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(54) **Title:** NUCLEIC ACID

(57) **Abstract:** The invention relates to nucleic acid derived from Perkinsus marinus which encodes a 9-elongase, a Δ 8-desaturase and a Δ 5-desaturase enzyme. All of the coding sequences can be transcribed as a single transcript.

NUCLEIC ACID

The present invention relates to nucleic acid derived from *Perkinsus marinus* which encodes a 9-elongase, Δ8-desaturase and a Δ5-desaturase enzyme. All of the coding sequences can be transcribed as a single transcript, which simplifies the process of transforming cells required to express all three proteins. The invention also relates to the individual coding sequences and to proteins encoded by these sequences as well as to a process for converting linoleic acid to arachidonic acid.

Fatty acids and triacylglycerides have a multiplicity of applications in the food industry, in animal nutrition, in cosmetics and in the pharmacological sector. Depending on whether they are free saturated or unsaturated fatty acids or else triacylglycerides with an elevated content of saturated or unsaturated fatty acids, they are suitable for very different applications. Polyunsaturated fatty acids such as linoleic acid and linolenic acid are essential for mammals, since they cannot be produced by the latter. Polyunsaturated ω3-fatty acids and ω6-fatty acids are therefore an important constituent in animal and human nutrition.

Hereinbelow, polyunsaturated fatty acids are referred to as PUFA, PUFAs, LCPUFA or LCPUFAs (**p**oly **u**nsaturated fatty **a**cids, **PUFA,**]ong **c**hain goly **u**nsaturated fatty **a**cids, **LCPUFA**).

The various fatty acids and triglycerides are mainly obtained from microorganisms such as Mortierella and Schizochytrium or from oil-producing plants such as soybean, oilseed rape, algae such as Crypthecodinium or Phaeodactylum and others, where they are obtained, as a rule, in the form of their triacylglycerides (= triglycerides = triglycerols). However, they can also be obtained from animals, such as, for example, fish. The free fatty acids are advantageously prepared by hydrolysis. Very long-chain polyunsaturated fatty acids such as docosahexaenoic acid (= DHA, C22:$6^{M,7,10,13,16,19}$), eicosapentaenoic acid (= EPA, C20:$5^{\Delta 5,8,11,14,17}$), arachidonic acid (= ARA, C20:$4^{\Delta 5,8,11,14}$), dihomo-κ-linolenic acid (C20:$3^{\Delta 8,n14}$) or docosapentaenoic acid (DPA, C22:$5^{\Delta 7,10,13,16,19}$) are not synthesized in oil crops such as oilseed rape, soybean, sunflower or safflower. Conventional natural sources of these fatty acids are fish such as herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, zander or tuna, or algae.

Depending on the intended use, oils with saturated or unsaturated fatty acids are preferred. In human nutrition, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred. The polyunsaturated ω3-fatty acids are said to have a positive effect on the cholesterol level in the blood and thus on the possibility of preventing heart disease. The risk of heart disease, stroke or hypertension can be reduced markedly by adding these ω3-fatty acids to the food. Also, ω3-fatty acids have a positive effect on inflammatory, specifically on chronically inflammatory, processes in association with immunological diseases such as rheumatoid arthritis. They are therefore added to foodstuffs, specifically to dietetic

foodstuffs, or are employed in medicaments. ω6-Fatty acids such as arachidonic acid tend to have a negative effect on these disorders in connection with these rheumatic diseases on account of our usual dietary intake.

ω3- and ω6-fatty acids are precursors of tissue hormones, known as eicosanoids, such as the prostaglandins, which are derived from dihomo-Hinolenic acid, arachidonic acid and eicosapentaenoic acid, and of the thromboxanes and leukotrienes, which are derived from arachidonic acid and eicosapentaenoic acid. Eicosanoids (known as the PG_2 series) which are formed from ω6-fatty acids generally promote inflammatory reactions, while eicosanoids (known as the PG_3 series) from ω3-fatty acids have little or no proinflammatory effect.

Owing to the positive characteristics of the polyunsaturated fatty acids, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of these fatty acids or triglycerides for the production of oils in various organisms with a modified content of unsaturated fatty acids. Thus, WO 91/13972 and its US equivalent describes a Δ9-desaturase. WO 93/11245 claims a Δ15-desaturase and WO 94/11516 a Δ12-desaturase. Further desaturases are described, for example, in EP-A-O 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A-O 794 250, Stukey *et al.,* J. Biol. Chem., 265, 1990: 20144-20149, Wada *et al.,* Nature 347, 1990: 200-203 or Huang *et al.,* Lipids 34, 1999: 649-659. However, the biochemical characterization of the various desaturases has been insufficient to date since the enzymes, being membrane-bound proteins, present great difficulty in their isolation and characterization (McKeon *et al.,* Methods in Enzymol. 71, 1981: 12141-12147, Wang *et al.,* Plant Physiol. Biochem., 26, 1988: 777-792). As a rule, membrane-bound desaturases are characterized by being introduced into a suitable organism which is subsequently analyzed for enzyme activity by analyzing the starting materials and the products. Δ6-Desaturases are described in WO 93/06712, US 5,614,393, US5614393, WO 96/21022, WO 00/21557 and WO 99/27111 and the application for the production of fatty acids in transgenic organisms is described in WO 98/46763, WO 98/46764 and WO 98/46765. In this context, the expression of various desaturases and the formation of polyunsaturated fatty acids is also described and claimed in WO 99/64616 or WO 98/46776. As regards the expression efficacy of desaturases and its effect on the formation of polyunsaturated fatty acids, it must be noted that the expression of a single desaturase as described to date has only resulted in low contents of unsaturated fatty acids/lipids such as, for example, Hinolenic acid and stearidonic acid. Moreover, a mixture of ω3- and ω6-fatty acids was obtained, as a rule.

Especially suitable microorganisms for the production of PUFAs are microalgae such as Phaeodactylum tricornutum, Porphiridium species, Thraustochytrium species, Schizochytrium species or Crypthecodinium species, ciliates such as Stylonychia or Colpidium, fungae such as Mortierella, Entomophthora or Mucor and/or mosses such as Physcomitrella, Ceratodon and Marchantia (R. Vazhappilly & F. Chen (1998) Botanica Marina 41: 553-558; K. Totani & K. Oba (1987) Lipids 22: 1060-1062; M.

Akimoto *et al.* (1998) Appl. Biochemistry and Biotechnology 73: 269-278). Strain selection has resulted in the development of a number of mutant strains of the microorganisms in question which produce a series of desirable compounds including PUFAs. However, the mutation and selection of strains with an improved production of a particular molecule such as the polyunsaturated fatty acids is a time-consuming and difficult process. This is why recombinant methods as described above are preferred whenever possible.

However, only limited amounts of the desired polyunsaturated fatty acids such as DPA, EPA or ARA can be produced with the aid of the abovementioned microorganisms, and, depending on the microorganism used, these are generally obtained as fatty acid mixtures of, for example, EPA, DPA and ARA.

A variety of synthetic pathways is being discussed for the synthesis of arachidonic acid, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (figure 1). Thus, EPA or DHA are produced in marine bacteria such as Vibrio sp. or Shewanella sp. *via* the polyketide pathway (Yu, R. *et al.* Lipids 35:1061-1064, 2000; Takeyama, H. *et al.* Microbiology 143:2725-2731, 1997).

An alternative strategy is the alternating activity of desaturases and elongases (Zank, T.K. *et al.* Plant Journal 31:255-268, 2002; Sakuradani, E. *et al.* Gene 238:445-453, 1999). A modification of the above-described pathway by Δ6-desaturase, Δ6-elongase, Δ5-desaturase, Δ5-elongase and Δ4-desaturase is the Sprecher pathway (Sprecher 2000, Biochim. Biophys. Acta 1486:219-231) in mammals. Instead of the Δ4-desaturation, a further elongation step is effected here to give C_{2A}, followed by a further Δ6-desaturation and finally /?-oxidation to give the C_{22} chain length. Thus what is known as Sprecher pathway (see figure 1) is, however, not suitable for the production in plants and microorganisms since the regulatory mechanisms are not known.

Depending on their desaturation pattern, the polyunsaturated fatty acids can be divided into two large classes, viz. *ω6-* or *ω3*-fatty acids, which differ with regard to their metabolic and functional activities (fig. 1).

The starting material for the *ω6*-metabolic pathway is the fatty acid linoleic acid (18:$2^{\Delta 9,12}$) while the *ω3*-pathway proceeds *via* linolenic acid (18:$3^{\Delta 9,12,15}$). Linolenic acid is formed by the activity of an *ω3*-desaturase (Tocher *et al.* 1998, Prog. Lipid Res. 37, 73-1 17 ; Domergue *et al.* 2002, Eur. J. Biochem. 269, 4105-4113).

Mammals, and thus also humans, have no corresponding desaturase activity (Δ12- and *ω3*-desaturase) and must take up these fatty acids (essential fatty acids) *via* the food. Starting with these precursors, the physiologically important polyunsaturated fatty acids arachidonic acid (= ARA, 20:$4^{\Delta 5,8,11,14}$), an *ω6*-fatty acid and the two *ω3*-fatty acids eicosapentaenoic acid (= EPA, 20:$5^{\Delta 5,8,11,1iM7}$) and docosahexaenoic acid (DHA, 22:$6^{M,7,10,13,17,19}$) are synthesized *via* the sequence of desaturase and elongase reactions. The application of *ω3*-fatty acids shows the therapeutic activity described

above in the treatment of cardiovascular diseases (Shimikawa 2001 , World Rev. Nutr. Diet. 88, 100-108), Entzündungen (Calder 2002, Proc. Nutr. Soc. 61, 345-358) and Arthritis (Cleland and James 2000, J. Rheumatol. 27, 2305-2307).

The elongation of fatty acids, by elongases, by 2 or 4 C atoms is of crucial importance for the production of C_{20}- and C_{22}-PUFAs, respectively. This process proceeds *via* 4 steps. The first step is the condensation of malonyl-CoA with the fatty-acid-acyl-CoA by ketoacyl-CoA synthase (KCS, hereinbelow referred to as elongase). This is followed by a reduction step (ketoacyl-CoA reductase, KCR), a dehydratation step (dehydratase) and a final reduction step (enoyl-CoA reductase). It has been postulated that the elongase activity affects the specificity and rate of the entire process (Millar and Kunst, 1997 Plant Journal 12:121-131).

There have been a large number of attempts in the past to obtain elongase genes. Millar and Kunst, 1997 (Plant Journal 12:121-131) and Millar *et* a/. 1999, (Plant Cell 11:825-838) describe the characterization of plant elongases for the synthesis of monounsaturated long-chain fatty acids (C22:1) and for the synthesis of very long-chain fatty acids for the formation of waxes in plants (C_{28}-C_{32}). Descriptions regarding the synthesis of arachidonic acid and EPA are found, for example, in WO0159128, WO0012720, WO02077213 and WO0208401. The synthesis of polyunsaturated C_{24}-fatty acids is described, for example, in Tvrdik *et al.* 2000, JCB 149:707-717 or WO0244320.

Higher plants comprise polyunsaturated fatty acids such as linoleic acid (18:2$^{\Delta 9,12}$) and linolenic acid (18:3$^{\Delta 9,12,15}$). ARA, EPA and DHA are found not at all in the seed oil of higher plants, or only in miniscule amounts (E. Ucciani: Nouveau Dictionnaire des Huiles Vegetales [New Dictionary of Vegetable Oils]. Technique & Documentation - Lavoisier, 1995. ISBN: 2-7430-0009-0). However, the production of LCPUFAs in higher plants, preferably in oil crops such as oilseed rape, linseed, sunflower and soybeans, would be advantageous since large amounts of high-quality LCPUFAs for the food industry, animal nutrition and pharmaceutical purposes might be obtained economically. To this end, it is advantageous to introduce, into oil crops, genes which encode enzymes of the LCPUFA biosynthesis *via* recombinant methods and to express them therein. These genes may encode for example Δ9-elongases, Δ8-desaturases and/or Δ5-desaturases. These genes can advantageously be isolated from microorganisms and lower plants which produce LCPUFAs and incorporate them in the membranes or triacylglycerides. Thus, it has already been possible to isolate Δ6-desaturase genes from the moss Physcomitrella patens and Δ6-elongase genes from *P. patens* and from the nematode C. *elegans.*

The first transgenic plants which comprise and express genes encoding LCPUFA biosynthesis enzymes and which, as a consequence, produce LCPUFAs were described for the first time, for example, in DE-A-102 19 203 (process for the production of polyunsaturated fatty acids in plants). However, these plants produce LCPUFAs in amounts which require further optimization for processing the oils which

are present in the plants.

As can be seen from Figure 1, products of the ω6-pathway can be modified using appropriate desaturases and, if necessary, elongases to give ω3 fatty acids. Therefore, it would be exceedingly valuable to develop a product which makes possible the production of ARA in a genetically modified organism.

The oyster protozoan parasite *Perkinsus marinusi* is capable of synthesizing saturated and unsaturated fatty acids, including the essential fatty acid, arachidonic acid [20:4(n-6), *via* the Δ-8 desaturase pathway. Surprisingly the present inventors have found that *P. marinusi* contains nucleic encoding a Δ9-elongase, a Δ8-desaturase and a Δ5-desaturase which can all be transcribed as a single transcript. The full length sequence is shown as SEQ ID NO: 1.

Therefore, in a first aspect of the invention, there is provided an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity and which is selected from the group consisting of:

a) Anuclei acid sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 or a homolog thereof;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity, wherein the polypeptides are selected from the group consisting of SEQ ID NOS 2, 3 and 4;

d) A derivative of a nucleic acid sequence of SEQ ID NO: 1 which encodes polypeptides with at least 40% identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4; wherein said polypeptides have Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity.

The advantage of the nucleic acid sequence of the present invention is that, although it encodes three separate enzymes, it can be transcribed as a single sequence, which makes it much simpler to prepare cloning and expression vectors expressing all three enzymes.

Preferably, the isolated nucleic acid sequence according to the invention is not identical to SEQ ID No 1 (sequence 1047306867) itself.

In the context of the present invention "hybridizes under stringent conditions" is intended to describe hybridization and washing conditions under which nucleotide sequences with at least 60% homology to one another usually remain hybridized with one another. Conditions are preferably such that sequences with at least approximately

65%, preferably at least approximately 70% and especially preferably at least 75% or more homology to one another usually remain hybridized to one another. These stringent conditions are known to the skilled worker and described, for example, in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6. A preferred nonlimiting example of stringent hybridization conditions is hybridizations in 6 x sodium chloride/sodium citrate (= SSC) at approximately 45°C, followed by one or more washing steps in 0.2 x SSC, 0.1% SDS at 50 to 65°C. The skilled worker knows that these hybridization conditions differ depending on the type of nucleic acid and, for example when organic solvents are present, regarding temperature and buffer concentration. Under "standard hybridization conditions", for example, the hybridization temperature is, depending on the type of nucleic acid, between 42°C and 58°C in aqueous buffer with a concentration of 0.1 to 5 x SSC (pH 7.2). If organic solvents, for example 50% formamide, are present in the abovementioned buffer, the temperature under standard conditions is approximately 42°C. The hybridization conditions for DNA:DNA hybrids, for example, are 0.1 x SSC and 20°C to 45°C, preferably 3O^{0}C to 45°C. The hybridization conditions for DNA: RNA hybrids are, for example, 0.1 x SSC and 30°C to 55^{0}C, preferably 45°C to 55°C. The abovementioned hybridization conditions are determined by way of example for a nucleic acid with approximately 100 bp (= base pairs) in length and with a G + C content of 50% in the absence of formamide. The skilled worker knows how to determine the required hybridization conditions on the basis of the abovementioned textbooks or textbooks such as Sambrook *et al.,* "Molecular Cloning", Cold Spring Harbor Laboratory, 1989; Hames and Higgins (Ed.) 1985, "Nucleic Acids Hybridization: A Practical Approach", IRL Press at Oxford University Press, Oxford; Brown (Ed.) 1991, "Essential Molecular Biology: A Practical Approach", IRL Press at Oxford University Press, Oxford.

