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CA 2621214 A1 2007/04/19

(21) 2 621 214

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2006/10/10
(87) Date publication PCT/PCT Publication Date: 2007/04/19
(85) Entrée phase nationale/National Entry: 2008/02/29
(86) N° demande PCT/PCT Application No.: EP 2006/067223
(87) N° publication PCT/PCT Publication No.: 2007/042510
(30) Priorité/Priority: 2005/10/13 (GB0520843.4)

(51) Cl.Int./Int.Cl. *C12N 9/02* (2006.01), *C12N 15/82* (2006.01), *C12N 9/10* (2006.01)

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(54) Titre : PROCEDE DE PRODUCTION D'ACIDE ARACHIDONIQUE ET/OU D'ACIDE EICOSAPENTAENOIQUE
(54) Title: PROCESS FOR THE PRODUCTION OF ARACHIDONIC ACID AND/OR EICOSAPENTAENOIC ACID

(57) Abrégé/Abstract:
vectors and transgenic plants containing said nucleic acid sequences.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2007 (19.04.2007)

PCT

(10) International Publication Number
WO 2007/042510 A3

(51) International Patent Classification:
C12N 9/02 (2006.01) *C12N 15/82* (2006.01)
C12N 9/10 (2006.01)

(21) International Application Number:
PCT/EP2006/067223

(22) International Filing Date: 10 October 2006 (10.10.2006)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0520843.4 13 October 2005 (13.10.2005) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) Date of publication of the international search report:
2 August 2007

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 2007/042510 A3

(54) Title: PROCESS FOR THE PRODUCTION OF ARACHIDONIC ACID AND/OR EICOSAPENTAENOIC ACID

(57) Abstract: vectors and transgenic plants containing said nucleic acid sequences.

DEMANDE OU BREVET VOLUMINEUX

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NOTE POUR LE TOME / VOLUME NOTE:

Process for the production of arachidonic acid and/or eicosapentaenoic acid

Description

The present invention relates to a new process for the production of arachidonic acid and/or eicosapentaenoic acid in plants through the co-expression of a Δ-12-/Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and a Δ-5-desaturase and a process for the production of lipids or oils having an increased content of unsaturated fatty acids, in particular ω-3 and ω-6 fatty acids having at least two double bonds and a 18 or 20 carbon atom chain length. Preferably the arachidonic acid and eicosapentaenoic acid are produced in at least a 1:2 ratio.

The invention furthermore relates to the production of a transgenic plants, preferably a transgenic crop plant, having an increased content of arachidonic acid and/or eicosapentaenoic acid, oils or lipids containing C_{18}- or C_{20}- fatty acids with a double bond in position $\Delta_{5, 8, 9, 11, 12, 14, 15 \text{ or } 17}$ of the fatty acid produced, respectively due to the expression of the Δ-12-/Δ-15-desaturase, of the Δ-9-elongase, of the Δ-8-desaturase and of the Δ-5-desaturase in the plant. The expression of the inventive Δ-12-/Δ-15-desaturase leads preferably to linoleic acid and α-linolenic acid as products having a double bond in the position $\Delta_{9, 12 \text{ and } 15}$ of the fatty acid.

The invention additionally relates to specific nucleic acid sequences encoding for proteins with Δ-12-/Δ-15-desaturase-, Δ-9-elongase-, Δ-8-desaturase- or Δ-5-desaturase-activity, nucleic acid constructs, vectors and transgenic plants containing said nucleic acid sequences.

Plants and especially oil crops have been used for centuries as sources for edible and non-edible products. There are written records and archaeological excavations that oil crops such as linseed, olive and sesame were widespread use at least six thousand years ago.

Non-edible products of oilseed crops such as rapeseed were used and included in lubricants, oil lamps, and cosmetics such as soaps. Oil crops differ in their cultural, economic and utilization characteristics, for example rapeseed and linseed are adapted to relatively cool climates, whereas oil palm and coconut are adapted to warm and damp climates. Some plants are a real oilseed plant that means the main product of such plants is the oil, whereas in case of others such as cotton or soybean

the oil is more or less a side product. The oils of different plants are basically characterized by their individual fatty acid pattern.

Fatty acids and triglycerides have numerous applications in the food industry, animal nutrition, cosmetics and in the drug sector. Depending on whether they are free saturated or unsaturated fatty acids or triglycerides with an increased content of saturated or unsaturated fatty acids, they are suitable for the most varied applications; thus, for example, **l**ong **c**hain **p**oly**u**nsaturated **f**atty **a**cids (= LCPUFAs) are added to infant formula to increase its nutritional value. The various fatty acids and triglycerides are mainly obtained from microorganisms such as Mortierella or from oil-producing plants such as soybean, oilseed rape, sunflower and others, where they are usually obtained in the form of their triacylglycerides. Alternatively, they are obtained advantageously from animals, such as fish. The free fatty acids are prepared advantageously by hydrolysis.

Whether oils with unsaturated or with saturated fatty acids are preferred depends on the intended purpose; thus, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred in human nutrition since they have a positive effect on the cholesterol level in the blood and thus on the possibility of heart disease. They are used in a variety of dietetic foodstuffs or medicaments. In addition PUFAs are commonly used in food, feed and in the cosmetic industry. Poly unsaturated ω-3- and/or ω-6-fatty acids are an important part of animal feed and human food. Because of the common composition of human food poly unsaturated ω-3-fatty acids, which are an essential component of fish oil, should be added to the food to increase the nutritional value of the food; thus, for example, poly unsaturated fatty acids such as Docosahexaenoic acid (= DHA, $C_{22:6}^{\Delta 4,7,10,13,16,19}$)or Eicosapentaenoic acid (= EPA, $C_{20:5}^{\Delta 5,8,11,14,17}$) are added as mentioned above to infant formula to increase its nutritional value. Whereas DHA has a positive effect on the brain development of babies. The addition of poly unsaturated ω-3-fatty acids is preferred as the addition of poly unsaturated ω-6-fatty acids like Arachidonic acid (= ARA, $C_{20:4}^{\Delta 5,8,11,14}$) to common food have an undesired effect for example on rheumatic diseases such as rheumatoid arthritis. Poly unsaturated ω-3- and ω-6-fatty acids are precursor of a family of paracrine hormones called eicosanoids such as prostaglandins which are products of the metabolism of Dihomo-γ-linoleic acid, ARA or EPA. Eicosanoids are in-

volved in the regulation of lipolysis, the initiation of inflammatory responses, the regulation of blood circulation and pressure and other central functions of the body. Eicosanoids comprise prostaglandins, leukotrienes, thromboxanes, and prostacyclins. ω-3-fatty acids seem to prevent artherosclerosis and cardiovascular diseases primarily by regulating the levels of different eicosanoids. Other Eicosanoids are the thromboxanes and leukotrienes, which are products of the metabolism of ARA or EPA.

Principally microorganisms such as Mortierella or oil producing plants such as soybean, rapeseed or sunflower or algae such as Crypthecodinium or Phaeodactylum are a common source for oils containing PUFAs, where they are usually obtained in the form of their triacyl glycerides. Alternatively, they are obtained advantageously from animals, such as fish. The free fatty acids are prepared advantageously by hydrolysis with a strong base such as potassium or sodium hydroxide.

Plant oils are in general rich in fatty acids such as monounsaturated fatty acids like oleic acid or **p**oly **u**nsaturated **f**atty **a**cids (= PUFA) like linoleic or linolenic acid. LCPUFAs like arachidonic acid or eicosapentaenoic acid are rarely found in plants exceptions are some Nephelium and Salvia species in which arachidonic acid is found and some Santalum species in which eicosapentaenoic acid is found. The LCPUFA Docosahexaenoic acid is not found in plants. LCPUFAs such as DHA, EPA, ARA, Dihomo-γ-linoleic acid ($C_{20:3}^{\Delta 8,11,14}$) or Docosapentaenoic acid (= DPA, $C_{22:5}^{\Delta 7,10,13,16,19}$) are not produced by oil producing plants such as soybean, rapeseed, safflower or sunflower. A natural sources for said fatty acids are fish for example herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, pike-perch, tuna or algae.

Approximately 80% of the oils and fats are used in the food industry. Nearly about 84 % of all world wide used vegetable oils are stemming from only six crops/oil crops, which are soybean, oil palm, rapeseed, sunflower, cottonseed, and groundnut.

On account of their positive properties there has been no shortage of attempts in the past to make available genes which participate in the synthesis of fatty acids or triglycerides for the production of oils in various organisms having a modified content of unsaturated fatty acids. Thus, in WO 91/13972 and its US equivalent a Δ-9-desaturase is described. In WO 93/11245 a Δ-15-desaturase and in WO 94/11516 a Δ-12-desaturase is claimed. WO 00/34439 discloses a Δ-5- and a Δ-8-desaturase.

Other desaturases are described, for example, in EP–A–0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP–A–0 794 250, 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. To date, however, the various desaturases have been only inadequately characterized biochemically since the enzymes in the form of membrane-bound proteins are isolable and characterizable only with very great difficulty (McKeon et al., Methods in Enzymol. 71, 1981: 275–277, Wang et al., Plant Physiol. Biochem., 26, 1988: 777–792). Generally, membrane-bound desaturases are characterized by introduction into a suitable organism, which is then investigated for enzyme activity by means of analysis of starting materials and products. Δ-6-Desaturases are described in WO 93/06712, US 5,614,393, US 5614393, WO 96/21022, WO0021557 and WO 99/27111 and their application to production in transgenic organisms is also described, e.g. in WO 9846763, WO 9846764 and WO 9846765. At the same time the expression of various fatty acid biosynthesis genes, as in WO 9964616 or WO 9846776, and the formation of poly-unsaturated fatty acids is also described and claimed. With regard to the effectiveness of the expression of desaturases and their effect on the formation of polyunsaturated fatty acids it may be noted that through expression of a desaturases and elongases as described to date only low contents of poly-unsaturated fatty acids/lipids, such as by way of example eicosapentaenoic or arachidonic acid, have been achieved. Therefore, an alternative and more effective pathway with higher product yield is desirable.

Accordingly, there is still a great demand for new and more suitable genes, which encode enzymes, which participate in the biosynthesis of unsaturated fatty acids and make it possible to produce certain fatty acids specifically on an industrial scale without unwanted byproducts forming. In the selection of genes for biosynthesis two characteristics above all are particularly important. On the one hand, there is as ever a need for improved processes for obtaining the highest possible contents of polyunsaturated fatty acids. Advantageously genes should be as selective as possible and should if possible have more than one activity in the fatty acid biosynthesis chain.

Accordingly, it is an object of the present invention to provide further genes of desaturase and elongase enzymes for the synthesis of polyunsaturated fatty acids in plants preferably in oilseed plants and to use them in a commercial process for the produc-

tion of PUFAs especially LCPUFAs. Said process should increase LCPUFA content in plants as much as possible preferably in seeds of an oil producing plant.

We have found that a process for the production of arachidonic acid or eicosapentaenoic acid achieves this object or arachidonic acid and eicosapentaenoic acid in transgenic plants that produces mature seeds with a content of at least 1 % by weight of said compounds referred to the total lipid content of said organism, which comprises the following steps:

a) introduction of at least one nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-12-desaturase and Δ-15-desaturase activity, and

b) introduction of at least one second nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-9-elongase activity, and

c) introduction of at least one third nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-8-desaturase activity, and

d) introduction of at least a one fourth nucleic acid sequence, which encodes a polypeptide having a Δ-5-desaturase activity, and

e) cultivating and harvesting of said transgenic plant.

According to the invention the used nucleic acid sequences are isolated nucleic sequences coding for polypeptides having a Δ-12-desaturase- and Δ-15-desaturase-, Δ9-elongase-, Δ-8 desaturase- or Δ5-desaturase-activity.

Advantageously nucleic acid sequences are used in the abovementioned process of the invention, which encode polypeptides having Δ-12-desaturase and Δ-15-desaturase activity, Δ-8-desaturase, Δ-9-elongase or Δ-5-desaturase activity and which are selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO:13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 and SEQ ID NO: 23, and

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 according to the degeneracy of the genetic code,

c) derivatives of the nucleic acid sequences depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, , SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 which encode polypeptides having at least 50 % homology to the sequence as depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 and which polypeptides having Δ-12-desaturase and Δ-15-desaturase activity, Δ-8-desaturase, Δ-9-elongase or Δ-5-desaturase activity.

In the inventive process the nucleic acid sequence encoding the bifunctional Δ-12-desaturase- and Δ-15-desaturase-enzyme leads to an increased flux from oleic acid (C18:1Δ9) to linolenic acid (C18:3 $^{\Delta 9,12,15}$) and thereby to an increase of ω-3-fatty acids in comparison to the ω-6-fatty acids. Furthermore this bifunctional enzyme acts on C16-fatty acids having one double bond in the fatty acid molecule as well as on C18-fatty acids having one double bond in the fatty acid molecule. This leads to a further increase in flux from precursor fatty acids such as C18 fatty acids such as oleic acid towards C18 fatty acids such as linoleic and linolenic acid. This is especially of advantage in plants such as oilseed plants having a high content of oleic acid like such as those from the family of the Brassicaceae, such as the genus Brassica, for example oilseed rape or canola; the family of the Elaeagnaceae, such as the genus Elaeagnus, for example the genus and species *Olea europaea*, or the family Fabaceae, such as the genus Glycine, for example the genus and species *Glycine max,* which are high in oleic acid. But also in other plants such oilseed plants like Brassica juncea, Camelina sativa, sunflower or safflower and all other plants mentioned herein this leads to a higher amount of ω-3-fatty acids. By using said inventive nucleic acid sequence and the activity of its gene product ω-3-fatty acids to the ω-6-fatty acids are produced in at least a 1:2 ratio, preferably in at least a 1:3 or 1:4 ratio, more prefera-

bly in at least a 1:5 or 1:6 ratio. That means especially arachidonic acid and eicosapentaenoic acid are produced in at least a 1:2 ratio, preferably in at least a 1:3 or 1:4 ratio, more preferably in at least a 1:5 or 1:6 ratio.

In particular ω-3-fatty acids or ω-6-fatty acids molecules are produced in the inventive process, arachidonic acid and eicosapentaenoic acid are most preferred produced. We have found that this object is advantageously achieved by the combined expression of four isolated nucleic acid sequences according to the invention which encode for polypeptides having the following activities: a polypeptide with Δ-12-desaturase- and Δ-15-desaturase-activity, a polypeptide with a C18-Δ-9-elongase-activity, a polypeptide with C20-Δ-8-desaturase-activity and a C20-Δ-5-desaturase-activity. This objective was achieved in particular by the co-expression of the isolated nucleic acid sequences according to the invention. C18 fatty acids with a single double bond in Δ-9-position are desaturated a first time to linoleic acid by the Δ-12-desaturase and Δ-15-desaturase and thereafter a second time to linolenic acid by the same enzyme advantageously used in the inventive process. The produced C18 fatty acids linoleic and linolenic acid both having a double bond in Δ-9-position are than elongated by the Δ-9-elongase, which is advantageously used in the inventive process. By the Δ-8-desaturase used in the process a double bond in Δ-8-position is introduced into C20 fatty acids. In addition a double bond is introduced into the produced fatty acid molecules in Δ-5-position by the Δ-5-desaturase. The end products of the whole enzymatic reaction are arachidonic acid and eicosapentaenoic acid.

The ω-3-fatty acids or ω-6-fatty acids, preferably ω-3-fatty acids produced in the process are advantageously bound in membrane lipids and/or triacylglycerides or mixtures of different glycerides, but may also occur in the plants as free fatty acids or else bound in the form of other fatty acid esters.

The fatty acid esters with ω-3-fatty acids or ω-6-fatty acids especially arachidonic acid and eicosapentaenoic acid molecules can be isolated in the form of an oil or lipid, for example in the form of compounds such as sphingolipids, phosphoglycerides, lipids, glycolipids such as glycosphingolipids, phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol, monoacylglycerides, diacylglycerides, triacylglycerides or other fatty acid esters such as the acetyl-coenzyme A esters from the plants

which have been used for the preparation of the fatty acid esters; preferably, they are isolated in the form of their diacylglycerides, triacylglycerides and/or in the form of phosphatidylcholine, especially preferably in the form of the triacylglycerides. In addition to these esters, the LCPUFAs are also present in the plants, advantageously in the oilseed plants as free fatty acids or bound in other compounds. As a rule, the various abovementioned compounds (fatty acid esters and free fatty acids) are present in the plants with an approximate distribution of 80 to 90% by weight of triglycerides, 2 to 5% by weight of diglycerides, 5 to 10% by weight of monoglycerides, 1 to 5% by weight of free fatty acids, 2 to 8% by weight of phospholipids, the total of the various compounds amounting to 100% by weight.

In the inventive process(es) [the singular shall include the plural and vice versa] the LCPUFAs are produced in a content of at least 1 % by weight, preferably at least 2, 3, 4 or 5 % by weight, more preferably at least 6, 7, 8, or 9 % by weight, most preferably 10, 20 or 30 % by weight referred to the total lipid content of the plant used in the process. That means Arachidonic acid and eicosapentaenoic acid are produced in a content of at least 1 % by weight, preferably at least 2, 3, 4 or 5 % by weight, more preferably at least 6, 7, 8, or 9 % by weight, most preferably 10, 20 or 30 % by weight referred to the total lipid content. Preferred starting material for the inventive process is oleic acid (C18:1), which is transformed to the preferred end products ARA or EPA. As for the inventive process plants are used the product of the process is not a product of one pure substance per se. It is a mixture of different substances where one or more compounds are the major product and others are only contained as side products. Advantageously the side products shall not exceed 20 % by weight referred to the total lipid content of the plant, preferably the side products shall not exceed 15 % by weight, more preferably they shall not exceed 10 % by weight, most preferably they shall not exceed 5 % by weight. In the event that a mixture of different fatty acids such as ARA and EPA are the product of the inventive process said fatty acids can be further purified by method known by a person skilled in the art such as distillation, extraction, crystallization at low temperatures, chromatography or a combination of said methods. These chemically pure fatty acids or fatty acid compositions are advantageous for applications in the food industry sector, the cosmetic sector and especially the pharmacological industry sector.

Fatty acid esters or fatty acid mixtures produced by the process according to the invention advantageously comprise 6 to 15% of palmitic acid, 1 to 6% of stearic acid, 7 to 85% of oleic acid, 0.5 to 8% of vaccenic acid, 0.1 to 1% of arachic acid, 7 to 25% of saturated fatty acids, 8 to 85% of monounsaturated fatty acids and 60 to 85% of polyunsaturated fatty acids including LCPUFAs, in each case based on 100% and on the total fatty acid content of the organisms. Advantageous LCPUFAs, which are present in the fatty acid esters or fatty acid mixtures are preferably at least 1%, 2%, 3%, 4% or 5% by weight of arachidonic acid and/or preferably at least 5%, 6%, 7%, 8%, 9% or 10% by weight of eicosapentaenoic acid, based on the total fatty acid content.

Moreover, the fatty acid esters or fatty acid mixtures which have been produced by the process of the invention advantageously comprise fatty acids selected from the group of the fatty acids erucic acid (13-docosaenoic acid), sterculic acid (9,10-methyleneoctadec-9-enoic acid), malvalic acid (8,9-methyleneheptadec-8-enoic acid), chaulmoogric acid (cyclopentenedodecanoic acid), furan fatty acid (9,12-epoxyocta-deca-9,11-dienoic acid), vernolic acid (9,10-epoxyoctadec-12-enoic acid), tariric acid (6-octadecynoic acid), 6-nonadecynoic acid, santalbic acid (t11-octadecen-9-ynoic acid), 6,9-octadecenynoic acid, pyrulic acid (t10-heptadecen-8-ynoic acid), crepenynic acid (9-octadecen-12-ynoic acid), 13,14-dihydrooropheic acid, octadecen-13-ene-9,11-diynoic acid, petroselenic acid (cis-6-octadecenoic acid), 9c,12t-octadecadienoic acid, calendulic acid (8t10t12c-octadecatrienoic acid), catalpic acid (9t11t13c-octadecatrienoic acid), eleostearic acid (9c11t13t-octadecatrienoic acid), jacaric acid (8c10t12c-octadecatrienoic acid), punicic acid (9c11t13c-octadecatrienoic acid), parinaric acid (9c11t13t15c-octadecatetraenoic acid), pinolenic acid (all-cis-5,9,12-octadecatrienoic acid), laballenic acid (5,6-octadecadienallenic acid), ricinoleic acid (12-hydroxyoleic acid) and/or coriolic acid (13-hydroxy-9c,11t-octadecadienoic acid). The abovementioned fatty acids are, as a rule, advantageously only found in traces in the fatty acid esters or fatty acid mixtures produced by the process according to the invention, that is to say that, based on the total fatty acids, they occur to less than 30%, preferably to less than 25%, 24%, 23%, 22% or 21%, especially preferably to less than 20%, 15%, 10%, 9%, 8%, 7%, 6% or 5%, very especially preferably to less than 4%, 3%, 2% or 1%. In a further preferred form of the invention, these abovementioned fatty acids occur to less than 0.9%, 0.8%, 0.7%, 0.6% or 0.5%, especially preferably to less than 0.4%, 0.3%, 0.2%, 0.1%, based on the total fatty acids. The fatty

acid esters or fatty acid mixtures produced by the process according to the invention advantageously comprise less than 0.1%, based on the total fatty acids, and/or no butyric acid, no cholesterol, no clupanodonic acid (= docosapentaenoic acid, C22:5$^{\Delta 4,8,12,15,21}$) and no nisinic acid (tetracosahexaenoic acid, C23:6$^{\Delta 3,8,12,15,18,21}$).

