Metabolic Engineering of ω3-Very Long Chain Polyunsaturated Fatty Acid Production by an Exclusively Acyl-CoA-dependent Pathway

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ω3-Very long chain polyunsaturated fatty acids (VLCPUFA) are essential for human development and brain function and, thus, are indispensable components of the human diet. The current main source of VLCPUFAs is represented by ocean fish stocks, which are in severe decline, and the development of alternative, sustainable sources of VLCPUFAs is urgently required. Our research aims at exploiting the powerful infrastructure available for the large scale culture of oilseed crops, such as rapeseed, to produce VLCPUFAs such as eicosapentaenoic acid in transgenic plants. VLCPUFA biosynthesis requires repeated desaturation and repeated elongation of long chain fatty acid substrates. In previous experiments the production of eicosapentaenoic acid in transgenic plants was found to be limited by an unexpected bottleneck represented by the acyl exchange between the site of desaturation, endoplasmic reticulum-associated phospholipids, and the site of elongation, the cytosolic acyl-CoA pool. Here we report on the establishment of a coordinated, exclusively acyl-CoA-dependent pathway, which avoids the rate-limiting transesterification steps between the acyl lipids and the acyl-CoA pool during VLCPUFA biosynthesis. The pathway is defined by previously uncharacterized enzymes, encoded by cDNAs isolated from the microalga Mantoniella squamata. The conceptual enzymatic pathway was established and characterized first in yeast to provide proof-of-concept data for its feasibility and subsequently in seeds of Arabidopsis thaliana. The comparison of the acyl-CoA-dependent pathway with the known lipid-linked pathway for VLCPUFA biosynthesis showed that the acyl-CoA-dependent pathway circumvents the bottleneck of switching the Δ6-desaturated fatty acids between lipids and acyl-CoA in Arabidopsis seeds.

Human development and health depend in many respects on the availability of long chain multiply unsaturated fatty acids of 20 or 22 carbons in length that contain up to 6 methylene-flanked cis-double bonds. These fatty acids are classified under the designation very long chain polyunsaturated fatty acids (VLCPUFAs). Nutritionally important VLCPUFAs include arachidonic acid (ARA, 20:4ω6,8,11,14), an ω6-fatty acid, and the ω3-fatty acids eicosapentaenoic acid (EPA, 20:5ω3,8,11,14,17) and docosahexaenoic acid (DHA, 22:6ω3,4,7,10,13,16,19). ω3-VLCPUFAs are of particular interest from a nutritional standpoint since the uptake of these fatty acids is considered to be low in Western diets (1). ω3-VLCPUFAs have long been investigated for their importance during human fetal development and the formation and function of the central nervous system, brain, and retina. In addition to their structural functions in membranes, several medical studies indicate that ω3-fatty acids have anti-inflammatory properties and, therefore, might be useful in the management of inflammatory and autoimmune diseases such as cardiovascular disease, major depression, arthritis, inflammatory bowel disease, asthma, and psoriasis (2).

Whereas mammals, including humans, can convert the essential precursors ω6-18:2 and ω3-18:3 (α-linolenic acid) to VLCPUFAs, a considerable proportion of VLCPUFA has to be taken up directly as components of the diet (3). Naturally occurring producers and sources of ω3-VLCPUFAs are microorganisms, including marine bacteria and microalgae. These organisms represent the starting point of the aquatic food chain by which VLCPUFAs ultimately accumulate in fish oils. At the moment the main source of ω3-VLCPUFAs for human consumption are fatty ocean fish, such as salmon, mackerel, tuna, or herring. Unfortunately, the increased demand for fish and fish oils has led to a depletion of fish stocks in vast ocean areas worldwide. Thus, as an exclusive source of VLCPUFAs, fish cannot cover the needs of a growing world population (4). Altogether, the increasing interest to find alternative sources of ω3-VLCPUFAs has led to various attempts to produce ω3-VLCPUFAs via the biotechnological introduction of new biosynthetic pathways in plants, ultimately aiming for VLCPUFA-production in annual oilseeds, e.g. in rapeseed or linseed.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) AM949596 and AM949597.
VLCPUFA synthesis in transgenic plants established so far starts from the plant endogenous fatty acids \(\omega 6-18:2^{9,12}\) or \(\omega 3-18:3^{12,15}\) and requires two distinct catalytic activities, desaturases and elongases. Both activities work together in an alternating manner to establish the final product. Genes for the biosynthesis of VLCPUFAs have been isolated from organisms of different kingdoms (algae, amoeba, fungi, moss, and flowering plants) and have been characterized by heterologous expression in various model systems regarding the properties of the encoded enzymes.