Furthermore, when the present specification refers to isolated nucleic acid molecules of a nucleotide sequence which hybridize with one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3 or with a part thereof under stringent conditions, "a part thereof is understood as meaning, in accordance with the invention, that at least 25 base pairs (= bp), 50 bp, 75 bp, 100 bp, 125 bp or 150 bp, preferably at least 175 bp, 200 bp, 225 bp, 250 bp, 275 bp or 300 bp, especially preferably 350 bp, 400 bp, 450 bp, 500 bp or more base pairs are used for the hybridization.

In the context of the present invention "Homologs" of the nucleic acid sequence with the sequence SEQ ID NO: 1 means, for example, allelic variants with at least approximately 50 or 60%, preferably at least approximately 60 or 70%, more preferably at least approximately 70 or 80%, 90% or 95% and even more preferably at least approximately 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95 %, 96%, 97%, 98%, 99% or more identity or homology with a nucleotide sequence shown in SEQ ID NO: 1.

"Allelic variants" comprise in particular functional variants which can be obtained by deletion, insertion or substitution of nucleotides from/into the sequence, it being intended, however, that the enzyme activity of the resulting proteins which are

synthesized is advantageously retained for the insertion of one or more genes.

"Homologs" also means bacterial, fungal and plant homologs, truncated sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence and derivatives such as, for example, promoter variants. The promoters upstream of the nucleotide sequences detailed can be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without the functionality or activity of the promoters being adversely affected, however. It is furthermore possible that the modification of the promoter sequence enhances their activity or that they are replaced entirely by more active promoters, including those from heterologous organisms.

In order to determine the percentage of homology (= identity) of two amino acid sequences, the sequences are written one under the other for an optimal comparison (for example, gaps may be introduced into the sequence of a protein or of a nucleic acid in order to generate an optimal alignment with the other protein or the other nucleic acid). Then, the amino acid residue or nucleotides at the corresponding amino acid positions or nucleotide positions are compared. If a position in a sequence is occupied by the same amino acid residue or the same nucleotide as the corresponding position in the other sequence, then the molecules are homologous at this position (i.e. amino acid or nucleic acid "homology" as used in the present context corresponds to amino acid or nucleic acid "identity"). The percentage of homology between the two sequences is a function of the number of positions which the sequences share (i.e. % homology = number of identical positions/total number of positions x 100). The terms homology and identity are therefore to be considered as synonymous.

The homology was calculated over the entire amino acid or nucleic acid sequence region. The skilled worker has available a series of programs which are based on various algorithms for the comparison of various sequences. Here, the algorithms of Needleman and Wunsch or Smith and Waterman give particularly reliable results. The program PileUp (J. Mol. Evolution., 25, 351-360, 1987, Higgins *et al.,* CABIOS, 5 1989: 151-153) or the programs Gap and BestFit [Needleman and Wunsch (J. Mol. Biol. 48; 443-453 (1970) and Smith and Waterman (Adv. Appl. Math. 2; 482-489 (1981)], which are part of the GCG software packet [Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 5371 1 (1991)], were used for the sequence alignment. The sequence Ogy values which are indicated above as a percentage were determined over the entire sequence region using the program GAP and the following settings: Gap Weight: 50, Length Weight: 3, Average Match: 10.000 and Average Mismatch: 0.000. Unless otherwise specified, these settings were always used as standard settings for the sequence alignments.

In the context of the present invention "Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity" is understood as meaning that a protein encoded by a derivative of SEQ ID NO:1 or nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 retains an enzymatic activity of at least 10%, preferably 20%, especially preferably 30% and very especially 40% in comparison with the proteins/enzymes encoded by the sequence

SEQ ID NO: 1 or nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 and can thus catalyse the conversion of linoleic acid to arachidonic acid.

Although it is often extremely useful to transcribe nucleic acid encoding polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity as a single sequence, there may be some circumstances in which it is preferable to make use of nucleic acid encoding a single enzyme, i.e. a Δ9-elongase, a Δ8-desaturase or a Δ5-desaturase.

Therefore, in a second aspect of the invention there is provided an isolated nucleic acid sequence which encodes a polypeptide with Δ9-elongase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1, SEQ ID NO: 9 or a homolog of one of these;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, activity, wherein the polypeptide comprises SEQ ID NO: 2 or SEQ ID NO: 10;

d) A derivative of a a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9, which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 2 or SEQ ID NO: 10; wherein said polypeptide has Δ9-elongase activity.

In a third aspect of the invention, there is provided an isolated nucleic acid sequence which encodes a polypeptide with Δ8-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 9351 to 10724 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ8-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 3;

d) A derivative of a a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 3; wherein said polypeptide has Δ8-desaturase activity.

In a fourth aspect of the invention, there is provided an isolated nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 10842 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ5-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 4;

d) A derivative of a a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 4; wherein said polypeptide has Δ5-desaturase activity.

In still another aspect of the invention there is provided a polypeptide which is encoded by a nucleic acid sequence of any of the first to fourth aspects of the invention.

Advantageously, the polypeptide encoded by these nucleic acid molecules have at least approximately 50%, preferably at least approximately 60% and more preferably at least approximately 70%, 80% or 90% and most preferably at least approximately 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more identity with the amino acid sequences shown in SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4 or SEQ ID NO: 9.

The nucleic acid sequences used in the process are advantageously introduced into an expression cassette which makes possible the expression of the nucleic acids in organisms such as microorganisms or plants.

Therefore, in another aspect of the invention there is provided a gene construct comprising a nucleic acid sequence which encodes one or more polypeptides with Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase activity as set out above, operably linked with one or more regulatory sequences.

In the expression cassette, the nucleic acid sequence which encodes Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase, is linked operably with one or more regulatory sequences, advantageously for enhancing gene expression. These regulatory sequences are intended to make possible the specific expression of the genes and proteins. Depending on the host organism, this may mean, for example, that the gene is expressed and/or overexpressed only after induction has taken place, or else that it is expressed and/or overexpressed immediately. For example, these regulatory sequences take the form of sequences to which inductors or repressors bind, thus controlling the expression of the nucleic acid. In addition to these novel regulatory sequences, or instead of these sequences, the natural regulatory elements of these sequences may still be present before the actual structural genes and, if appropriate, may have been genetically modified in such a way that their natural regulation is eliminated and the expression of the genes is enhanced. However, the expression cassette (= expression construct = gene construct) can also be simpler in construction,

that is to say no additional regulatory signals have been inserted before the nucleic acid sequence or its derivatives, and the natural promoter together with its regulation was not removed. Instead, the natural regulatory sequence has been mutated in such a way that regulation no longer takes place and/or gene expression is enhanced. These modified promoters can also be positioned on their own before the natural gene in the form of part-sequences (= promotor with parts of the nucleic acid sequences used in accordance with the invention) in order to enhance the activity. Moreover, the gene construct may advantageously also comprise one or more what are known as enhancer sequences in operable linkage with the promoter, which make possible an enhanced expression of the nucleic acid sequence.

Additional advantageous sequences, such as further regulatory elements or terminator sequences, may also be inserted at the 3' end of the DNA sequences. One or more sequences encoding enzymes which catalyse the conversion of ARA to an ω3-unsaturated fatty acid such as EPA or DHA may also be present. Thus, for example, sequences encoding a Δ5-elongase, ω3-desaturase and/or Δ4-desaturase, may be present in one or more copies of the expression cassette (= gene construct). Preferably, only one copy of the genes is present in each expression cassette. This gene construct or the gene constructs can be expressed together in the host organism. In this context, the gene construct(s) can be inserted in one or more vectors and be present in the cell in free form, or else be inserted in the genome. It is advantageous for the insertion of further genes in the genome when the genes to be expressed are present together in one gene construct.

In this context, the regulatory sequences or factors can, as described above, preferably have a positive effect on the gene expression of the genes introduced, thus enhancing it. Thus, an enhancement of the regulatory elements, advantageously at the transcriptional level, may take place by using strong transcription signals such as promoters and/or enhancers. In addition, however, enhanced translation is also possible, for example by improving the stability of the mRNA.

The regulatory sequences include, in particular, plant sequences such as promoter and terminator sequences. The constructs can advantageously be stably propagated in microorganisms, in particular in *E. coli* and *Agrobacterium tumefaciens,* under selective conditions and make possible the transfer of heterologous DNA into plants or microorganisms.

Useful regulatory sequences are present, for example, in promoters such as the cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, laclq, *17,* T5, T3, gal, trc, ara, SP6, *A-PR* or *A-PL* promoter and are advantageously employed in Gram-negative bacteria. Further advantageous regulatory sequences are, for example, present in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFa, AC, P-60, CYC1 , GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck *et al.,* Cell 21 (1980) 285-294], PRP1 [Ward *et al.,* Plant. Mol. Biol. 22 (1993)], SSU, OCS, iib4, usp, STLS1, B33, nos or in the ubiquitin or phaseolin promoter. Advantageous in

this context are also inducible promoters, such as the promoters described in EP-A-O 388 186 (benzenesulfonamide-inducible), Plant J. 2, 1992:397-404 (Gatz *et ai,* tetracycline-inducible), EP-A-O 335 528 (abscissic acid-inducible) or WO 93/21334 (ethanol- or cyclohexenol-inducible) promoters. Further suitable plant promoters are the cytosolic FBPase promoter or the ST-LSI promoter of potato (Stockhaus *et al.,* EMBO J. 8, 1989, 2445), the glycine max phosphoribosylpyrophosphate amidotransferase promoter (Genbank Accession No.. U87999) or the node-specific promoter described in EP-A-O 249 676.

Especially advantageous promoters are promoters which make possible the expression in tissues which are involved in the biosynthesis of fatty acids. Very especially advantageous are seed-specific promoters, such as the USP promoter as described, but also other promoters such as the LeB4, DC3, phaseolin or napin promoter. Further especially advantageous promoters are seed-specific promoters which can be used for monocotyledonous or dicotyledonous plants and which are described in US 5,608,152 (oilseed rape napin promoter), WO 98/45461 (Arabidopsis oleosin promoter), US 5,504,200 (Phaseolus vulgaris phaseolin promoter), WO 91/13980 (Brassica Bce4 promoter), by Baeumlein *et al.,* Plant J., 2, 2, 1992:233-239 (LeB4 promoter from a legume), these promoters being suitable for dicots. Examples of promoters which are suitable for monocots are the barley lpt-2 or lpt-1 promoter (WO 95/1 5389 and WO 95/23230), the barley hordein promoter and other suitable promoters described in WO 99/16890.

In principle, it is possible to use all natural promoters together with their regulatory sequences, such as those mentioned above. It is also possible and advantageous to use synthetic promoters, either in addition or alone, in particular when they mediate seed-specific expression, such as those described in WO 99/16890.

In order to achieve a particularly high ARA content, especially in transgenic plants, the genes should advantageously be expressed in oil crops in a seed-specific manner. To this end, seed-specific promoters can be used, or those promoters which are active in the embryo and/or in the endosperm. In principle, seed-specific promoters can be isolated both from dicotyledonous and from monocotyledonous plants. Preferred promoters are listed hereinbelow: USP (= unknown seed protein) and vicilin (Vicia faba) [Baumlein *et al.,* Mol. Gen Genet, 1991, 225(3)], napin (oilseed rape) [US 5,608,152], acyl carrier protein (oilseed rape) [US 5,315,001 and WO 92/18634], oleosin (Arabidopsis thaliana) [WO 98/45461 and WO 93/20216], phaseolin (Phaseolus vulgaris) [US 5,504,200], Bce4 [WO 91/13980], legumines B4 (LegB4 promoter) [Baumlein *et al.,* Plant J., 2,2, 1992], Lpt2 and lpt1 (barley) [WO 95/15389 and WO95/23230], seed-specific promoters from rice, maize and wheat [WO 99/16890], Amy32b, Amy 6-6 and aleurain [US 5,677,474], Bce4 (oilseed rape) [US 5,530,149], glycinin (soybean) [EP 571 741], phosphoenol pyruvate carboxylase (soybean) [JP 06/62870], ADR12-2 (soybean) [WO 98/08962], isocitrate lyase (oilseed rape) [US 5,689,040] or α-amylase (barley) [EP 781 849].

Plant gene expression can also be facilitated *via* a chemically inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that gene expression should take place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracycline-inducible promoter (Gatz *et al.* (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

To ensure the stable integration of the biosynthesis genes into the transgenic plant over a plurality of generations, it is usually necessary for each of the nucleic acids which encodes a protein of interest to be expressed under the control of a separate promoter, preferably a promoter which differs from the other promoters, since repeating sequence motifs can lead to instability of the T-DNA, or to recombination events. This is one reason why the nucleic acid of the present invention is particularly advantageous since sequences encoding Δ9-elongase, Δ8-desaturase and Δ5-desaturase can be transcribed as a single unit needing only one promoter. It will, of course, be necessary for other genes encoding, for example, Δ5-elongase, ω3-desaturase and/or Δ4-desaturase to be under the control of separate promoters.

In this context, the expression cassette is advantageously constructed in such a way that a promoter is followed by a suitable cleavage site, advantageously in a polylinker, for insertion of the nucleic acid to be expressed and, if appropriate, a terminator sequence is positioned behind the polylinker. This sequence is repeated several times, preferably three, four or five times, so that up to five genes can be combined in one construct and introduced into the transgenic plant in order to be expressed. Advantageously, the sequence is repeated up to three times. To express the nucleic acid sequences, the latter are inserted behind the promoter *via* a suitable cleavage site, for example in the polylinker. Advantageously, each nucleic acid sequence has its own promoter and, if appropriate, its own terminator sequence. Such advantageous constructs are disclosed, for example, in DE 101 02 337 or DE 101 02 338. However, it is also possible to insert a plurality of nucleic acid sequences behind a promoter and, if appropriate, before a terminator sequence. Here, the insertion site, or the sequence, of the inserted nucleic acids in the expression cassette is not of critical importance, that is to say a nucleic acid sequence can be inserted at the first or last position in the cassette without its expression being substantially influenced thereby. Advantageously, different promoters such as, for example, the USP, LegB4 or DC3 promoter, and different terminator sequences can be used in the expression cassette. However, it is also possible to use only one type of promoter in the cassette. This, however, may lead to undesired recombination events.

As described above, the transcription of the genes which have been introduced should advantageously be terminated by suitable terminator sequences at the 3' end of the biosynthesis genes which have been introduced (behind the stop codon). An example of a sequence which can be used in this context is the OCS 1 terminator sequence. As is the case with the promoters, different terminator sequences should be used for each gene.

The gene construct of the present invention may also comprise biosynthesis genes of the fatty acid or lipid metabolism selected from the group acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s),fatty acid acyltransferase(s), acyl-CoA:lysophospholipid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, allenoxide synthases, hydroperoxide lyases or fatty acid elongase(s) and desaturase(s) such as Δ4-desaturase, Δ5-desaturase, Δ6-desaturase, Δδ-desaturase, Δ9-desaturase, Δ12-desaturase or Δ6-elongase.

These additional nucleic acids or genes can be cloned into the expression cassettes, which are then used for transforming plants with the aid of vectors such as Agrobacterium.

Here, the regulatory sequences or factors can, as described above, preferably have a positive effect on, and thus enhance, the expression genes which have been introduced. Thus, enhancement of the regulatory elements can advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. However, an enhanced translation is also possible, for example by improving the stability of the mRNA. In principle, the expression cassettes can be used directly for introduction into the plants or else be introduced into a vector.

Therefore, in yet another aspect of the invention, there is provided a vector comprising a nucleic acid or a gene construct in any of the aspects of the invention described above.

In one embodiment, the vector may be a cloning vector.

The nucleic acid sequence(s) of the invention may be introduced alone, or preferably, in combination with an expression cassette (nucleic acid construct) into an organism. To introduce the nucleic acids, the latter are advantageously amplified and ligated in the known manner. Preferably, a procedure following the protocol for Pfu DNA polymerase or a Pfu/Taq DNA polymerase mixture is followed. The primers are selected taking into consideration the sequence to be amplified. The primers should advantageously be chosen in such a way that the amplificate comprises the entire codogenic sequence from the start codon to the stop codon. After the amplification, the amplificate is expediently analyzed. For example, a gel-electrophoretic separation can be carried out, which is followed by a quantitative and a qualitative analysis. Thereafter, the amplificate can be purified following a standard protocol (for example Qiagen). An aliquot of the purified amplificate is then available for the subsequent cloning step.