The isolated nucleic acid sequences used in the process according to the invention encode proteins or parts of these, where the proteins or the individual protein or parts thereof comprise(s) an amino acid sequence with sufficient homology to an amino acid sequence which is shown in the sequences SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 and SEQ ID NO: 24 so that the proteins or parts thereof retain a Δ-12-desaturase and Δ-15-desaturase-, Δ-9-elongase-, Δ-8-desaturase- and/or Δ-5-desaturase activity. The proteins or parts thereof which is/are encoded by the nucleic acid molecule(s) preferably retains their essential enzymatic activity and the ability of participating in the metabolism of compounds required for the synthesis of cell membranes or lipid bodies in organisms, advantageously in plants, or in the transport of molecules across these membranes. Advantageously, the proteins encoded by the 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: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 and SEQ ID NO: 24. For the purposes of the invention, homology or homologous is understood as meaning identity or identical, respectively.

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 53711 (1991)], were used for the sequence alignment. The sequence homology 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.

Moreover, in the process of the invention advantageously nucleic acid sequences are used which differ from one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO:13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 and SEQ ID NO: 23 (and parts thereof) owing to the degeneracy of the genetic code and which thus encode the same Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase as those encoded by the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO:13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 and SEQ ID NO: 23.

Suitable plants for the production in the process according to the invention are, in principle all plants that produces mature seeds especially crop plants such as oilseed plants.

Plants which are suitable are, in principle, all those plants which are capable of synthesizing fatty acids and that produce mature seeds, such as all dicotyledonous or monocotyledonous plants. Advantageous plants are selected from the group consisting of the plant families Anacardiaceae, Asteraceae, Apiaceae, Boraginaceae, Brassicaceae, Cannabaceae, Elaeagnaceae, Euphorbiaceae, Fabaceae, Geraniaceae, Gramineae, Juglandaceae, Leguminosae, Linaceae, Lythrarieae, Malvaceae, Onagraceae, Palmae, Poaceae, Rubiaceae, Scrophulariaceae, Solanaceae, Sterculiaceae and Theaceae or vegetable plants or ornamentals. More preferred plants are selected from the group consisting of the plant genera of Pistacia, Mangifera, Anacardium, Calendula, Carthamus, Centaurea, Cichorium, Cynara, Helianthus, Lactuca, Locusta, Tagetes, Valeriana, Borago, Daucus, Brassica, Camelina, Melanosinapis, Sinapis, Arabadopsis, Orychophragmus, Cannabis, Elaeagnus, Manihot, Janipha,

Jatropha, Ricinus, Pisum, Albizia, Cathormion, Feuillea, Inga, Pithecolobium, Acacia, Mimosa, Medicajo, Glycine, Dolichos, Phaseolus, Pelargonium, Cocos, Oleum, Juglans, Wallia, Arachis, *Linum,* Punica, Gossypium, Camissonia, Oenothera, Elaeis, Hordeum, Secale, Avena, Sorghum, Andropogon, Holcus, Panicum, Oryza, Zea, Triticum, Coffea, Verbascum, Capsicum, Nicotiana, Solanum, Lycopersicon, Theobroma and Camellia.

Examples which may be mentioned are the following plants selected from the group consisting of 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 french marigold], Apiaceae, such as the genus Daucus, for example the genus and species Daucus carota [carrot], 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, Cannabaceae, such as the genus Cannabis, such as the genus and species Cannabis sativa [hemp], Elaeagnaceae, such as the genus Elaeagnus, for example the genus and species Olea europaea [olive], 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*], 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], 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], 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], 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], 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 blattaria, Verbascum chaixii, Verbascum densiflorum, Verbascum lagurus, Verbascum longifolium, 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. glabriusculum, 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].

Plants which are especially advantageously used in the process according to the invention are plants which belong to the oil-producing plants, that is to say which are

used for the production of oil, such as 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 are plants which are high in C18:1-, C18:2- and/or C18:3-fatty acids, such as oilseed rape, canola, Brassica juncea, Camelina sativa, Orychophragmus, sunflower, safflower, tobacco, verbascum, sesame, cotton, pumpkin/squash, poppy, evening primrose, walnut, linseed, hemp or thistle. Very especially preferred plants are plants such as rapeseed, canola, safflower, sunflower, poppy, mustard, hemp, evening primrose, walnut, linseed or hemp. Other preferred plants are castor bean, sesame, olive, calendula, punica, hazel nut, maize, almond, macadamia, cotton, avocado, pumpkin, laurel, pistachio, oil palm, peanut, soybean, marigold, coffee, tobacco, cacao and borage

For the production of further ω-6- and/or ω-3-fatty acids it is advantageously to introduce further nucleic fatty acid sequences, which encode other enzymes of the fatty acids synthesis chain such as preferably Δ-5-elongase(s) and/or Δ-4-desaturase(s) [for the purposes of the present invention, the plural is understood as comprising the singular and vice versa]. Other Genes of the fatty acid or lipid metabolism, which can be introduced are selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s), fatty acid acyl transferase(s), acyl-CoA:lysophospholipid acyltransferases, 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). Preferred nucleic acid sequences, which can be used in addition in the inventive

process, are disclosed in the sequence protocol of WO2005/012316 and in Table 1 of the specification of said application, these sequences are hereby incorporated by reference.

Transgenic plants are to be understood as meaning single plant cells, certain tissues, organs or parts of plants and their cultures on solid media or in liquid culture, parts of plants and entire plants such as plant cell cultures, protoplasts from plants, callus cultures or plant tissues such as leafs, stem, shoots, seeds, flowers, roots, tubers etc. Said transgenic plants can be cultivated for example on solid or liquid culture medium, in soil or in hydroponics. Plants in the sense of the invention also 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 such as seeds 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.

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, gene constructs or vectors as described herein 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 plant 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 Δ12-desaturase and Δ15-desaturase-, Δ-9-elongase-, Δ-8-desaturase- and/or Δ5-desaturase-genes – 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 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 a plant, 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 a plant, 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 oilseed crops.

After cultivation transgenic plants which are used in the inventive process can be brought to the market without isolating the ω-6- and/or ω-3-fatty acids preferably the arachidonic and/or eicosapentaenoic acid. Preferably the ω-6- and/or ω-3-fatty acids are isolated from the plant in the form of their free fatty acids, their lipids or oils. The purification can be done by conventional methods such as squeezing and extraction of the plants or other methods instead of the extraction such as distillation, crystallization at low temperatures, chromatography or a combination of said methods. Advantageously the plants are grinded, heated and/or vaporized before the squeezing and extraction procedure. As solvent for the extraction solvents such as hexane or other solvents having a similar extraction behavior are used. The isolated oils are further purified by acidification with for example phosphoric acid. The free fatty acids are produced from said oils or lipids by hydrolysis. Charcoal or diatom earth is used to remove dyes from the fluid. In another preferred embodiment of the inventive process the alkyl ester of the fatty acids are produced from the oils and lipids by transesterification with an enzyme of with conventional chemistry. A preferred method is the production of the alkyl ester in the presence of alcoholates of the corresponding lower alcohols (C1 to C10 alcohols such as methanol, ethanol, propanol, butanol, hexanol etc.) such as methanolate or ethanolate. Therefore as the skilled worker knows the alcohol in the presence of a catalytic amount of a base such as NaOH or KOH is added to the oils or lipids.

In a preferred form of the inventive process the lipids can be obtained in the usual manner after the plants have been grown. To this end, the organisms can first be harvested and then disrupted, or they can be used directly. 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. It is advantageous to extract the lipids with suitable solvents such as apolar solvents, for example hexane, or polar solvents, for example ethanol, isopropanol, or mixtures such as hexane/isopropanol, phenol/chloroform/isoamyl alcohol, at temperatures between 0°C and 80°C, preferably between 20°C and 50°C. As a rule, the biomass is extracted with an excess of solvent, for example with an excess of solvent to biomass of 1:4. The solvent is subsequently removed, for example by distillation. The extraction may also be carried out with supercritical CO_2. After the extraction, the remainder of the biomass can be removed, for example, by filtration. Standard methods for the extraction of fatty acids from plants and microorganisms are described in Bligh et al. (Can. J. Biochem. Physiol. 37, 1959: 911-917) or Vick et al. (Plant Physiol. 69, 1982: 1103-1108).

The crude oil thus obtained can then be purified further, for example by removing cloudiness by adding polar solvents such as acetone or apolar solvents such as chloroform, followed by filtration or centrifugation. Further purification via columns or other techniques is also possible.

To obtain the free fatty acids from the triglycerides, the latter are hyrolyzed in the customary manner, for example using NaOH or KOH.

In the inventive process oils, lipids and/or free fatty acids or fractions thereof are produced. Said products can be used for the production of feed and food products, cosmetics or pharmaceuticals.

The oils, lipids, LCPUFAs or fatty acid compositions produced according to the inventive process 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 and/or microbial oils such as from Mortierella or Crypthecodinium. These oils, lipids, fatty acids or fatty acid mixtures, which are

composed of vegetable, microbial and/or animal constituents, may also be used for the preparation of feedstuffs, foodstuffs, cosmetics or pharmaceuticals.

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, fat, fatty acid and/or fatty acid composition is preferably high in polyunsaturated (PUFA and/or LCPUFA) free and/or, advantageously, esterified fatty acid(s), in particular oleic acid, linoleic acid, α-linolenic acid, arachidonic acid and/or eicosatetraenoic acid.

Transgenic plants which comprise the LCPUFAs synthesized in the process according to the invention can also advantageously be marketed directly without there being any need for the oils, lipids or fatty acids synthesized to be isolated.

However, the LCPUFAs produced in the process according to the invention can also be isolated from the plants as described above, 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 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 preferred biosynthesis site of the fatty acids, oils, lipids or fats in the plants which are advantageously used is, for example, in general the seed or cell strata of the seed, so that seed-specific expression of the nucleic acids used in the process makes sense. However, it is obvious that the biosynthesis of fatty acids, oils or lipids need not be limited to the seed tissue, but can also take place in a tissue-specific manner in all the other parts of the plant, for example in epidermal cells or in the tubers.

In principle, the LCPUFAs produced by the process according to the invention in the organisms used in the process can be increased in two different ways. Advantageously, the pool of free polyunsaturated fatty acids and/or the content of the esterified polyunsaturated fatty acids produced via the process can be enlarged. Advantageously, the pool of esterified polyunsaturated fatty acids in the transgenic plants is enlarged by the process according to the invention.

In principle all nucleic acids encoding polypeptides with Δ-8-desaturase, Δ-9-elongase and/or Δ-5-desaturase activity can be used in the inventive process. Preferably the nucleic acid sequences can be isolated for example from microorganism or plants such as fungi like Mortierella, algae like Euglena, Crypthecodinium or Isochrysis, diatoms like Phaeodactylum, protozoa like amoeba such as Acanthamoeba or Perkinsus or mosses like Physcomitrella or Ceratodon, but also non-human animals such as Caenorhabditis are possible as source for the nucleic acid sequences. Advantageous nucleic acid sequences according to the invention which encode polypeptides having a Δ-8-desaturase, Δ-9-elongase and/or Δ-5-desaturase activity are originate from microorganisms or plants, advantageously Phaeodactylum tricornutum, Ceratodon purpureus, Physcomitrella patens, Euglena gracilis, Acanthamoeba castellanii, Perkinsus marinus or Isochrysis galbana. Thus, the co expression of a C18-specific Δ-12-desaturase and Δ-15-desaturase, a C18-specific Δ-9 elongase, a C20-specific Δ-8-desaturase and a C20-specific Δ-5-desaturase leads to the formation of Arachidonic acid (C20:6n-4, Δ5, 8, 11, 14) and/or Eicosapentaenoic acid (C20:3n-5, Δ5, 8, 11, 14, 17). Most preferred are the sequences mentioned in the sequence protocol.

In another embodiment the invention furthermore relates to isolated nucleic acid sequences encoding polypeptides with Δ-12-desaturase and Δ-15-desaturase-, Δ-9-

elongase-, Δ-8-desaturase- and/or Δ-5-desaturase-acitivity.

In one embodiment the invention relates to an isolated nucleic acid sequence which encodes a polypeptide having a Δ-12-desaturase and Δ-15-desaturase activity selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 22 which encode polypeptides having at least 40 % homology to the sequence as depicted in SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 and which polypeptides having Δ-12-desaturase and Δ-15-desaturase activity.

This inventive Δ-12-desaturase and Δ-15-desaturase is able to desaturate C16-fatty acids having at least one double bond in the fatty acid chain and/or C18-fatty acids having at least one double bond in the fatty acid chain. Preferably C16- and/or C18-fatty acids having only one double bond in the fatty acid chain are desaturated. This activity leads to an increase in flux from precursor fatty acids such as C18-fatty acids towards C18-fatty acids having more than one double bond in the fatty acid chain such as linoleic and/or linolenic acid. C18-fatty acids are more preferred in the reaction than C16-fatty acids. C18-fatty acids are more than doubled preferred.

In another embodiment the invention relates to an isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-9-elongase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 11;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 12;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 11 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 12 and which polypeptides having Δ-9-elongase activity.

In yet another embodiment the invention relates to an isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-8-desaturase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and which polypeptides having Δ-8-desaturase activity.

Further in another embodiment the invention relates to an isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-5-desaturase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 15 or SEQ ID NO: 17;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 16 or SEQ ID NO: 18;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 15 or SEQ ID NO: 17 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 16 or SEQ ID NO: 18 and which polypeptides having Δ-5-desaturase activity.

By derivative(s) of the sequences according to the invention is meant, for example, functional homologues of the polypeptides or enzymes encoded by SEQ ID NO: 3,

SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 which exhibit the same said specific enzymatic activity. This specific enzymatic activity allows advantageously the synthesis of LCPUFAs of the ω-6- and/or ω-3-pathway of the fatty acid synthesis chain such as ARA and/or EPA. The said sequences encode enzymes which exhibit Δ-12-desaturase and Δ-15-desaturase-, Δ-9-elongase-, Δ-8-desaturase- and/or Δ-5-desaturase-activity.

The enzyme according to the invention, Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase, advantageously either elongates fatty acid chains with 18 carbon atoms (see SEQ ID NO: 11) or introduces a double bond into fatty acid residues of glycerolipids, free fatty acids or acyl-CoA fatty acids at position C_8-C_9 (see SEQ ID NO: 3, 5 or 7) or at position C_5-C_6 (see SEQ ID NO: 15 or 17) or at position C_{12}-C_{13} and C_{15}-C_{16} of the fatty acid chain (see SEQ ID NO: 19, 21 or 23).

The inventive nucleic acid molecules, for example a nucleic acid molecule with a nucleotide sequence of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 or of a part thereof 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 comprising a complete sequence of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 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: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 or with the aid of the amino acid sequences detailed in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24. A nucleic acid according to the invention can be amplified by standard PCR amplification techniques using cDNA or, alternatively, genomic DNA as template and suitable oligonucleotide primers. 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 nucleotide sequence can be generated by standard synthetic methods, for example using an automatic DNA synthesizer.

Homologs of the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase nucleic acid sequences with the sequence SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 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: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 or its homologs, derivatives or analogs or parts thereof. Furthermore, isolated nucleic acid molecules of a nucleotide sequence which hybridize with one of the nucleotide sequences shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 or with a part thereof, for example hybridized 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. It is also possible and advantageous to use the full sequence. Allelic variants comprise in particular functional variants which can be obtained by deletion, insertion or substitution of nucleotides from/into the sequence detailed in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23, 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. Proteins which retain the enzymatic activity of the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase, i.e. whose activity is essentially not reduced, means proteins with at least 10%, preferably 20%, especially preferably 30%, very especially preferably 40% of the original enzyme activity in comparison with the protein encoded by SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23. 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 53711 (1991)], were used for the sequence alignment. The sequence homology 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.

Homologs of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 means for example also bacterial, fungal and plant homologs, truncated sequences, single-

stranded DNA or RNA of the coding and noncoding DNA sequence.

Homologs of SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 also means 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 a further embodiment, derivatives of the nucleic acid molecule according to the invention represented in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 encode proteins with at least 40%, advantageously approximately 50 or 60%, advantageously at least approximately 60 or 70% and more preferably at least approximately 70 or 80%, 80 to 90%, 90 to 95% and most preferably at least approximately 96%, 97%, 98%, 99% or more homology (= identity) with a complete amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24. The homology was calculated over the entire amino acid or nucleic acid sequence region. 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 53711 (1991)], were used for the sequence alignment. The sequence homology values which are indicated above as a percentage were determined over the entire sequence region using the program BestFit 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.

Moreover, the invention comprises nucleic acid molecules which differ from one of the nucleotide sequences shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID

NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 (and parts thereof) owing to the degeneracy of the genetic code and which thus encode the same Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase as those encoded by the nucleotide sequences shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23.

In addition to the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase shown in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23, the skilled worker will recognize that DNA sequence polymorphisms which lead to changes in the amino acid sequences of the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase may exist within a population. These genetic polymorphisms in the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase gene may exist between individuals within a population owing to natural variation. These natural variants usually bring about a variance of 1 to 5% in the nucleotide sequence of the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase or Δ-5-desaturase gene. Each and every one of these nucleotide variations and resulting amino acid polymorphisms in the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase,
Δ-8-desaturase or Δ-5-desaturase which are the result of natural variation and do not modify the functional activity are to be encompassed by the invention.

The nucleic acid sequence(s) according to the invention (for purposes of the application the singular encompasses the plural and vice versa) or fragments thereof may advantageously be used for isolating other genomic sequences via homology screening.

The said derivatives may be isolated, for example, from other organisms, eukaryotic organisms such as plants, especially mosses, algae, dinoflagellates, protozoa or fungi.

Allele variants include in particular functional variants obtainable by deletion, insertion or substitution of nucleotides in the sequences depicted in SEQ ID NO: 3, SEQ ID

NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 the enzymatic activity of the derived synthesized proteins being retained.

Starting from the DNA sequence described in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 or parts of said sequences such DNA sequences can be isolated using, for example, normal hybridization methods or the PCR technique from other eukaryotes such as those identified above for example. These DNA sequences hybridize under standard conditions with the said sequences. For hybridization use is advantageously made of short oligonucleotides of the conserved regions of an average length of about 15 to 70 bp, preferably of about 17 to 60 bp, more preferably of about 19 to 50 bp, most preferably of about 20 to 40 bp, for example, which can be determined by comparisons with other desaturase or elongase genes in the manner known to those skilled in the art. The histidine box sequences are advantageously employed. However, longer fragments of the nucleic acids according to the invention or the complete sequences may also be used for hybridization. Depending on the nucleic acid employed: oligonucleotide, longer fragment or complete sequence, or depending on which type of nucleic acid, DNA or RNA, is used for hybridization these standard conditions vary. Thus, for example, the melting temperatures of DNA:DNA hybrids are approximately 10°C lower than those of DNA:RNA hybrids of the same length.

By standard conditions is meant, for example, depending on the nucleic acid in question temperatures between 42°C and 58°C in an aqueous buffer solution having a concentration of between 0.1 and 5 x SSC (1 X SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.2) or additionally in the presence of 50 % formamide, such as by way of example 42°C in 5 x SSC, 50 % formamide. Hybridization conditions for DNA:DNA hybrids are advantageously 0.1 x SSC and temperatures between approximately 20°C and 45°C, preferably between approximately 30°C and 45°C. For DNA:RNA hybrids the hybridization conditions are advantageously 0.1 x SSC and temperatures between approximately 30°C and 55°C, preferably between approximately 45°C and 55°C. These specified temperatures for hybridization are melting temperature values calculated by way of example for a nucleic acid having a length of approximately 100 nu-

cleotides and a G + C content of 50 % in the absence of formamide. The experimental conditions for DNA hybridization are described in relevant genetics textbooks such as by way of example Sambrook et al., „Molecular Cloning“, Cold Spring Harbor Laboratory, 1989, and may be calculated by formulae known to those skilled in the art, for example as a function of the length of the nucleic acids, the nature of the hybrids or the G + C content. Those skilled in the art may draw on the following textbooks for further information on hybridization: Ausubel et al. (eds), 1985, Current Protocols in Molecular Biology, John Wiley & Sons, New York; Hames and Higgins (eds), 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, by derivatives is meant homologues of the sequences SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23, for example eukaryotic homologues, truncated sequences, single-stranded DNA of the encoding and nonencoding DNA sequence or RNA of the encoding and nonencoding DNA sequence.

