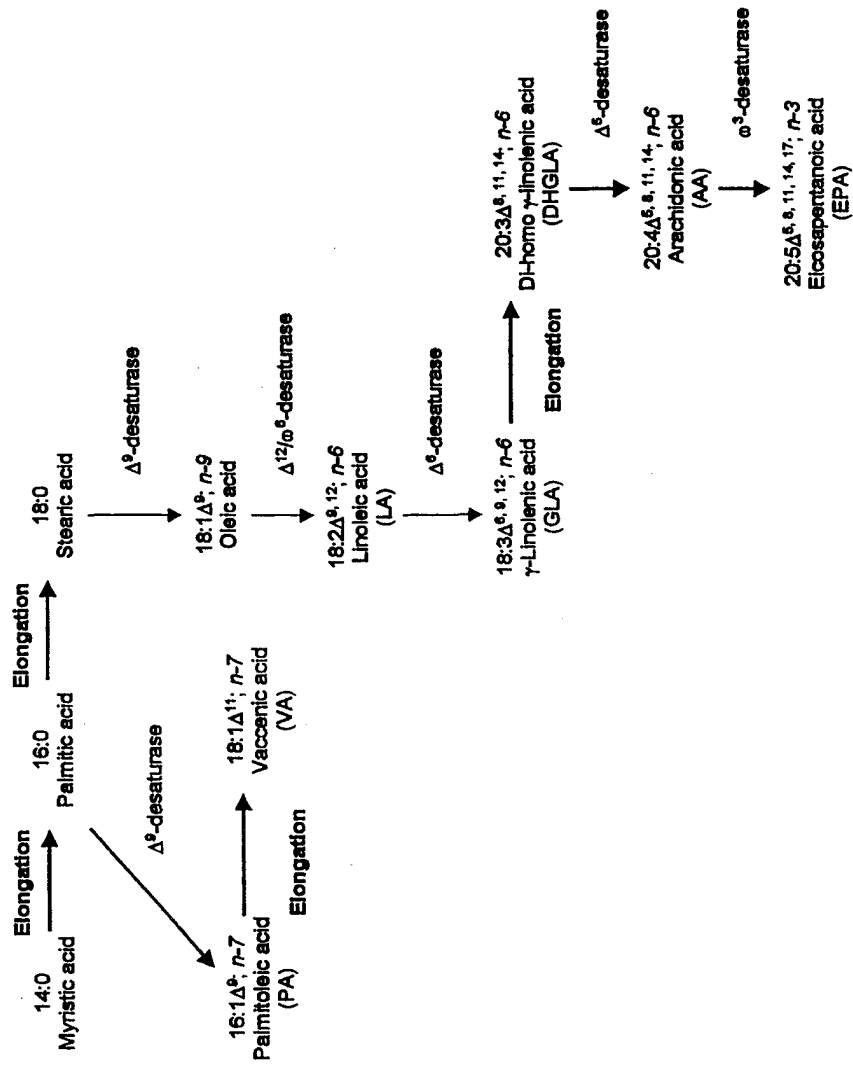


FIG. 1

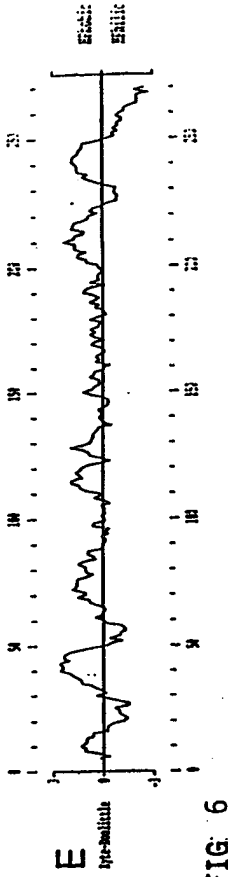


FIG 2

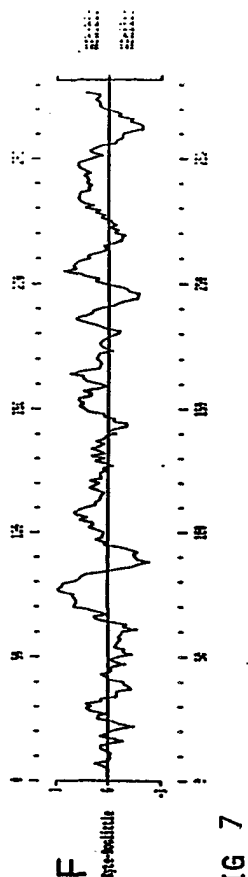


FIG 3

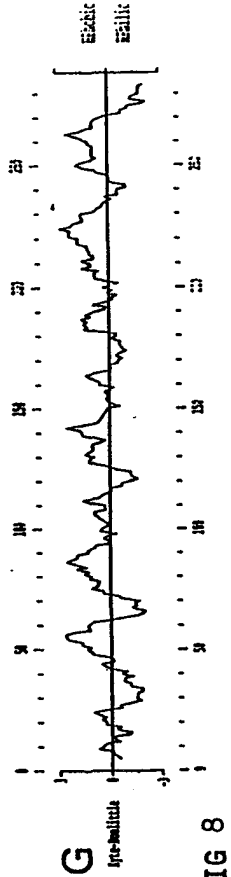


FIG 4

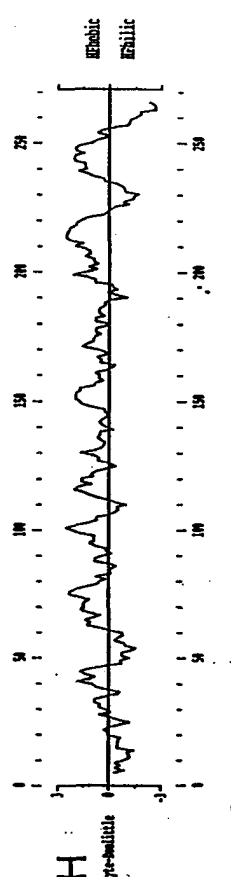


FIG 5

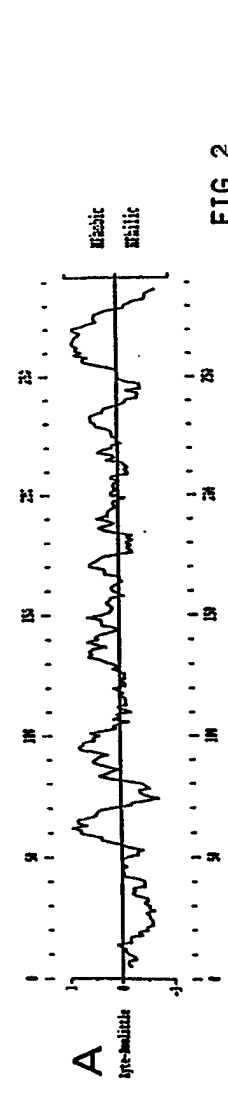


FIG 6

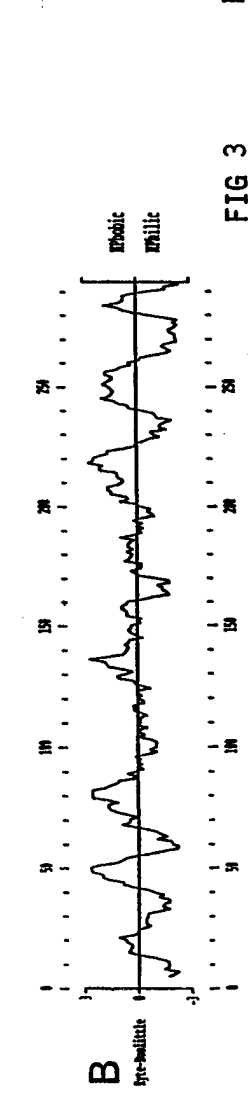


FIG 7

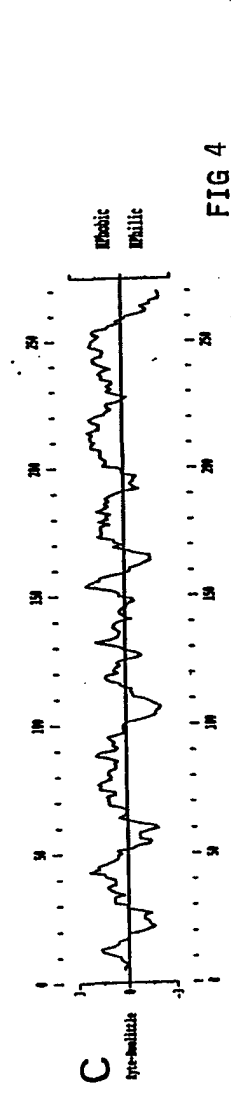


FIG 8

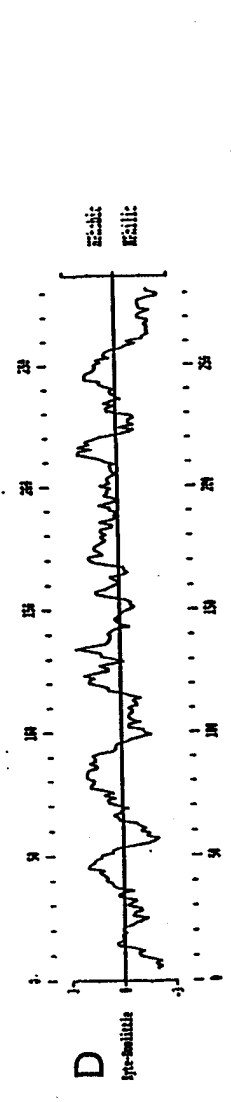


FIG 9