Suitable cloning vectors are generally known to the skilled worker. These include, in particular, vectors which are capable of replication in microbial systems, that is to say mainly vectors which ensure efficient cloning in yeasts or fungi and which make possible the stable transformation of plants. Those which must be mentioned in

particular are various binary and cointegrated vector systems which are suitable for the T-DNA-mediated transformation. Such vector systems are, as a rule, characterized in that they comprise at least the vir genes required for the Agrobacterium-mediated transformation and the T-DNA-delimiting sequences (T-DNA border). These vector systems advantageously also comprise further cis-regulatory regions such as promoters and terminator sequences and/or selection markers, by means of which suitably transformed organisms can be identified. While in the case of cointegrated vector systems vir genes and T-DNA sequences are arranged on the same vector, binary systems are based on at least two vectors, one of which bears vir genes, but no T-DNA, while a second one bears T-DNA, but no vir gene. Owing to this fact, the last-mentioned vectors are relatively small, easy to manipulate and to replicate both in *E. coli* and in Agrobacterium. These binary vectors include vectors from the series pBIB-HYG, pPZP, pBecks, pGreen. In accordance with the invention, Bin19, **pBI101**, pBinAR, pGPTV and pCAMBIA are used by preference. An overview of the binary vectors and their use is found in Hellens *et al,* Trends in Plant Science (2000) 5, 446-451 . In order to prepare the vectors, the vectors can first be linearized with restriction endonuclease(s) and then modified enzymatically in a suitable manner. Thereafter, the vector is purified, and an aliquot is employed for the cloning step. In the cloning step, the enzymatically cleaved and, if appropriate, purified amplificate is cloned with vector fragments which have been prepared in a similar manner, using ligase. In this context, a particular nucleic acid construct, or vector or plasmid construct, can have one or else more than one codogenic gene segment. The codogenic gene segments in these constructs are preferably linked operably with regulatory sequences. The regulatory sequences include, in particular, plant sequences such as the above-described promoters and terminator sequences. The constructs can advantageously be stably propagated in microorganisms, in particular in *E. coli* and Agrobacterium tumefaciens, under selective conditions and make possible the transfer of heterologous DNA into plants or microorganisms.

The nucleic acids of the invention can be introduced into organisms such as microorganisms or advantageously plants, advantageously using cloning vectors, and thus be used in the transformation of plants such as those which are published and cited in: Plant Molecular Biology and Biotechnology (CRC Press, Boca Raton, Florida), Chapter 6/7, p. 71-1 19 (1993); F.F. White, Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, 15-38; B. Jenes *et al.,* Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-143; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225. Thus, the nucleic acids, the inventive nucleic acids and nucleic acid constructs, and/or vectors used in the process can be used for the recombinant modification of a broad spectrum of organisms, advantageously plants, so that the latter become better and/or more efficient producers of ARA.

A series of mechanisms exist by which modification of the Δ9-elongase, Δ8-desaturase and Δ5-desaturase proteins is possible, so that the yield, production and/or production

efficiency of ARA in a plant, preferably in an oil crop plant or a microorganism, can be influenced directly owing to these modified proteins. The number or activity of the proteins or genes can be increased, so that greater amounts of the gene products and, ultimately, greater amounts of the compounds of the general formula I are produced. A *de novo* synthesis in an organism which has lacked the activity and ability to biosynthesize the compounds prior to introduction of the corresponding gene(s) is also possible. This applies analogously to the combination with further desaturases or elongases or further enzymes of the fatty acid and lipid metabolism. The use of various divergent sequences, i.e. sequences which differ at the DNA sequence level, may also be advantageous in this context, or else the use of promoters for gene expression which make possible a different gene expression in the course of time, for example as a function of the degree of maturity of a seed or an oil-storing tissue.

Owing to the introduction of a gene encoding Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase into an organism, alone or in combination with other genes in a cell, it is not only possible to increase biosynthesis flux towards the end product, but also to increase, or to create *de novo* the corresponding triacylglycerol composition. Likewise, the number or activity of other genes which are involved in the import of nutrients which are required for the biosynthesis of one or more fatty acids, oils, polar and/or neutral lipids, can be increased, so that the concentration of these precursors, cofactors or intermediates within the cells or within the storage compartment is increased, whereby the ability of the cells to produce ARA as described below is enhanced further. By optimizing the activity or increasing the number of one or more genes encoding Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase which are involved in the biosynthesis ARA, or by destroying the activity of one or more genes which are involved in the degradation of ARA, an enhanced yield, production and/or efficiency of production of fatty acid and lipid molecules in organisms, advantageously in plants, is made possible.

Nucleic acids which can advantageously be used in the process are derived from bacteria, fungi, diatoms, animals such as Caenorhabditis or Oncorhynchus or plants such as algae or mosses, such as the genera Shewanella, Physcomitrella, Thraustochytrium, Fusarium, Phytophthora, Ceratodon, Mantoniella, Ostreococcus, Isochrysis, Aleurita, Muscarioides, Mortierella, Borago, Phaeodactylum, Crypthecodinium, specifically from the genera and species *Oncorhynchus mykiss, Xenopus laevis, Ciona intestinalis, Thalassiosira pseudonona, Mantoniella squamata, Ostreococcus sp., Ostreococcus tauri, Euglena gracilis, Physcomitrella patens, Phytophtora infestans, Fusarium graminaeum, Cryptocodinium cohnii, Ceratodon purpureus, Isochrysis galbana, Aleurita faήnosa, Thraustochytrium sp., Muscarioides viallii, Mortierella alpina, Borago officinalis, Phaeodactylum tricornutum, Caenorhabditis elegans or especially advantageously from Oncorhynchus mykiss, Euglena gracilis, Thalassiosira pseudonona or Crypthecodinium cohnii.*

In an alternative embodiment, the vector may be an expression vector designed to transform an organism in which the nucleic acid is to be expressed and linoleic acid converted to ARA.

These advantageous vectors, preferably expression vectors, comprise the nucleic acid(s) which encode the Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase and which are described in the first to fourth aspects of the invention.

As used in the present context, the term "vector" refers to a nucleic acid molecule which is capable of transporting another nucleic acid to which it is bound. One type of vector is a "plasmid", a circular double-stranded DNA loop into which additional DNA segments can be ligated. A further type of vector is a viral vector, it being possible for additional DNA segments to be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they have been introduced (for example bacterial vectors with bacterial replication origin). Other vectors are advantageously integrated into the genome of a host cell when they are introduced into the host cell, and thus replicate together with the host genome. Moreover, certain vectors can govern the expression of genes with which they are in operable linkage. These vectors are referred to in the present context as "expression vectors". Usually, expression vectors which are suitable for DNA recombination techniques take the form of plasmids.

In the present description, where the term "plasmid" is used, it should be understood that plasmids can be substituted for other types of expression vector, such as viral vectors, which exert similar functions. Furthermore, the term "vector" is also intended to comprise other vectors with which the skilled worker is familiar, such as phages, viruses such as SV40, CMV, TMV, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA.

The recombinant expression vectors advantageously used in the process comprise the nucleic acids described below or the above-described gene construct in a form which is suitable for expressing the nucleic acids used in a host cell, which means that the recombinant expression vectors comprise one or more regulatory sequences, selected on the basis of the host cells used for the expression, which regulatory sequence(s) is/are linked operably with the nucleic acid sequence to be expressed. In a recombinant expression vector, "linked operably" means that the nucleotide sequence of interest is bound to the regulatory sequence(s) in such a way that the expression of the nucleotide sequence is possible and they are bound to each other in such a way that both sequences carry out the predicted function which is ascribed to the sequence (for example in an in-vitro transcription/translation system, or in a host cell if the vector is introduced into the host cell). The term "regulatory sequence" is intended to comprise promoters, enhancers and other expression control elements (for example polyadenylation signals). These regulatory sequences are described, for example, in Goeddel: Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), or see: Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnolgy, CRC Press, Boca Raton, Florida, Ed.: Glick and Thompson, Chapter 7, 89-108, including the references cited therein. Regulatory sequences comprise those which govern the constitutive expression of a nucleotide sequence in many types of host cell and those which govern the direct expression of the nucleotide

sequence only in specific host cells under specific conditions. The skilled worker knows that the design of the expression vector can depend on factors such as the choice of host cell to be transformed, the desired expression level of the protein and the like.

The recombinant expression vectors used can be designed for the expression of Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase in prokaryotic or eukaryotic cells. This is advantageous since intermediate steps of the vector construction are frequently carried out in microorganisms for the sake of simplicity. For example, the Δ9-elongase, Δδ-desaturase and/or Δ5-desaturase gene can be expressed in bacterial cells, insect cells (using Baculovirus expression vectors), yeast and other fungal cells (see Romanos, M.A., *et al.* (1992) "Foreign gene expression in yeast: a review", Yeast 8:423-488; van den Hondel, CAM. J.J., *et al.* (1991) "Heterologous gene expression in filamentous fungi", in: More Gene Manipulations in Fungi, J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego; and van den Hondel, C.A.M. J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of Fungi, Peberdy, J.F., *et al.,* Ed., pp. 1-28, Cambridge University Press: Cambridge), algae (Falciatore *et al.,* 1999, Marine Biotechnology. 1, 3:239-251), ciliates of the types: Holotrichia, Peritrichia, Spirotrichia, Suctoria, Tetrahymena, Paramecium, Colpidium, Glaucoma, Platyophrya, Potomacus, Desaturaseudocohnilembus, Euplotes, Engelmaniella and Stylonychia, in particular of the genus Stylonychia lemnae, using vectors in a transformation method as described in WO 98/01572 and, preferably, in cells of multi-celled plants (see Schmidt, R. and Willmitzer, L. (1988) "High efficiency Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana leaf and cotyledon explants" Plant Cell Rep.:583-586; Plant Molecular Biology and Biotechnology, C Press, Boca Raton, Florida, Chapter 6/7, pp.71-1 19 (1993); F.F. White, B. Jenes *et al.,* Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-43; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225 (and references cited therein)). Suitable host cells are furthermore discussed in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). As an alternative, the recombinant expression vector can be transcribed and translated *in vitro,* for example using T7-promoter regulatory sequences and T7-polymerase.

In most cases, the expression of proteins in prokaryotes involves the use of vectors comprising constitutive or inducible promoters which govern the expression of fusion or nonfusion proteins. Typical fusion expression vectors are, inter alia, pGEX (Pharmacia Biotech Inc; Smith, D.B., and Johnson, K.S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) und pRIT5 (Pharmacia, Piscataway, NJ), where glutathione S-transferase (GST), maltose-E binding protein and protein A, respectively, is fused with the recombinant target protein.

Examples of suitable inducible nonfusion *E. coli* expression vectors are, inter alia, pTrc (Amann *etal.* (1988) Gene 69:301-315) and pET 11d (Studier *et al.,* Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California

(1990) 60-89). The target gene expression from the pTrc vector is based on the transcription from a hybrid trp-lac fusion promoter by the host RNA polymerase. The target gene expression from the vector pET 11d is based on the transcription of a T7-gn10-lac fusion promoter, which is mediated by a viral RNA polymerase (T7 gn1), which is coexpressed. This viral polymerase is provided by the host strains BL21 (DE3) or HMS1 74 (DE3) from a resident Λ-prophage which harbors a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

Other vectors which are suitable for prokaryotic organisms are known to the skilled worker, these vectors are, for example in *E. coli* pLG338, pACYC184, the pBR series such as pBR322, the pUC series such as pUC1 8 or pUC1 9, the M1 13mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, **pIN-HM 13-B1,** Λgt1 1 or pBdCI, in Streptomyces plJ101 , plJ364, plJ702 or plJ361 , in Bacillus pUB1 10, pC194 or pBD214, in Corynebacterium pSA77 or pAJ667.

In a further embodiment, the expression vector is a yeast expression vector. Examples for vectors for expression in the yeast *S.cerevisiae* comprise pYeDesaturased (Baldari *et al.* (1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz (1982) Cell 30:933-943), pJRY88 (Schultz *et al.* (1987) Gene 54:1 13-123) and pYES2 (Invitrogen Corporation, San Diego, CA). Vectors and processes for the construction of vectors which are suitable for use in other fungi, such as the filamentous fungi, comprise those which are described in detail in: van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of fungi, J.F. Peberdy *et al.,* Ed., pp. 1-28, Cambridge University Press: Cambridge, or in: More Gene Manipulations in Fungi [J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego]. Further suitable yeast vectors are, for example, pAG-1 , YEp6, YEpI 3 or pEMBLYe23.

As an alternative, Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase can be expressed in insect cells using Baculovirus vectors. Baculovirus expression vectors which are available for the expression of proteins in cultured insect cells (for example Sf9 cells) comprise the pAc series (Smith *et al.* (1983) MoI. Cell Biol.. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

The abovementioned vectors are only a small overview over suitable vectors which are possible. Further plasmids are known to the skilled worker and are described, for example, in: Cloning Vectors (Ed. Pouwels, P.H., *et al.,* Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). For further suitable expression systems for prokaryotic and eukaryotic cells, see the Chapters 16 and 17 in Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2. edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In a further embodiment of the process, the Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase can be expressed in single-celled plant cells (such as algae), see

Falciatore *et al.,* 1999, Marine Biotechnology 1 (3):239-251 and references cited therein, and in plant cells from higher plants (for example spermatophytes such as arable crops). Examples of plant expression vectors comprise those which are described in detail in: Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992) "New plant binary vectors with selectable markers located proximal to the left border", Plant Mol. Biol. 20:1 195-1 197; and Bevan, M.W. (1984) "Binary Agrobacterium vectors for plant transformation", Nucl. Acids Res. 12:871 1-8721; Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, p. 15-38.

A plant expression cassette preferably comprises regulatory sequences which are capable of governing the expression of genes in plant cells and which are linked operably so that each sequence can fulfill its function, such as transcriptional termination, for example polyadenylation signals. Preferred polyadenylation signals are those which are derived from Agrobacterium tumefaciens T-DNA, such as gene 3 of the Ti plasmid pTiACH5 (Gielen *et al.,* EMBO J. 3 (1984) 835 et seq.), which is known as octopine synthase, or functional equivalents thereof, but all other terminator sequences which are functionally active in plants are also suitable.

Since plant gene expression is very often not limited to the transcriptional level, a plant expression cassette preferably comprises other sequences which are linked operably, such as translation enhancers, for example the overdrive sequence, which enhances the tobacco mosaic virus 5' - untranslated leader sequence, which increases the protein/RNA ratio (Gallie *et al.,* 1987, Nucl. Acids Research 15:8693-871 1).

As described above, plant gene expression must be linked operably with a suitable promoter which triggers gene expression with the correct timing or in a cell- or tissue-specific manner. Utilizable promoters are constitutive promoters (Benfey *et al.,* EMBO J. 8 (1989) 2195-2202), such as those which are derived from plant viruses, such as 35S CaMV (Franck *et al.,* Cell 21 (1980) 285-294), 19S CaMV (see also US 5352605 and WO 84/02913), or plant promoters, such as the promoter of the Rubisco subunit, which is described in US 4,962,028.

Other preferred sequences for use in operable linkage in plant gene expression cassettes are targeting sequences, which are required for steering the gene product into its corresponding cell compartment (see a review in Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423 and references cited therein), for example into the vacuole, into the nucleus, all types of plastids, such as amyloplasts, chloroplasts, chromoplasts, the extracellular space, the mitochondria, the endoplasmid reticulum, elaioplasts, peroxisomes and other compartments of plant cells.