In addition, by homologues of the sequences SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 is meant derivatives such as by way of example promoter variants. These variants may be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without, however, adversely affecting the functionality or efficiency of the promoters. Furthermore, the promoters can have their efficiency increased by altering there sequence or be completely replaced by more effective promoters even of foreign organisms.

By derivatives is also advantageously meant variants whose nucleotide sequence has been altered in the region from –1 to –2000 ahead of the start codon in such a way that the gene expression and/or the protein expression is modified, preferably increased. Furthermore, by derivatives is also meant variants, which have been modified at the 3’ end.

The nucleic acid sequences according to the invention which encode a Δ-12-desaturase and Δ-15-desaturase, a Δ-9-elongase, a Δ-8-desaturase and/or a Δ-5-

desaturase may be produced by synthesis or obtained naturally or contain a mixture of synthetic and natural DNA components as well as consist of various heterologous Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase gene segments from different organisms. In general, synthetic nucleotide sequences are produced with codons, which are preferred by the corresponding host organisms, plants for example. This usually results in optimum expression of the heterologous gene. These codons preferred by plants may be determined from codons having the highest protein frequency, which are expressed in most of the plant species of interest. An example concerning the bacterium Corynebacterium glutamicum is provided in Wada et al. (1992) *Nucleic Acids Res.* 20:2111-2118). Such experiments can be carried out using standard methods and are known to the person skilled in the art.

Functionally equivalent sequences which encode the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase gene are those derivatives of the sequence according to the invention which despite differing nucleotide sequence still possess the desired functions, that is to say the enzymatic activity and specific selectivity of the proteins. That means such functionally equivalent sequences have an biological or enzymatic activity, which is at least 10%, preferably at least 20%, 30%, 40% or 50% especially preferably at least 60%, 70%, 80% or 90% and very especially at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or more of the activity of the proteins/enzymes encoded by the inventive sequences. Thus, functional equivalents include naturally occurring variants of the sequences described herein as well as artificial ones, e.g. artificial nucleotide sequences adapted to the codon use of a plant which have been obtained by chemical synthesis.

In addition, artificial DNA sequences are suitable, provided, as described above, they mediate the desired property, for example an increase in the content of Δ-12, Δ-15, Δ-8 and/or Δ-5-double bonds in fatty acids and an elongation of C18-fatty acids having a Δ-9-double bond in fatty acids, oils or lipids in plants that produce mature seeds preferably in crop plants by over expression of the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase gene. Such artificial DNA sequences can exhibit Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase activity, for example by back-translation of pro-

teins constructed by means of molecular modeling, or be determined by in vitro selection. Possible techniques for in vitro evolution of DNA to modify or improve the DNA sequences are described in Patten, P.A. et al., Current Opinion in Biotechnology 8, 724-733(1997) or in Moore, J.C. et al., Journal of Molecular Biology 272, 336–347 (1997). Particularly suitable are encoding DNA sequences which are obtained by back-translation of a polypeptide sequence in accordance with the codon use specific to the host plant. Those skilled in the art familiar with the methods of plant genetics can easily determine the specific codon use by computer analyses of other known genes of the plant to be transformed.

Other suitable equivalent nucleic acid sequences, which may be mentioned are sequences that encode fusion proteins, a component of the fusion protein being a Δ-12-desaturase and Δ-15-desaturase, Δ-8-desaturase and/or Δ-5-desaturase polypeptide and/or a Δ-9 elongase polypeptide or a functionally equivalent part thereof. The second part of the fusion protein can be, for example, another polypeptide having enzymatic activity or an antigenic polypeptide sequence by means of which it is possible to demonstrate Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase expression (e.g. myc tag or his tag). Preferably, however, this is a regulatory protein sequence, such as by way of example a signal sequence for the endoplasmic reticulum (= ER) which directs the Δ-12-desaturase and Δ-15-desaturase, Δ-8-desaturase and/or Δ-5-desaturase protein and/or the Δ-9-elongase protein to the desired point of action, or regulatory sequences which influence the expression of the nucleic acid sequence according to the invention, such as promoters or terminators. In another preferred embodiment the second part of the fusion protein is a plastidial targeting sequence as described by Napier J.A. [Targeting of foreign proteins to the chloroplast, Methods Mol. Biol., 49, 1995: 369 - 376]. A preferred used vector comprising said plastidial targeting sequence is disclosed by Colin Lazarus [Guerineau F., Woolston S., Brooks L., Mullineaux P. “An expression cassette for targeting foreign proteins into chloroplast; Nucleic. Acids Res., Dec 9, 16 (23), 1988: 11380].

Advantageously, the Δ-12-desaturase and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase genes in the method according to the invention may be combined with other genes for fatty acid biosynthesis as described above.

Examples of such genes are the acyl transferases, other desaturases or elongases such as Δ-4-desaturases or ω-3- and/or ω-6-specific desaturases) and/or such as Δ-5-elongases to mention only some of them. For in vivo and especially in vitro synthesis combination with e.g. NADH cytochrome B5 reductases, which can take up or release reduction equivalents is advantageous.

By the amino acid sequences according to the invention is meant proteins which contain an amino acid sequence depicted in the sequences SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 or a sequence obtainable there from by substitution, inversion, insertion or deletion of one or more amino acid groups (such sequences are derivatives of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24), whereas the enzymatic activities of the proteins depicted in SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 12, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 being retained or not substantially reduced, that is they still possess the same enzymatic specificity. By „not substantially reduced“ or „the same enzymatic activity“ is meant all enzymes which still exhibit at least 10%, 20%, 30%, 40% or 50%, preferably at least 60%, 70%, 80% or 90% particularly preferably at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more, of the enzymatic activity of the initial enzyme obtained from the wild type source organism such as organisms of the genus Physcomitrella, Ceratodon, Borago, Thraustochytrium, Schizochytrium, Phytophtora, Mortierella, Caenorhabditis, Aleuritia, Muscariodides, Isochrysis, Phaeodactylum, Crypthecodinium, Acanthamoeba or Euglena preferred source organisms are organisms such as the species Euglena gracilis, Isochrysis galbana, Phaeodactylum tricornutum, Caenorhabditis elegans, Thraustochytrium , Phytophtora infestans, Ceratodon purpureus, Isochrysis galbana, Aleuritia farinosa, Muscariodides vialii, Mortierella alpina, Borago officinalis or Physcomitrella patens. For the estimation of an enzymatic activity, which is “not substantially reduced” or which has the “same enzymatic activity” the enzymatic activity of the derived sequences are determined and compared with the wild type enzyme activities. In doing this, for example, certain amino acids may be replaced by others having similar physicochemical properties (space filling, basicity, hydrophobicity, etc.). For example, arginine residues are exchanged for lysine residues, valine residues for isoleucine

residues or aspartic acid residues for glutamic acid residues. However, one or more amino acids may also be swapped in sequence, added or removed, or a plurality of these measures may be combined with one another.

By derivatives is also meant functional equivalents, which in particular also contain natural or artificial mutations of an originally isolated sequence encoding a Δ-12-desaturase and Δ-15-desaturase, a Δ-9-elongase, a Δ-8-desaturase and/or a Δ-5-desaturase, which continue to exhibit the desired function, that is the enzymatic activity and substrate selectivity thereof is not substantially reduced. Mutations comprise substitutions, additions, deletions, exchanges or insertions of one or more nucleotide residues. Thus, for example, the present invention also encompasses those nucleotide sequences, which are obtained by modification of the Δ-12-desaturase and Δ-15-desaturase nucleotide sequence, the Δ-8-desaturase nucleotide sequence, the Δ-5-desaturase nucleotide sequence and/or the Δ-9-elongase nucleotide sequence used in the inventive processes. The aim of such a modification may be, e.g., to further bind the encoding sequence contained therein or also, e.g., to insert further restriction enzyme interfaces.

Functional equivalents also include those variants whose function by comparison as described above with the initial gene or gene fragment is weakened (= not substantially reduced) or reinforced (= enzyme activity higher than the activity of the initial enzyme, that is activity is higher than 100%, preferably higher than 110%, 120%, 130%, 140% or 150%, particularly preferably higher than 200% or more).

At the same time the nucleic acid sequence may, for example, advantageously be a DNA or cDNA sequence. Suitable encoding sequences for insertion into an expression cassette according to the invention include by way of example those which encode a Δ-12-desaturase and Δ-15-desaturase, a Δ-8-desaturase and/or a Δ-5-desaturase with the sequences described above and lend the host the ability to overproduce fatty acids, oils or lipids having double bonds in the Δ-12-, Δ-15-, Δ-8-position and Δ-5-position, it being advantageous when at the same time fatty acids having at least four double bonds are produced. These sequences may be of homologous or heterologous origin.

By the gene construct (= nucleic acid construct or fragment or expression cassette) according to the invention is meant the sequences specified in SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 which result from the genetic code and/or derivatives thereof which are functionally linked with one or more regulation signals advantageously to increase the gene expression and which control the expression of the encoding sequence in the host cell. These regulatory sequences should allow the selective expression of the genes and the protein expression. Depending on the host plant this may mean, for example, that the gene is expressed and/or overexpressed only after induction or that it is expressed and/or overexpressed immediately. Examples of these regulatory sequences are sequences to which inductors or repressors bind and in this way regulate the expression of the nucleic acid. In addition to these new regulation sequences or instead of these sequences the natural regulation of these sequences ahead of the actual structural genes may still be present and optionally have been genetically modified so that natural regulation was switched off and the expression of the genes increased. However, the gene construct can also be built up more simply, that is no additional regulation signals have been inserted ahead of the nucleic acid sequence or derivatives thereof and the natural promoter with its regulation has not been removed. Instead of this the natural regulation sequence was mutated in such a way that no further regulation ensues and/or the gene expression is heightened. These modified promoters in the form of part sequences (= promoter containing parts of the nucleic acid sequences according to the invention) can also be brought on their own ahead of the natural gene to increase the activity. In addition, the gene construct may advantageously also contain one or more so-called enhancer sequences functionally linked to the promoter which allow enhanced expression of the nucleic acid sequence. At the 3' end of the DNA sequences additional advantageous sequences may also be inserted, such as further regulatory elements or terminators. The SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 and/or SEQ ID NO: 23 gene may be present in one or more copies in the gene construct (=expression cassette).

As described above, the regulatory sequences or factors can preferably positively influence and so increase the gene expression of the introduced genes. Thus, reinforcement of the regulatory elements advantageously on the transcription level may

be effected by using powerful transcription signals such as promoters and/or enhancers. However, in addition reinforcement of translation is also possible, for example by improving the stability of the mRNA.

Suitable promoters in the expression cassette are in principle all promoters which can control the expression of foreign genes in microorganisms like protozoa such as amoeba, ciliates, algae such as green, brown, red or blue algae such as Euglena, bacteria such as gram-positive or gram-negative bacteria, yeasts such as Saccharomyces, Pichia or Schizosaccharomyces or fungi such as Mortierella, Thraustochytrium or Schizochytrium or plants such as Aleuritia, advantageously in plants or fungi. Such microorganisms are generally used to clone the inventive genes and possible other genes of the fatty acid biosynthesis chain for the production of fatty acids according to the inventive process. Use is preferably made in particular of plant promoters or promoters derived from a plant virus. Advantageous regulation sequences for the method according to the invention are found for example in promoters such as cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, $lacI^{q-}$, T7, T5, T3, gal, trc, ara, SP6, λ-P_R or in λ-P_L promoters which are employed advantageously in gram-negative bacteria. Other advantageous regulation sequences are found, for example, in the gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFα, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck et al., Cell 21(1980) 285-294], SSU, OCS, lib4, STLS1, B33, nos (= Nopalin Synthase Promoter) or in the ubiquintin or phaseolin promoter. The expression cassette may also contain a chemically inducible promoter by means of which the expression of the exogenous Δ-12- and Δ-15-,
Δ-8- and/or Δ-5-desaturase gene and/or the Δ-9-elongase gene in the microorganism and/or plant can be controlled advantageously in the plants at a particular time. Advantageous plant promoters of this type are by way of example the PRP1 promoter [Ward et al., Plant.Mol. Biol. 22 (1993), 361-366], a promoter inducible by benzenesulfonamide (EP 388 186), a promoter inducible by tetracycline [Gatz et al., (1992) Plant J. 2, 397 - 404], a promoter inducible by salicylic acid (WO 95/19443), a promoter inducible by abscisic acid (EP 335 528) and a promoter inducible by ethanol or cyclohexanone (WO 93/21334). Other examples of plant promoters, which can advantageously be used are the promoter of cytosolic FBPase from potato, the ST-LSI promoter from potato (Stockhaus et al., EMBO J. 8 (1989) 2445-245), the promoter of

phosphoribosyl pyrophosphate amidotransferase from Glycine max (see also gene bank accession number U87999) or a nodiene-specific promoter as described in EP 249 676. Particularly advantageous are those plant promoters, which ensure expression in tissues or plant parts/organs in which fatty acid biosynthesis or the precursor stages thereof occurs, as in endosperm or in the developing embryo for example. Particularly noteworthy are advantageous promoters, which ensure seed-specific expression such as by way of example the USP promoter or derivatives thereof, the LEB4 promoter, the phaseolin promoter or the napin promoter. The particularly advantageous USP promoter cited according to the invention or its derivatives mediate very early gene expression in seed development [Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459-67]. Other advantageous seed-specific promoters which may be used for monocotylodonous or dicotylodonous plants are the promoters suitable for dicotyledons such as napin gene promoters, likewise cited by way of example, from oilseed rape (US 5,608,152), the oleosin promoter from Arabidopsis (WO 98/45461), the phaseolin promoter from Phaseolus vulgaris (US 5,504,200), the Bce4 promoter from Brassica (WO 91/13980) or the leguminous B4 promoter (LeB4, Baeumlein et al., Plant J., 2, 2, 1992: 233 – 239) or promoters suitable for monocotylodons such as the promoters of the lpt2 or lpt1 gene in barley (WO 95/15389 and WO 95/23230) or the promoters of the barley hordeine gene, the rice glutelin gene, the rice oryzin gene, the rice prolamin gene, the wheat gliadin gene, the white glutelin gene, the corn zein gene, the oats glutelin gene, the sorghum kasirin gene or the rye secalin gene which are described in WO99/16890.

Furthermore, particularly preferred are those promoters, which ensure the expression in tissues or plant parts in which, for example, the biosynthesis of fatty acids, oils and lipids or the precursor stages thereof takes place. Particularly noteworthy are promoters, which ensure a seed-specific expression. Noteworthy are the promoter of the napin gene from oilseed rape (US 5,608,152), the USP promoter from Vicia faba (USP = unknown seed protein, Baeumlein et al., Mol Gen Genet, 1991, 225 (3): 459 - 67), the promoter of the oleosin gene from Arabidopsis (WO 98/45461), the phaseolin promoter (US 5,504,200) or the promoter of the legumin B4 gene (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2): 233-9). Other promoters to be mentioned are that of the lpt2 or lpt1 gene from barley (WO 95/15389 and WO 95/23230), which mediate seed-specific expression in monocotyledonous plants. Other advantageous seed specific

promoters are promoters such as the promoters from rice, corn or wheat disclosed in WO 99/16890 or Amy32b, Amy6-6 or aleurain (US 5,677,474), Bce4 (rape, US 5,530,149), glycinin (soy bean, EP 571 741), phosphoenol pyruvat carboxylase (soy bean, JP 06/62870), ADR12-2 (soy bean, WO 98/08962), isocitratlyase (rape, US 5,689,040) or β–amylase (barley, EP 781 849).

As described above, the expression construct (= gene construct, nucleic acid construct) may contain yet other genes, which are to be introduced into the microorganism or plant. These genes can be subject to separate regulation or be subject to the same regulation region as the Δ-12- and Δ-15-desaturase gene and/or the Δ-8- and/or Δ-5--desaturase gene and/or the Δ-9-elongase gene. These genes are by way of example other biosynthesis genes, advantageously for fatty acid biosynthesis, which allow increased synthesis. Examples which may be mentioned are the genes for example of the Δ-9–, Δ-4-desaturase, Δ-5-elongase, α–ketoacyl reductases, α–ketoacyl synthases, elongases or the various hydroxylases and acyl–ACP thioesterases. The desaturase and elongase genes are advantageously used in the nucleic acid construct.

In principle all natural promoters with their regulation sequences can be used like those named above for the expression cassette according to the invention and the method according to the invention. Over and above this, synthetic promoters may also advantageously be used.

In the preparation of an a gene construct various DNA fragments can be manipulated in order to obtain a nucleotide sequence, which usefully reads in the correct direction and is equipped with a correct reading raster. To connect the DNA fragments (= nucleic acids according to the invention) to one another adaptors or linkers may be attached to the fragments.

The promoter and the terminator regions can usefully be provided in the transcription direction with a linker or polylinker containing one or more restriction points for the insertion of this sequence. Generally, the linker has 1 to 10, mostly 1 to 8, preferably 2 to 6, restriction points. In general the size of the linker inside the regulatory region is less than 100 bp, frequently less than 60 bp, but at least 5 bp. The promoter may be native or homologous as well as foreign or heterologous to the host organism, for ex-

ample to the host plant. In the 5'-3' transcription direction the expression cassette contains the promoter, a DNA sequence which encodes a Δ-12- and Δ-15-desaturase gene, a Δ-8-desaturase gene, a Δ-5-desaturase gene and/or a Δ-9-elongase gene and a region for transcription termination. Different termination regions can be exchanged for one another in any desired fashion.

Furthermore, manipulations, which provide suitable restriction interfaces or which remove excess DNA or restriction interfaces can be employed. Where insertions, deletions or substitutions, such as transitions and transversions, come into consideration, *in vitro* mutagenesis, primer repair, restriction or ligation may be used. In suitable manipulations such as restriction, chewing back or filling of overhangs for blunt ends complementary ends of the fragments can be provided for the ligation.

For an advantageous high expression the attachment of the specific ER retention signal SEKDEL inter alia can be of importance (Schouten, A. et al., Plant Mol. Biol. 30 (1996), 781-792). In this way the average expression level is tripled or even quadrupled. Other retention signals, which occur naturally in plant and animal proteins located in the ER may also be employed for the construction of the cassette. In another preferred embodiment a plastidial targeting sequence is used as described by Napier J.A. [Targeting of foreign proteins to the chloroplast, Methods Mol. Biol., 49, 1995: 369 - 376]. A preferred used vector comprising said plastidial targeting sequence is disclosed by Colin Lazarus [Guerineau F., Woolston S., Brooks L., Mullineaux P. "An expression cassette for targeting foreign proteins into chloroplast; Nucleic. Acids Res., Dec 9, 16 (23), 1988: 11380].

Preferred polyadenylation signals are plant polyadenylation signals, preferably those which substantially correspond to T-DNA polyadenylation signals from Agrobacterium tumefaciens, in particular gene 3 of the T-DNA (octopin synthase) of the Ti plasmid pTiACH5 (Gielen et al., EMBO J.3 (1984), 835 et seq.) or corresponding functional equivalents.

An expression cassette/gene construct is produced by fusion of a suitable promoter with a suitable Δ-12- and Δ-15-desaturase DNA sequence, a suitable Δ-8- and/or Δ-5-desaturase DNA sequence and/or a suitable Δ-9-elongase DNA sequence together with a polyadenylation signal by common recombination and cloning techniques as

described, for example, in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989) as well as in T.J. Silhavy, M.L. Berman and L.W. Enquist, Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984) and in Ausubel, F.M. et al., Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience (1987).

The DNA sequences encoding the nucleic acid sequences used in the inventive processes such as the Δ-12- and Δ-15-desaturase from Acanthamoeba castellanii or Perkinsus marinus, Δ-8-desaturase from Euglena gracilis, Acanthamoeba castellanii or Perkinsus marinus, the Δ-9-elongase from Isochrysis galbana or Acanthamoeba castellanii and/or the Δ-5-desaturase for example from Thraustrochytrium, Acanthamoeba castellanii or Perkinsus marinus or other organisms such as Caenorhabditis elegans, Mortierella alpina, Borage officinalis or Physcomitrella patens contain all the sequence characteristics needed to achieve correct localization of the site of fatty acid, lipid or oil biosynthesis. Accordingly, no further targeting sequences are needed per se. However, such localization may be desirable and advantageous and hence artificially modified or reinforced so that such fusion constructs are also a preferred advantageous embodiment of the invention.

Particularly preferred are sequences, which ensure targeting in plastids. Under certain circumstances targeting into other compartments (reported in: Kermode, Crit. Rev. Plant Sci. 15, 4 (1996), 285-423) may also be desirable, e.g. into vacuoles, the mitochondrium, the endoplasmic reticulum (ER), peroxisomes, lipid structures or due to lack of corresponding operative sequences retention in the compartment of origin, the cytosol.

