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TRANSLATOR CERTIFICATION

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The document is designated as:

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Method for producing polyunsaturated fatty acids

[0001] The present invention relates to a process for the production of eicosapentaenoic acid, docosapentaenoic acid and/or docosahexaenoic acid in transgenic plants, providing in the plant at least one nucleic acid sequence which codes for a polypeptide having a Δ6-desaturase activity; at least one nucleic acid sequence which codes for a polypeptide having a Δ6-elongase activity; at least one nucleic acid sequence which codes for a polypeptide having a Δ5-desaturase activity; and at least one nucleic acid sequence which codes for a polypeptide having a Δ5-elongase activity, where the nucleic acid sequence which codes for a polypeptide having a Δ5-elongase activity is modified by comparison with the nucleic acid sequence in the organism from which the sequence is derived in that it is adapted to the codon usage in one or more plant species.

[0002] In a preferred embodiment there is additionally provision of further nucleic acid sequences which code for a polypeptide having the activity of an ω3-desaturase and/or of a Δ4-desaturase in the plant.

[0003] In a further preferred embodiment there is provision of further nucleic acid sequences which code for acyl-CoA dehydrogenase(s), acyl-ACP (acyl carrier protein) desaturase(s), acyl-ACP thioesterase(s), fatty acid acyl transferase(s), acyl-CoA:lysophospholipid acyl transferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, allene oxide synthases, hydroperoxide lyases or fatty acid elongase(s) in the plant.

[0004] The invention furthermore relates to recombinant nucleic acid molecules comprising at least one nucleic acid sequence which codes for a polypeptide having a Δ6-desaturase activity; at least one nucleic acid sequence which codes for a polypeptide having a Δ5-desaturase activity; at least one nucleic acid sequence which codes for a polypeptide having a Δ6-elongase activity; and at least one nucleic acid sequence which codes for a polypeptide having a Δ5-elongase activity and which is modified by comparison with the nucleic acid sequence in the organism from which the sequence originates in that it is adapted to the codon usage in one or more plant species.

[0005] A further part of the invention relates to oils, lipids and/or fatty acids which have been produced by the process according to the invention, and to their use.

[0006] Finally, the invention also relates to transgenic plants which have been produced by the process of the invention or which comprise a recombinant nucleic acid molecule of the invention, and to the use thereof as foodstuffs or feedstuffs.

[0007] Lipid synthesis can be divided into two sections: the synthesis of fatty acids and their binding to sn-glycerol-3-phosphate, and the addition or modification of a polar head group. Usual lipids which are used in membranes comprise phospholipids, glycolipids, sphingolipids and phosphoglycerides. Fatty acid synthesis starts with the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase or into acetyl-ACP by acetyl transacylase. After condensation reaction, these two product molecules together form acetoacetyl-ACP, which is converted via a series of condensation, reduction and dehydration reactions so that a saturated fatty acid molecule with the desired chain length is obtained. The production of the unsaturated fatty acids from these molecules is catalyzed by specific desaturases, either aerobically by means of molecular oxygen or anaerobically (regarding the fatty acid synthesis in

microorganisms, see F. C. Neidhardt et al. (1996) E. coli and Salmonella. ASM Press: Washington, D.C., p. 612-636 and references cited therein; Lengeler et al. (Ed.) (1999) Biology of Procaryotes. Thieme: Stuttgart, New York, and the references therein, and Magnuson, K., et al. (1993) Microbiological Reviews 57:522-542 and the references therein). To undergo the further elongation steps, the resulting phospholipid-bound fatty acids must be returned to the fatty acid CoA ester pool. This is made possibly by acyl-CoA:lysophospholipid acyltransferases. Moreover, these enzymes are capable of transferring the elongated fatty acids from the CoA esters back to the phospholipids. If appropriate, this reaction sequence can be followed repeatedly.

[0008] Furthermore, fatty acids must subsequently be transported to various modification sites and incorporated into the triacylglycerol storage lipid. A further important step during lipid synthesis is the transfer of fatty acids to the polar head groups, for example by glycerol fatty acid acyltransferase (see Frentzen, 1998, Lipid, 100(4-5):161-166).

[0009] An overview of the biosynthesis of fatty acids in plants, desaturation, the lipid metabolism and the membrane transport of lipidic compounds, beta-oxidation, the modification of fatty acids, cofactors and the storage and assembly of triacylglycerol, including the references is given by the following papers: Kinney (1997) Genetic Engineering, Ed.: J K Setlow, 19:149-166; Ohlrogge and Browse (1995) Plant Cell 7:957-970; Shanklin and Cahoon (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49: 611–641; Voelker (1996) Genetic Engeneering, Ed.: J K Setlow, 18: 111–13; Gerhardt (1992) Prog. Lipid R. 31: 397–417; Guhnemann-Schafer & Kindl (1995) Biochim Biophys Acta 1256: 181–186; Kunau et al. (1995) Prog. Lipid Res. 34: 267–342; Stymne et al. (1993) in: Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants, Ed.: Murata and Somerville, Rockville, American Society of Plant Physiologists, 150–158; Murphy & Ross (1998) Plant Journal. 13(1) :1–16.