In recent years a range of tests was done to establish the biosynthesis of VLCPUFAs in plants by introducing a variety of different front-end desaturases and elongases (5, 6). Two previous approaches are of particular relevance for the study presented here. First, the so called alternative pathway for the biosynthesis of VLCPUFAs was successfully tested in Arabidopsis leaves (7). This pathway is based on the sequential action of a \(\Delta^9\) elongase (from *Isoschizys galbana*) (8), a \(\Delta^8\) desaturase (from *Euglena gracilis*) (9), and a \(\Delta^5\) desaturase (from the fungus *Maritirrella alpina*) (10). The \(\Delta^9\) elongase converts CoA-bound 18:2\(^{9,12}\) and/or \(\omega 3-18:3\) into 20:2\(^{11,14}\) and/or 20:3\(^{11,14,17}\), which could be further converted to ARA and EPA via lipid-bound \(\Delta^8\) and \(\Delta^5\) desaturation, respectively. In this fashion a rate-limiting \(\Delta^6\)-elongation step was avoided, and EPA accumulation of up to 3% of the leaf total lipid content of *Arabidopsis thaliana* was achieved. It must be noted, however, that leaf tissue is not oleogenic and that the fatty acids are directly incorporated into membrane lipids rather than stored as oils as is the case in seeds and as would be desirable for VLCPUFA production. Moreover, a crucial problem was observed. The \(\Delta^9\)-elongation products (20:2\(^{11,14}\) and 20:3\(^{11,14,17}\)) accumulated to very high levels in the acyl-CoA pool of the transgenic *Arabidopsis* plants (11), indicating an inefficient transfer of these non-native fatty acids out of the acyl-CoA pool.

In the second approach the coexpression of \(\Delta^6\) and \(\Delta^5\) desaturases of the diatom *Phaeodactylum tricornutum* with a \(\Delta^6\) elongase from the moss *Physcomitrella patens* in lineese showed that the enzymes carried out \(\Delta^5\) and \(\Delta^6\) desaturation on lipid-bound substrates in the plant with a positional specificity for the sn-2 position of phosphatidylcholine (PC), whereas the \(\Delta^6\) elongase preferred acyl-CoA-species as substrates (12, 13). Comprehensive acyl-CoA and lipid analysis of the EPA-producing transgenic lineese plants demonstrated that production of VLCPUFAs in plants requires not only the interplay of desaturases and elongases but also the transfer of acyl groups from the PC pool into the CoA pool and vice versa. This transfer is very likely catalyzed by the enzymatic activity of an acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT). The LPCAT endogenous to lineese does not accept the newly formed \(\Delta^6\)-desaturated fatty acids, and thus, the \(\Delta^6\)-desaturated fatty acids accumulated to high levels. In brief, the back-and-forth trans-acylation of fatty acids between the PC and CoA pools by the action of LPCAT represents a rate-limiting factor for VLCPUFA production by this transgenic approach in lineese (12).

In the research mentioned as well as in several other studies, e.g. Refs. 14 and 15, desaturases from various organisms were analyzed for their feasibility to produce VLCPUFAs. The accumulated evidence indicates that the front-end desaturases characterized from algae, fungi, and moss are lipid-dependent desaturases and accept glycerol lipid-linked substrates. In contrast to these lipid-dependent desaturases, a number of mammalian front-end desaturases accept acyl-CoA species as substrates (16–18). Recently, one acyl-CoA-dependent \(\Delta^6\) desaturase from the microalga *Ostreococcus tauri* was identified (19). From fatty acid analyses performed on microalgae like O. tauri, however, it could be concluded that further desaturases with a preference for acyl-CoA substrates can be isolated from those organisms. Such plant-like acyl-CoA-dependent enzymes with the correct substrate specificities may allow bypassing the rate-limiting transport and exchange of intermediates of VLCPUFA synthesis between lipid-bound desaturation in the PC pool and elongation steps in the acyl-CoA pool. Many vascular plants synthesize 18:2\(^{9,12}\) and 18:3\(^{9,12,15}\) in their seed oils but do not produce or incorporate VLCPUFAs with chain lengths above 18 carbon atoms or more than three double bonds into triacylglycerol (TAG). To produce the \(\omega 3\)-fatty acid, \(\omega 3-20:5^{9,6,11,14,17}\) (EPA), in seed oils, it is necessary to introduce one additional elongation and two desaturation steps. To avoid the accumulation of \(\omega 6\) byproducts of desaturation, the enzymes selected should be specific for \(\omega 3\) substrates. A second known constraint to circumvent is the rate-limiting shuffling of fatty acids between PC and CoA pools observed by Abbadi et al. (12).