Advantageously, the nucleic acid sequences according to the invention or the gene construct together with at least one reporter gene are cloned into a gene construct, which is introduced into the organism via a vector or directly into the genome. This reporter gene should allow easy detection via a growth, fluorescence, chemical, bioluminescence or resistance assay or via a photometric measurement. Examples of reporter genes which may be mentioned are antibiotic- or herbicide-resistance genes, hydrolase genes, fluorescence protein genes, bioluminescence genes, sugar or nucleotide metabolic genes or biosynthesis genes such as the Ura3 gene, the Ilv2 gene,

the luciferase gene, the β-galactosidase gene, the gfp gene, the 2–desoxyglucose–6–phosphate phosphatase gene, the β-glucuronidase gene, β-lactamase gene, the neomycin phosphotransferase gene, the hygromycin phosphotransferase gene or the BASTA (= gluphosinate-resistance) gene. These genes permit easy measurement and quantification of the transcription activity and hence of the expression of the genes. In this way genome positions may be identified which exhibit differing productivity.

In a preferred embodiment an gene construct comprises upstream, i.e. at the 5' end of the encoding sequence, a promoter and downstream, i.e. at the 3' end, a polyadenylation signal and optionally other regulatory elements which are operably linked to the intervening encoding sequence for Δ-12- and Δ-15-desaturase, Δ-8-desaturase, Δ-9-elongase and/or Δ-5-desaturase DNA sequence. By an operable linkage is meant the sequential arrangement of promoter, encoding sequence, terminator and optionally other regulatory elements in such a way that each of the regulatory elements can fulfill its function in the expression of the encoding sequence in due manner. The sequences preferred for operable linkage are targeting sequences for ensuring subcellular localization in plastids. However, targeting sequences for ensuring subcellular localization in the mitochondrium, in the endoplasmic reticulum (= ER), in the nucleus, in oil corpuscles or other compartments may also be employed as well as translation promoters such as the 5' lead sequence in tobacco mosaic virus (Gallie et al., Nucl. Acids Res. 15 (1987), 8693 -8711).

An expression cassette/gene construct may, for example, contain a constitutive promoter or a tissue-specific promoter (preferably the USP or napin promoter) the gene to be expressed and the ER retention signal. For the ER retention signal the KDEL amino acid sequence (lysine, aspartic acid, glutamic acid, leucine) or the KKX amino acid sequence (lysine-lysine-X-stop, wherein X means every other known amino acid) is preferably employed.

For expression in a prokaryotic or eukaryotic host organism, for example a microorganism such as a fungus or a plant such as an oil crop the expression cassette is advantageously inserted into a vector such as by way of example a plasmid, a phage or other DNA which allows optimum expression of the genes in the host organism. Examples of suitable plasmids are: in E. coli pLG338, pACYC184, pBR series such as

e.g. pBR322, pUC series such as pUC18 or pUC19, M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III^{113}-B1, λgt11 or pBdCI; in Streptomyces pIJ101, pIJ364, pIJ702 or pIJ361; in Bacillus pUB110, pC194 or pBD214; in Corynebacterium pSA77 or pAJ667; in fungi pALS1, pIL2 or pBB116; other advantageous fungal vectors are described by Romanos, M.A. et al., [(1992) „Foreign gene expression in yeast: a review“, *Yeast* 8: 423-488] and by van den Hondel, C.A.M.J.J. et al. [(1991) „Heterologous gene expression in filamentous fungi“ as well as in More Gene Manipulations in Fungi [J.W. Bennet & L.L. Lasure, eds., pp. 396-428: Academic Press: San Diego] and in „Gene transfer systems and vector development for filamentous fungi“ [van den Hondel, C.A.M.J.J. & Punt, P.J. (1991) in: Applied Molecular Genetics of Fungi, Peberdy, J.F. et al., eds., pp. 1-28, Cambridge University Press: Cambridge]. Examples of advantageous yeast promoters are 2µM, pAG-1, YEp6, YEp13 or pEMBLYe23. Examples of algal or plant promoters are pLGV23, pGHlac^{+}, pBIN19, pAK2004, pVKH or pDH51 (see Schmidt, R. and Willmitzer, L., 1988). The vectors identified above or derivatives of the vectors identified above are a small selection of the possible plasmids. Further plasmids are well known to those skilled in the art and may be found, for example, in the book Cloning Vectors (Eds. Pouwels P.H. et al. Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). Suitable plant vectors are described inter alia in „Methods in Plant Molecular Biology and Biotechnology“ (CRC Press), Ch. 6/7, pp. 71-119. Advantageous vectors are known as shuttle vectors or binary vectors which replicate in E. coli and Agrobacterium.

By vectors is meant with the exception of plasmids all other vectors known to those skilled in the art such as by way of example phages, viruses such as SV40, CMV, baculovirus, adenovirus, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA. These vectors can be replicated autonomously in the host organism or be chromosomally replicated, chromosomal replication being preferred.

In a further embodiment of the vector the gene construct according to the invention may also advantageously be introduced into the organisms in the form of a linear DNA and be integrated into the genome of the host organism by way of heterologous or homologous recombination. This linear DNA may be composed of a linearized

plasmid or only of the expression cassette as vector or the nucleic acid sequences according to the invention.

In a further advantageous embodiment the nucleic acid sequence according to the invention can also be introduced into an organism on its own.

If in addition to the nucleic acid sequence according to the invention further genes are to be introduced into the organism, all together with a reporter gene in a single vector or each single gene with a reporter gene in a vector in each case can be introduced into the organism, whereby the different vectors can be introduced simultaneously or successively.

The vector advantageously contains at least one copy of the nucleic acid sequences according to the invention and/or the expression cassette (= gene construct) according to the invention.

By way of example the plant expression cassette can be installed in the pRT transformation vector ((a) Toepfer et al., 1993, Methods Enzymol., 217: 66-78; (b) Toepfer et al. 1987, Nucl. Acids. Res. 15: 5890 ff.).

Alternatively, a recombinant vector (= expression vector) can also be transcribed and translated in vitro, e.g. by using the T7 promoter and the T7 RNA polymerase.

Expression vectors employed in prokaryotes frequently make use of inducible systems with and without fusion proteins or fusion oligopeptides, wherein these fusions can ensue in both N-terminal and C-terminal manner or in other useful domains of a protein. Such fusion vectors usually have the following purposes: i.) to increase the RNA expression rate; ii.) to increase the achievable protein synthesis rate; iii.) to increase the solubility of the protein; iv.) or to simplify purification by means of a binding sequence usable for affinity chromatography. Proteolytic cleavage points are also frequently introduced via fusion proteins, which allow cleavage of a portion of the fusion protein and purification. Such recognition sequences for proteases are recognized, e.g. factor Xa, thrombin and enterokinase.

Typical advantageous fusion and expression vectors are pGEX [Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67: 31-40], pMAL (New England Bio-

labs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which contains glutathione S-transferase (GST), maltose binding protein or protein A.

Other examples of E. coli expression vectors are pTrc [Amann et al., (1988) *Gene* 69:301-315] and pET vectors [Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89; Stratagene, Amsterdam, The Netherlands].

Other advantageous vectors for use in yeast are pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), and pYES derivatives (Invitrogen Corporation, San Diego, CA). Vectors for use in filamentous fungi are described 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., eds., pp. 1-28, Cambridge University Press: Cambridge.

Alternatively, insect cell expression vectors can also be advantageously utilized, e.g. for expression in Sf 9 cells. These are e.g. the vectors of the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

Furthermore, plant cells or algal cells can advantageously be used for gene expression. Examples of plant expression vectors may be found in Becker, D., et al. (1992) „New plant binary vectors with selectable markers located proximal to the left border“, *Plant Mol. Biol.* 20: 1195-1197 or in Bevan, M.W. (1984) „Binary *Agrobacterium* vectors for plant transformation“, *Nucl. Acid. Res*. 12: 8711-8721.

The host plant (= transgenic plant) advantageously contains at least one copy of the nucleic acid according to the invention and/or of the gene construct according to the invention.

The introduction of the nucleic acids according to the invention, the gene construct or the vector into organisms, plants for example, can in principle be done by all of the methods known to those skilled in the art. The introduction of the nucleic acid sequences gives rise to recombinant or transgenic plants.

To introduce the nucleic acids used in the process, 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 mentioned above and 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, again herein 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 used in the process, the inventive nucleic acids and gene constructs, 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. 71-119 (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 PUFA and/or LCPUFA producers.

In the case of microorganisms, those skilled in the art can find appropriate methods for the introduction of the inventive nucleic acid sequences, the gene construct or the vector in the textbooks by Sambrook, J. et al. (1989) Molecular cloning: A laboratory manual, Cold Spring Harbor Laboratory Press, by F.M. Ausubel et al. (1994) Current protocols in molecular biology, John Wiley and Sons, by D.M. Glover et al., DNA Cloning Vol.1, (1995), IRL Press (ISBN 019-963476-9), by Kaiser et al. (1994) Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press or Guthrie et al. Guide to Yeast Genetics and Molecular Biology, Methods in Enzymology, 1994, Academic Press.

The transfer of foreign genes into the genome of a plant is called transformation. In doing this the methods described for the transformation and regeneration of plants from plant tissues or plant cells are utilized for transient or stable transformation. Suitable methods are protoplast transformation by poly(ethylene glycol)-induced DNA uptake, the „biolistic“ method using the gene cannon – referred to as the particle bombardment method, electroporation, the incubation of dry embryos in DNA solution, microinjection and gene transfer mediated by Agrobacterium. Said methods are described by way of example in B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, eds. S.D. Kung and R. Wu, Academic Press (1993) 128-143 and in Potrykus Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991) 205-225). The nucleic acids or the construct to be expressed is preferably cloned into a vector, which is suitable for transforming Agrobacterium tumefaciens, for example pBin19 (Bevan et al., Nucl. Acids Res. 12 (1984) 8711). Agrobacteria transformed by such a vector can then be used in known manner for the transformation of plants, in particular of crop plants such as by way of example tobacco plants, for example by bathing bruised leaves or chopped leaves in an agrobacterial solution and then culturing them in suitable media. The transformation of plants by means of Agrobacterium tumefaciens is described, for example, by Höfgen and Willmitzer in Nucl. Acid Res. (1988) 16, 9877 or is known inter alia from F.F. White, Vectors for Gene Transfer in Higher Plants; in Transgenic Plants, Vol. 1, Engineering and Utilization, eds. S.D. Kung and R. Wu, Academic Press, 1993, pp. 15-38.

Agrobacteria transformed by an expression vector according to the invention may likewise be used in known manner for the transformation of plants such as test plants like Arabidopsis or crop plants such as cereal crops, corn, oats, rye, barley, wheat, soybean, rice, cotton, sugar beet, canola, sunflower, flax, hemp, potatoes, tobacco, tomatoes, carrots, paprika, oilseed rape, tapioca, cassava, arrowroot, tagetes, alfalfa, lettuce and the various tree, nut and vine species, in particular of oil-containing crop plants such as soybean, peanut, castor oil plant, sunflower, corn, cotton, flax (linseed), oilseed rape, poppy, mustard, sesame, almond, macadamia, olive, calendula, punica, hazel nut, avocado, pumpkin, walnut, laurel, pistachio, Orychophragmus, marigold, borage, primrose, canola, evening primrose, hemp, coconut, oil palm, safflower (Carthamus tinctorius), coffee or cocoa bean, e.g. by bathing bruised leaves or chopped leaves in an agrobacterial solution and then culturing them in suitable media.

For the production of LCPUFAs, for example arachidonic acid and/or eicosapentaenoic acid, borage, linseed, sunflower, safflower, Brassica napus, Brassica juncea, Camelina sativa or Orychophragmus are advantageously suitable.

The genetically modified plant cells may be regenerated by all of the methods known to those skilled in the art. Appropriate methods can be found in the publications referred to above by S.D. Kung and R. Wu, Potrykus or Höfgen and Willmitzer.

Accordingly, a further aspect of the invention relates to transgenic organisms transformed by at least one nucleic acid sequence, expression cassette or vector according to the invention as well as cells, cell cultures, tissue, parts – such as, for example, leaves, roots, etc. in the case of plant organisms – or reproductive material derived from such organisms. The terms „host organism“, „host cell“, „recombinant (host) organism“ and „transgenic (host) cell“ are used here interchangeably. Of course these terms relate not only to the particular host organism or the particular target cell but also to the descendants or potential descendants of these organisms or cells. Since, due to mutation or environmental effects certain modifications may arise in successive generations, these descendants need not necessarily be identical with the parental cell but nevertheless are still encompassed by the term as used here.

Suitable organisms or host organisms for the nucleic acid, gene construct or vector according to the invention are advantageously in principle all plants, which are able to synthesize fatty acids, especially unsaturated fatty acids or are suitable for the expression of recombinant genes as described above. Further 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, corn, cotton, flax, oilseed rape, coconut, oil palm, safflower (Carthamus tinctorius) or cocoa bean, bacteria such as the genus Escherichia, yeasts such as the genus Saccharomyces. Preference is given to organisms which can naturally synthesize oils in relatively large quantities such as fungi like Mortierella alpina, Pythium insidiosum or plants such as soybean, oilseed rape, coconut, oil palm, safflower, flax, castor oil plant, Calendula, peanut, cocoa bean or sunflower, or yeasts such as Saccharomyces cerevisiae and particular preference is given to the family of the Brassicaceae such as oilseed rape, soybean, flax, sunflower, Calendula, Mortierella or Saccharomyces cerevisiae.

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

Usable expression strains, e.g. those exhibiting a relatively low protease activity, are described in: Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128.

A further object of the invention as described relates to the use of an expression cassette containing DNA sequences encoding a Δ-12- and Δ-15-desaturase, a Δ-9-elongase, a Δ-8-desaturase and/or a Δ-5-desaturase gene or DNA sequences hybridizing therewith for the transformation of plant cells, tissues or parts of plants. The aim of use is to increase the content of fatty acids, oils or lipids having an increased content of double bonds.

In doing so, depending on the choice of promoter, the Δ-12- and Δ-15-desaturase, the Δ-9-elongase, the Δ-8-desaturase and/or the Δ-5-desaturase gene can be expressed specifically in the leaves, in the seeds, the nodules, in roots, in the stem or other parts of the plant, preferably in leaves and/or seeds. Those transgenic plants overproducing fatty acids, oils or lipids according to the invention, the reproductive material thereof, together with the plant cells, tissues or parts thereof are a further object of the present invention.

The expression cassette or the nucleic acid sequences according to the invention containing a Δ-12- and Δ-15-desaturase, a Δ-9-elongase, a Δ-8-desaturase and/or a Δ-5-desaturase gene sequence can, moreover, also be employed for the transformation of the organisms identified by way of example above such as bacteria, cyanobacteria, yeasts, filamentous fungi, ciliates and algae with the objective of increasing the content of fatty acids, oils or lipids according to the invention.

Within the framework of the present invention is the increase of the content of fatty acids, oils or lipids possessing a higher amount of ω-3-fatty acids in comparison to ω-6-fatty acids such as eicosapentaenoic acid in comparison to arachidonic acid, due to functional over expression of the Δ-12- and Δ-15-desaturase, the Δ-9-elongase, the Δ-8-desaturase and/or the Δ-5-desaturase gene in the plant according to the invention, advantageously in the transgenic oilseed plants according to the invention, by

comparison with the non genetically modified initial plants at least for the duration of at least one plant generation.

The preferred locus of biosynthesis, of fatty acids, oils or lipids for example, is generally the seed or cell layers of the seed so that a seed-specific expression of the Δ-12- and Δ-15-desaturase, the Δ-9-elongase, the Δ-8-desaturase and/or the Δ-5-desaturase gene is appropriate. It is, however, obvious that the biosynthesis of fatty acids, oils or lipids need not be limited to the seed tissue but rather can also occur in tissue-specific manner in all other parts of the plant – in epidermis cells or in the nodules for example.

A constitutive expression of the exogenous Δ-12- and Δ-15-desaturase, Δ-9-elongase, Δ-8-desaturase and/or Δ-5-desaturase gene is, moreover, advantageous. On the other hand, however, an inducible expression may also appear desirable.

The efficiency of the expression of the Δ-12- and Δ-15-desaturase, the Δ-9-elongase, the Δ-8-desaturase and/or the Δ-5-desaturase gene can be determined, for example, *in vitro* by shoot meristem propagation. In addition, an expression of the Δ-12- and Δ-15-desaturase, the Δ-9-elongase, the Δ-8-desaturase and/or the Δ-5-desaturase gene modified in nature and level and its effect on fatty acid, oil or lipid biosynthesis performance can be tested on test plants in greenhouse trials.

An additional object of the invention comprises transgenic plants transformed by an expression cassette containing a Δ-12- and Δ-15-desaturase, a Δ-9-elongase, a Δ-8-desaturase and/or a Δ-5-desaturase gene sequence according to the invention or DNA sequences hybridizing therewith, as well as transgenic cells, tissue, parts and reproduction material of such plants. Particular preference is given in this case to transgenic crop plants such as by way of example barley, wheat, rye, oats, corn, soybean, rice, cotton, sugar beet, the family of the Brassicaceae such as oilseed rape and canola, sunflower, flax, hemp, thistle, potatoes, tobacco, tomatoes, tapioca, cassava, arrowroot, alfalfa, lettuce and the various tree, nut and vine species.

For the purposes of the invention plants are mono- and dicotyledonous plants that produce mature seeds.

A further refinement according to the invention are transgenic plants as described above which contain the nucleic acid sequences, the gene construct and/or vector of the invention.

The invention is explained in more detail by the following examples.

Examples

Example 1: General cloning methods

The cloning methods, such as by way of example restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linkage of DNA fragments, transformation of Escherichia coli cells, culture of bacteria and sequence analysis of recombinant DNA, were carried out as described in Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6).

Example 2: Sequence analysis of recombinant DNA

Sequencing of recombinant DNA molecules was done using a laser fluorescence DNA sequencer from the ABI company by the method of Sanger (Sanger et al. (1977) Proc. Natl. Acad. Sci. USA74, 5463-5467). Fragments resulting from a polymerase chain reaction were sequenced and checked to prevent polymerase errors in the constructs to be expressed.

Example 3: Cloning of the PUFA specific desaturases from Acanthamoeba castellanii (= SEQ ID NO: 3, 5, 15, 19 and 21)

Acanthamoeba castellanii (Eukaryota; Protista; Sarcomastigophora; Sarcodina; Rhizopodea; Lobosa) is an amoeba species, which is a common species in the soil. Acanthamoeba castellanii can grow vegetative over a broad temperature range (10 to 32°C). A. castellanii is able to de novo synthesize linoleic acid and C20 n-6 fatty acids.

A. castellanii (ATTC 30010) was grown at 30°C on a medium containing 0,75% (w/v) peptone, 1,5% (w/v) glucose and 0,75% (w/v) yeast extract according to the reference of Jones et al. [Temperature-induced membrane-lipid adaptation in Acanthamoeba castellanii. Biochem J. 1993, 290:273-278]. The cell cultures were grown under shak-

ing (200 U/min) and harvested with a centrifuge at 250 x g, 5 min, 4°C, after they have reached a cell density of $5x10^6$-10^7 (measured in a Fuchs-Rosenthal Haemozytometer).

The total mRNA was isolated from said harvested cells with the aid of the RNeasy plant mini Kit (Qiagen). cDNA was synthesized from the total mRNA with the SMART RACE cDNA amplification kit (Clontech) according to the instructions of the manufacturer.

For the isolation of new desaturase genes the following degenerated primers were used for the amplification:

Deg1:
5'- GGITGG(C/T/A)TIGGICA(T/C) GA(T/C)(GT) (CT)I(GT) (GC)ICA-3'

Deg2:
5'- GG(A/G)AA(TCGA)AG(A/G)TG(A/G)TG(T/C)TC(A/G/T)AT(T/C)TG-3'

The aforementioned primers were used for the amplification in combination with the 3'-adapter-primer of the SMART RACE cDNA amplification kit.

The following protocol was used for the amplification:

a) 2 min at 95°C,

b) 30 sec at 94 °C
30 sec at 55-72°C
2 min at 72 °C
Number of cycles: 30

c) 10 min at 72 °C

PCR amplicons were cloned and sequenced according to the instructions of the manufacturer (pTOPO, Invitrogen). The sequence information was used for the production of full-length clones. For the cloning of the full-length clones 5'- and 3'-specific primers were synthesized. Said primers were used for the amplification in the SMART RACE cDNA amplification kit (Clontech) and the amplicons werecloned into the pTOPO vector (Invitrogen)

Three sequences were identified, which show low similarities to desaturase genes.

In addition according to [Zank et al. 2002, Plant Journal 31:255 268] sequence 9Ac (Δ-9-Elongase from Acanthamoeba, SEQ ID NO: 11) could be identified, which shows low similarities to elongase genes.

Table 1: Acanthamoeba castellanii desaturase sequences

Gene	Nucleotide bp	SEQ ID NO:
12Ac (Δ-12/ Δ15-Desaturase from Acanthamoeba)	1224 bp	19, 21
8Ac (Δ-8-Desaturase from Acanthamoeba)	1374 bp	3, 5
5Ac (Δ-5-Desaturase from Acanthamoeba)	1353 bp	15

Example 4: Cloning of the PUFA specific desaturases from Perkinsus marinus (= SEQ ID NO: 7, 17 and 23)

Perkinsus marinus, which belongs to the Protista, is a parasite in seashells. P. marinus is able to synthesize LCPUFAs such as arachidonic acid (20:4). The LCPUFAs are produced according to the present work over the Δ-8-/Δ-5-fatty acid pathway (see figure 1).