As described above, plant gene expression can also be achieved *via* a chemically inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that the gene expression takes place in a time-specific manner. Examples of such

promoters are a salicylioacid-inducible promoter (WO 95/19443), a tetracyclin-inducible promoter (Gatz *et al.* (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

Promoters which respond to biotic or abiotic stress conditions are also suitable, for example the pathogen-induced PRP1 gene promoter (Ward *etal.,* Plant. Mol. Biol. 22 (1993) 361-366), the heat-inducible tomato hspδOpromoter (US 5,187,267), the chill-inducible potato alpha-amylase promoter (WO 96/12814) or the wound-inducible pinll promoter (EP-A-O 375 091).

Especially preferred are those promoters which bring about the gene expression in tissues and organs in which the biosynthesis of fatty acids, lipids and oils takes place, in seed cells, such as cells of the endosperm and of the developing embryo. Suitable promoters are the oilseed rape napin promoter (US 5,608,152), the Vicia faba USP promoter (Baeumlein *et al.,* Mol Gen Genet, 1991 , 225 (3):459-67), 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 legumine B4 promoter (LeB4; Baeumlein *et al.,* 1992, Plant Journal, 2 (2):233-9), and promoters which bring about the seed-specific expression in monocotyledonous plants such as maize, barley, wheat, rye, rice and the like. Suitable noteworthy promoters are the barley lpt2 or lpt1 gene promoter (WO 95/15389 and WO 95/23230) or the promoters from the barley hordein gene, the rice glutelin gene, the rice oryzin gene, the rice prolamine gene, the wheat gliadine gene, the wheat glutelin gene, the maize zeine gene, the oat glutelin gene, the sorghum kasirin gene or the rye secalin gene, which are described in WO 99/16890.

As described above, it may be advantageous to include in an expression cassette nucleic acid encoding enzymes capable of coverting ARA to ω3-unsaturated fatty acids such as EPA or DHA. Thus, for example the expression cassette may also include nucleic acid encoding a Δ5-elongase, ω3-desaturase and/or Δ4-desaturase. Such expression cassettes can be introduced *via* the simultaneous transformation of a plurality of individual expression constructs or, preferably, by combining a plurality of expression cassettes on one construct. Also, a plurality of vectors can be transformed with in each case a plurality of expression cassettes and then transferred into the host cell.

Other promoters which are likewise especially suitable are those which bring about a plastid-specific expression, since plastids constitute the compartment in which the precursors and some end products of lipid biosynthesis are synthesized. Suitable promoters, such as the viral RNA polymerase promoter, are described in WO 95/16783 and WO 97/06250, and the clpP promoter from Arabidopsis, described in WO 99/46394.

Vector DNA can be introduced into prokaryotic and eukaryotic cells *via* conventional transformation or transfection techniques. The terms "transformation" and

"transfection", conjugation and transduction, as used in the present context, are intended to comprise a multiplicity of methods known in the prior art for the introduction of foreign nucleic acid (for example DNA) into a host cell, including calcium phosphate or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation or particle bombardment. Suitable methods for the transformation or transfection of host cells, including plant cells, can be found in Sambrook *et al.* (Molecular Cloning: A Laboratory Manual., 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) and other laboratory textbooks such as Methods in Molecular Biology, 1995, Vol. 44, Agrobacterium protocols, Ed.: Gartland and Davey, Humana Press, Totowa, New Jersey.

In a further aspect of the invention there is provided a transgenic non human organism comprising at least one nucleic acid, gene construct or vector according to a previous aspect of the invention.

The transgenic nonhuman organism may be a microorganism, a nonhuman animal or a plant.

Host cells which are suitable in principle for taking up the nucleic acid according to the invention, the gene product according to the invention or the vector according to the invention are all prokaryotic or eukaryotic organisms. The host organisms which are advantageously used are microorganisms such as fungi or yeasts, or plant cells, preferably plants or parts thereof. Fungi, yeasts or plants are preferably used, especially plants, for example plants such as oil crops, which are high in lipid compounds, such as oilseed rape, evening primrose, hemp, thistle, peanut, canola, linseed, soybean, safflower, sunflower, borage, or plants such as maize, wheat, rye, oats, thticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanacea plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut), and perennial grasses and fodder crops. Especially preferred plants according to the invention are oil crops such as soybean, peanut, oilseed rape, canola, linseed, hemp, evening primrose, sunflower, safflower, trees (oil palm, coconut).

In an advantageous embodiment, the term "nucleic acid (molecule)" as used in the present context additionally comprises the untranslated sequence at the 3' and at the 5' end of the coding gene region: at least 500, preferably 200, especially preferably 100 nucleotides of the sequence upstream of the 5' end of the coding region and at least 100, preferably 50, especially preferably 20 nucleotides of the sequence downstream of the 3' end of the coding gene region. An "isolated" nucleic acid molecule is separate from other nucleic acid molecules which are present in the natural source of the nucleic acid. An "isolated" nucleic acid preferably has no sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (for example sequences which are located at the 5' and 3' ends of the nucleic acid). In various embodiments, the isolated Δ9-elongase, Δ8-desaturase or Δ5-desaturase

molecule can comprise for example fewer than approximately 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in the genomic DNA of the cell from which the nucleic acid is derived. The same applies to other nucleic acid sequences which may be included in an expression cassette, for example sequences encoding a Δ5-elongase, ω3-desaturase and/or Δ4-desaturase

The nucleic acid molecules of the present invention, for example a nucleic acid molecule with a nucleotide sequence of SEQ ID NO: 1 or residues 7668 to 12077 thereof, or the parts of SEQ ID NO: 1 specified in the second to fourth aspects of the invention, can be isolated using molecular-biological standard techniques and the sequence information provided herein. Also, for example a homologous sequence or homologous, conserved sequence regions can be identified at the DNA or amino acid level with the aid of comparative algorithms. They can be used as hybridization probe and standard hybridization techniques (such as, for example, those described in Sambrook *et al.,* Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) for isolating further nucleic acid sequences which can be used in the process.

Moreover, a nucleic acid molecule from *Perkinsus marinus* comprising a complete sequence of SEQ ID NO: 1 or a part thereof can be isolated by polymerase chain reaction, where oligonucleotide primers which are used on the basis of this sequence or parts thereof (for example a nucleic acid molecule comprising the complete sequence or part thereof can be isolated by polymerase chain reaction using oligonucleotide primers which have been generated based on this same sequence). For example, mRNA can be isolated from cells (for example by means of the guanidinium thiocyanate extraction method of Chirgwin *et al.* (1979) Biochemistry 18:5294-5299) and cDNA by means of reverse transcriptase (for example Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase, available from Seikagaku America, Inc., St.Petersburg, FL).

Synthetic oligonucleotide primers for the amplification by means of polymerase chain reaction can be generated based on one of the sequences shown in SEQ ID NO: 1 or with the aid of the amino acid sequences detailed in SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4. Particularly suitable primers are shown in the Examples as SEQ ID NO: 5 and SEQ ID NO: 6.

A nucleic acid according to the invention can be amplified by standard PCR amplification techniques using cDNA or, alternatively, genomic DNA as template (SEQ ID NO 9) and suitable oligonucleotide primers (SEQ ID NO: 5 and SEQ ID NO: 6). The nucleic acid amplified thus can be cloned into a suitable vector and characterized by means of DNA sequence analysis. Oligonucleotides which correspond to a desaturase or elongase nucleotide sequence can be generated by standard synthetic methods, for example using an automatic DNA synthesizer.

The abovementioned nucleic acids and protein molecules with Δ9-elongase, Δ8-desaturase and/or Δ5-desaturase activity may be used in a process for the production of ARA from linoleic acid in transgenic organisms.

Therefore, in a further aspect of the invention, there is provided a process for the conversion of linoleic acid or a derivative thereof to arachidonic acid or a derivative thereof in an organism, the process comprising introducing into an organism which comprises linoleic acid at least one nucleic acid sequence comprising :

a) SEQ ID NO: 1 (Full sequence 1047306867), sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 or a homolog of one of these;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence of SEQ ID NO: 1 or a sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1;

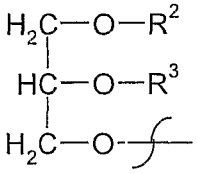
c) an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity, wherein the polypeptides are selected from the group consisting of SEQ ID NOS 2, 3 and 4;

d) A derivative of a nucleic acid sequence of SEQ ID NO: 1 which encodes polypeptides with at least 40% identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4; wherein said polypeptides have Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity.

and expressing said nucleic acid sequence.

In the context of the present invention, a "derivative" of linoleic or arachidonic acid is a compound in which the OH of the carboxylic acid moiety is replaced by a moiety R^1, wherein:

R^1 is coenzyme A (thioester), lysophosphatidylcholine, lysophosphatidylethanolamine, lysophosphatidylglycerol, lysodiphosphatidylglycerol, lysophosphatidylserine, lysophosphatidylinositol, sphingo base or a radical of the formula II



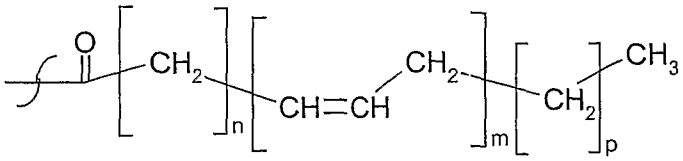
(II)

in which

R^2 = hydrogen, lysophosphatidyl choline, lysophosphatidylethanolamine, lysophosphatidylglycerol, lysodiphosphatidylglycerol, lysophosphatidylserine,

lysophosphatidylinositol or saturated or unsaturated C_2-C_{24}-alkylcarbonyl,

R^3 = hydrogen, saturated or unsaturated C_2-C_{24}-alkylcarbonyl, or R^2 and R^3 independently of one another are a radical of the formula Ia:



(Ia)

in which

n = 2, 3, 4, 5, 6, 7 or 9, m = 2, 3, 4, 5 or 6 and p = 0 or 3;

and wherein an oxygen in the R^1 radical may be replaced by sulphur such that R^1 is bonded to the remainder of the molecule *via* a thioester linkage.

The processes according to the invention preferably yields total ARA in a content of at least 1% by weight, advantageously at least 3% by weight, based on the total fatty acids in the transgenic organisms, preferably in a transgenic plant.

Since a plurality of reaction steps are performed by the starting compounds linoleic acid ($18:2^{\Delta 9,12}$) in the process according to the invention, ARA ($20:4^{\Delta 5,8,11,14}$) is not obtained as a pure product; minor traces of the precursors are always present in the end product.

Chemically pure ARA can also be synthesized by the process described above. To this end, ARA or a derivative thereof is isolated from the organisms, such as the microorganisms or the plants or the culture medium in or on which the organisms have been grown, or from the organism and the culture medium, in the known manner, for example *via* extraction, distillation, crystallization, chromatography or a combination of these methods. These chemically ARA or ARA derivatives are advantageous for applications in the food industry sector, the cosmetic sector and especially the pharmacological industry sector.

The process may include additional steps of converting the ARA to an ω-3 fatty acid by introducing into the organism nucleic acid encoding a ω-3 desaturase and optionally a Δ5-elongase and/or a Δ4-elongase and/or a Δ4-desaturase.

In a further aspect the invention comprises a process for the conversion of $18:2^{\Delta 9,12}$ (linoleic acid) to $20:2^{\Delta 11,14}$, the process comprising introducing into an organism which comprises linoleic acid at least one nucleic acid sequence which encodes a polypeptide having Δ9-elongase activity and which comprises :

a) a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO:

1, SEQ ID NO: 9 or a homolog of one of these;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9;

c) an isolated nucleic acid sequence which encodes a polypeptide with Δ9-elongase activity, wherein the polypeptide comprises SEQ ID NO: 2 or SEQ ID NO: 10;

d) A derivative of a a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 2 or SEQ ID NO: 10; wherein said polypeptide has Δ9-elongase activity;

and expressing said nucleic acid sequence.

In a further aspect of the invention there is provided a process for the conversion of 20:2$^{\Delta 11,14}$ to 20:3$^{\Delta 8,11,14}$, the process comprising introducing into an organism which comprises 20:2$^{\Delta 11,14}$, or which comprises linoleic acid and a Δ9 elongase, an isolated nucleic acid sequence which encodes a polypeptide with Δ8-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 9351 to 10724 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ8-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 3;

d) A derivative of a a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 3; wherein said polypeptide has Δ8-desaturase activity; and

expressing said nucleic acid sequence.

In a further aspect of the invention, there is provided a process for the conversion of 20:3 $^{\Delta 8,11,14}$ to 20:4 $^{\Delta 5,8,11,14}$ (ARA), the process comprising introducing into an organism which comprises 20:3 $^{\Delta 8,11,14}$ or which comprises 20:2 $^{\Delta 11,14}$ and a Δ8-desaturase, or which comprises linoleic acid, a Δ9 elongase and a Δ8-desaturase, an isolated nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID

NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 10842 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ5-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 4;

d) A derivative of a a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 4; wherein said polypeptide has Δ5-desaturase activity.

and expressing said nucleic acid sequence.

The process may include additional steps of converting the ARA to an ω-3 fatty acid by introducing into the organism nucleic acid encoding a ω-3 desaturase and optionally a Δ5-elongase and/or a Δ4-elongase and/or a Δ4-desaturase.

For the processes set out above, it has been found that expression has been most effectively achieved using induction with galactose.

Suitable organisms for the production in the process according to the invention are, in principle, any organisms such as microorganisms, nonhuman animals or plants.

Plants which are suitable are, in principle, all those plants which are capable of synthesizing fatty acids, such as all dicotyledonous or monocotyledonous plants, algae or mosses. Advantageous plants are selected from the group of the plant families Adelotheciaceae, Anacardiaceae, Asteraceae, Apiaceae, Betulaceae, Boraginaceae, Brassicaceae, Bromeliaceae, Caricaceae, Cannabaceae, Convolvulaceae, Chenopodiaceae, Crypthecodiniaceae, Cucurbitaceae, Ditrichaceae, Elaeagnaceae, Ericaceae, Euphorbiaceae, Fabaceae, Geraniaceae, Gramineae, Juglandaceae, Lauraceae, Leguminosae, Linaceae, Euglenaceae, Prasinophyceae or vegetable plants or ornamentals such as Tagetes.