To contribute to a solution for these problems, we report on the isolation and characterization of acyl-CoA-dependent desaturases from the microalgae *Mantoniella squamata* and *O. tauri*. Using the new enzymes, an entirely acyl-CoA-dependent \(\omega 3\)-VLCPUFA biosynthetic pathway consisting of \(\Delta^6\)- and \(\Delta^5\) desaturase enzymes was successfully established in *Saccharomyces cerevisiae* and in seeds of *A. thaliana* plants. This modified pathway may allow a more efficient flux during VLCPUFA biosynthesis and avoids the bottleneck after \(\Delta^6\) desaturation described by Abbadi et al. (12).

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and DNA-modifying enzymes were obtained from MBI Fermentas. Standards of fatty acids as well as all other chemicals were from Sigma; methanol, n-hexane, and isopropanol (all high performance liquid chromatography grade) were from Baker. Fatty acids and acyl-CoAs were either obtained from Cayman Chemicals or Laro-dan. Basic molecular biological and biochemical techniques were performed as described (20).

**Algae Material and Growth Conditions**—*M. squamata* SAG 65.90 (21) was obtained from the algae culture collection Göttingen (SAG, Germany). *O. tauri* strain OTTH0595-genome was obtained from the Roscoff Culture Collection. Non-axenic cultures were grown in batch cultures under long daylight (14 h) conditions with 45 \(\mu\)mol of photons m\(^{-2}\) s\(^{-1}\) in 200 ml of Brackish Water Medium (1/2 SWES) complemented with soil extract at 20 °C.

cDNA Library Construction and Random Sequencing of the cDNA Library—Total RNA from 7-day-old *M. squamata* cultures were isolated using the RNAeasy kit (Qiagen) per the manufacturer’s instructions. Poly A+ mRNAs were prepared...
using oligo-dT cellulose (22) (see above) and reverse-transcribed via a reverse transcription kit (Promega) and then used for the construction of the cDNA library with a Lambda ZAP Gold library construction kit (Stratagene). After in vivo mass excision of the cDNA library, plasmid recovery and transformation of Escherichia coli (Stratagene), plasmid DNA was prepared on a Qiagen DNA preparation robot (Qiagen) according to the manufacturer’s instructions and submitted to random sequencing by the chain termination method using the ABI PRISM Big Dye Termination Cycle Sequencing Ready Reaction kit (PerkinElmer Life Sciences). Analyses and annotations of the EST sequences between 100 and 500 base pairs resulted in a non-redundant EST data base. Data base screening of the cDNA library yielded two sequences that were annotated as putative desaturases.