P. marinus was grown at 28°C as disclosed by La Peyre et al. (J: Eurkaryot. Microbiol. 1993, 40: 304 – 310).

The total mRNA was isolated from said harvested cells with the aid of the RNeasy plant mini Kit (Qiagen). cDNA was synthesized from the total mRNA with the SMART RACE cDNA amplification kit (Clontech) according to the instructions of the manufacturer.

For the isolation of new desaturase genes the following degenerated primers were used for the amplification:

Deg1:

5'- GGITGG(C/T/A)TIGGICA(T/C) GA(T/C)(GT) (CT)I(GT) (GC)ICA-3'

Deg2:

5'- GG(A/G)AA(TCGA)AG(A/G)TG(A/G)TG(T/C)TC(A/G/T)AT(T/C)TG-3'

The aforementioned primers were used for the amplification in combination with the 3'-adapter-primer of the SMART RACE cDNA amplification kit.

The following protocol was used for the amplification:

d) 2 min at 95°C,

e) 30 sec at 94 °C
30 sec at 55-72°C
2 min at 72 °C
Number of cycles: 30

f) 10 min at 72 °C

PCR amplicons were cloned and sequenced according to the instructions of the manufacturer (pTOPO, Invitrogen). The sequence information was used for the production of full-length clones. For the cloning of the full-length clones 5'- and 3'-specific primers were synthesized. Said primers were used for the amplification in the SMART RACE cDNA amplification kit (Clontech) and the amplicons werecloned into the pTOPO vector (Invitrogen)Three sequences were identified, which show low similarities to desaturase genes.

Table 2: Perkinsus marinus desaturase sequences

Gene	**Nucleotide bp**	**SEQ ID NO:**
12Pm (Δ-12 -Desaturase from Perkinsus)	1254 bp	23
8Pm (Δ-8-Desaturase from Perkinsus)	1236 bp	7
5Pm (Δ-5-Desaturase from Perkinsus)	1374bp	17

Example 5: Cloning of expression plasmids for the heterologous expression of A. castellanii and P. marinus genes in yeasts

For the heterologous expression in yeasts the respective sequences were PCR amplified and with the restriction enzymes KpnI-SacI the resulting sequences were cloned into the yeast vector pYES2 (Invitrogen). For the amplification specific primers (see table 3 below) were used. Only the open reading frames of the PUFA genes were amplified. In addition restriction cleavage sides were attached to the nucleic acid sequences. At the 5'-end a KpnI side and a so named Kozak sequence (Cell, 1986, 44: 283 – 292) was added. To the 3'-end a SacI side was attached.

Table 3: Primers for the amplification of the nucleic acid sequences of the desaturases

Gen	**bp**	**primer**	**SEQ ID NO:**
12Ac	1224	Fwd : **GGTACC**ATGGCGATCACGACGACGCAGACAC	25
		Rvs : **GAGCTCC**TAGTGGGCCTTGCCGTGCTTGATCTCC	26
8Ac	1374	Fwd : **GGTACC**ATGGTCCTCACAACCCCGGCCCTC	27
		Rvs : **GGAGCT**CTCAGTTCTCAGCACCCATCTTC	28
5Ac	1353	Fwd: **GGTACC**ATGGCCACCGCATCTGCATC	29
		Rvs: **GGAGCT**TTAGCCGTAGTAGGCCTCCTT	30
9Ac	891	Fwd : **GGTACC**ATGGCGGCTGCGACGGCGAC	31
		Rvs: **GGAGCT**TTAGTCGTGCTTCCTCTTGGG	32
12Pm	1254	Fwd : **GGTACC**ATGACCCAAACTGAGGTCCA	33
		Rvs: **GGAGCT**CTAACGAGAAGTGCGAGCGT	34
8Pm	1236	Fwd : **GGTACC**ATGTCTTCTCTTACCCTCTA	35
		Rvs: **GGAGCT**CTATTCCACTATGGCAACAG	36
5Pm	1374	Fwd : **GGTACC**ATGACTACTTCAACCACTAC	37
		Rvs: **GGAGCT**CTACCTAGCAAGCAATCTCT	38

Composition of the PCR mix (50 µl)

5,00 µL	Template cDNA
5,00 µL	10x Puffer (Advantage-Polymerase)+ 25mM $MgCl_2$
5,00 µL	2mM dNTP
1,25 µL	each primer (10 pmol/µL of the 5'-ATG as well as of the 3'-stopp primer)
0,50 µL	Advantage polymerase

The Advantage polymerase from Clontech was employed.

PCR protocol

Addition temperature: 1 min at 55 °C
Denaturing temperature: 1 min at 94 °C
Elongation temperature: 2 min at 72 °C
Number of cycles: 35

The PCR products and the vector pYES2 were incubated with the restriction enzymes KpnI and SacI for 1 h at 37°C. Afterwards a ligation reaction was done with the Rapid Ligation Kit (Roche) according to the instructions of the manufacturer. The reaction mixture was than used for the transformation of E. coli DH5α cells (Invitrogen) again according to the instructions of the manufacturer. Positive clones were identified with PCR (reaction scheme as described above). The plasmid DNA was isolated (Qiagen Dneasy) and the resulting plasmids were checked by sequencing and transformed with the lithium acetate method into the Saccharomyces strain W303-1A. As a control the plasmid pYES2 (vector without insert) was transformed in parallel. The transformed yeasts were selected on complete minimal dropout uracil medium (CMdum) agar plates supplemented with 2% glucose, but without uracil.

To express the genes from A. castellanii and P. marinus, precultures consisting of in each case 5 ml of CMdum dropout uracil liquid medium supplemented with 2% (w/v) raffinose, but without uracil were initially inoculated with the selected transformants and incubated for 2 days at 30°C and 200 rpm. Then, 5 ml of CMdum (without uracil) liquid medium supplemented with 2% of raffinose and 300 µM of various fatty acids were inoculated with the precultures to an OD_{600} of 0.05. Expression was induced by the addition of 2% (w/v) of galactose. The cultures were incubated for a further 96 hours at 22°C.

Example 6: Cloning of expression plasmids for the expression in plants

To transform plants, a further transformation vector based on pBIN19-35S (Bevan M. (1984) Binary Agrobacterium vectors for plant transformation. Nucl. Acids Res. 18:203) was generated. To this end, BamHI-XbaI cleavage sites were inserted at the 5' and 3' end of the coding sequences, using PCR. The corresponding primer sequences were derived from the 5' and 3' regions of the respective nucleic acid sequence (see table 4).

Table 4: Primers for the expression in plants

Gen	**bp**	**primer**	**SEQ ID NO:**
12Ac	1224	Fwd : **GGATCC**ACCATGGCGATCACGACGACGCAGACAC	39
		Rvs : **GGTCTAGA**CTAGTGGGCCTTGCCGTGCTTGATCTCC	40
8Ac	1374	Fwd : **GGATCC**AGGATGGTCCTCACAACCCCGGCCCTC	41
		Rvs : **GGTCTAGA**TCAGTTCTCAGCACCCATCTTC	42
5Ac	1353	Fwd: **GGATCC**ATGGCCACCGCATCTGCATC	43
		Rvs: **GGTCTAGA**TTAGCCGTAGTAGGCCTCCTT	44
9Ac	891	Fwd : **GGATCC**ATGGCGGCTGCGACGGCGAC	45
		Rvs: **GGTCTAGA**TTAGTCGTGCTTCCTCTTGGG	46
12Pm	1254	Fwd : **GGATCC**ATGACCCAAACTGAGGTCCA	47
		Rvs: **GGTCTAGA**CTAACGAGAAGTGCGAGCGT	48
8Pm	1236	Fwd : **GGATCC**ATGTCTTCTCTTACCCTCTA	49
		Rvs: **GGTCTAGA**CTATTCCACTATGGCAACAG	50
5Pm	1374	Fwd : **GGATCC**ATGACTACTTCAACCACTAC	51
		Rvs: **GGTCTAGA**CTACCTAGCAAGCAATCTCT	52

Composition of the PCR mix (50 µl):

5.00 µl template cDNA
5.00 µl 10x buffer (Advantage polymerase)+ 25mM $MgCl_2$
5.00 µl 2mM dNTP
1.25 µl of each primer (10 pmol/µl)
0.50 µl Advantage polymerase

The Advantage polymerase from Clontech was employed.

PCR reaction conditions:
Annealing temperature: 1 min 55°C
Denaturation temperature: 1 min 94°C
Elongation temperature: 2 min 72°C
Number of cycles: 35

The PCR products as well as the vector pBin19-35S were incubated with the restriction enzymes BamHI and XbaI for 16 hours at 37°C. Afterwards a ligation reaction was done with the Rapid Ligation Kit (Roche) according to the instructions of the manufacturer. The reaction mixture was than used for the transformation of E. coli DH5α cells (Invitrogen) again according to the instructions of the manufacturer. Positive clones were identified with PCR (reaction scheme as described above) and the plasmid DNA was isolated (Qiagen Dneasy). The resulting plasmids were checked by sequencing and transformed by electroporation into *Agrobacterium tumefaciens* GC3101. Afterwards the transformants were plated on 2% YEB Medium agar plates with kanamycin. Kanamycin tolerant cells were picked and used for the transformation of Arabidopsis thaliana.

Example 7: Expression of A. castellanii and P. marinus genes in yeasts

Yeasts which had been transformed with the plasmids pYES2, pYES-12Ac, pYES-8Ac, pYES2-5Ac, pYES2-9Ac, pYES2-12Pm, pYES2-8Pm and pYES2-5Pm as described in Example 5 were analyzed as follows:

The yeast cells from the main cultures were harvested by centrifugation (100 x g, 5 min, 20°C) and washed with 100 mM $NaHCO_3$, pH 8.0 to remove residual medium and fatty acids. Starting with the yeast cell sediments, fatty acid methyl esters (FAMEs) were prepared by acid methanolysis. To this end, the cell sediments were incubated for one hour at 80°C together with 2 ml of 1 N methanolic sulfuric acid and

2% (v/v) of dimethoxypropane. The FAMEs were extracted twice with petroleum ether (PE). To remove nonderivatized fatty acids, the organic phases were washed in each case once with 2 ml of 100 mM $NaHCO_3$, pH 8.0 and 2 ml of distilled water. Thereafter, the PE phases were dried with Na_2SO_4, evaporated under argon and taken up in 100 µl of PE. The samples were separated on a DB-23 capillary column (30 m, 0.25 mm, 0.25 µm, Agilent) in a Hewlett-Packard 6850 gas chromatograph equipped with flame ionization detector. The conditions for the GLC analysis were as follows: the oven temperature was programmed from 50°C to 250°C with a rate of 5°C/min and finally 10 min at 250°C (holding).

The signals were identified by comparing the retention times with corresponding fatty acid standards (Sigma). The methodology is described for example in Napier and Michaelson, 2001, Lipids. 36 (8):761-766; Sayanova et al., 2001, Journal of Experimental Botany. 52 (360):1581-1585, Sperling et al., 2001, Arch. Biochem. Biophys. 388 (2):293-298 and Michaelson et al., 1998, FEBS Letters. 439 (3):215-218.

Example 8: Functional characterization of the genes of A. castellanii

The substrate activity and specificity of the genes were determined after expression and after feeding various fatty acids. The substrate specificity of the desaturases after expressions in yeasts can be determined by feeding various different fatty acids. Specific examples for the determination of the specificity and activity are disclosed for example in WO 93/11245, WO 94/11516, WO 93/06712, US 5,614,393, US5614393, WO 96/21022, WO0021557 und WO 99/27111, Qiu et al. 2001, J. Biol. Chem. 276, 31561-31566 for Δ4-desaturases, Hong et al. 2002, Lipids 37,863-868 for Δ5-desaturases. WO2005/012316 teaches such a method for example in example 18 in more detail.

a) Characterization of the gene 12Ac:

First the construct pYES-12Ac was tested in yeasts without feeding fatty acids. Astonishingly it was shown in comparison to the control vector pYES2 (vector without insert) that even without feeding fatty acids new fatty acids are detectable in the yeasts (Figure 2 A and B).

Figure 2 A and B show a comparison of the fatty acid profile between the control (construct pYES2 without insert, Figure 2A) and the construct pYES2-12Ac (Figure 2B), which contains the Acanthamoeba castellanii gene for the Δ-12-/Δ-15-desaturase. The fatty acids are marked. The new fatty acids synthesized are in case of construct pYES2-12Ac (2B) the fatty acids C16:2, C16:3, C18:2 and C18:3, whereas the unusual fatty acids 16:2n-4 and 16:3n-1 are formed for the C16 fatty acids. For the C18 fatty acids linoleic and linolenic acid (18:2n-6 and 18:2n-3) are formed.

According to the new synthesized fatty acids it is possible to identify the gene product of the nucleic acid sequence as a Δ-12-desaturase. The enzyme is able to desaturate C18:1 and C16:1 as substrate to the corresponding C18:2 and C16:2 fatty acids. The conversion rate of C18:1 (40,0%) is higher than the rate of the C16:1 (15,8%) conversion. That means the conversion rate of C18:1 is more than double than the conversion rate of the C16:1.

The conversion rate of the desaturase was calculated according to the following formula:

$$\frac{\text{Substrate}}{(\text{Substrate} + \text{Product}) \times 100}$$

The result of the formula is given as percentage value.

Furthermore the enzyme shows in addition a clear Δ-15-desaturase-activity. That means also that products of the Δ-12-desaturase reaction, which are C16:2 and/or C18:2 are further desaturated to C16:3 and/or C18:3.

b) Characterization of the gene 8Ac:

According to different sequence alignments (Blast) performed with the sequence SEQ ID NO: 3 (8Ac sequence) with different data bases (NCBI-BLAST: http://www.ncbi.nlm.nih.gov/BLAST/) the encoded protein sequence is most likely a putative Δ-5-desaturase.

Sequences with significant similarities	(bits)	Value
gi|16033740|gb|AAL13311.1| delta-5 fatty acid desaturase [P...	176	1e-42
gi|50882495|gb|AAT85663.1| polyunsaturated fatty acid delta...	170	6e-41
gi|4150956|dbj|BAA37090.1| delta 5 fatty acid desaturase [D...	156	9e-37
gi|23894018|emb|CAD53323.1| delta 5 fatty acid desaturase [...	156	1e-36
gi|33466346|gb|AAQ19605.1| delta-4 fatty acid desaturase [E...	150	7e-35
gi|5263169|dbj|BAA81814.1| fatty acid desaturase [Dictyoste...	149	1e-34
gi|25956288|gb|AAN75707.1| delta 4-desaturase [Thraustochyt...	142	1e-32
gi|25956290|gb|AAN75708.1| delta 4-desaturase [Thraustochyt...	139	1e-31
gi|25956294|gb|AAN75710.1| delta 4-desaturase [Thraustochyt...	139	1e-31
gi|25956292|gb|AAN75709.1| delta 4-desaturase [Thraustochyt...	138	2e-31
gi|20069125|gb|AAM09688.1| delta-4 fatty acid desaturase [T...	138	3e-31
gi|39545945|gb|AAR28035.1| delta-5 desaturase [Mortierella ...	136	9e-31
gi|3859488|gb|AAC72755.1| delta-5 fatty acid desaturase [Mo...	135	2e-30
gi|41017070|sp|O74212|FAD5_MORAP Delta-5 fatty acid desatur...	130	7e-29
gi|48854274|ref|ZP_00308437.1| COG3239: Fatty acid desatura...	114	4e-24
gi|48854276|ref|ZP_00308439.1| COG3239: Fatty acid desatura...	114	7e-24

According to this putative activity different fatty acids were fed (18:2, 18:3, 20:3n-6, 20:4n-3). None of said fatty acids were desaturated by the enzyme. This result clearly shows that the protein encoded by the 8Ac gene has neither a Δ-5-desaturase activity nor a Δ-6-desaturase activity.

Unexpectedly after feeding of the fatty acids 20:2n-6 und 20:3n-3 it could be shown, that the 8Ac sequence encodes a Δ-8-desaturase (see figures 3 A, 3 B, 4 A and 4 B).

Figure 3 A and B shows the fatty acid profile of yeasts transformed with the construct pYES2 as control (Figure 3 A) and pYES2-8Ac (Figure 3 B) and fed with the fatty acid C20:$2^{\Delta 11,14}$. The respective fatty acids are market.

Figure 4 A and B shows the fatty acid profile of yeast transformed with the construct pYES2 (Figure 4 A) as control and pYES2-8Ac (Figure 4 B) and fed with the fatty acid C20:$3^{\Delta 11,14,17}$. The respective fatty acids are market.

The protein encoded by 8Ac sequence is therefore a Δ-8-desaturase. The conversion rates for the fatty acids C20:2 and C20:3 are 15,2% and 17,5% respectively. This is absolutely astonishing as the 8Ac sequence, which has some similarities to "front-end" desaturases, has a different conserved region of the characteristic Cyt b5 motiv His-Pro-Gly-Gly (HPGG), which is necessary for building the Heme domain. In general mutations in said domain lead to depletion of the enzymatic acitivty (Sayanova et al. 1999, Plant Physiol 121(2):641-646). The amino acid sequence of this new Δ-8-desaturase shows unexpected differences to known "front-end" desaturases. Instead of the HPGG motive this desaturase shows the motive HPAG, which is due to an alanine in position 44 of the sequence. Sayanova et al. 1999, Plant Physiol 121(2):641-646 has shown that such a change of the motive from HPPG to HPAG leads to inactive enzymes. Therefore the activity of the new Δ-8-desaturase is even more astonishing.

For the further improvement of the activity of the Δ-8-desaturase, the sequence of the enzyme was mutagenized. The following primer.

8AcMf CAAGTACCACCCGGGCGGCAGCAGGGCCA and
8AcMr TGGCCCTGCTGCCGCCCGGGTGGTACTTG

were used together with the site directed mutagenesis Kit (Stratagene) for the mutagenesis according to the instructions of the manufacturer of the Δ-8-desaturase. The mutagenesis was afterwards checked by sequencing. Due to the mutagenesis the nucleotide sequences 124-CACCCGGCCGGC was changed to 124-CACCCGGGCGGC, which leads to a change from Alanine to Glycine in position 44 of the nucleic acid sequence shown in SEQ ID NO: 3. The resulting sequence is shown in SEQ ID NO: 5. As already described for the sequence of 8Ac the mutated sequence 8AcM was also cloned into the vector pYES2 and transformed into yeast. Yeast transformed either with the vector pYES-8Ac or pYES2-8AcM were grown and fed in parallel with different fatty acids (see table 5). The results of the feeding are shown in table 5. The mutated enzyme 8AcM shows in comparison to the wild type enzyme 8Ac an increased activity towards the fatty acid C20:2. This is a two fold increase of the activity. The mutation has no influence of the activity with the fatty acid

C20:3 as substrate. This clearly shows that with the mutation the activity of the Δ-8-desaturase can be influenced in a very specific manner.

Table 5. Fatty acid conversion rate of yeasts transformed with pYES-8Ac or pYES2-8AcM

Plasmid	**Fatty acid C20:2**	**Fatty acid C20:3**
pYES-8Ac	15,2 %	17,5 %
pYES2-8AcM	30,0 %	17,2 %

The mutated Δ-8-desaturase 8AcM and its derivatives are especially useful alone or in combination with the Δ-12- and Δ-15-desaturase, the Δ-9-elongase and the Δ-5-desaturase for the synthesis of arachidonic acid.

c) Characterization of the gene 5Pm:

The constructs pYES2 and pYES-5Pm were transformed into yeasts grown in paralell as described. Afterwards 250 µM of different fatty acids were fed. During this feeding experiments it can be shown that fatty acids such as C16:0, C16:1, C18:0, C18:1, C18:2n-6, C20:2n-6 or C22:4n-6 are not desaturated by the protein encoded by the 5Pm sequence. Whereas the substrate C20:3n-6 was desaturated by the enzyme (see figures 5 A and 5 B). Figures 5 A and 5 B clearly shows that the enzyme produces arachidonic acid during the transformation of the fatty acid substrate C20:3n-6. No new fatty acid is produced by the control (Figure 5 A). The desaturation of the fatty acid substrate C20:3n-6 to arachidonic acid is due to a Δ-5-desaturase activity, which is encoded by the 5Pm sequence (SEQ ID NO: 17). The conversion rate calculated according to the equation mentioned above is 15,4%.

Figure 5 A and 5 B shows the comparison of the fatty acid profile of yeasts transformed with the construct pYES2 as control and fed with the fatty acid C20:3n-6 (Figure 5 A) and with the construct pYES2-5Pm fed with the fatty acid C20:3n-6 (Figure 5 B). The fatty acids are marked. The new synthesized fatty acid is C20:4n-6 (arachidonic acid).

d) Characterization of the genes 5Ac, 9Ac, 12Pm und 8Pm:

According to sequence comparisons it was able to identify the sequences 5Ac, 12Pm and 8Pm as desaturases having a Δ-5-desaturase, Δ-12-desaturase and Δ-8-desaturase activity. For the sequence 9Ac we were able to show a Δ-9-elongase activity.