[0010] Depending on the desaturation pattern, two large classes of polyunsaturated fatty acids, the ω6 and the ω3 fatty acids, which differ with regard to their metabolism and their function, can be distinguished.

[0011] In the text which follows, polyunsaturated fatty acids are referred to as PUFA, PUFAs, LCPUFA or LCPUFAs (poly unsaturated fatty acids, PUFA, long chain poly unsaturated fatty acids, LCPUFA).

[0012] The fatty acid linoleic acid ($18:2^{\Delta 9,12}$) acts as starting material for the ω6 metabolic pathway, while the ω3 pathway proceeds via linolenic acid ($18:3^{\Delta 9,12,15}$). Linolenic acid is formed from linoleic acid by the activity of an ω3-desaturase (Tocher et al. (1998) Prog. Lipid Res. 37: 73–117; Domergue et al. (2002) Eur. J. Biochem. 269: 4105–4113).

[0013] Mammals, and thus also humans, have no corresponding desaturase activity (Δ12- and ω3-desaturase) for the formation of the starting materials and must therefore take up these fatty acids (essential fatty acids) via the food. Starting with these precursors, the physiologically important polyunsaturated fatty acids arachidonic acid (=ARA, $20:4^{\Delta 5,8,11,14}$), an ω6-fatty acid and the two ω3-fatty acids eicosapentaenoic acid (=EPA, $20:5^{\Delta 5,8,11,14,17}$) and docosahexaenoic acid (DHA, $22:6^{\Delta 4,7,10,13,17,19}$) are synthesized via a sequence of desaturase and elongase reactions.

[0014] The elongation of fatty acids, by elongases, by 2 or 4 C atoms is of crucial importance for the production of C_{20}- and C_{22}-PUFAs, respectively. This process proceeds via 4 steps. The first step is the condensation of malonyl-CoA onto the fatty acid acyl-CoA by ketoacyl-CoA synthase (KCS, hereinbelow

referred to as elongase). This is followed by a reduction step (ketoacyl-CoA reductase, KCR), a dehydration step (dehydratase) and a final reduction step (enoyl-CoA reductase). It has been postulated that the elongase activity affects the specificity and rate of the entire process (Millar and Kunst (1997) Plant Journal 12:121-131).

[0015] Fatty acids and triacylglycerides have a multiplicity of applications in the food industry, in animal nutrition, in cosmetics and the pharmacological sector. Depending on whether they are free saturated or unsaturated fatty acids or else triacylglycerides with an elevated content of saturated or unsaturated fatty acids, they are suitable for very different applications. Thus, for example, lipids with unsaturated, specifically with polyunsaturated fatty acids, are preferred in human nutrition. The polyunsaturated ω3-fatty acids are supposed to have a positive effect on the cholesterol level in the blood and thus on the prevention of heart disease. The risk of heart disease, strokes or hypertension can be reduced markedly by adding these ω3-fatty acids to the food (Shimikawa (2001) World Rev. Nutr. Diet. 88: 100-108).

[0016] ω3-fatty acids also have a positive effect on inflammatory, specifically on chronically inflammatory, processes in association with immunological diseases such as rheumatoid arthritis (Calder (2002) Proc. Nutr. Soc. 61: 345-358; Cleland and James (2000) J. Rheumatol. 27: 2305-2307). They are therefore added to foodstuffs, specifically to dietetic foodstuffs, or are employed in medicaments. ω6-fatty acids such as arachidonic acid tend to have a negative effect in connection with these rheumatological diseases.

[0017] ω3- and ω6-fatty acids are precursors of tissue hormones, known as eicosanoids, such as the prostaglandins, which are derived from dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid, and of the thromboxanes and leukotrienes, which are derived from arachidonic acid and eicosapentaenoic acid. Eicosanoids (known as the PG_2 series) which are formed from the ω6-fatty acids, generally promote inflammatory reactions, while eicosanoids (known as the PG_3 series) from ω3-fatty acids have little or no proinflammatory effect.

[0018] Polyunsaturated long-chain ω3-fatty acids such as eicosapentaenoic acid (=EPA, C20:$5^{\Delta 5,8,11,14,17}$) or docosahexaenoic acid (=DHA, C22:$6^{\Delta 4,7,10,13,16,19}$) are important components of human nutrition owing to their various roles in health aspects, including the development of the child brain, the functionality of the eyes, the synthesis of hormones and other signal substances, and the prevention of cardiovascular disorders, cancer and diabetes (Poulos, A (1995) Lipids 30:1-14; Horrocks, L A and Yeo Y K (1999) Pharmacol Res 40:211-225).