Isolation and Cloning of Desaturase Sequences—To obtain full-length cDNA sequences, the rapid amplification of cDNA ends (RACE) technique was used. Therefore, 5 μl of total RNA isolated from M. squamata were reverse-transcribed and ligated to adaptor-ligated double-stranded cDNA by using the Marathon cDNA amplification kit (BD Bioscience). Adaptor-ligated double-stranded cDNA was used as template for 5’- and 3’-RACE PCRs to obtain the missing 5’-prime and 3’-prime ends of the coding sequences for several desaturases. For RACE reactions, general-specific primers were designed from EST sequence information. Primers used in 5’- and 3’-RACE were as follows: for MsΔ6 (MsI) as 5’-RACE primer, 5’-CATCCGCAGCGCATCTCTAC-3’, and as 3’-RACE primer, 5’-GGAGAAGAGGTGGTGGATGACCTGG-3’, and as 3’-RACE primer, 5’-GGAGAAGAGGTGGTGGATGACCTGG-3’; for MsΔ5 (MsII) as 5’-RACE primer, 5’-CCAGTGAGGGGAGTAGCTGGCCGGG-3’, and as 3’-RACE primer, 5’-CACTCTCCGCGGGGCTCACTAC-3’. A 50-μl standard reaction contained 1× Advantage2 DNA polymerase buffer, 1 μl of Advantage 2 DNA Polymerase (BD Bioscience), 0.2 mM concentrations of each dNTP, 0.5 μM 5’ or 3’ primer, 0.5 μM adaptor primer 1 (API), and 5 μl of adaptor-ligated double-stranded cDNA. RACE-PCR amplification was performed as follows: 30 s at 94 °C; 5 cycles of 5 s at 94 °C, 4 min at 72 °C; 5 cycles of 5 s at 94 °C, 4 min at 70 °C; 20 cycles of 5 s at 94 °C, 4 min at 68 °C. Amplified products were isolated from agarose gels, purified using a gel extraction kit (GE Healthcare), and subsequently cloned into pGEM-T (Promega). Cloned inserts were sequenced using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Cloning cDNA for Desaturases into Yeast Expression Vectors—From the 5’- and 3’-cDNA sequence data obtained by RACE-PCR, the putative translation initiation codons and stop codons were identified, and this sequence information was used to obtain full-length cDNA clones of the putative desaturases from M. squamata. Gene-specific primers were designed to the 5’- and the 3’-ends of the coding regions of the corresponding nucleotide sequences, introducing restriction sites for cloning into the yeast expression vectors and the yeast consensus sequence for enhanced translation in front of the start codons (23). The open reading frames of the MsΔ6 (MsI) and MsΔ5 (MsII) and of OtΔ5 (OtII) were amplified and modified by PCR. The following pairs of primers were used (restrictions sites are in bold, translation initiation sequences are in italics, and start or stop codons are underlined): for MsΔ6 as forward primer, 5’-ATGCCGGCCGCACATATGTGTCTCCTCCCAAGGAAT-3’, and as reverse primer 5’-ATCGAGATCTCTAGTGAAGCTTGAGCCTTC-3’; for MsΔ5 as forward primer, 5’-ATGCCGGCCGCCACATATGCGCCCCCGCGACGAAC-3’, and as reverse primer, 5’-ATGCAGATCTTACCGATTTTGAAGGAC-3’; and for OtΔ5 as forward primer, 5’-ATGCCGGCCGCACATATGGGAGCAAGCCGCAGCGAC-3’, and as reverse primer, 5’-ATGCAGATCTTACCGAGGTTTGGAGGACGG-3’. The amplified cDNAs were cloned into the pGEM-T vector (Promega) before being released and cloned into the yeast expression vector (pESC-LEU or pESC-TRP, respectively, Stratagene) using the restriction sites inserted by PCR, yielding pESC-LEU-MsΔ6 and pESC-TRP-MsΔ5.

For comparison with the known front-end desaturases from P. tricornutum, PtΔ6 and PtΔ5, or with the known acyl-CoA-dependent front-end desaturase from O. tauri, OtΔ6, the P. tricornutum or O. tauri cDNA clones were cloned into yeast expression vectors as described for the M. squamata desaturases, yielding pESC-LEU-PtΔ6-PSE1 and pESC-TRP-PtΔ5 using the primers (restrictions sites are in bold; start or stop codons are underlined): for OtΔ6 as forward primer, 5’-ATGCAGATCTTACCGCGTTTCCGAGGTG-3’; for PtΔ6 as forward primer, 5’-ATCCGGGCCCCACATATGGGACGGGAGAGGCGAAT-3’, and as reverse primer, 5’-ATGCAGATCTTACCGCGTTTCCGAGGTG-3’; for PtΔ5 as forward primer, 5’-ATGCAGATCTTACCGCGTTTCCGAGGTG-3’, and as reverse primer, 5’-ATGCAGATCTTACCGCGTTTCCGAGGTG-3’. To obtain pESC-LEU-MsΔ6-PSE1, pESC-LEU-OtΔ6-PSE1, and pESC-LEU-PtΔ6-PSE1, the Δ6 elongase from P. patens, PSE1 (24), was cloned into the yeast expression vector. Primers used to amplify PSE1: the forward primer was 5’-ATGCCGATCTTACCGCGTTTCCGAGGTG-3’, and as reverse primer, 5’-ATGCAGATCTTACCGCGTTTCCGAGGTG-3’. The Δ6 primer was designed based on the vector pUC19, and three OCS terminators, and three specific USP promoters (26), three OCS terminators, and three specific USP promoters (26), and three different polylinkers between each promoter and terminator was first introduced to the vector pUC19 (Pharmacia Corp.), yielding the USP123OCS plasmid. The open reading frames of the different desaturases and elongases were modified by PCR to create appropriate restriction sites adjacent to the start and stop codons, cloned into the pGEM-T vector (Promega), and sequenced to confirm their accuracy. The primers used were (restrictions sites are in bold, and start or stop codons are underlined): MsΔ6, forward, 5’-ATGCCGGCCGCACATA-
Acyl-CoA-dependent VLCPUFA Biosynthesis in Yeast and Plants