In combination with the 12Ac and 8Ac gene the complete set of enzymes from A. castellanii, which is necessary for the synthesis for arachidonic (C20:4n-6) or eicosapentaenoic acid could be identified. In addition further genes for the synthesis of said aforementioned fatty acids are isolated from P. marinus. With the aid of said genes the PUFA and/or LCPUFA content can be further improved. For the synthesis of arachidonic acid or eicosapentaenoic acid said genes can be introduced in plants or microorganism (see example 8).

Example 8: Generation of transgenic plants

a) Generation of transgenic oilseed rape plants (modified method of Moloney et al., 1992, Plant Cell Reports, 8:238-242)

Binary vectors in Agrobacterium tumefaciens C58C1:pGV2260 or Escherichia coli (Deblaere et al, 1984, Nucl. Acids. Res. 13, 4777-4788) can be used for generating transgenic oilseed rape plants. To transform oilseed rape plants (Var. Drakkar, NPZ Nordeutsche Pflanzenzucht, Hohenlieth, Germany), a 1:50 dilution of an overnight culture of a positively transformed agrobacterial colony in Murashige-Skoog medium (Murashige and Skoog 1962 Physiol. Plant. 15, 473) supplemented with 3% sucrose (3MS medium) is used. Petiols or hypocotyls of freshly germinated sterile oilseed rape plants (in each case approx. 1 cm^2) are incubated with a 1:50 agrobacterial dilution for 5-10 minutes in a Petri dish. This is followed by 3 days of coincubation in the dark at 25°C on 3MS medium supplemented with 0.8% Bacto agar. The cultures are then grown for 3 days at 16 hours light/8 hours dark and the cultivation is continued in a weekly rhythm on MS medium supplemented with 500 mg/l Claforan (cefotaxim sodium), 50 mg/l kanamycin, 20 μM benzylaminopurine (BAP), now supplemented with 1.6 g/l of glucose. Growing shoots are transferred to MS medium supplemented with 2% sucrose, 250 mg/l Claforan and 0.8% Bacto agar. If no roots develop after three weeks, 2-indolebutyric acid was added to the medium as growth hormone for

rooting.

Regenerated shoots are obtained on 2MS medium supplemented with kanamycin and Claforan; after rooting, they are transferred to compost and, after growing on for two weeks in a controlled-environment cabinet or in the greenhouse, allowed to flower, and mature seeds are harvested and analyzed by lipid analysis for elongase and/or desaturasse expression, such as Δ-12- and Δ-15-desaturase, Δ-8-desaturase, Δ-9-elongase or Δ-5-desaturase activity. In this manner, lines with elevated contents of PUFAs and/or LCPUFAs can be identified.

b) Generation of transgenic linseed plants

Transgenic linseed plants can be generated for example by the method of Bell et al., 1999, In Vitro Cell. Dev. Biol.-Plant. 35(6):456-465 by means of particle bombardment. In general, linseed was transformed by an agrobacteria-mediated transformation, for example by the method of Mlynarova et al. (1994), Plant Cell Report 13: 282-285.

c) Generation of transgenic Arabidopsis plants

Binary plasmids were transferred to A. tumefaciens strainGV3101 by electroporation and kanamycin-resistant colonies were selected in all cases. Wildtype Col0 or transgenic line CA1-9, containing the coding region of *I. galbana* elongating activity, *IgASE1* [Qi, B., Beaudoin, F., Fraser, T., Stobart, A.K., Napier, J.A. and Lazarus, C.M. (2002) Identification of a cDNA encoding a novel C18-D9 polyunsaturated fatty acid-specific elongating activity from the docosahexaenoic acid (DHA)-producing micro-alga, *Isochrysis galbana*. *FEBS Lett*. 510, 159-65] was used as the host for transformation with A. castellanii Δ^8–desaturase gene. *A.* tumefaciens–mediated transformation was performed as described in Bechthold et al. [(1993) In planta Agrobacterium-mediated gene transfer by infiltration of Arabidopsis thaliana plants. C.R. Acad. Sci. Ser. III Sci.Vie., 316, 1194-1199.] and seeds from dipped plants were spread on Murashige and Skoog medium containing 50 µg ml^{-1} kanamycin.

Example 9: Lipid extraction from leafs

The effect of the genetic modification in plants, fungi, algae, ciliates or on the

production of a desired compound (such as a fatty acid) can be determined by growing the modified microorganisms or the modified plant under suitable conditions (such as those described above) and analyzing the medium and/or the cellular components for the elevated production of desired product (i.e. of the lipids or a fatty acid). These analytical techniques are known to the skilled worker and comprise spectroscopy, thin-layer chromatography, various types of staining methods, enzymatic and microbiological methods and analytical chromatography such as high-performance liquid chromatography (see, for example, Ullman, Encyclopedia of Industrial Chemistry, Vol. A2, p. 89-90 and p. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) “Applications of HPLC in Biochemistry“ in: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 17; Rehm et al. (1993) Biotechnology, Vol. 3, Chapter III: “Product recovery and purification“, p. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F., and Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., and Henry, J.D. (1988) Biochemical Separations, in: Ullmann’s Encyclopedia of Industrial Chemistry, Vol. B3; Chapter 11, p. 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

In addition to the abovementioned processes, plant lipids are extracted from plant material as described by Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22):12935-12940 and Browse et al. (1986) Analytic Biochemistry 152:141-145. The qualitative and quantitative analysis of lipids or fatty acids is described by Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 pp. (Oily Press Lipid Library; 1); “Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) - 16 (1977) under the title: Progress in the Chemistry of Fats and Other Lipids CODEN.

One example is the analysis of fatty acids (abbreviations: FAME, fatty acid methyl ester; GC-MS, gas liquid chromatography/mass spectrometry; TAG, triacylglycerol; TLC, thin-layer chromatography).

The unambiguous detection for the presence of fatty acid products can be obtained by analyzing recombinant organisms using analytical standard methods: GC, GC-MS

or TLC, as described on several occasions by Christie and the references therein (1997, in: Advances on Lipid Methodology, Fourth Edition: Christie, Oily Press, Dundee, 119-169; 1998, Gaschromatographie-Massenspektrometrie-Verfahren [Gas chromatography/mass spectrometric methods], Lipide 33:343-353).

The material to be analyzed can be disrupted by sonication, grinding in a glass mill, liquid nitrogen and grinding or via other applicable methods. After disruption, the material must be centrifuged. The sediment is resuspended in distilled water, heated for 10 minutes at 100°C, cooled on ice and recentrifuged, followed by extraction for one hour at 90°C in 0.5 M sulfuric acid in methanol with 2% dimethoxypropane, which leads to hydrolyzed oil and lipid compounds, which give transmethylated lipids. These fatty acid methyl esters are extracted in petroleum ether and finally subjected to a GC analysis using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 µm, 0.32 mm) at a temperature gradient of between 170°C and 240°C for 20 minutes and 5 minutes at 240°C. The identity of the resulting fatty acid methyl esters must be defined using standards, which are available from commercial sources (i.e. Sigma).

Plant material is initially homogenized mechanically by comminuting in a pestle and mortar to make it more amenable to extraction.

This is followed by heating at 100°C for 10 minutes and, after cooling on ice, by resedimentation. The cell sediment is hydrolyzed for one hour at 90°C with 1 M methanolic sulfuric acid and 2% dimethoxypropane, and the lipids are transmethylated. The resulting fatty acid methyl esters (FAMEs) are extracted in petroleum ether. The extracted FAMEs are analyzed by gas liquid chromatography using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm) and a temperature gradient of from 170°C to 240°C in 20 minutes and 5 minutes at 240°C. The identity of the fatty acid methyl esters is confirmed by comparison with corresponding FAME standards (Sigma). The identity and position of the double bond can be analyzed further by suitable chemical derivatization of the FAME mixtures, for example to give 4,4-dimethoxyoxazoline derivatives (Christie, 1998) by means of GC-MS.

Leaf material from transgenic *Arabidopsis thaliana* Col0 and super-transformants of

transgenic line CA1-9 both transformed with the construct pBIN1935S-8Ac were analyzed ba gas chromatography of methyl ester derivates as described above. Identities were confirmed by GC-MS and co-migration with authentic standards. The conversion rates are shown in the following table 6:

5

Table 6: Conversion rate with AcD8 (delta-8-desaturase from Acanthamoeba castellanii) of different substrates

fatty acids	% of total fatty acids	% conversion of substrate
$20:2^{\Delta 11, 14}$	1.1	-
$20:3^{\Delta 8, 11, 14}$	1.9	63
$20:2^{\Delta 11, 14, 17}$	1.3	-
$20:2^{\Delta 8, 11, 14, 17}$	0.8	40

Figure 6 shows the result with the line CA1-9. In the double transgenic Arabidopsis a clear activity of Ac8 can be shown by the conversion of the present $20:2^{\Delta 11,14}$ or $20:3^{\Delta 11, 14, 17}$ into $20:3^{\Delta 8, 11, 14}$ or $20:4^{\Delta 8, 11, 14, 17}$, the precursors of arachidonic acid or eicosapentaenoic acid.

Additionally Acyl-CoA profiles were done from the Arabidopsis leaves of Arabidopsis wild type (Figure 7 A), Arabidopsis Δ9elo (Figure 7 B) and Arabidopsis Δ9eloΔ8des (Figure 7 C) using the method of Larson et al. [Plant J. 2002 Nov;32(4):519-27]. Results from the measurements are shown in Figure 7 and demonstrate again the functionality of 8Ac in plants.

Equivalents:

Many equivalents of the specific embodiments according to the invention described herein can be identified or found by the skilled worker resorting simply to routine experiments. These equivalents are intended to be within the scope of the patent claims.

DEMANDE OU BREVET VOLUMINEUX

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CECI EST LE TOME	1	DE	2
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JUMBO APPLICATIONS/PATENTS

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NOM DU FICHIER / FILE NAME :

NOTE POUR LE TOME / VOLUME NOTE:

What is claimed is:

1. A process for the production of arachidonic acid or eicosapentaenoic acid or arachidonic acid and eicosapentaenoic acid in transgenic plants that produce mature seeds with a content of at least 1 % by weight of said compounds referred to the total lipid content of said organism which comprises the following steps:

a) introduction of at least one nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-12-desaturase- and Δ-15-desaturase-activity, and

b) introduction of at least one second nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-9-elongase-activity, and

c) introduction of at least one third nucleic acid sequence in said transgenic plant, which encodes a polypeptide having a Δ-8-desaturase-activity, and

d) introduction of at least a one fourth nucleic acid sequence, which encodes a polypeptide having a Δ-5-desaturase-activity, and

e) cultivating and harvesting of said transgenic plant.

2. The process as claimed in claim 1, wherein the nucleic acid sequences which encode polypeptides having Δ-12-desaturase and Δ-15-desaturase activity, Δ-8-desaturase, Δ-9-elongase or Δ-5-desaturase activity are selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO:13, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 and SEQ ID NO: 23, and

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18,

SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 according to the degeneracy of the genetic code,

c) derivatives of the nucleic acid sequences depicted in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, , SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 which encode polypeptides having at least 50 % homology to the sequence as depicted in SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 and which polypeptides having Δ-12-desaturase and Δ-15-desaturase activity, Δ-8-desaturase, Δ-9-elongase or Δ-5-desaturase activity.

3. The process as claimed in claim 1 or 2, wherein the transgenic plant that produces mature seeds is an oilseed plant.

4. The process as claimed in claim 1 or 2, wherein the transgenic plant that produces mature seeds is selected from the group consisting of the plant families of Anacardiaceae, Asteraceae, Apiaceae, Boraginaceae, Brassicaceae, Cannabaceae, Elaeagnaceae, Euphorbiaceae, Fabaceae, Geraniaceae, Gramineae, Juglandaceae, Leguminosae, Linaceae, Lythrarieae, Malvaceae, Onagraceae, Palmae, Poaceae, Rubiaceae, Scrophulariaceae, Solanaceae, Sterculiaceae and Theaceae.

5. The process as claimed in claim 1 or 2, wherein the transgenic plant that produces mature seeds is selected from the group consisting of the plant genera of Pistacia, Mangifera, Anacardium, Calendula, Carthamus, Centaurea, Cichorium, Cynara, Helianthus, Lactuca, Locusta, Tagetes, Valeriana, Borago, Daucus, Brassica, Camelina, Melanosinapis, Sinapis, Arabadopsis, Orychophragmus, Cannabis, Elaeagnus, Manihot, Janipha, Jatropha, Ricinus, Pisum, Albizia, Cathormion, Feuillea, Inga, Pithecolobium, Acacia, Mimosa, Medicajo, Glycine, Dolichos, Phaseolus, Pelargonium, Cocos, Oleum, Juglans, Wallia, Arachis, *Linum,* Punica, Gossypium, Camissonia, Oenothera, Elaeis, Hordeum, Secale, Avena, Sorghum, Andropogon, Holcus, Panicum, Oryza, Zea, Triticum, Coffea,

Verbascum, Capsicum, Nicotiana, Solanum, Lycopersicon, Theobroma and Camellia.

6. The process as claimed in any of the claims 1 to 5, wherein the transgenic plant is selected from the group consisting of rapeseed, poppy, mustard, hemp, castor bean, sesame, olive, calendula, punica, hazel nut, maize, almond, macadamia, cotton, avocado, pumpkin, walnut, laurel, pistachio, primrose, canola, evening primrose, oil palm, peanut, linseed, soybean, safflower, marigold, coffee, tobacco, cacao, sunflower and borage.

7. The process as claimed in any of the claims 1 to 6, wherein the arachidonic acid or eicosapentaenoic acid or arachidonic acid and eicosapentaenoic acid is isolated in the form of their oils, lipids of free fatty acids.

8. The process as claimed in any of the claims 1 to 7, wherein arachidonic acid and eicosapentaenoic acid is produced in at least a 1:2 ratio.

9. The process as claimed in any of the claims 1 to 8, wherein the arachidonic acid and eicosapentaenoic acid are produced in a content of at least 5 % by weight referred to the total lipid content.

10. The process as claimed in any of the claims 1 to 9, wherein the Δ-12-desaturase- and Δ-15-desaturase used in the process desaturates C16 or C18-fatty acids having one double bond in the fatty acid chain or C16 and C18-fatty acids having one double bond in the fatty acid chain.

11. An isolated nucleic acid sequence which encodes a polypeptide having a Δ-12-desaturase and Δ-15-desaturase activity selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 22 which encode polypeptides having at least 40 % homology to the sequence as depicted in SEQ ID NO: 20, SEQ ID NO: 22 or SEQ ID NO: 24 and which polypeptides having Δ-12-desaturase and Δ-15-desaturase activity.

12. An isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-9-elongase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 11;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 12;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 11 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 12 and which polypeptides having Δ-9-elongase activity.

13. An isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-8-desaturase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 3, SEQ ID NO: 5 or SEQ ID NO: 7 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8 and which polypeptides having Δ-8-desaturase activity.

14. An isolated nucleic acid sequence comprising a nucleotide sequence which encodes a Δ-5-desaturase selected from the group consisting of

a) a nucleic acid sequence depicted in SEQ ID NO: 15 or SEQ ID NO: 17;

b) a nucleic acid sequence, which, as a result of the degeneracy of the genetic code, can be derived from a polypeptide sequence as depicted in SEQ ID NO: 16 or SEQ ID NO: 18;

c) derivatives of the nucleic acid sequence depicted in SEQ ID NO: 15 or SEQ ID NO: 17 which encode polypeptides having at least 70 % homology to the sequence as depicted in SEQ ID NO: 16 or SEQ ID NO: 18 and which polypeptides having Δ-5-desaturase activity.

15. A polypeptide encoded by an isolated nucleic acid sequence as claimed in claims 11 to 14.

16. A gene construct comprising an isolated nucleic acid having the sequence SEQ ID NO: 3, SEQ ID NO: 5, SEQ ID NO: 7, SEQ ID NO: 11, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21 or SEQ ID NO: 23 as claimed in claims 11 to 14, where the nucleic acid is functionally linked to one or more regulatory signals.

17. A gene construct as claimed in claim 16, whose gene expression is increased by the regulatory signals.

18. A vector comprising a nucleic acid as claimed in claims 11 to 14 or a gene construct as claimed in claim 17.

19. A transgenic plant comprising at least one nucleic acid as claimed in claims 11 to 14, a gene construct as claimed in claim 17 or a vector as claimed in claim 18.