Examples which may be mentioned are the following plants selected from the group consisting of: Adelotheciaceae such as the genera Physcomitrella, for example the genus and species *Physcomitrella patens,* Anacardiaceae such as the genera Pistacia, Mangifera, Anacardium, for example the genus and species *Pistacia vera* [pistachio], *Mangifer indica* [mango] or *Anacardium occidentale* [cashew], Asteraceae, such as the genera Calendula, Carthamus, Centaurea, Cichorium, Cynara, Helianthus, Lactuca, Locusta, Tagetes, Valeriana, for example the genus and species Calendula officinalis [common marigold], Carthamus tinctorius [safflower], *Centaurea cyanus* [cornflower], *Cichorium intybus* [chicory], Cynara scolymus [artichoke], *Helianthus annus* [sunflower], *Lactuca sativa, Lactuca crispa, Lactuca esculenta, Lactuca scariola L. ssp.*

sativa, Lactuca scariola L. var. integrata, Lactuca scariola L. var. integrifolia, Lactuca sativa subsp. romana, Locusta communis, Valeriana locusta [salad vegetables], *Tagetes lucida, Tagetes erecta* or *Tagetes tenuifolia* [african or trench marigold], Apiaceae, such as the genus Daucus, for example the genus and species *Daucus carota* [carrot], Betulaceae, such as the genus Corylus, for example the genera and species *Corylus avellana* or *Corylus colurna* [hazelnut], Boraginaceae, such as the genus Borago, for example the genus and species *Borago officinalis* [borage], Brassicaceae, such as the genera Brassica, Camelina, Melanosinapis, Sinapis, Arabadopsis, for example the genera and species *Brassica napus, Brassica rapa* ssp. [oilseed rape], *Sinapis arvensis Brassica juncea, Brassica juncea var. juncea, Brassica juncea var. crispifolia, Brassica juncea var. foliosa, Brassica nigra, Brassica sinapioides,* Camelina sativa, *Melanosinapis communis* [mustard], *Brassica oleracea* [fodder beet] or Arabidopsis thaliana, Bromeliaceae, such as the genera Anana, Bromelia (pineapple), for example the genera and species *Anana comosus, Ananas ananas* or *Bromelia comosa* [pineapple], Caricaceae, such as the genus Carica, such as the genus and species *Carica papaya* [pawpaw], Cannabaceae, such as the genus Cannabis, such as the genus and species *Cannabis sativa* [hemp], Convolvulaceae, such as the genera Ipomea, Convolvulus, for example the genera and species *lpomoea batatus, lpomoea pandurata, Convolvulus batatas, Convolvulus tiliaceus, lpomoea fastigiata, lpomoea tiliacea, lpomoea triloba* or *Convolvulus panduratus* [sweet potato, batate], Chenopodiaceae, such as the genus Beta, such as the genera and species *Beta vulgaris, Beta vulgaris var. altissima, Beta vulgaris var. vulgaris, Beta maritima, Beta vulgaris var. perennis, Beta vulgaris var. conditiva* or *Beta vulgaris var. esculenta* [sugarbeet], Crypthecodiniaceae, such as the genus Crypthecodinium, for example the genus and species *Cryptecodinium cohnii,* Cucurbitaceae, such as the genus Cucurbita, for example the genera and species *Cucurbita maxima, Cucurbita mixta, Cucurbita pepo* or *Cucurbita moschata* [pumpkin/squash], Cymbellaceae, such as the genera Amphora, Cymbella, Okedenia, Phaeodactylum, Reimeria, for example the genus and species *Phaeodactylum tricornutum,* Ditrichaceae, such as the genera Ditrichaceae, Astomiopsis, Ceratodon, Chrysoblastella, Ditrichum, Distichium, Eccremidium, Lophidion, Philibertiella, Pleuridium, Saelania, Trichodon, Skottsbergia, for example the genera and species *Ceratodon antarcticus, Ceratodon columbiae, Ceratodon heterophyllus, Ceratodon purpurascens, Ceratodon purpureus, Ceratodon purpureus ssp. convolutus, Ceratodon purpureus ssp. stenocarpus, Ceratodon purpureus var. rotundifolius, Ceratodon ratodon, Ceratodon stenocarpus, Chrysoblastella chilensis, Ditrichum ambiguum, Ditrichum brevisetum, Ditrichum crispatissimum, Ditrichum difficile, Ditrichum falcifolium, Ditrichum flexicaule, Ditrichum giganteum, Ditrichum heteromallum, Ditrichum lineare, Ditrichum lineare, Ditrichum montanum, Ditrichum montanum, Ditrichum pallidum, Ditrichum punctulatum, Ditrichum pusillum, Ditrichum pusillum var. tortile, Ditrichum rhynchostegium, Ditrichum schimperi, Ditrichum tortile, Distichium capillaceum, Distichium hagenii, Distichium inclinatum, Distichium macounii, Eccremidium floridanum, Eccremidium whiteleggei, Lophidion stήctus, Pleuridium acuminatum, Pleuridium altemifoiium, Pleuridium holdridgei, Pleuridium mexicanum, Pleuridium ravenelii, Pleuridium subulatum,*

Saelania glaucescens, Trichodon borealis, Trichodon cylindήcus or Tή chodon cylindricus var. oblongus, Elaeagnaceae, such as the genus Elaeagnus, for example the genus and species *Olea europaea* [olive], Ericaceae, such as the genus Kalmia, for example the genera and species *Kalmia latifolia, Kalmia angustifolia, Kalmia microphylla, Kalmia polifolia, Kalmia occidentalis, Cistus chamaerhodendros* or *Kalmia lucida* [mountain laurel], Euglenaceae, such as the genera Ascoglena, Astasia, Colacium, Cyclidiopsis, Euglena, Euglenopsis, Hyalaphacus, Khawkinea, Lepocinclis, Phacus, Strombomonas, Trachelomonas, for example the genus and species *Euglena gracilis;* Euphorbiaceae, such as the genera Manihot, Janipha, Jatropha, Ricinus, for example the genera and species *Manihot utilissima, Janipha manihot, Jatropha manihot, Manihot aipil, Manihot dulcis, Manihot manihot, Manihot melanobasis, Manihot esculenta* [cassava] or *Ricinus communis* [castor-oil plant], Fabaceae, such as the genera Pisum, Albizia, Cathormion, Feuillea, Inga, Pithecolobium, Acacia, Mimosa, Medicajo, Glycine, Dolichos, Phaseolus, soybean, for example the genera and species *Pisum sativum, Pisum arvense, Pisum humile* [pea], *Albizia berteriana, Albizia julibrissin, Albizia lebbeck, Acacia berteriana, Acacia littoralis, Albizia berteriana, Albizzia berteriana, Cathormion berteriana, Feuillea berteriana, Inga fragrans, Pithecellobium berterianum, Pithecellobium fragrans, Pithecolobium berterianum, Pseudalbizzia berteriana, Acacia julibrissin, Acacia nemu, Albizia nemu, Feuilleea julibrissin, Mimosa julibrissin, Mimosa speciosa, Sericanrda julibrissin, Acacia lebbeck, Acacia macrophylla, Albizia lebbeck, Feuilleea lebbeck, Mimosa lebbeck, Mimosa speciosa, Medicago sativa, Medicago falcata, Medicago varia* [alfalfa] *Glycine max Dolichos soja, Glycine gracilis, Glycine hispida, Phaseolus max, Soja hispida* or *Soja max* [soybean], Funariaceae, such as the genera Aphanorrhegma, Entosthodon, Funaria, Physcomitrella, Physcomitrium, for example the genera and species *Aphanorrhegma serratum, Entosthodon attenuatus, Entosthodon bolanderi, Entosthodon bonplandii, Entosthodon califomicus, Entosthodon drummondii, Entosthodon jamesonii, Entosthodon leibergii, Entosthodon neoscoticus, Entosthodon rubήsetus, Entosthodon spathulifolius, Entosthodon tucsoni, Funaria americana, Funaria bolanderi, Funaria calcarea, Funaria californica, Funaria calvescens, Funaria convoluta, Funaria flavicans, Funaria groutiana, Funaria hygrometήca, Funaria hygrometrica var. arctica, Funaria hygrometrica var. calvescens, Funaria hygrometrica var. convoluta, Funaria hygrometrica var. muralis, Funaria hygrometήca var. utahensis, Funaria microstoma, Funaria microstoma var. obtusifolia, Funaria muhlenbergii, Funaria orcuttii, Funaria plano-convexa, Funaria polaris, Funaria ravenelii, Funaria rubriseta, Funaria serrata, Funaria sonorae, Funaria sublimbatus, Funaria tucsoni, Physcomitrella californica, Physcomitrella patens, Physcomitrella reader!, Physcomitrium australe, Physcomitrium californicum, Physcomitrium collenchymatum, Physcomitrium coloradense, Physcomitrium cupuliferum, Physcomitrium drummondii, Physcomitrium eurystomum, Physcomitrium flexifolium, Physcomitrium hookeri, Physcomitrium hookeri var. serratum, Physcomitrium immersum, Physcomitrium kellermanii, Physcomitrium megalocarpum, Physcomitrium pyriforme, Physcomitrium pyriforme var. serratum, Physcomitrium rufipes, Physcomitrium sandbergii, Physcomitrium subsphaericum, Physcomitrium washingtoniense,* Geraniaceae, such

as the genera Pelargonium, Cocos, Oleum, for example the genera and species *Cocos nucifera, Pelargonium grossularioides* or *Oleum cocois* [coconut], Gramineae, such as the genus Saccharum, for example the genus and species Saccharum officinarum, Juglandaceae, such as the genera Juglans, Wallia, for example the genera and species *Juglans regia, Juglans ailanthifolia, Juglans sieboldiana, Juglans cinerea, Wallia cinerea, Juglans bixbyi, Juglans californica, Juglans hindsii, Juglans intermedia, Juglans jamaicensis, Juglans major, Juglans microcarpa, Juglans nigra* or *Wallia nigra* [walnut], Lauraceae, such as the genera Persea, Laurus, for example the genera and species *Laurus nobilis* [bay], *Persea americana, Persea gratissima* or *Persea persea* [avocado], Leguminosae, such as the genus Arachis, for example the genus and species *Arachis hypogaea* [peanut], Linaceae, such as the genera Adenolinum, for example the genera and species *Linum usitatissimum, Linum humile, Linum austriacum, Linum bienne, Linum angustifolium, Linum catharticum, Linum flavum, Linum grandiflorum, Adenolinum grandiflorum, Linum lewisii, Linum narbonense, Linum perenne, Linum perenne var. lewisii, Linum pratense* or *Linum trigynum* [linseed], Lythrarieae, such as the genus Punica, for example the genus and species *Punica granatum* [pomegranate], Malvaceae, such as the genus Gossypium, for example the genera and species *Gossypium hirsutum, Gossypium arboreum, Gossypium barbadense, Gossypium herbaceum* or *Gossypium thurberi* [cotton], Marchantiaceae, such as the genus Marchantia, for example the genera and species *Marchantia berteroana, Marchantia foliacea, Marchantia macropora,* Musaceae, such as the genus Musa, for example the genera and species *Musa nana, Musa acuminata, Musa paradisiaca, Musa* spp. [banana], Onagraceae, such as the genera Camissonia, Oenothera, for example the genera and species *Oenothera biennis* or *Camissonia brevipes* [evening primrose], Palmae, such as the genus Elaeis, for example the genus and species *Elaeis guineensis* [oil palm], Papaveraceae, such as, for example, the genus Papaver, for example the genera and species *Papaver orientate, Papaver rhoeas, Papaver dubium* [poppy], Pedaliaceae, such as the genus Sesamum, for example the genus and species *Sesamum indicum* [sesame], Piperaceae, such as the genera Piper, Artanthe, Peperomia, Steffensia, for example the genera and species *Piper aduncum, Piper amalago, Piper angustifolium, Piper auritum, Piper betel, Piper cubeba, Piper longum, Piper nigrum, Piper retrofractum, Artanthe adunca, Artanthe elongata, Peperomia elongata, Piper elongatum, Steffensia elongata* [cayenne pepper], Poaceae, such as the genera Hordeum, Secale, Avena, Sorghum, Andropogon, Holcus, Panicum, Oryza, Zea (maize), Triticum, for example the genera and species *Hordeum vulgare, Hordeum jubatum, Hordeum murinum, Hordeum secalinum, Hordeum distichon Hordeum aegiceras, Hordeum hexastichon, Hordeum hexastichum, Hordeum irregulare, Hordeum sativum, Hordeum secalinum* [barley], *Secale cereale* [rye], Avena sativa, *Avena fatua, Avena byzantina, Avena fatua var. sativa, Avena hybrida* [oats], *Sorghum bicolor, Sorghum halepense, Sorghum saccharatum, Sorghum vulgare, Andropogon drummondii, Holcus bicolor, Holcus sorghum, Sorghum aethiopicum, Sorghum arundinaceum, Sorghum caffrorum, Sorghum cernuum, Sorghum dochna, Sorghum drummondii, Sorghum durra, Sorghum guineense, Sorghum lanceolatum, Sorghum nervosum, Sorghum saccharatum, Sorghum*

subglabrescens, Sorghum verticilliflorum, Sorghum vulgare, Holcus halepensis, Sorghum miliaceum, Panicum militaceum [millet], Oryza sativa, Oryza latifolia [rice], *Zea mays* [maize] *Triticum aestivum, Triticum durum, Triticum turgidum, Triticum hybernum, Triticum macha, Triticum sativum* or *Triticum vulgare* [wheat], Porphyridiaceae, such as the genera Chroothece, Flintiella, Petrovanella, Porphyridium, Rhodella, Rhodosorus, Vanhoeffenia, for example the genus and species *Porphyridium cruentum,* Proteaceae, such as the genus Macadamia, for example the genus and species *Macadamia intergrifolia* [macadamia], Prasinophyceae, such as the genera Nephroselmis, Prasinococcus, Scherffelia, Tetraselmis, Mantoniella, Ostreococcus, for example the genera and species *Nephroselmis olivacea, Prasinococcus capsulatus, Scherffelia dubia, Tetraselmis chui, Tetraselmis suecica,* Mantoniella squamata, Ostreococcus tauri, Rubiaceae, such as the genus Coffea, for example the genera and species Coffea spp., *Coffea arabica, Coffea canephora* or *Coffea liberica* [coffee], Scrophulariaceae, such as the genus Verbascum, for example the genera and species *Verbascum blattaήa, Verbascum chaixii, Verbascum densiflorum, Verbascum lagurus, Verbascum Iongifolium, Verbascum lychnitis, Verbascum nigrum, Verbascum olympicum, Verbascum phlomoides, Verbascum phoenicum, Verbascum pulverulentum* or *Verbascum thapsus* [verbascum], Solanaceae, such as the genera Capsicum, Nicotiana, Solanum, Lycopersicon, for example the genera and species *Capsicum annuum, Capsicum annuum var. glabήusculum, Capsicum frutescens* [pepper], *Capsicum annuum* [paprika], *Nicotiana tabacum, Nicotiana alata, Nicotiana attenuata, Nicotiana glauca, Nicotiana langsdorffii, Nicotiana obtusifolia, Nicotiana quadrivalvis, Nicotiana repanda, Nicotiana rustica, Nicotiana sylvestris* [tobacco], *Solanum tuberosum* [potato], *Solanum melongena* [eggplant] *Lycopersicon esculentum, Lycopersicon lycopersicum, Lycopersicon pyriforme, Solanum integrifolium* or *Solanum lycopersicum* [tomato], Sterculiaceae, such as the genus Theobroma, for example the genus and species *Theobroma cacao* [cacao] or Theaceae, such as the genus Camellia, for example the genus and species *Camellia sinensis* [tea].

Advantageous microorganisms are, for example, fungi selected from the group of the families Chaetomiaceae, Choanephoraceae, Cryptococcaceae, Cunninghameliaceae, Demetiaceae, Moniliaceae, Mortierellaceae, Mucoraceae, Pythiaceae, Sacharomycetaceae, Saprolegniaceae, Schizosacharomycetaceae, Sodariaceae or Tuberculariaceae.