Expression in S. cerevisiae—S. cerevisiae cells strain INVSc1 (Invitrogen) were transformed as described (27). For induction, expression cultures were grown for 48 h to 72 h at 22 °C in the presence of 2% (w/v) galactose supplemented with 150–350 μM concentrations of appropriate fatty acid substrate and in the presence of 1% Igepal CA630 (Nonidet P-40) from Sigma-Aldrich. Cells were harvested by centrifugation at 1200×g for 5 min, and the pellets were washed twice with H2O before being used for further analysis. The host strain transformed with the empty vector(s) was used as negative control in all experiments.

Arabidopsis Transformation—A. thaliana ecotype Columbia (Col-0) plants were transformed by floral dipping (37). T2 seeds were collected from individual T1 plants resistant to ammonium glutosinate and analyzed individually by GC.

Fatty Acid Analysis—Fatty acid methyl esters (FAMEs) were obtained by methylation of microalgae or yeast cell sediments with 0.5 M sulfuric acid in methanol containing 2% (v/v) dioxoxygenpane at 80 °C for 1 h. FAMEs were extracted in 2 ml of n-hexane, dried under N2, and analyzed by gas chromatography (GC). FAMEs of single or pooled Arabidopsis seeds were prepared by transesterification with trimethylsulfonium hydroxide (28). FAMEs of TLC-separated individual lipids were obtained by transesterification with 333 μl of toluol/methanol (1:2 v/v) and 167 μl of 0.5 M NaOCH3 at room temperature for 20 min. FAMEs were extracted in 500 μl of NaCl, 50 μl of HCl (37%), and 2 ml of n-hexane, dried under N2, and analyzed by GC. The GC analysis was performed with an Agilent GC 6890 system coupled with a flame ionization detector equipped with a capillary 122-2332 DB-23 column (30 m × 0.32 mm; 0.5 μm coating thickness; Agilent). Helium was used as carrier gas (1 ml min−1). Samples were injected at 220 °C. The temperature gradient was 150 °C for 1 min, 150 to 200 °C at 15 °C min−1, 200 to 250 °C at 2 °C min−1, and 250 °C for 10 min. Data were processed using the HP ChemStation Rev. A09.03. FAMEs were identified by comparison with appropriate reference substances.

Lipid Analysis—For lipid analysis, 10 mg of seeds were homogenized in 4 ml of chloroform/methanol 1:2 (v/v), and lipids were extracted on a shaker for 4 h and then for 20 h with 5 ml of chloroform/methanol 2.1 (v/v) at 4 °C. The resulting organic phases were combined and dried under N2. The remaining lipids were dissolved in 1 ml of chloroform. Separation of lipid classes (neutral lipids and phospholipids) was achieved using a silica column (Bond Elut SI, 100 mg/ml; Varian). Lipid extracts were loaded on the silica column pre-equilibrated with chloroform and then fractionated into the lipid classes by elution as follows: neutral lipids with chloroform and phospholipids with methanol/glacial acetic acid (9:1. v/v). Isolation of individual components of the phospholipid class was achieved by thin layer chromatography using appropriate standards and with methanol/chloroform/glacial acetic acid (25:65:8, v/v) as developing solvent.

Lipid Analysis of Arabidopsis Seeds—For lipid analysis, 10 mg of seeds were homogenized in 4 ml of chloroform/methanol/glacial acetic acid (2:1:0.1 v/v/v) and incubated for 24 h at 4 °C. Seed residues were pelleted (2 min, 3000 × g). The supernatant was collected, and the pelleted seed residues were incubated with 2 ml of n-hexane for 30 min at room temperature. The resulting organic phases were combined and dried under N2. The dried lipids were dissolved in 200 μl of chloroform. Separation of lipid classes (TAG and different phospholipids) was achieved by thin layer chromatography with methanol/chloroform/glacial acetic acid (25:65:8, v/v) as a developing solvent. Lipids (TAG, PC, phosphatidylinositol (PI)/phosphatidylerine (PS), and phosphatidylethanolamine (PE)) were identified according to authentic standards (Avanti), scraped out from the thin layer chromatography plates, and reextracted with developing solvent for subsequent fatty acid analysis.