20. The transgenic plant as claimed in claim 19, wherein the plant is an oilseed plant.

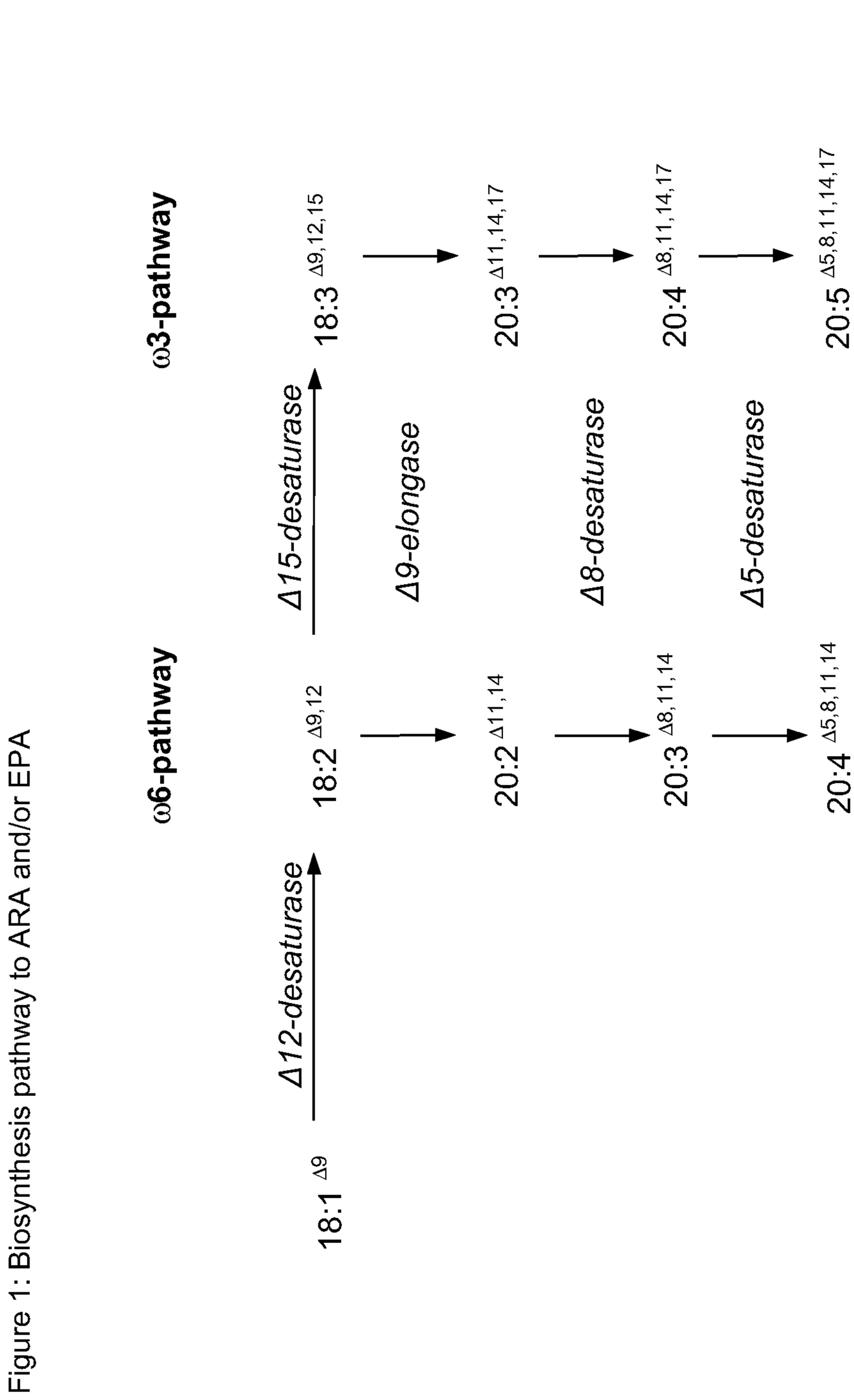


Figure 1: Biosynthesis pathway to ARA and/or EPA

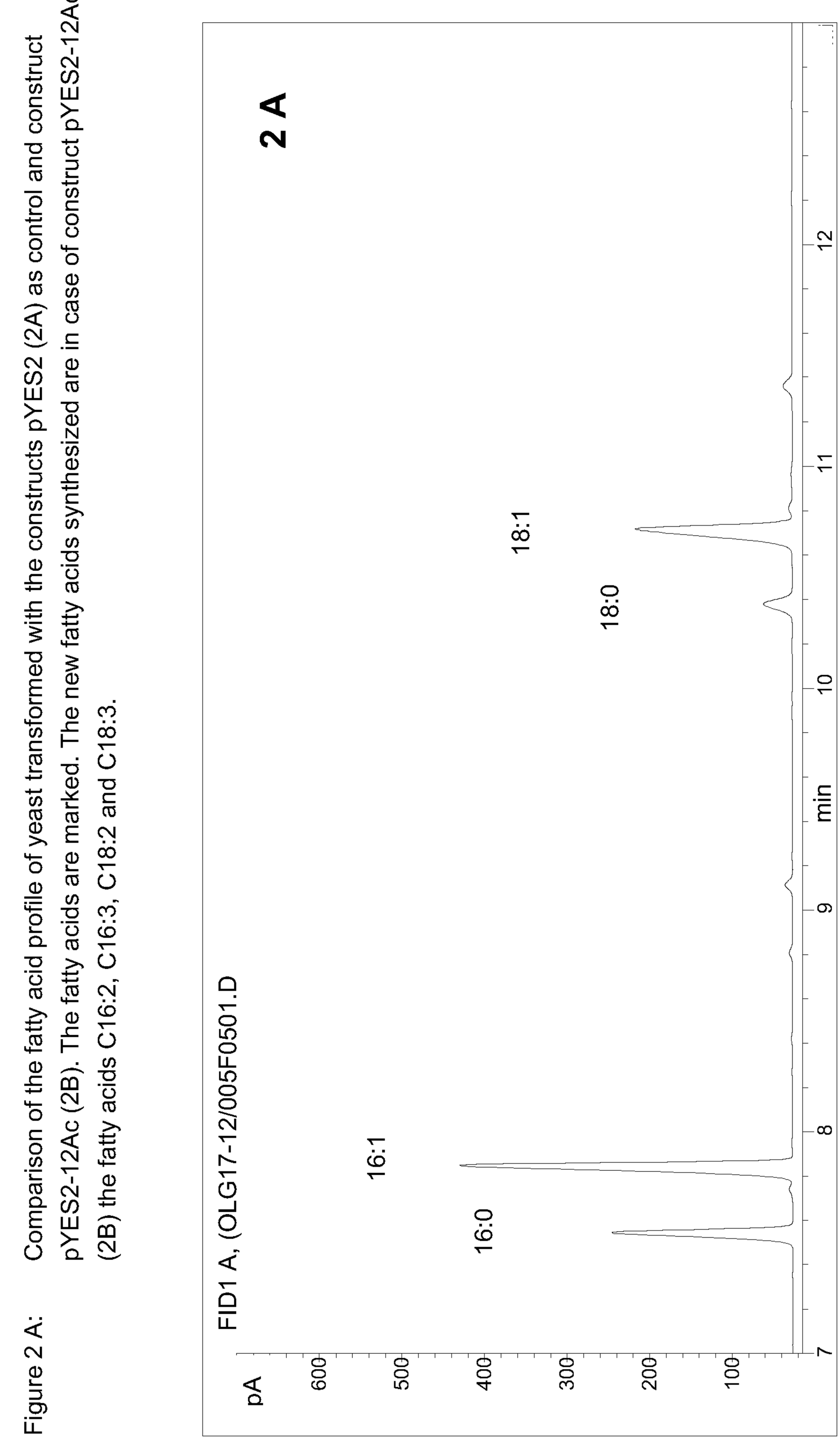


Figure 2 A: Comparison of the fatty acid profile of yeast transformed with the constructs pYES2 (2A) as control and construct pYES2-12Ac (2B). The fatty acids are marked. The new fatty acids synthesized are in case of construct pYES2-12Ac (2B) the fatty acids C16:2, C16:3, C18:2 and C18:3.

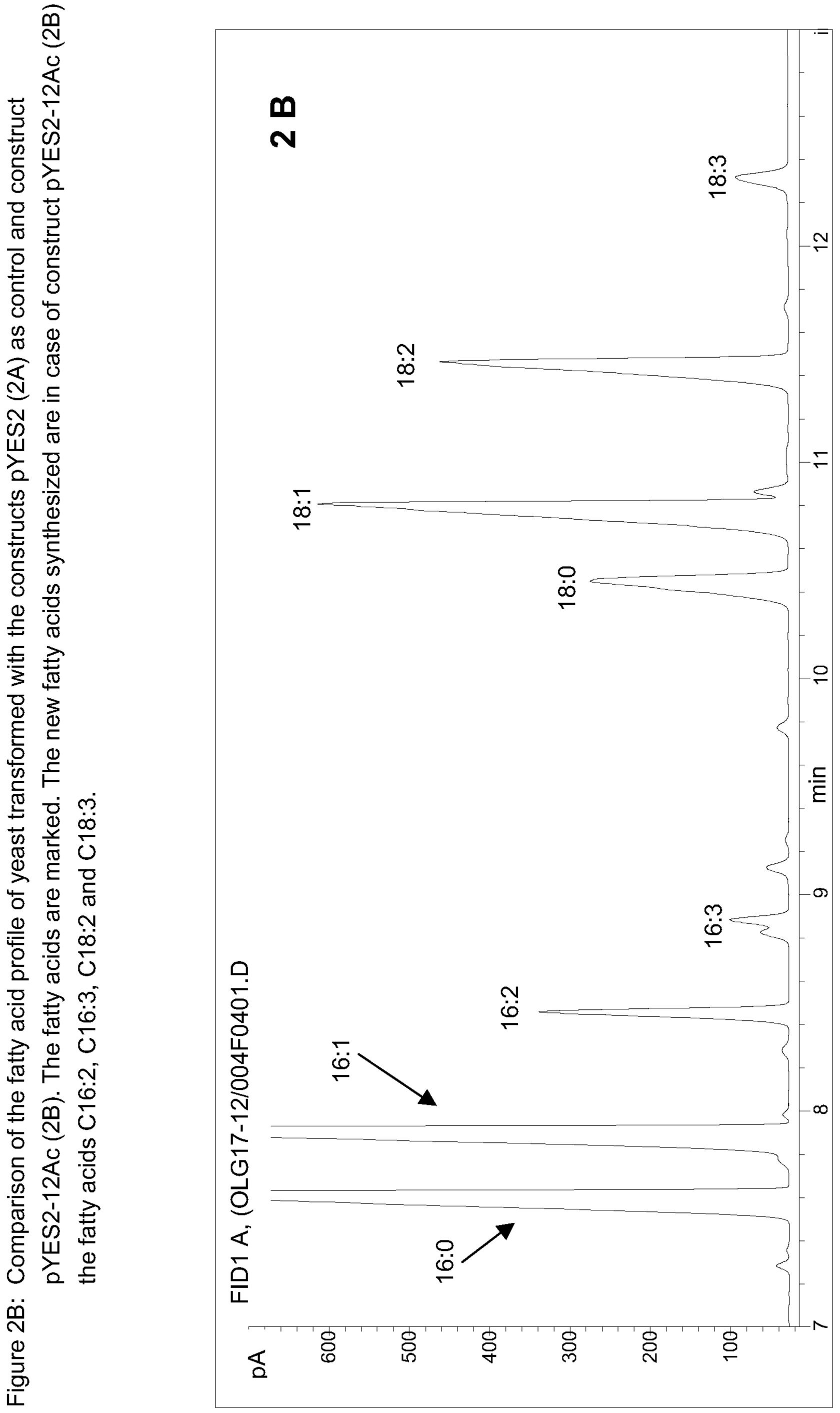


Figure 2B: Comparison of the fatty acid profile of yeast transformed with the constructs pYES2 (2A) as control and pYES2-12Ac (2B). The fatty acids are marked. The new fatty acids synthesized are in case of construct pYES2-12Ac (2B) fatty acids C16:2, C16:3, C18:2 and C18:3.

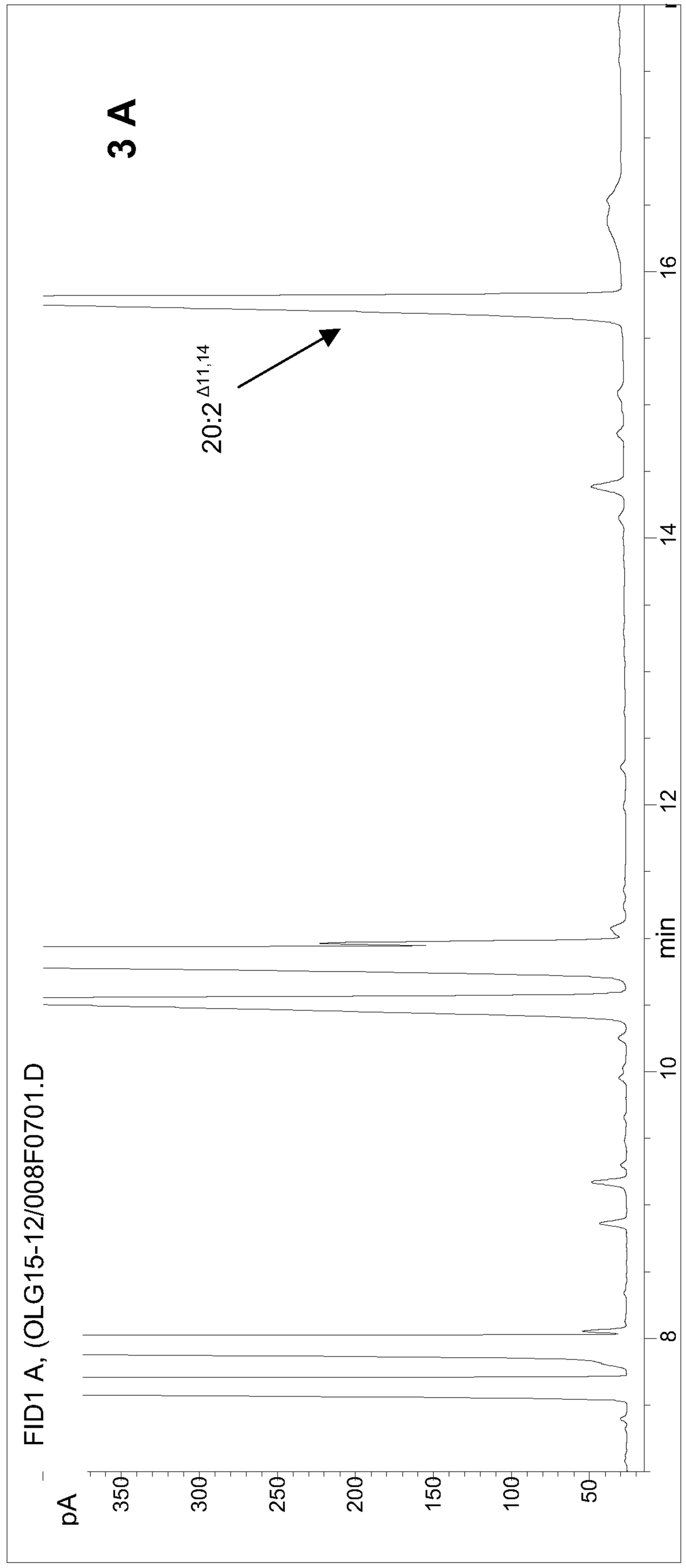


Figure 3 A: Fatty acid profile of yeasts transformed with the construct pYES2 as control (Figure 3 A) and pYES2-8Ac (Figure 3 B) and fed with the fatty acid C20:2$^{\Delta 11,14}$. The respective fatty acids are marked.

3 B

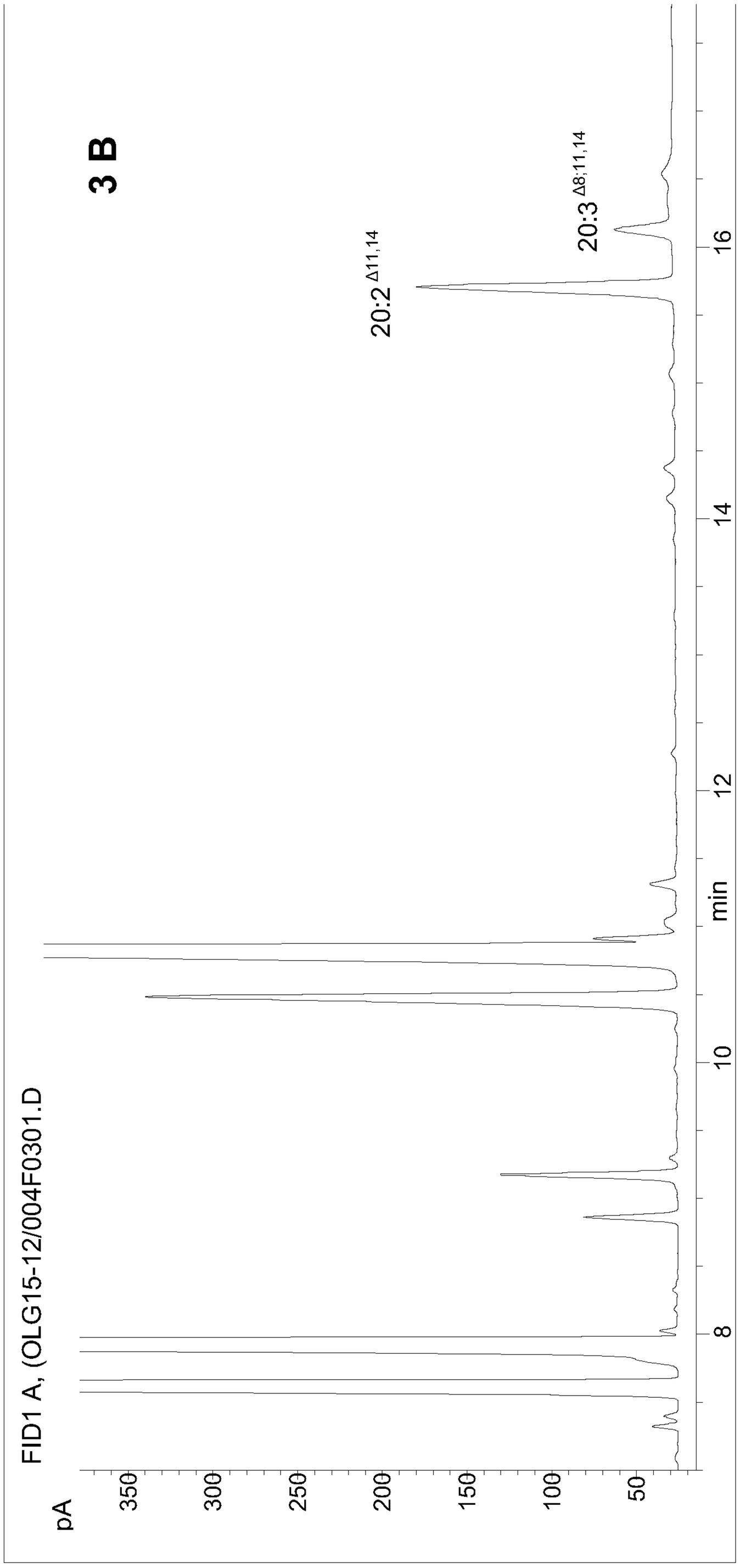


Figure 3 B: Fatty acid profile of yeasts transformed with the construct pYES2 as control (Figure 3 A) and pYES2-8Ac (Figure 3 B) and fed with the fatty acid C20:2$^{\Delta 11,14}$. The respective fatty acids are marked.

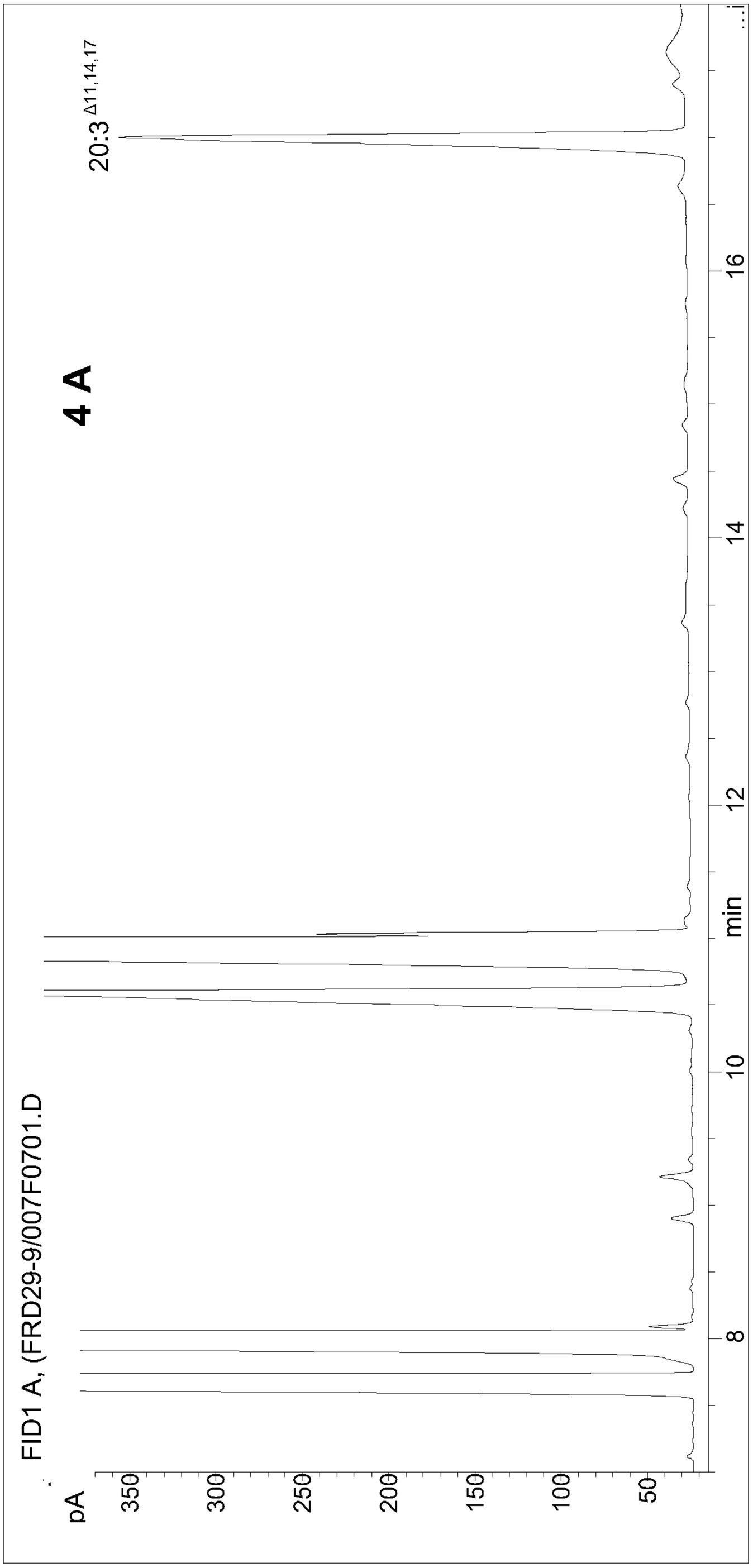
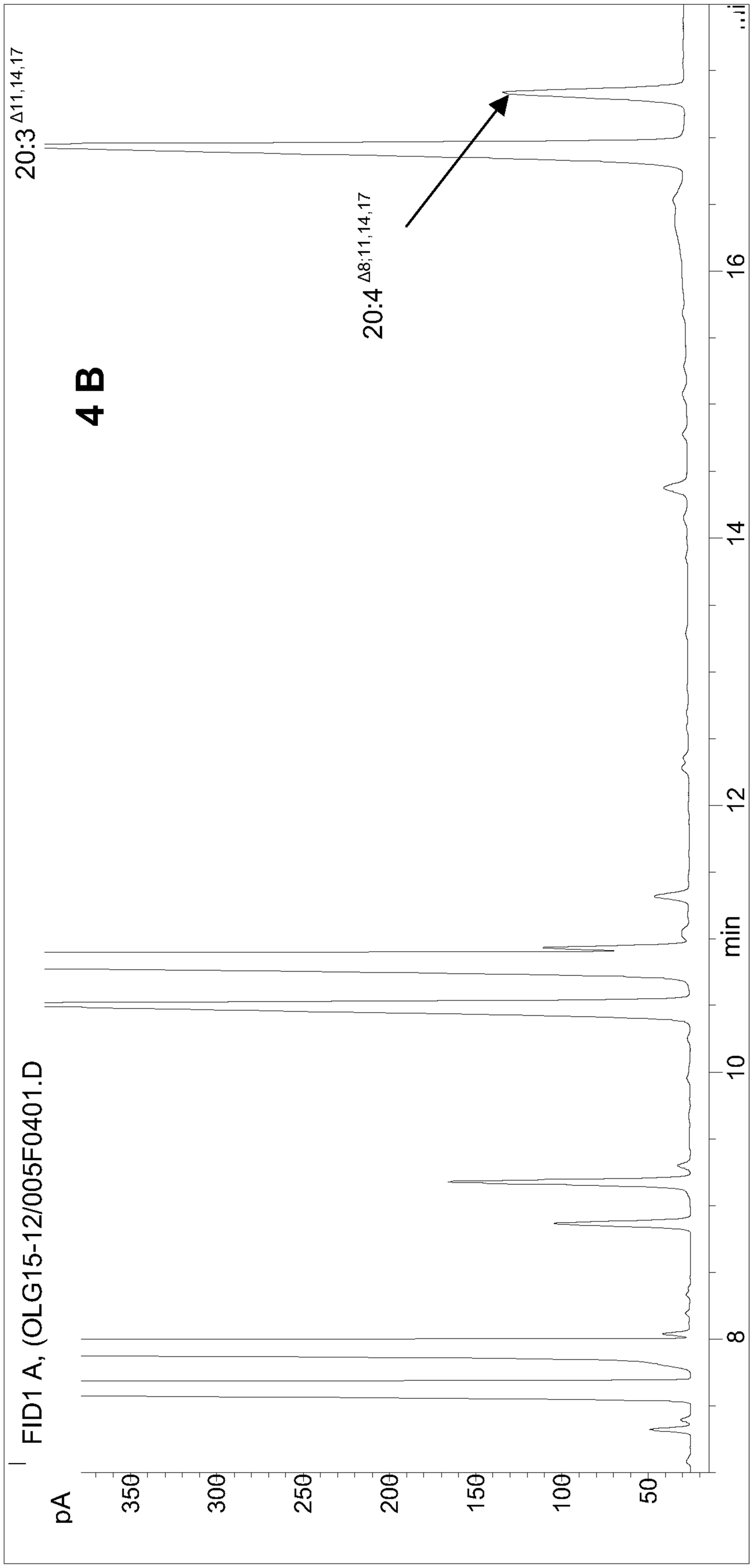


Figure 4 A: Fatty acid profile of yeast transformed with the construct pYES2 (Figure 4 A) as control and pYES2-8Ac (Figure 4 B) and fed with the fatty acid C20:3$^{\Delta 11,14,17}$. The respective fatty acids are market.