FIG. 10

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Elo1 -----MVSDWKNFCLEKASR---FRPTIDRPFNNIYLWDYFNRAVWGWA TAGRFQ
Elo2 -----MNSLVTQYAAPLFFERYPQLHDYLPTEERPFFNISLWEHFDDVVTRV TNGRFV
Elo3 MNTT TSTVIAAVADQFQSLNSSSCFLKVHVPSEENP-FGIELWPIFSKVFEYFSG--YP
Cig30 -----MDTSMNFSRGLKMDLMQ
B207d4 -----
F56H11.4 -----MAQHPLVQRLLDVKFDTKRFVAIATHG
F41H10.8 -----MPQGEVSFFFEVLTTA
F56H11.3 -----MYLNYFATEIFHRSAVCETEACRSSKIMIADVFKWKF DANELWSLLTNQ

Elo1 PKDFEFTVGKQPLSEPR--PVLLFIAMYYMIFGGRSLVK--SCKPLKRFISQVHMLM
Elo2 PSEFQFIAGELPLSTLP--PVLYAITAYMIFGGRFILS--KSKPKLNGLFQDHLNVL
Elo3 AEQFEFIHNKTFLANGY--HAVSITIVYIIFGGQAILRALNASPLKFKLLEIHNLF
Cig30 PYDFETFDLRPFLEEWVSSFLIVVVYLLLVVCGTYR--TRKSPSLQRPLILVSSFL
B207d4 -----
F56H11.4 PKNFPDAEG-RKFFADHFDTIQASILMIVVFGTKWFR--NRQPFQITIPNLINRFL
F41H10.8 P--FSHELK-KKHIAQTQAAFWISMAVYVIFGLKAVT--NRKPFDLTGPLNLVWAGL
F56H11.3 DEVFPHIR-ARRFIQEHFGLFVQMAIAYVILVFSIKRFMR--DREPPQLTTALRLVWFFL