Acyl-CoA Species, Synthesis, Extraction, and Analysis—Authentic standards for saturated and monounsaturated acyl-CoA esters with acyl-chain lengths from C12 to C18 were obtained from Sigma. Standards for polyunsaturated acyl-CoAs (18:3Δ9,12,15, 18:4Δ6,9,12,15, 20:3Δ6,11,14, 20:4Δ8,11,14,17, and 20:5Δ8,11,14,17) were synthesized enzymatically using a recombinant acyl-CoA synthetase from Pseudomonas sp. (Sigma). The reaction mixture (200 μl), containing 100 mM Tris·HCl, pH 8.1, 10 mM MgCl2, 5 mM ATP, 5 mM CoASH, 2 mM dithiothreitol, 25 μM free fatty acids, and 2.5 units of acyl-CoA synthetase, was incubated at 37 °C for 2 h. The reaction was stopped with 50 μl of glacial acetic acid/ethanol 1:1 (v/v), and the desired aqueous phases were washed with petroleum ether to remove residual free fatty acids. After purification of the acyl-CoA species on a Sep-Pak column (Strata C18-E; Phenomenex) using acetonitrile as the eluting solvent, the samples were dried under argon and dissolved in 50 mM MES, pH 5.0.
For acyl-CoA analysis of yeast cells, 20 ml of liquid cultures were harvested at an A₆₀₀ of 1.5–2.0, and acyl-CoA-species were extracted as described (19). The conversion of acyl-CoA esters to their etheno derivatives and acyl-CoA analysis was performed as described in Larson and Graham (29).

Accession Numbers—Sequences reported in this study (supplemental Fig. 1) were deposited to GenBank™ as AM949597 (MsΔ6) and AM949596 (MsΔ5).

RESULTS AND DISCUSSION

The Marine Microalga, M. squamata, Accumulates High Levels of VLCPUFAs—The biosynthesis of VLCPUFAs in various plant species has previously been achieved by the introduction of genes from different organisms that encode enzymes responsible for fatty acid desaturation, elongation, and trans-esterification (7, 12, 14, 15). In this study it was the aim to discover enzymes that exhibit greater specificity for ω3 substrates and could be used in an exclusively acyl-CoA-dependent pathway for VLCPUFA biosynthesis, which abolishes a need for rate-limiting acyl shuttling.

To test whether M. squamata was suitable as a donor organism for the isolation of genes encoding acyl-CoA-dependent Δ6 and Δ5 desaturases, the fatty acid composition of the alga was analyzed. M. squamata was found to accumulate particularly ω3-VLCPUFAs up to 20 mol% of the total fatty acids, which were predominantly associated with TAG but were also found in phospholipids and the acyl-CoA pool. These observations prompted us to pursue further attempts to isolate candidate genes encoding enzymes with the desired characteristics.

Isolation of Putative Acyl-CoA-dependent Front-end Desaturases from M. squamata and O. tauri—Using an EST data base established from M. squamata cDNA, two partial sequences, MsI and MsII, were annotated as putative desaturases. Based on the EST information, full-length clones were isolated by a RACE-PCR approach (supplemental Fig. 1). The amino acid sequence deduced from the MsI cDNA shared 66.5% amino acid identity with the sequence from an acyl-CoA-dependent Δ6 desaturase from the related microalga, O. tauri (MsΔ6, GenBank™ accession number AY746357), 64% identity with an unknown partial sequence of a putative desaturase sequence from Ostreococcus lucimarinus CCE9901 (GenBank™ accession number XM_001421036), and 42% identity with the Δ5 desaturase from Pavlova salina (GenBank™ accession number DQ995517). The deduced amino acid sequence of the second clone, MsII, shared 64% identity with an uncharacterized putative Δ5 desaturase from O. lucimarinus CCE9901 (GenBank™ accession number XM_001420818), 66% identity with a putative Δ5 desaturase from O. tauri (GenBank™ accession number CR954212), 51% identity with the known Δ5 desaturase from P. patens (GenBank™ accession number D554492), and 32% identity with the previously described Δ5 desaturase from Marchantia polymorpha (GenBank™ accession numberAY583465).