Examples of microorganisms which may be mentioned are those from the groups: Choanephoraceae, such as the genera Blakeslea, Choanephora, for example the genera and species *Blakeslea trispora, Choanephora cucurbitarum, Choanephora infundibulifera* var. *cucurbitarum,* Mortierellaceae, such as the genus Mortierella, for example the genera and species *Mortierella isabellina, Mortierella polycephala , Mortierella ramanniana , Mortierella vinacea, Mortierella zonata,* Pythiaceae, such as the genera Phytium, Phytophthora, for example the genera and species *Pythium debaryanum, Pythium intermedium, Pythium irregulare, Pythium megalacanthum, Pythium paroecandrum, Pythium sylvaticum, Pythium ultimum, Phytophthora cactorum,*

Phytophthora cinnamomi, Phytophthora citricola, Phytophthora citrophthora, Phytophthora cryptogea, Phytophthora drechsleri, Phytophthora erythroseptica, Phytophthora lateralis, Phytophthora megasperma, Phytophthora nicotianae, Phytophthora nicotianae var. parasitica, Phytophthora palmivora, Phytophthora parasitica, Phytophthora syringae, Saccharomycetaceae, such as the genera Hansenula, Pichia, Saccharomyces, Saccharomycodes, Yarrowia, for example the genera and species *Hansenula anomala, Hansenula californica, Hansenula canadensis, Hansenula capsulata, Hansenula ciferrii, Hansenula glucozyma, Hansenula henricii, Hansenula holstii, Hansenula minuta, Hansenula nonfermentans, Hansenula philodendri, Hansenula polymorpha, Hansenula saturnus, Hansenula subpelliculosa, Hansenula wickerhamii, Hansenula wingei, Pichia alcoholophila, Pichia angusta, Pichia anomala, Pichia bispora, Pichia burtonii, Pichia canadensis, Pichia capsulata, Pichia carsonii, Pichia cellobiosa, Pichia ciferrii, Pichia farinosa, Pichia fermentans, Pichia finlandica, Pichia glucozyma, Pichia guilliermondii, Pichia haplophila, Pichia henricii, Pichia holstii, Pichia jadinii, Pichia lindnerii, Pichia membranaefaciens, Pichia methanolica, Pichia minuta var. minuta, Pichia minuta var. nonfermentans, Pichia norvegensis, Pichia ohmeri, Pichia pastoris, Pichia philodendri, Pichia pini, Pichia polymorpha, Pichia quercuum, Pichia rhodanensis, Pichia sargentensis, Pichia stipitis, Pichia strasburgensis, Pichia subpelliculosa, Pichia toletana, Pichia trehalophila, Pichia vini, Pichia xylosa, Saccharomyces aceti, Saccharomyces bailii, Saccharomyces bayanus, Saccharomyces bisporus, Saccharomyces capensis, Saccharomyces carlsbergensis, Saccharomyces cerevisiae, Saccharomyces cerevisiae var. ellipsoideus, Saccharomyces chevalieri, Saccharomyces delbrueckii, Saccharomyces diastaticus, Saccharomyces drosophilarum, Saccharomyces elegans, Saccharomyces ellipsoideus, Saccharomyces fermentati, Saccharomyces florentinus, Saccharomyces fragilis, Saccharomyces heterogenicus, Saccharomyces hienipiensis, Saccharomyces inusitatus, Saccharomyces italicus, Saccharomyces kluyveri, Saccharomyces krusei, Saccharomyces lactis, Saccharomyces marxianus, Saccharomyces microellipsoides, Saccharomyces montanus, Saccharomyces norbensis, Saccharomyces oleaceus, Saccharomyces paradoxus, Saccharomyces pastorianus, Saccharomyces pretoriensis, Saccharomyces rosei, Saccharomyces rouxii, Saccharomyces uvarum, Saccharomycodes ludwigii, Yarrowia lipolytica,* Schizosacharomycetaceae such as the genera Schizosaccharomyces e.g. the species *Schizosaccharomyces japonicus var. japonicus, Schizosaccharomyces japonicus var. versatilis, Schizosaccharomyces malidevorans, Schizosaccharomyces octosporus, Schizosaccharomyces pombe var. malidevorans, Schizosaccharomyces pombe var. pombe,* Thraustochytriaceae such as the genera Althornia, Aplanochytrium, Japonochytrium, Schizochytrium, Thraustochytrium e.g. the species *Schizochytrium aggregatum, Schizochytrium limacinum, Schizochytrium mangrove!, Schizochytrium minutum, Schizochytrium octosporum, Thraustochytrium aggregatum, Thraustochytrium amoeboideum, Thraustochytrium antacticum, Thraustochytrium arudimentale, Thraustochytrium aureum, Thraustochytrium benthicola, Thraustochytrium globosum, Thraustochytrium indicum, Thraustochytrium kerguelense, Thraustochytrium kinnei, Thraustochytrium*

motivum, Thraustochytrium multirudimentale, Thraustochytrium pachydermum, Thraustochytrium proliferum, Thraustochytrium roseum, Thraustochytrium rossii, Thraustochytrium striatum or *Thraustochytrium visurgense.*

Further advantageous microorganisms are, for example, bacteria selected from the group of the families Bacillaceae, Enterobacteriacae or Rhizobiaceae.

Examples which may be mentioned are the following microorganisms selected from the group consisting of: Bacillaceae, such as the genus Bacillus, for example the genera and species *Bacillus acidocaldarius, Bacillus acidoterrestris, Bacillus alcalophilus, Bacillus amyloliquefaciens, Bacillus amylolyticus, Bacillus brevis, Bacillus cereus, Bacillus circulans, Bacillus coagulans, Bacillus sphaericus subsp. fusiformis, Bacillus galactophilus, Bacillus globisporus, Bacillus globisporus subsp. marinus, Bacillus halophilus, Bacillus lentimorbus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus polymyxa, Bacillus psychrosaccharolyticus, Bacillus pumilus, Bacillus sphaericus, Bacillus subtilis subsp. spizizenii, Bacillus subtilis subsp. subtilis* or *Bacillus thuringiensis;* Enterobacteriacae such as the genera Citrobacter, Edwardsiella, Enterobacter, Erwinia, Escherichia, Klebsiella, Salmonella or Serratia, for example the genera and species *Citrobacter amalonaticus, Citrobacter diversus, Citrobacter freundii, Citrobacter genomospecies, Citrobacter gillenii, Citrobacter intermedium, Citrobacter koseri, Citrobacter murliniae, Citrobacter sp., Edwardsiella hoshinae, Edwardsiella ictaluri, Edwardsiella tarda, Erwinia alni, Erwinia amylovora, Erwinia ananatis, Erwinia aphidicola, Erwinia billingiae, Erwinia cacticida, Erwinia cancerogena, Erwinia carnegieana, Erwinia carotovora* subsp. *atroseptica, Erwinia carotovora* subsp. *betavasculorum, Erwinia carotovora* subsp. *odorifera, Erwinia carotovora* subsp. *wasabiae, Erwinia chrysanthemi, Erwinia cypripedii, Erwinia dissolvens, Erwinia herbicola, Erwinia mallotivora, Erwinia milletiae, Erwinia nighfluens, Erwinia nimipressuralis, Erwinia persicina, Erwinia psidii, Erwinia pyrifoliae, Erwinia quercina, Erwinia rhapontici, Erwinia rubrifaciens, Erwinia salicis, Erwinia stewartii, Erwinia tracheiphila, Erwinia uredovora, Escherichia adecarboxylata, Escherichia anindolica, Escherichia aurescens, Escherichia blattae, Escherichia coli, Escherichia coli var. communior, Escherichia coli-mutabile, Escherichia fergusonii, Escherichia hermannii, Escherichia sp., Escherichia vulneris, Klebsiella aerogenes, Klebsiella edwardsii subsp. atlantae, Klebsiella ornithinolytica, Klebsiella oxytoca, Klebsiella planticola, Klebsiella pneumoniae, Klebsiella pneumoniae subsp. pneumoniae, Klebsiella sp., Klebsiella terrigena, Klebsiella trevisanii, Salmonella abony, Salmonella arizonae, Salmonella bongori, Salmonella choleraesuis subsp. arizonae, Salmonella choleraesuis subsp. bongori, Salmonella choleraesuis subsp. cholereasuis, Salmonella choleraesuis subsp. diarizonae, Salmonella choleraesuis subsp. houtenae, Salmonella choleraesuis subsp. indica, Salmonella choleraesuis subsp. salamae, Salmonella daressalaam, Salmonella enterica subsp. houtenae, Salmonella enterica subsp. salamae, Salmonella enteritidis, Salmonella gallinarum, Salmonella heidelberg, Salmonella panama, Salmonella senftenberg, Salmonella typhimurium, Serratia entomophila, Serratia ficaria, Serratia fonticola, Serratia grimesii, Serratia liquefaciens, Serratia marcescens, Serratia marcescens subsp. marcescens, Serratia marinorubra,*

Serratia odorifera, Serratia plymouthensis, Serratia piymuthica, Serratia proteamaculans, Serratia proteamaculans subsp. quinovora, Serratia quinivorans or *Serratia rubidaea;* Rhizobiaceae, such as the genera Agrobacterium, Carbophilus, Cheiatobacter, Ensifer, Rhizobium, Sinorhizobium, for example the genera and species *Agrobacterium atlanticum, Agrobacterium ferrugineum, Agrobacterium gelatinovorum, Agrobacterium larrymoorei, Agrobacterium meteori, Agrobacterium radiobacter, Agrobacterium rhizogenes, Agrobacterium rubi, Agrobacterium stellulatum, Agrobacterium tumefaciens, Agrobacterium vitis, Carbophilus carboxidus, Cheiatobacter heintzii, Ensifer adhaerens, Ensifer arboris, Ensifer fredii, Ensifer kostiensis, Ensifer kummerowiae, Ensifer medicae, Ensifer meliloti, Ensifer saheli, Ensifer terangae, Ensifer xinjiangensis, Rhizobium cicer*ή *Rhizobium etli, Rhizobium fredii, Rhizobium galegae, Rhizobium gallicum, Rhizobium giardinii, Rhizobium hainanense, Rhizobium huakuif, Rhizobium huautlense, Rhizobium indigoferae, Rhizobium japonicum, Rhizobium leguminosarum, Rhizobium loessense, Rhizobium loti, Rhizobium lupini, Rhizobium mediterraneum, Rhizobium meliloti, Rhizobium mongolense, Rhizobium phaseoli, Rhizobium radiobacter, Rhizobium rhizogenes, Rhizobium rubi, Rhizobium sullae, Rhizobium tianshanense, Rhizobium trifolii, Rhizobium tropici, Rhizobium undicola, Rhizobium vitis, Sinorhizobium adhaerens, Sinorhizobium arboris, Sinorhizobium fredii, Sinorhizobium kostiense, Sinorhizobium kummerowiae, Sinorhizobium medicae, Sinorhizobium meliloti, Sinorhizobium morelense, Sinorhizobium saheli* or *Sinorhizobium xinjiangense.*

Further examples of advantageous microorganisms for the process according to the invention are protists or diatoms selected from the group of the families Dinophyceae, Turaniellidae or Oxytrichidae, such as the genera and species: *Crypthecodinium cohnii, Phaeodactylum tricornutum, Stylonychia mytilus, Stylonychia pustulata, Stylonychia putrina, Stylonychia notophora, Stylonychia sp.,* Colpidium campylum or Colpidium sp.

Those which are advantageously applied in the process according to the invention are transgenic organisms such as fungi, such as mortierella or thraustrochytrium, yeasts such as Saccharomyces or Schizosaccharomyces, mosses such as Physcomitrella or Ceratodon, nonhuman animals such as Caenorhabditis, algae such as Nephroselmis, Pseudoscourfielda, Prasinococcus, Scherffelia, Tetraselmis, Mantoniella, Ostreococcus, Crypthecodinium or Phaeodactylum or plants such as dicotyledonous or monocotyledonous plants. Organisms which are especially advantageously used in the process according to the invention are organisms which belong to the oil-producing organisms, that is to say which are used for the production of oil, such as fungi, such as Mortierella or Thraustochytrium, algae such as Nephroselmis, Pseudoscourfielda, Prasinococcus, Scherffelia, Tetraselmis, Mantoniella, Ostreococcus, Crypthecodinium, Phaeodactylum, or plants, in particular plants, preferably oilseed or oil crop plants which comprise large amounts of lipid compounds, such as peanut, oilseed rape, canola, sunflower, safflower (Carthamus tinctoria), poppy, mustard, hemp, castor-oil plant, olive, sesame, Calendula, Punica, evening primrose, verbascum, thistle, wild roses, hazelnut, almond, macadamia, avocado, bay, pumpkin/squash, linseed, soybean, pistachios, borage, trees (oil palm, coconut or walnut) or arable crops such

as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa or bushy plants (coffee, cacao, tea), Salix species, and perennial grasses and fodder crops. Preferred plants according to the invention are oil crop plants such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, Calendula, Punica, evening primrose, pumpkin/squash, linseed, soybean, borage, trees (oil palm, coconut). Especially preferred for the process are plants which are high in C18:2-fatty acids, such as sunflower, safflower, tobacco, verbascum, sesame, cotton, pumpkin/squash, poppy, evening primrose, walnut, linseed, hemp or thistle. Very especially preferred plants are plants such as safflower, sunflower, poppy, evening primrose, walnut, linseed or hemp.

It is also be advantageous for the above-described method according to the invention additionally to introduce, into the organism, further nucleic acids which encode enzymes of the fatty acid or lipid metabolism, in addition to the nucleic acids of the first to fourth aspects of the invention.

Such nucleic acids are advantageously derived from plants such as algae, for example algae of the family of the Prasinophyceae such as the genera Heteromastix, Mammella, Mantoniella, Micromonas, Nephroselmis, Ostreococcus, Prasinocladus, Prasinococcus, Pseudoscourfielda, Pycnococcus, Pyramimonas, Scherffelia or Tetraselmis such as the genera and species Heteromastix longifillis, Mamiella gilva, Mantoniella squamata, Micromonas pusilla, Nephroselmis olivacea, Nephroselmis pyriformis, Nephroselmis rotunda, Ostreococcus tauri, Ostreococcus sp. Prasinocladus ascus, Prasinocladus lubricus, Pycnococcus provasolii, Pyramimonas amylifera, Pyramimonas disomata, Pyramimonas obovata, Pyramimonas orientalis, Pyramimonas parkeae, Pyramimonas spinifera, Pyramimonas sp., Tetraselmis apiculata, Tetraselmis carteriaformis, Tetraselmis chui, Tetraselmis convolutae, Tetraselmis desikacharyi, Tetraselmis gracilis, Tetraselmis hazeni, Tetraselmis impellucida, Tetraselmis inconspicua, Tetraselmis levis, Tetraselmis maculata, Tetraselmis marina, Tetraselmis striata, Tetraselmis subcordiformis, Tetraselmis suecica, Tetraselmis tetrabrachia, Tetraselmis tetrathele, Tetraselmis verrucosa, Tetraselmis verrucosa fo. rubens or Tetraselmis sp. or from algae of the family Euglenaceae such as the genera Ascoglena, Astasia, Colacium, Cyclidiopsis, Euglena, Euglenopsis, Hyalophacus, Khawkinea, Lepocinclis, Phacus, Strombomonas or Trachelomonas, such as the genera and species Euglena acus, Euglena geniculate, Euglena gracilis, Euglena mixocylindracea, Euglena rostrifera, Euglena viridis, Colacium stentorium, Trachelomonas cylindrica or Trachelomonas volvocina. The nucleic acids used are advantageously derived from algae of the genera Euglena, Mantoniella or Ostreococcus.

Further advantageous plants are algae such as Isochrysis or Crypthecodinium, algae/diatoms such as Thalassiosira or Phaeodactylum, mosses such as Physcomitrella or Ceratodon, or higher plants such as the Primulaceae such as Aleuritia, Calendula stellata, Osteospermum spinescens or Osteospermum

hyoseroides, microorganisms such as fungi, such as Aspergillus, Thraustochytrium, Phytophthora, Entomophthora, Mucor or Mortierella, bacteria such as Shewanella, yeasts or animals such as nematodes such as Caenorhabditis, insects, frogs, abalone, or fish. The isolated nucleic acid sequences according to the invention are advantageously derived from an animal of the order of the vertebrates. Preferably, the nucleic acid sequences are derived from the classes of the Vertebrata; Euteleostomi, Actinopterygii; Neopterygii; Teleostei; Euteleostei, Protacanthopterygii, Salmoniformes; Salmonidae or Oncorhynchus or Vertebrata, Amphibia, Anura, Pipidae, Xenopus or Evertebrata such as Protochordata, Tunicata, Holothuroidea, Cionidae such as Amaroucium constellatum, Botryllus schlosseri, Ciona intestinalis, Molgula citrina, Molgula manhattensis, Perophora viridis or Styela partita. The nucleic acids are especially advantageously derived from fungi, animals, or from plants such as algae or mosses, preferably from the order of the Salmoniformes, such as the family of the Salmonidae, such as the genus Salmo, for example from the genera and species Oncorhynchus mykiss, Trutta trutta or Salmo trutta fario, from algae, such as the genera Mantoniella or Ostreococcus, or from the diatoms such as the genera Thalassiosira or Phaeodactylum or from algae such as Crypthecodinium.

In a preferred embodiment, the process furthermore comprises the step of obtaining a cell or an intact organism which comprises the nucleic acid sequences used in the process, where the cell and/or the organism is transformed with a nucleic acid sequence according to the invention which encodes the Δ9-elongase, Δ8-desaturase and/or the Δ5-desaturase, a gene construct or a vector as described above, alone or in combination with further nucleic acid sequences which encode proteins of the fatty acid or lipid metabolism. In a further preferred embodiment, this process furthermore comprises the step of obtaining the oils, lipids or free fatty acids from the organism or from the culture. The culture can, for example, take the form of a fermentation culture, for example in the case of the cultivation of microorganisms, such as, for example, Mortierella, Thalassiosira, Mantoniella, Ostreococcus, Saccharomyces or Thraustochytrium, or a greenhouse- or field-grown culture of a plant. The cell or the organism produced thus is advantageously a cell of an oil-producing organism, such as an oil crop, such as, for example, peanut, oilseed rape, canola, linseed, hemp, peanut, soybean, safflower, hemp, sunflowers or borage.