Elo1 TSVSFLWLILVEQMLP:IVYRHGLYFAVGNVESWTQPMETIY-YENMNTFVEFADTVLM
Elo2 TSLSLTLLELVEQLVP:IVQGLYFALGNIGAWTQPLVTIY-YMNYLVKFIETIDTFFL
Elo3 TSI SLVLWLLVLEQLVPMVYHNGLFWBISKEAEAPKLVTIY-YLNLYTKFVELIDTVFL
Cig30 AIFSI:LGTLRWKFMATVMETVGLKQTVCFAIYTDAMVRFWSF:FLLSKVVELGDTAFI
B207d4 -----
F56H11.4 AAFS:LAGAVK:TPPEFGT:IANKG:IVASY:KVDFD:TKGENGYVWV:FMASKLFE:LVDTIFL
F41H10.8 AIFSTLGS:ATTFGL:LHEFFSRG:FFESYIH:IGDFYNG:LSGMFTW:FMVLSKVAE:FGDTLFI
F56H11.3 SVFSI:YGSWT:FPFMVQQ:IRLYG:YGGCG:EA:LSN:LPSQAEY:WLF:FLITLSKAVE:RVDITFFL

Elo1 VLKHKRKLITFLHTYHHGATA:LCYNQLVQYTAVTWVPM:ENLAVHV:YVWYFLLSAS:STIRV
Elo2 VLKHKRKLITFLHTYHHGATA:LCYQQLMGTTSISWVPI:ENLGVHVM:YVWYFLLAAR:GIRV
Elo3 VLRRKKLITFLHTYHHGATA:LCYTQLIGRTSVEWV:VLLNGLGVHV:YVWYFLLS:CGSIRV
Cig30 ILRKRPLITFVHWYHHSTVLLFETSGYKKNVPSGGWFM:TMNFGVHSM:YTYTMKA:AKLKH
B207d4 ILRKKPLITFLHWYHHITVLLYSWYSYKDMVAGGGWFM:TMNYGVHAM:YYSYALRAA:GFRV
F56H11.4 VLRKRPLMFLHWYHHITLMTIYAWYSHPLTPGFNRYG:YLNFFVHAF:YYSYFLRSMKIRV
F41H10.8 ILRKKPLMFLHWYHHVLTMNYAFMSFEANLGFNTWI:TWNFVSHS:YVGYMLR:SFVQV
F56H11.3 VLRKKPLITFLHWYHHMATFVFFCSNYPTSSQSRVGY:VNLFFVHAF:YVYVYFTR:SMN:KVV