In addition, the putative Δ5 desaturase from O. tauri (GenBank™ accession number CR954212) was cloned by a PCR-based approach, taking advantage of available genomic information, and named OtII. A comparison of the putative desaturase-like sequences from the microalgae revealed properties characteristic for front-end desaturases, including the presence of an N-terminal HPGG motif (cytochrome b₅ binding domain) (30, 31), three conserved histidine boxes most likely involved in the coordination of the diiron center of the active site (32), and the presence of a typical His to Gln substitution in the more variable third histidine box.

Biosynthetic Capacity of M. squamata Enzymes Heterologously Expressed in Yeast in Comparison to Known Lipid-dependent Desaturases—To confirm catalytic activity and substrate specificities of the putative desaturases encoded by MsI, MsII, and OtI, the sequences were individually expressed in the S. cerevisiae strain INVSc1 in the presence of potential fatty acid substrates for Δ6- or Δ5-fatty acid desaturases (Table 1).

With expression of empty vector controls, only yeast-endogenous fatty acids and the added substrates were detected. With expression of MsI, a new fatty acid product was observed only when 18:3<sup>n-3</sup>,12,15 (ω-3-linolenic acid) was supplied (Table 1). The product fatty acid was identified as 18:4<sup>Δ6,9,12,15</sup> (stearidonic acid), indicating Δ6-desaturation by MsI. Therefore, MsI is referred to as MsΔ6 from this point on. Fatty acids other than α-linolenic acid, including yeast-endogenous fatty acids, were not accepted by MsΔ6 (Table 1), indicating that only a narrow range of substrates are accepted by MsΔ6. This interesting substrate preference differs from that of the previously identified OtΔ6. Whereas OtΔ6 accepts both 18:3<sup>n-3</sup>,12,15 and 18:3<sup>n-3</sup>,9,12,15 (α-linolenic acid) as substrates, MsΔ6 is more selective for the ω3-substrate, α-linolenic acid (Table 1). The pronounced preference of MsΔ6 for ω3 substrates is a desirable trait for an ω3-VLCPUFA pathway in plants, because both ω6 and ω3 substrates may be competitively available for conversion. A preference for ω3 substrates has previously been described for enzymes from Primula luteola (34) and Echium species (35); however, the reported ratio of 5.8 of converted ω3 to ω6 fatty acids for the Primula enzyme (34) was much lower than that of 114 observed here for MsΔ6.

When MsII was expressed, new fatty acid products were detected in the presence of 20:3<sup>Δ6,9,12,15</sup> (di-homo-γ-linolenic acid), 20:3<sup>Δ11,14,17</sup> or 20:4<sup>Δ8,11,14,17</sup> substrates. The products were identified as ARA and EPA, respectively. The conversion of 20:3<sup>Δ11,14,17</sup> into the corresponding Δ5 desaturation product was observed only in minor amounts. Therefore, MsII is referred to as MsΔ5 from this point. Other fatty acids supplied

### TABLE 1

| Substrate Desaturation | Substrate Desaturation |
|------------------------|------------------------|
| MsI = MsΔ6             | MsI = MsΔ5             |
| Substrate              | %                      | Substrate              | %                      |
| 16:1<sup>Δ9</sup>      | n.d.                   | 16:1<sup>Δ9</sup>      | n.d.                   |
| 18:1<sup>Δ9</sup>      | n.d.                   | 18:1<sup>Δ9</sup>      | n.d.                   |
| 18:2<sup>Δ9,12</sup>   | 0.3 ± 0.01             | 18:2<sup>Δ9,12</sup>   | n.d.                   |
| 18:3<sup>Δ9,12,15</sup>| 34.2 ± 4.9             | 18:3<sup>Δ9,12,15</sup>| n.d.                   |
| 20:3<sup>Δ11,14,17</sup>| 0.13 ± 0.02            | 20:3<sup>Δ11,14,17</sup>| 1.0 ± 0.04             |
| 20:4<sup>Δ8,11,14,17</sup>| 0.1 ± 0.01             | 20:4<sup>Δ8,11,14,17</sup>| 1.0 ± 0.4              |
| 20:5<sup>Δ8,11,14,17</sup>| 8.2 ± 0.5              | 20:5<sup>Δ8,11,14,17</sup>| 9.5 ± 2.2              |

To test the substrate specificity, yeast cultures expressing MsI or MsII were supplemented with different fatty acid species (250 μM). Desaturation (%) was calculated as product × 100/(educt + product) using values corresponding to percent of total fatty acids. The values are either the mean of three or six independent experiments. n.d., not detectable.
to the cultures were not converted (Table 1). Thus, in contrast to MsΔ6, MsΔ5 exhibited a slightly broader range of accepted substrates, which is comparable with those of known lipid-dependent Δ5 desaturases, which show roughly equal activities against ω3 and ω6 substrates (Table 1).