In the case of plant cells, plant tissue or plant organs, "growing" is understood as meaning, for example, the cultivation on or in a nutrient medium, or of the intact plant on or in a substrate, for example in a hydroponic culture, potting compost or on arable land.

For the purposes of the invention, "transgenic" or "recombinant" means with regard to, for example, a nucleic acid sequence, an expression cassette (= gene construct) or a vector comprising the nucleic acid sequence or an organism transformed with the nucleic acid sequences, expression cassettes or vectors according to the invention, all those constructions brought about by recombinant methods in which either

a) the nucleic acid sequence according to the invention, or

b) a genetic control sequence which is operably linked with the nucleic acid sequence according to the invention, for example a promoter, or

c) a) and b)

are not located in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to take the form of, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The natural genetic environment is understood as meaning the natural genomic or chromosomal locus in the original organism or the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, most preferably at least 5000 bp. A naturally occurring expression cassette - for example the naturally occurring combination of the natural promoter of the nucleic acid sequences with the corresponding Δ5-desaturase gene - becomes a transgenic expression cassette when this expression cassette is modified by non-natural, synthetic ("artificial") methods such as, for example, mutagenic treatment. Suitable methods are described, for example, in US 5,565,350 or WO 00/15815.

A transgenic organism or transgenic plant for the purposes of the invention is therefore understood as meaning, as above, that the nucleic acids used in the process are not at their natural locus in the genome of an organism, it being possible for the nucleic acids to be expressed homologously or heterologously. However, as mentioned, transgenic also means that, while the nucleic acids according to the invention are at their natural position in the genome of an organism, the sequence has been modified with regard to the natural sequence, and/or that the regulatory sequences of the natural sequences have been modified. Transgenic is preferably understood as meaning the expression of the nucleic acids according to the invention at an unnatural locus in the genome, i.e. homologous or, preferably, heterologous expression of the nucleic acids takes place. Preferred transgenic organisms are fungi such as Mortierella or Phytophtora, mosses such as Physcomitrella, algae such as Mantoniella, Euglena, Crypthecodinium or Ostreococcus, diatoms such as Thalassiosira or Phaeodactylum, or plants such as the oil crops.

Organisms or host organisms for the nucleic acids, the expression cassette or the vector used in the process according to the invention are, in principle, advantageously all organisms which are capable of synthesizing fatty acids, specifically unsaturated fatty acids, and/or which are suitable for the expression of recombinant genes. Examples which may be mentioned are plants such as Arabidopsis, Asteraceae such as Calendula or crop plants such as soybean, peanut, castor-oil plant, sunflower, maize, cotton, flax, oilseed rape, coconut, oil palm, safflower (Carthamus tinctorius) or

cacao bean, microorganisms, such as fungi, for example the genus Mortierella, Thraustochytrium, Saprolegnia, Phytophtora or Pythium, bacteria, such as the genus Escherichia or Shewanella, yeasts, such as the genus Saccharomyces, cyanobacteria, ciliates, algae such as Mantoniella, Euglena, Thalassiosira or Ostreococcus, or protozoans such as dinoflagellates, such as Crypthecodinium. Preferred organisms are those which are naturally capable of synthesizing substantial amounts of oil, such as fungi, such as Mortierella alpina, Pythium insidiosum, Phytophtora infestans, or plants such as soybean, oilseed rape, coconut, oil palm, safflower, flax, hemp, castor-oil plant, Calendula, peanut, cacao bean or sunflower, or yeasts such as Saccharomyces cerevisiae with soybean, flax, oilseed rape, safflower, sunflower, Calendula, Mortierella or Saccharomyces cerevisiae being especially preferred. In principle, host organisms are, in addition to the abovementioned transgenic organisms, also transgenic animals, advantageously nonhuman animals, for example C. elegans, Ciona intestinalis or Xenopus laevis.

Further utilizable host cells are detailed in: Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

Expression strains which can be used, for example those with a lower protease activity, are described in: Gottesman, Sr, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 119-128.

These include plant cells and certain tissues, organs and parts of plants in all their phenotypic forms such as anthers, fibers, root hairs, stalks, embryos, calli, cotelydons, petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the actual transgenic plant and/or can be used for bringing about the transgenic plant.

Transgenic plants which comprise the polyunsaturated fatty acids synthesized in the process according to the invention can advantageously be marketed directly without there being any need for the oils, lipids or fatty acids synthesized to be isolated. Plants for the process according to the invention are listed as meaning intact plants and all plant parts, plant organs or plant parts such as leaf, stem, seeds, root, tubers, anthers, fibers, root hairs, stalks, embryos, calli, cotelydons, petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the actual transgenic plant and/or can be used for bringing about the transgenic plant. In this context, the seed comprises all parts of the seed such as the seed coats, epidermal cells, seed cells, endosperm or embryonic tissue. However, the compounds produced in the process according to the invention can also be isolated from the organisms, advantageously plants, in the form of their oils, fats, lipids and/or free fatty acids. Polyunsaturated fatty acids produced by this process can be obtained by harvesting the organisms, either from the crop in which they grow, or from the field. This can be done *via* pressing or extraction of the plant parts, preferably the plant seeds. In this context, the oils, fats, lipids and/or free fatty acids can be obtained by what is known as cold-beating or cold-pressing without applying heat. To allow for greater ease of

disruption of the plant parts, specifically the seeds, they are previously comminuted, steamed or roasted. The seeds which have been pretreated in this manner can subsequently be pressed or extracted with solvents such as warm hexane. The solvent is subsequently removed. In the case of microorganisms, the latter are, after harvesting, for example extracted directly without further processing steps or else, after disruption, extracted *via* various methods with which the skilled worker is familiar. In this manner, more than 96% of the compounds produced in the process can be isolated. Thereafter, the resulting products are processed further, i.e. refined. In this process, substances such as the plant mucilages and suspended matter are first removed. What is known as desliming can be effected enzymatically or, for example, chemico-physically by addition of acid such as phosphoric acid. Thereafter, the free fatty acids are removed by treatment with a base, for example sodium hydroxide solution. The resulting product is washed thoroughly with water to remove the alkali remaining in the product and then dried. To remove the pigment remaining in the product, the products are subjected to bleaching, for example using filler's earth or active charcoal. At the end, the product is deodorized, for example using steam.

The fatty acids produced by the processes of the present invention can be isolated from the organism in the form of an oil, a lipid or a free fatty acid. Suitable organisms are, for example, those mentioned above. Preferred organisms are transgenic plants.

One embodiment of the invention is therefore oils, lipids or fatty acids of formula I or fractions thereof which have been produced by the above-described process, especially preferably oil, lipid or a fatty acid composition comprising a compound of formula I and being derived from transgenic plants.

A further embodiment according to the invention is the use of the oil, lipid, the fatty acids and/or the fatty acid composition in feedstuffs, foodstuffs, cosmetics or pharmaceuticals. The oils, lipids, fatty acids or fatty acid mixtures according to the invention can be used in the manner with which the skilled worker is familiar for mixing with other oils, lipids, fatty acids or fatty acid mixtures of animal origin, such as, for example, fish oils. These oils, lipids, fatty acids or fatty acid mixtures, which are composed of vegetable and animal constituents, may also be used for the preparation of feedstuffs, foodstuffs, cosmetics or pharmacologicals.

The term "oil", "lipid" or "fat" is understood as meaning a fatty acid mixture comprising unsaturated, saturated, preferably esterified, fatty acid(s). The oil, lipid or fat is preferably high in polyunsaturated free or, advantageously, esterified fatty acid(s), in particular linoleic acid, Hinolenic acid, dihomo-Hinolenic acid, arachidonic acid, α-linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid.

The amount of unsaturated esterified fatty acids preferably amounts to approximately 30%, a content of 50% is more preferred, a content of 60%, 70%, 80% or more is even more preferred. For the analysis, the fatty acid content can, for example, be determined

by gas chromatography after converting the fatty acids into the methyl esters by transesterification. The oil, lipid or fat can comprise various other saturated or unsaturated fatty acids, for example calendulic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and the like. The content of the various fatty acids in the oil or fat can vary, in particular depending on the starting organism.

The ARA produced in the process may be, as described above, in the form of fatty acid derivatives, for example sphingolipids, phosphoglycerides, lipids, glycolipids, phospholipids, monoacylglycerol, diacylglycerol, triacylglycerol or other fatty acid esters.

The ARA and other polyunsaturated fatty acids which are present can be liberated for example *via* treatment with alkali, for example aqueous KOH or NaOH, or acid hydrolysis, advantageously in the presence of an alcohol such as methanol or ethanol, or *via* enzymatic cleavage, and isolated *via,* for example, phase separation and subsequent acidification *via,* for example, H_2SO_4. The fatty acids can also be liberated directly without the above-described processing step.

After their introduction into an organism, advantageously a plant cell or plant, the nucleic acids used in the process can either be present on a separate plasmid or, advantageously, integrated into the genome of the host cell. In the case of integration into the genome, integration can be random or else be effected by recombination such that the native gene is replaced by the copy introduced, whereby the production of the desired compound by the cell is modulated, or by the use of a gene in trans, so that the gene is linked operably with a functional expression unit which comprises at least one sequence which ensures the expression of a gene and at least one sequence which ensures the polyadenylation of a functionally transcribed gene. The nucleic acids are advantageously introduced into the organisms *via* multiexpression cassettes or constructs for multiparallel expression, advantageously into the plants for the multiparallel seed-specific expression of genes.

If microorganisms such as yeasts, such as Saccharomyces or Schizosaccharomyces, fungi such as Mortierella, Aspergillus, Phytophtora, Entomophthora, Mucor or Thraustochytrium, algae such as Isochrysis, Mantoniella, Euglena, Ostreococcus, Phaeodactylum or Crypthecodinium are used as organisms in the process according to the invention, these organisms are advantageously grown in fermentation cultures.

If microorganisms are used as organisms in the process according to the invention, they are grown or cultured in the manner with which the skilled worker is familiar, depending on the host organism. As a rule, microorganisms are grown in a liquid medium comprising a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins, at temperatures of between 0^0C and 100^0C, preferably between 10^0C and $6O^0$C, while passing in oxygen. The pH of the liquid medium can

either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batchwise, semi-batchwise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semicontinuously or continuously. The polyunsaturated fatty acids produced can be isolated from the organisms as described above by processes known to the skilled worker, for example by extraction, distillation, crystallization, if appropriate precipitation with salt, and/or chromatography. To this end, the organisms can advantageously be disrupted beforehand.

If the host organisms are microorganisms, the process according to the invention is advantageously carried out at a temperature of between 0^{0}C and 95°C, preferably between 10^{0}C and 85^{0}C, especially preferably between 15^{0}C and 75^{0}C, very especially preferably between 15^{0}C and 45°C.

In this process, the pH value is advantageously kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8.

The process according to the invention can be operated batchwise, semibatchwise or continuously. An overview over known cultivation methods can be found in the textbook by Chmiel (Bioproze βtechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to Bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must suitably meet the requirements of the strains in question. Descriptions of culture media for various microorganisms can be found in the textbook "Manual of Methods fur General Bacteriology" of the American Society for Bacteriology (Washington D. C , USA, 1981).

As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements.

Preferred carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of very good carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media *via* complex compounds such as molasses or other by¬ products from sugar raffination. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil, peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

Nitrogen sources are usually organic or inorganic nitrogen compounds or materials

comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as comsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a mixture.

Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly suitable chelating agents include dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

The fermentation media used according to the invention for culturing microorganisms usually also comprise other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, pantothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium. The exact composition of the media compounds heavily depends on the particular experiment and is decided upon individually for each specific case. Information on the optimization of media can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Editors P.M. Rhodes, P.F. Stanbury, IRL Press (1997) pp. 53-73, ISBN 0 19 963577 3). Growth media can also be obtained from commercial suppliers, for example Standard 1 (Merck) or BHI (brain heart infusion, DIFCO) and the like.

All media components are sterilized, either by heat (20 min at 1.5 bar and 121^{0}C) or by filter sterilization. The components may be sterilized either together or, if required, separately. All media components may be present at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45^{0}C, preferably at from 25°C to 40^{0}C, and may be kept constant or may be altered during the experiment. The pH of the medium should be in the range from 5 to 8.5, preferably around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic

compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids it is possible to add to the medium suitable substances having a selective effect, for example antibiotics. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, ambient air into the culture. The temperature of the culture is normally 20° to 40°C and preferably 25°C to 40°C. The culture is continued until formation of the desired product is at a maximum. This aim is normally achieved within 10 to 160 hours.

The fermentation broths obtained in this way, in particular those containing polyunsaturated fatty acids, usually contain a dry mass of from 7.5 to 25% by weight.

The fermentation broth can then be processed further. The biomass may, according to requirement, be removed completely or partially from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decanting or a combination of these methods or be left completely in said broth. It is advantageous to process the biomass after its separation.

However, the fermentation broth can also be thickened or concentrated without separating the cells, using known methods such as, for example, with the aid of a rotary evaporator, thin-film evaporator, falling-film evaporator, by reverse osmosis or by nanofiltration. Finally, this concentrated fermentation broth can be processed to obtain the fatty acids present therein.

The fatty acids obtained in the process are also suitable as starting material for the chemical synthesis of further products of interest. For example, they can be used in combination with one another or alone for the preparation of pharmaceuticals, foodstuffs, animal feeds or cosmetics.

All of the nucleic acid sequences used in the process according to the invention are advantageously derived from a eukaryotic organism such as a plant, a microorganism or an animal. The nucleic acid sequences are preferably derived from the order Salmoniformes, algae such as Mantoniella, Crypthecodinium, Euglena or Ostreococcus, fungi such as the genus Phytophthora or from diatoms such as the genera Thalassiosira or Phaeodactylum.

The invention will now be described in greater detail with reference to the following Examples and to the drawings in which:

FIGURE 1 shows various synthetic pathways for the biosynthesis of ω-6 and ω-3 fatty acids.

FIGURE 2 is a gas chromatography trace showing the conversion of Δ9,12-18:2 (linoleic acid) to Δ11,14-20:2 by heterologous expression of the *P. marinus* Δ9-elongase sequence (SEQ ID NO: 1, residues 7668 to 9200) in yeast induced either by galactose (Figure 2A) or glucose (Figure 2B).

Example 1 - Cloning of a FAE1 elongase from *Perkinsus marinus*

Perkinsus marinusi is an oyster protozoan parasite capable of synthesizing saturated and unsaturated fatty acids including the essential fatty acid, arachidonic acid [20:4(n-6)]. *P. marinus* employs the delta-8 (Δ-8) desaturase pathway to synthesize arachidonic acid.

Materials and Methods.

Growth and harvesting of P. marinus.

Perkinsus marinus meronts were cultivated at 28°C in a medium prepared as described by La Peyre et al. (J Eukaryot Microbiol 1993;40:304-10) and contained amino acids, nucleotides, carbohydrates, and vitamins, but no fetal bovine serum.

Nucleic acid manipulation and PCR-based cloning.

DNA was extracted from cells using a DNeasy DNA mini kit (Qiagen). DNA were amplified with primers specific to delta5 desaturase gene as follows: the reactions were heated to 95 C for 2 min followed by 35 cycles at 95 C for 1 min, 2 min at 52 and 72 C for 4 min, then a single step at 72 C for 5 min. PCR amplification products were cloned into TOPO vector (Invitrogen) and verified by sequencing. FAE elongase gen was amplified with gene-specific primers (Tablel) designed to the 5' and 3' ends of the coding region, with restriction sites to facilitate cloning into the yeast vector (Table I). Forward primers for cloning into yeast expression vector pYES2 (Invitrogen) were designed to contain a G/A at position -3 and a G at position +4 to improve translation initiation in eukaryotic cells.

Oligonucleotide primers used in this study.