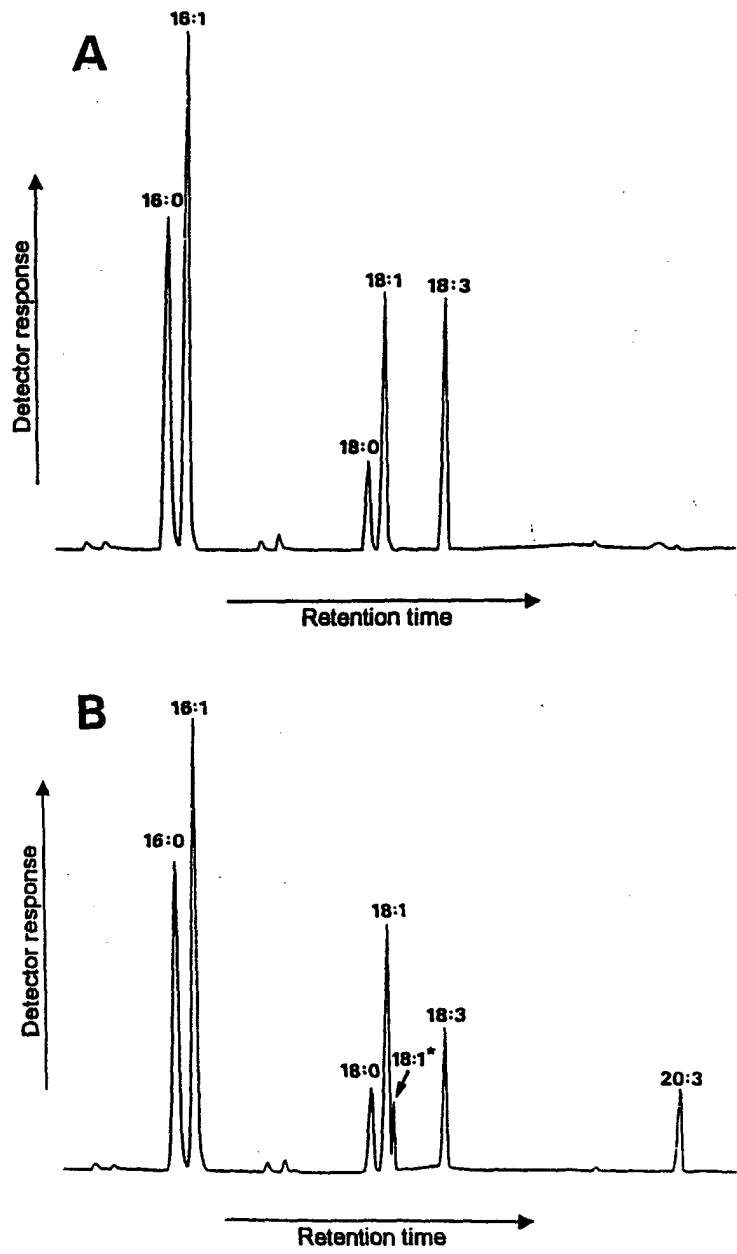
Elo1 --WVKAWVTRLO:VQFMDL:IVVYVY:YQKIVAAV:FKNACTPQCED:GLGSMTA:IAAGAA:
Elo2 --WVKWVTRFO:IQFVLD:IGFIYFAVYQKAVHLYF--PI:PHCGD:QVGSSTATFAGCA:
Elo3 --WVKQWVTRFO:IQFL:IDLVFVYFATYTFYAHKYL-DGILPNKGT:QYGTQAAAAYGY:
Cig30 PNL:LPV:ITSLQ:LOMVLGT-IFG-IL-----NYIW--RQEKGCHTTTEHF:FW-SFML
B207d4 SRKFAMF:ITLSQ:ITQMLMGC-VVN-YL-----VFCW--MQHDQCHSHFQN:IFW-SSLM
F56H11.4 PGF:IAQA:ITSLQ:VQF:ISCAVLA-HL-----GYLM--HFTNAN:QFEP:SVFKLAVFM
F41H10.8 PAK:IAKN:ITMQ:LOFV:ITHF:ILE-HV-----GYLA--VTGQ:SV:STPGY:YWFCLM
F56H11.3 PAK:ISMA:ITVLQ:LTQFM--CFIYGCT-----MYYS--LATN:QARY:PSNT:PATLQCL:

Elo1 LTSYLFLL:ISFY:EVYKRGSA:SGKK:INKNN-----
Elo2 ISSYLVLFF:ISFY:INVYKRC:KTRTSRVVKRAHGGVA:AKVNEYVNV:DLKNVPTPSP:SPKPKQ
Elo3 LTSYLLLF:ISFY:IQSYKKG:KTKVK:SEVSGSVA:SGSSTGVKTSNTKV:SSRKA-----
Cig30 YGTYFILLFAH:FFHRAVLRPKG:IVAS:SQ-----
B207d4 YLSYLVLFF:CHFF:FEAYI-----
F56H11.4 DITTYLALF:VNF:FLQSYVLR:SGKDY:KAVPKKKN-----
F41H10.8 EISYVVLFGNF:YQSY:IKGSGK--KFNAEKKTEK:IE-----
F56H11.3 YTLHL-----

Elo1 -----
Elo2 RRKR
Elo3 -----
Cig30 -----
B207d4 -----
F56H11.4 -----
F41H10.8 -----
F56H11.3 -----