Figure 4 B: Fatty acid profile of yeast transformed with the construct pYES2 (Figure 4 A) as control and pYES2-8Ac (Figure 4 B) and fed with the fatty acid C20:3$^{\Delta 11,14,17}$. The respective fatty acids are market.



5 A

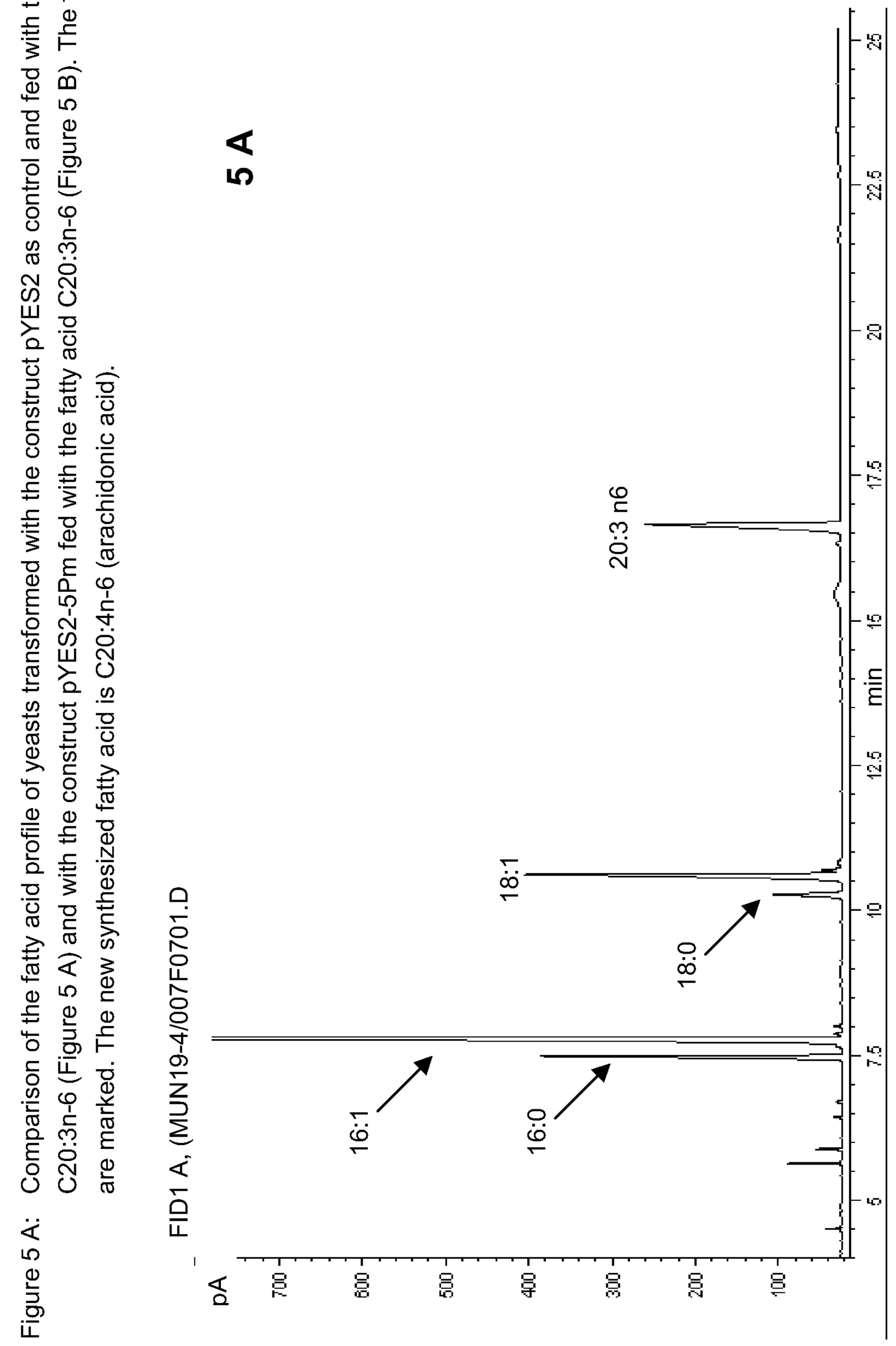


Figure 5 A: Comparison of the fatty acid profile of yeasts transformed with the construct pYES2 as control and fed with the fatty acid C20:3n-6 (Figure 5 B) and with the construct pYES2-5Pm fed with the fatty acid C20:3n-6 (Figure 5 A) and with the construct pYES2-5Pm fed with the fatty acid C20:3n-6 (Figure 5 B). The fatty acids are marked. The new synthesized fatty acid is C20:4n-6 (arachidonic acid).

5 B

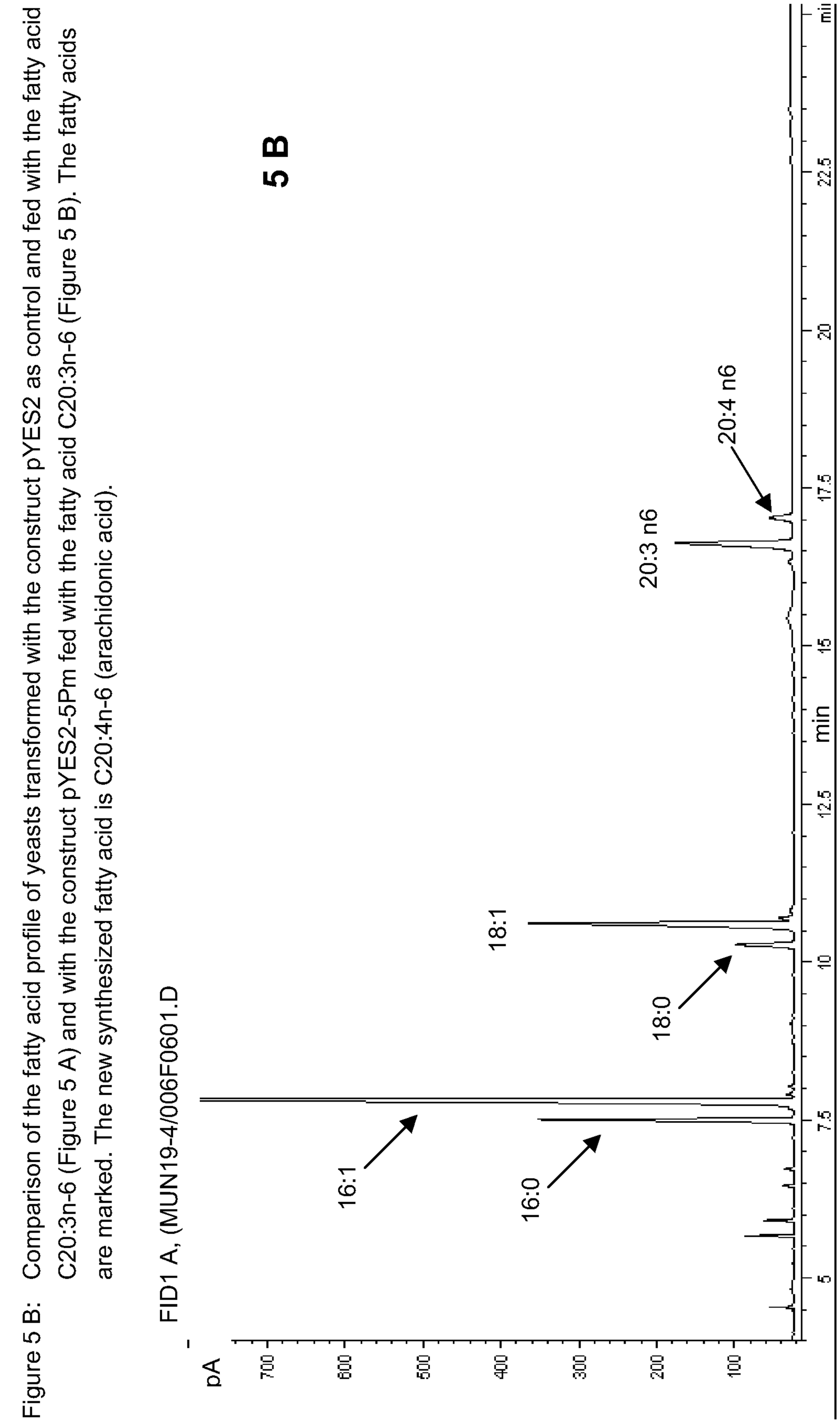


Figure 5 B: Comparison of the fatty acid profile of yeasts transformed with the construct pYES2 as control and fed with the fatty acid C20:3n-6 (Figure 5 A) and with the construct pYES2-5Pm fed with the fatty acid C20:3n-6 (Figure 5 B). The fatty acids are marked. The new synthesized fatty acid is C20:4n-6 (arachidonic acid).

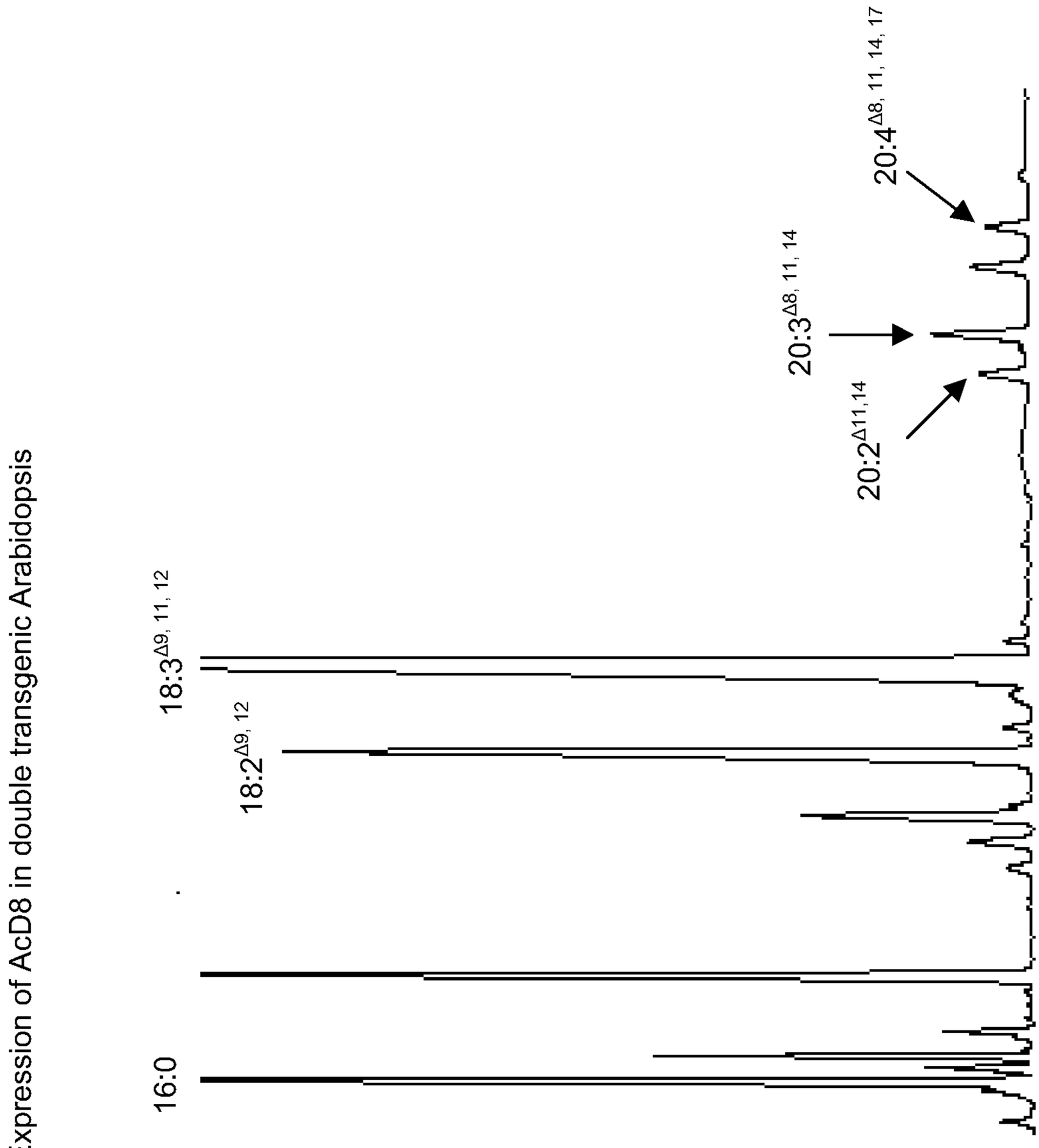


Figure 6: Expression of AcD8 in double transgenic Arabidopsis

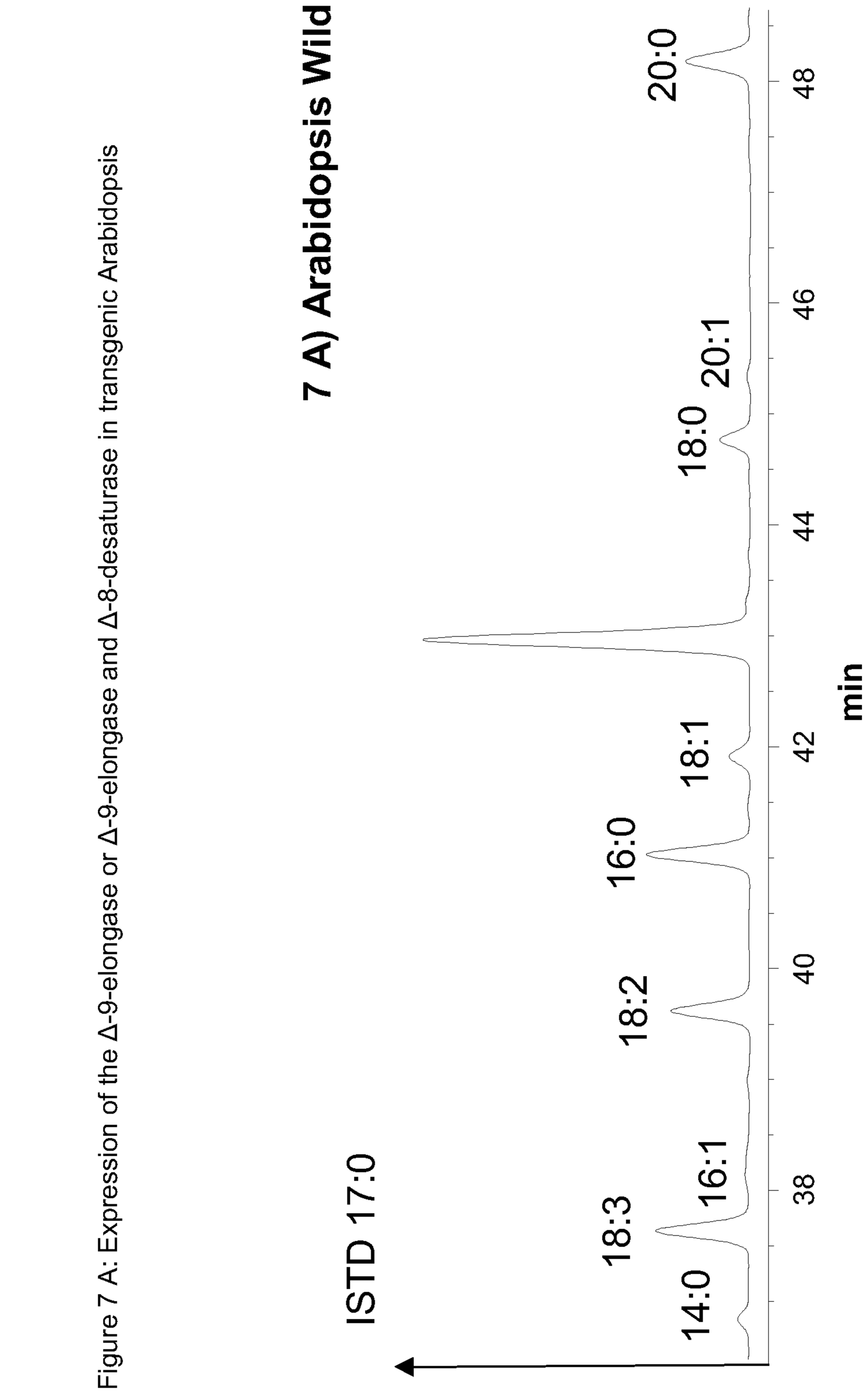


Figure 7 A: Expression of the Δ-9-elongase or Δ-9-elongase and Δ-8-desaturase in transgenic Arabidopsis

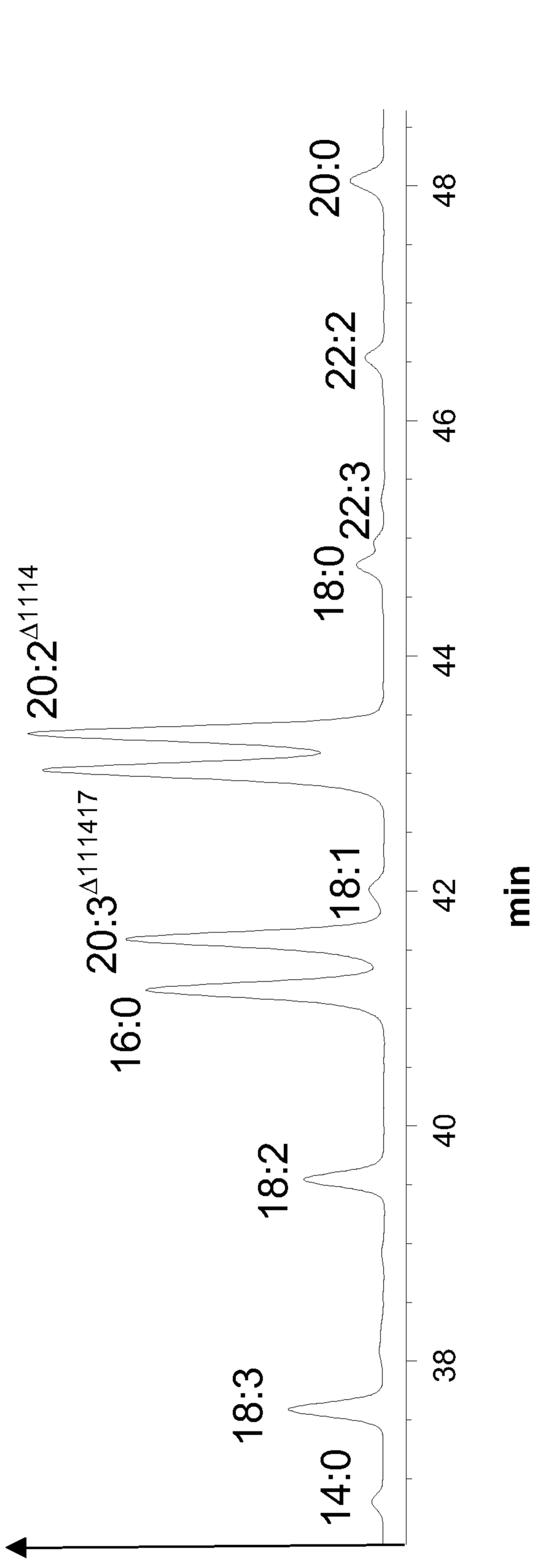


Figure 7 B: Expression of the Δ-9-elongase or Δ-9-elongase and Δ-8-desaturase in transgenic Arabidopsis

Figure 7 C: Expression of the Δ-9-elongase or Δ-9-elongase and Δ-8-desaturase in transgenic Arabidopsis

7 C) Arabidopsis Δ9eloΔ8des