Transcripts of *Perkinsus marinus* were analyzed by reverse transcriptase PCR (RT-PCR). Total RNA was extracted from cells using an RNeasy plant mini kit (Qiagen). First strand cDNA was synthesised from total RNA using the SMART RACE cDNA Amplification kit (BD-Clontech, Basingstoke, UK) according to the manufacture's instructions. Single -stranded cDNAs were amplified with following primers.

FAEoperon forward 5'-

GGMTTCGAGGAGTAGGATCTTATCTGAGGATAGTCACACTAGTCGTACT-S'
FAEoperon reverse 5'-CATCTGCGAATACTAACCATACATT

The reactions were heated to 95 C for 2 min followed by 30 cycles at 94 C for 30 s, 30 s at temperatures ranging from 55 to 72 according to the primer design and 72 C for 2 min, then a single step at 72 C for 10 min. PCR amplification products were cloned into TOPO vector (Invitrogen) and verified by sequencing. Surprisingly it was shown that the transcripts of the Δ9-elongase, Δ8-desaturase and Δ5-desaturase are all found on the the same mRNA. This is the first example showing PUFA genes to be organized in an operon-like structure.

In a further investigation the specificity of the Δ9-elongase was analyzed. For this purpose the coding sequence of this gene was amplified by RT-PCR as described above using following primers.

Elo2For: 5'- ATGCAAGTTCCCGCGGAGCATCACTCC -3'
Elo2Rev: 5'- CGTTACGCATCAATATTATGCATAGCCAACC -3'

The amplified PCR product was then cloned into a pCRscript vector according to manufacture's recommendations (Stratagen). In a second PCR step the modified sequences for yeast expression were introduced using following primers.

Yeast expression.

Kpn Elo2For 5'- TTGGTACCATGGGATTTCCTGCGGAG -3'
Sac Elo1 Rev 5'- GGGAGCTCTTACGCATCAATATTATGCATAGC-3'

Sequence of the primers is given in the 5' to 3' orientation
Restriction sites used for cloning are in bold.

RESULTS

Isolation of FAE1 elongase from *P.marinus.*

Using publicly available data derived from an *P.marinus* genome sequencing project carried out by TIGR (http://www.tigr.org/tdb/e2k1/pmg/) we identified one contig (1047306867) which showed significant homology to known elongases, with the target sequence (designated Elol For, SEQ ID NO: 9) consisting of an open reading frame of 511 residues and no introns. The putative amino acid derived from the target sequence is SEQ ID NO: 10.

Functional characterisation in Saccharomyces cerevisiae.

The full-length cDNA corresponding to putative Δ9 fatty acid elongase (SEQ ID NO: 9) was cloned into yeast expression vector pYES2 to give a construct designated pYPmFAE. *S.cerevisiae* strain W303-1A was transformed with the pYPmFAE or the empty vector as a control. Transformed cell were grown in a minimal medium containing raffinose and induced with 2% galactose. After 48h of growth total yeast fatty acids were extracted and the resulting FAMEs analysed by GC.

GC analysis (Figure 2) revealed that yeast cells transformed with pYPmFAE produced an additional fatty acid, which was identified as eicosadienoic acid indicating that the gene we had cloned encoded a delta 9 fatty acid elongase. Yeast cells expressing the *P. marinus* delta 9 fatty acid elongase is capable of recognizing C18:2 (c9,12) substrate with a 8.2% percentage of conversion rate.

Table 1 shows the fatty acid content of the yeast cells after transformation with pYPmFAE (+) or with the empty vector pYES2 (-) and induction with 2% galactose. The percentage conversion for $18:2^{\Delta 9,12}$ to $20:2^{\Delta 11,14}$, for example is calculated by the equation:

$$\%\,\text{conversion} = \frac{[20:2^{\Delta 11,14}]}{[18:2^{\Delta SM2}] + [20:2^{\Delta 11,14}]}$$

TABLE 1

	FATTY ACIDS										
%	16:0	$16:1^{\Delta 9}$	18:0	$18:1^{\Delta 9}$	$18:2^{\Delta 9,12}$	$20:2^{\Delta 11,14}$	$18:3^{\Delta 6,9,12}$	$20:3^{\Delta 8,11,14}$	$20:4^{\Delta 5,8,11,14}$	$22:4^{\Delta 7,10,13,16}$	% con
FAE 18:2+	19.05	22.79	5.15	12.54	**37.14**	**3.33**	0.00	0.00	0.00	0.00	**8.2**
FAE 18:2-	20.81	19.50	5.50	12.11	**42.07**	**0.00**	0.00	0.00	0.00	0.00	**0.0**
FAE 18:3+	18.86	19.77	4.81	11.15	0.00	0.00	**44.93**	**0.48**	0.00	0.00	**1.1**
FAE 18:3-	20.35	18.15	4.84	10.33	0.00	0.00	**46.27**	**0.07**	0.00	0.00	**0.1**
FAE 20:4+	20.84	31.09	5.48	15.91	0.00	0.00	0.00	0.00	**26.68**	**0.00**	**0.0**
FAE 20:4-	22.13	31.00	4.55	14.65	0.00	0.00	0.00	0.00	**27.67**	**0.00**	**0.0**

The results presented in Table 1 show that no elongase activity was detected with

20:4$^{\Delta 5,8,11,14}$, and a minimal activity (1% conversion) for 18:3$^{\Delta 6,9,12}$. It therefore appears that the $\Delta 9$ fatty acid elongase is selective for linoleic acid and does not act to elongate other PUFAs.

CLAIMS

1. An isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity and which is selected from the group consisting of:

a) a nucleic acid sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 or a homolog thereof;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity, wherein the polypeptides are selected from the group consisting of SEQ ID NOS 2, 3 and 4;

d) a derivative of a nucleic acid sequence of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4; wherein said polypeptides have Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity.

2. An isolated nucleic acid sequence which encodes a polypeptide with Δ9-elongase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1, SEQ ID NO: 9 or a homolog of one of these;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9;

c) an isolated nucleic acid sequence which encodes a polypeptide with Δ9-elongase, activity, wherein the polypeptide comprises SEQ ID NO: 2 or SEQ ID NO: 10;

d) a derivative of a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 2 or SEQ ID NO: 10; wherein said polypeptide has Δ9-elongase activity.

3. An isolated nucleic acid sequence which encodes a polypeptide with Δ8-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 or a homolog thereof;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising residues 9351 to 10724 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes a polypeptide with Δ8-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 3;

d) a derivative of a a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 3; wherein said polypeptide has Δ8-desaturase activity.

4. An isolated nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 or a homolog thereof;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising residues 10842 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 4;

d) a derivative of a a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 4; wherein said polypeptide has Δ5-desaturase activity.

5. A polypeptide which is encoded by a nucleic acid sequence as claimed in any one of claims 1 to 4.

6. A gene construct comprising a nucleic acid sequence as claimed in any one of claims 1 to 4 operably linked with one or more regulatory sequences.

7. A gene construct as claimed in claim 6, further comprising one or more sequences encoding enzymes which catalyse the conversion of ARA to an ω3-unsaturated fatty acid.

8 A gene construct as claimed in claim 7, comprising sequences encoding a Δ5-elongase, ω3-desaturase and/or a Δ4-desaturase.

9. A gene construct as claimed in any one of claims 6 to 8, further comprising one or more biosynthesis genes of the fatty acid or lipid metabolism selected from the group acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s),fatty acid acyltransferase(s), acyl-CoA:lysophospholipid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid

acetylenases-, lipoxygenases, triacylglycerol lipases, allenoxide synthases, hydroperoxide lyases or fatty acid elongase(s) and desaturase(s).

10. A vector comprising nucleic acid as claimed in any one of claims 1 to 4 or a gene construct as claimed in any one of claims 6 to 9

11. A transgenic nonhuman organism comprising at least one nucleic acid according to any one of claims 1 to 4, a gene construct according to any one of claims 6 to 9 or a vector according to claim 10.

12. A transgenic nonhuman organism as claimed in claim 11, which organism is a microorganism, a nonhuman animal or a plant

13. A transgenic nonhuman organism according to claim 22 or claim 12, which organism is a plant.

14. A process for the conversion of linoleic acid or a derivative thereof to arachidonic acid or a derivative thereof in an organism, the process comprising introducing into an organism which comprises linoleic acid at least one nucleic acid sequence comprising :

a) SEQ ID NO: 1 (Full sequence 1047306867), sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1 or a homolog of one of these;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence of SEQ ID NO: 1 or a sequence comprising nucleic acid residues 7668 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity, wherein the polypeptides are selected from the group consisting of SEQ ID NOS 2, 3 and 4;

d) A derivative of a nucleic acid sequence of SEQ ID NO: 1 which encodes polypeptides with at least 40% identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 3 and SEQ ID NO: 4; wherein said polypeptides have Δ9-elongase, Δ8-desaturase and Δ5-desaturase activity.

and expressing said nucleic acid sequence.

15. A process as claimed in claim 14, further including the additional step of converting the ARA to an ω-3 fatty acid by introducing into the organism nucleic acid encoding a ω-3 desaturase and optionally a Δ5-elongase and/or a Δ4-elongase and/or a Δ4-desaturase.

16. A process for the conversion of $18:2^{\Delta 9,12}$ (linoleic acid) to $20:2^{\Delta 11,14}$, the process comprising introducing into an organism which comprises linoleic acid at least one nucleic acid sequence which encodes a polypeptide having Δ9-elongase activity and which comprises :

a) a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1, SEQ ID NO: 9 or a homolog of one of these;

b) a nucleic acid sequence which hybridizes under stringent conditions with a nucleic acid sequence comprising residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9;

c) an isolated nucleic acid sequence which encodes a polypeptide with Δ9-elongase, activity, wherein the polypeptide comprises SEQ ID NO: 2 or SEQ ID NO: 10;

d) a derivative of a sequence comprising nucleic acid residues 7668 to 9200 of SEQ ID NO: 1 or SEQ ID NO: 9 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 2 or SEQ ID NO: 10; wherein said polypeptide has Δ9-elongase activity.

and expressing said nucleic acid sequence.

17. A process for the conversion of 20:$2^{\Delta 11,14}$ to 20:$3^{\Delta 8,11,14}$, the process comprising introducing into an organism which comprises 20:$2^{\Delta 11,14}$, or which comprises linoleic acid and a Δ9 elongase, an isolated nucleic acid sequence which encodes a polypeptide with Δ8-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 9351 to 10724 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ8-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 3;

d) A derivative of a a sequence comprising nucleic acid residues 9351 to 10724 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 3; wherein said polypeptide has Δ8-desaturase activity; and

expressing said nucleic acid sequence.

18. A process for the conversion of 20:3 $^{\Delta 8,11,14}$ to 20:4 $^{\Delta 5,8,11,14}$ (ARA), the process comprising introducing into an organism which comprises 20:3 $^{\Delta 8,11,14}$ or which comprises 20:2 $^{\Delta 11,14}$ and a Δ8-desaturase, or which comprises linoleic acid, a Δ9 elongase and a Δ8-desaturase, an isolated nucleic acid sequence which encodes a polypeptide with Δ5-desaturase activity and which is selected from the group consisting of:

a) a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 or a homolog thereof;

b) nucleic acid sequences which hybridize under stringent conditions with a nucleic acid sequence comprising residues 10842 to 12077 of SEQ ID NO: 1;

c) an isolated nucleic acid sequence which encodes polypeptides with Δ5-desaturase activity, wherein the polypeptide comprises SEQ ID NO: 4;

d) A derivative of a a sequence comprising nucleic acid residues 10842 to 12077 of SEQ ID NO: 1 which encodes a polypeptide with at least 40% identity at the amino acid level with SEQ ID NO: 4; wherein said polypeptide has Δ5-desaturase activity.

and expressing said nucleic acid sequence.

19. A process as claimed in claim 18, further including the step of converting the ARA to an ω-*3* fatty acid by introducing into the organism nucleic acid encoding a ω-*3* desaturase and optionally a Δ5-elongase and/or a Δ4-elongase and/or a Δ4-desaturase.

20. A process as claimed in any one of claims 14 to 19, further including the step of induction with galactose.

21. A process as claimed in any one of claims 14 to 20, wherein the organism is a microorganisms, a nonhuman animal or a plant.

FIG. 1

Various synthetic pathways for the biosynthesis of DHA (docosahexaenoic acid)

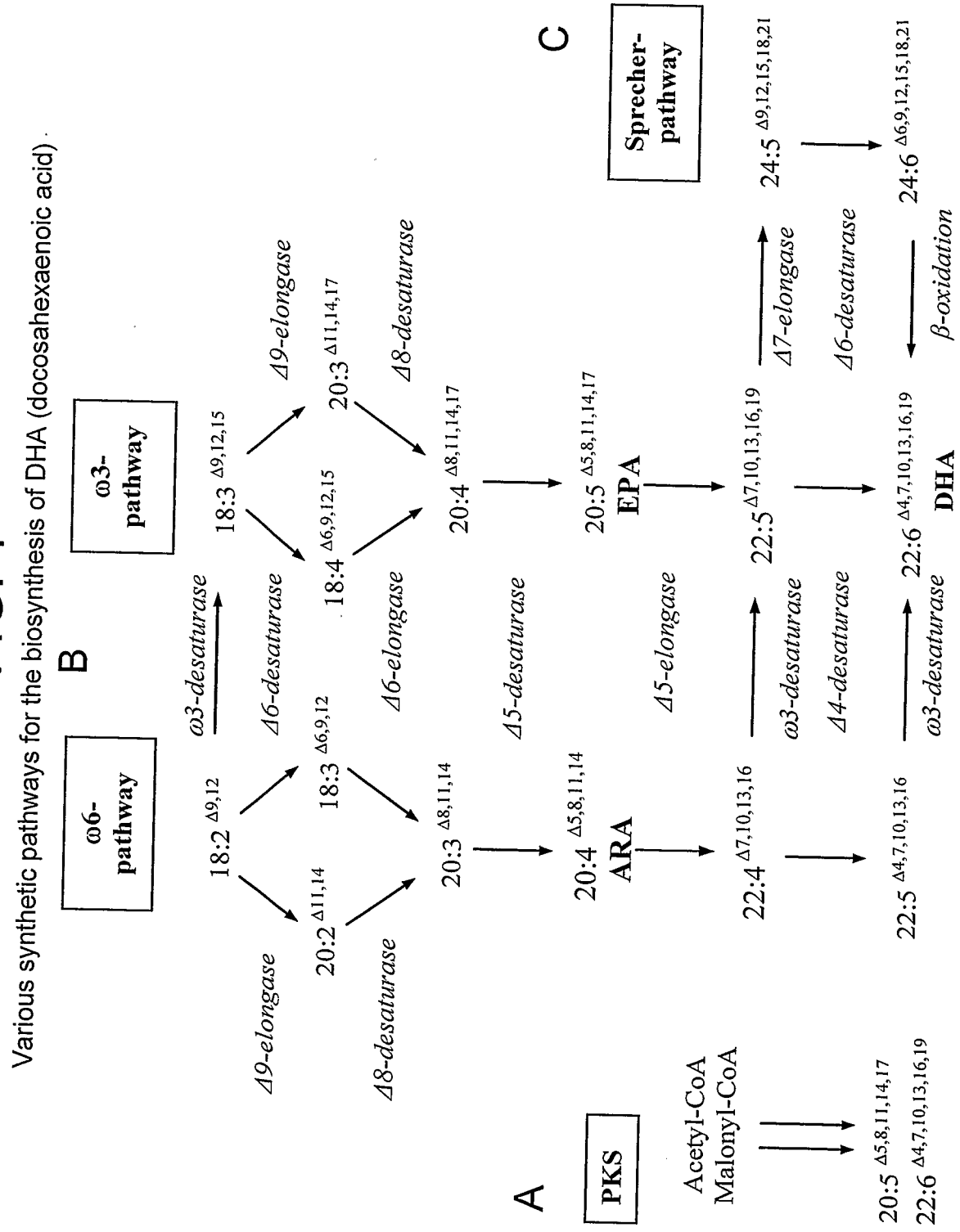


FIG. 2

Converson of 18:2 n-6 to 20:2 n-6 by heterologous expression of the P. marinus sequence PmFAE in yeast