[0019] Owing to the present-day composition of human food, an addition of polyunsaturated ω3-fatty acids, which are preferentially found in fish oils, to the food is particularly important. Thus, for example, polyunsaturated fatty acids such as docosahexaenoic acid (=DHA, C22:$6^{\Delta 4,7,10,13,16,19}$) or eicosapentaenoic acid (=EPA, C20:$5^{\Delta 5,8,11,14,17}$) are added to infant formula to improve the nutritional value. There is therefore a demand for the production of polyunsaturated long-chain fatty acids.

[0020] The various fatty acids and triglycerides are mainly obtained from microorganisms such as *Mortierella* or *Schizochytrium* or from oil-producing plants such as soybeans, oilseed rape, and algae such as *Crypthecodinium* or *Phaeodactylum* and others, being obtained, as a rule, in the form of their triacylglycerides (=triglycerides=triglycerols). However, they can also be obtained from animals, for example, fish. The free fatty acids are advantageously prepared by hydrolyzing the triacylglycerides. Very long-chain polyunsaturated fatty acids such as DHA, EPA, arachidonic acid (ARA, C20:$4^{\Delta 5,8,11,14}$),

dihomo-γ-linolenic acid (DHGL, C20:3$^{\Delta 8,11,14}$) or docosapentaenoic acid (DPA, C22:5$^{\Delta 7,10,13,16,19}$) are, however, not synthesized in oil crops such as oilseed rape, soybeans, sunflowers and safflower. Conventional natural sources of these fatty acids are fish such as herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, zander or tuna, or algae.

[0021] Owing to the positive characteristics of the polyunsaturated fatty acids, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of these fatty acids or triglycerides for the production of oils in various organisms with a modified content of unsaturated fatty acids. Thus, WO 91/13972 and its US equivalent describe a Δ9-desaturase. WO 93/11245 claims a Δ15-desaturase and WO 94/11516 a Δ12-desaturase. Further desaturates 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. (1990) J. Biol. Chem., 265: 20144-20149, Wada et al. (1990) Nature 347: 200-203 or Huang et al. (1999) Lipids 34: 649-659. However, the biochemical characterization of the various desaturases has been insufficient to date since the enzymes, being membrane-bound proteins, present great difficulty in their isolation and characterization (McKeon et al. (1981) Methods in Enzymol. 71: 12141-12147, Wang et al. (1988) Plant Physiol. Biochem., 26: 777-792).

[0022] As a rule, membrane-bound desaturases are characterized by being introduced into a suitable organism which is subsequently analyzed for enzyme activity by analyzing the starting materials and the products. Δ6-Desaturases are described in WO 93/06712, U.S. Pat. No. 5,614,393, WO 96/21022, WO 00/21557 and WO 99/27111. The application of this enzyme for the production of fatty acids in transgenic organisms is described in WO 98/46763, WO 98/46764 and WO 98/46765. The expression of various desaturases and the formation of polyunsaturated fatty acids is also described and claimed in WO 99/64616 or WO 98/46776. As regards the expression efficacy of desaturases and its effect on the formation of polyunsaturated fatty acids, it must be noted that the expression of a single desaturase as described to date has only resulted in low contents of unsaturated fatty acids/lipids such as, for example, γ-linolenic acid and stearidonic acid.

[0023] There have been a number of attempts in the past to obtain elongase genes. Millar and Kunst (1997) Plant Journal 12: 121-131 and Millar et al. (1999) Plant Cell 11:825-838 describe the characterization of plant elongases for the synthesis of monounsaturated long-chain fatty acids (C22:1) and for the synthesis of very long-chain fatty acids for the formation of waxes in plants (C_{28}-C_{32}). The synthesis of arachidonic acid and EPA is described, for example, in WO 01/59128, WO 00/12720, WO 02/077213 and WO 02/08401. The synthesis of polyunsaturated C24-fatty acids is described, for example, in Tvrdik et al. (2000) J. Cell Biol. 149: 707–718 or in WO 02/44320.

[0024] Especially suitable microorganisms for the production of PUFAs are microalgae such as *Phaeodactylum tricornutum*, *Porphiridium* species, *Thraustochytrium* species, *Schizochytrium* species or *Crypthecodinium* species, ciliates such as *Stylonychia* or *Colpidium*, fungi such as *Mortierella*, *Entomophthora* or *Mucor* and/or mosses such as *Physcomitrella*, *Ceratodon* and *Marchantia* (R. Vazhappilly & F. Chen (1998) Botanica Marina 41: 553-558; K. Totani & K. Oba (1987) Lipids 22: 1060-1062; M. Akimoto et al. (1998) Appl. Biochemistry and

Biotechnology 73: 269-278). Strain selection has resulted in the development of a number of mutant strains of the microorganisms in question which produce a series of desirable compounds including PUFAs. However, the mutation and selection of strains with an improved production of a particular molecule such as the polyunsaturated fatty acids is a time-consuming and difficult process. Moreover,

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