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FIG. 11



INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01035

| | | | | |
|--|---|--|---|---|
| A. CLASSIFICATION OF SUBJECT MATTER | | | | |
| IPC 7 | C12N15/54 | C12N9/10 | | |
| | C07C57/12 | A61K38/45 | | |
| | | C12N1/21 | | |
| | | A61K31/202 | | |
| | | A01H5/00 | | |
| | | A23L1/30 | | |
| C07C57/03 | | | | |
| According to International Patent Classification (IPC) or to both national classification and IPC | | | | |
| B. FIELDS SEARCHED | | | | |
| Minimum documentation searched (classification system followed by classification symbols) | | | | |
| IPC 7 C12N | | | | |
| Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched | | | | |
| Electronic data base consulted during the international search (name of data base and, where practical, search terms used) | | | | |
| EPO-Internal, WPI Data, PAJ, BIOSIS, STRAND | | | | |
| C. DOCUMENTS CONSIDERED TO BE RELEVANT | | | | |
| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. | | |
| X | DATABASE SWALL 'Online! EMBL Heidelberg, Germany; ID: YLF4_CAEEL, AC: Q03574, 1 February 1994 (1994-02-01) WILSON R ET AL.: "2.2 Mb of contiguous nucleotide sequence from chromosome III of C. elegans" XP002143744 abstract <div style="text-align: center;">--- -/--</div> | 1-12, 18, 21, 24 | | |
| <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. | | | | |
| ° Special categories of cited documents : <table style="width: 100%; border: none;"> <tr> <td style="width: 50%; border: none; vertical-align: top;"> *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; border: none; vertical-align: top;"> *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family </td> </tr> </table> | | | *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family |
| *A* document defining the general state of the art which is not considered to be of particular relevance *E* earlier document but published on or after the international filing date *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) *O* document referring to an oral disclosure, use, exhibition or other means *P* document published prior to the international filing date but later than the priority date claimed | *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. *&* document member of the same patent family | | | |
| Date of the actual completion of the international search | | Date of mailing of the international search report | | |
| 28 July 2000 | | 17. 08. 00 | | |
| Name and mailing address of the ISA | | Authorized officer | | |
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | | Oderwald, H | | |

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International Application No

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| X | --- JAMES D W ET AL: "DIRECTED TAGGING OF THE ARABIDOPSIS FATTY ACID ELONGATION I (FAE1) GENE WITH THE MAIZE TRANSPOREN ACTIVATOR" PLANT CELL, US, AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS, ROCKVILLE, MD, vol. 7, March 1995 (1995-03), pages 309-319, XP002911493 ISSN: 1040-4651 cited in the application the whole document | 1-3,7,8, 16-18, 21-23, 27-29 |
| A | --- WATTS J L AND BROWNE J: "Isolation and characterization of a delta5-fatty acid desaturase from Caenorhabditis elegans" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 362, no. 1, 1 February 1999 (1999-02-01), pages 175-182, XP002143742 the whole document | 22 |
| A | --- NAPIER ET AL: "Identification of a Caenorhabditis elegans delta6-fatty-acid-desaturase by heterologous expression in Saccharomyces cerevisiae" BIOCHEMICAL JOURNAL, GB, PORTLAND PRESS, LONDON, vol. 330, no. 2, March 1998 (1998-03), pages 611-614-614, XP002099453 ISSN: 0264-6021 the whole document | 23 |
| A | --- SALEM N ET AL: "Arachidonic and docosahexaenoic acids are biosynthesized from their 18-carbon precursors in human infants" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, US, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 93, no. 93, January 1996 (1996-01), pages 49-54-54, XP002131822 ISSN: 0027-8424 the whole document | |
| | --- -/-- | |

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 00/01035

| C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT | | |
|--|---|-----------------------|
| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| A | OH ET AL: "ELO2 and ELO3, homologs of the Saccharomyces cerevisiae ELO1 gene, function in fatty acid elongation and are required for sphingolipid formation" JOURNAL OF BIOLOGICAL CHEMISTRY, US, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 28, 11 July 1997 (1997-07-11), pages 17376-17384, XP002119019 ISSN: 0021-9258 cited in the application the whole document | |
| P, X | WO 00 12720 A (ABBOTT LAB) 9 March 2000 (2000-03-09) the whole document | 1-3, 7-31, 35 |

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 00/01035

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 38-40 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

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

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

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/GB 00/01035

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO 0012720 A | 09-03-2000 | AU 5696499 A | 21-03-2000 |