When OtII was expressed in yeast, trace amounts of Δ5-desaturated fatty acid products were detected in the presence of 20:3Δ5,11,14 or 20:4Δ8,11,14,17, which were identified as ARA and EPA, respectively (data not shown), and OtII was consequently termed OtΔ5. Because of the low activity of OtΔ5 with expression in yeast, experiments described in the following paragraphs were performed with MsΔ6 and MsΔ5 only.

The desaturation efficiencies of the newly discovered enzymes were compared with those of the known acyl-CoA-dependent Δ6 desaturase from O. tauri, OtΔ6 (19), and the lipid-dependent enzymes from P. tricornutum, PtΔ6 and PtΔ5, (33). To avoid the influence of different expression levels of transgenes in yeast, at least six individual expression cultures were set up in parallel and individually analyzed, and results were averaged. This experimental design minimizes the possibility that differences in fatty acid product accumulation originate solely from differences in transgene expression. Fig. 1A shows the efficiency of MsΔ6 conversion of 18:3Δ9,12,15 to 18:4Δ6,9,12,15 in comparison to those of the known acyl-CoA-dependent Δ6 desaturase from O. tauri and the lipid-dependent Δ6 desaturase from P. tricornutum. As shown in Fig. 1A, MsΔ6 was equally efficient as OtΔ6 under identical conditions, and about 34% of the available ω3–18:3 substrate was desaturated by both enzymes. In contrast, the lipid-dependent PtΔ6 converted only 15% of the supplied α-linolenic acid to stearidonic acid, resulting in a substantially lower efficiency of conversion. An important characteristic of MsΔ6 is the high desaturation efficiency, which exceeded that of the O. tauri acyl-CoA-dependent Δ6 desaturase.

The efficiency of MsΔ5 converting 20:4Δ8,11,14,17 to EPA was compared with that of the lipid-dependent Δ5 desaturase from P. tricornutum, PtΔ5 (Fig. 1C). Only ~8% of the supplied substrate was converted by MsΔ5 in comparison to ~26% by PtΔ5, indicating poor efficiency of MsΔ5 under the conditions used. The respective desaturation efficiencies of MsΔ5 and PtΔ5 with 20:3Δ5,11,14 as a substrate were similar to those observed with 20:4Δ8,11,14,17 substrate (Fig. 1, B versus C).

The low desaturation efficiency of MsΔ5 upon expression in yeast (Fig. 1, B and C) could neither be increased by changes of expression conditions nor by optimization of codon usage of the algal enzyme (data not shown). A very similar problem arose for OtΔ5, which exhibited only trace activity upon expression in yeast.

**Uniform Distribution of MsΔ6 and MsΔ5 Desaturation Products in Different Yeast Lipid Classes Indicates Acyl-CoA Dependence**—Because the distribution of product fatty acids in different lipid classes can serve as an indication for desaturation in the acyl-CoA pool, yeast cultures expressing MsΔ6 and MsΔ5 were supplied with exogenous 18:3Δ9,12,15 or 20:3Δ5,11,14, respectively, and the distribution of desaturation products in individual lipid classes was analyzed. Analogous experiments were performed in parallel with the acyl-CoA-dependent OtΔ6 and the lipid-dependent PtΔ6 and PtΔ5.

For all Δ6 desaturases tested, 18:4Δ6,9,12,15, produced by Δ6 desaturation of 18:3Δ9,12,15 substrate, was detected predominantly in the neutral lipid fraction and in PC and to a lesser degree in other phospholipids analyzed (Fig. 2A). As expected for a lipid-dependent desaturase, expression of PtΔ6 resulted in accumulation of the 18:4Δ6,9,12,15 product predominantly in PC, whereas 18:4Δ6,9,12,15...
was roughly equally distributed between neutral lipids and PC with expression of the CoA-dependent OtΔ6. Expression of MsΔ6 resulted in an even more pronounced accumulation of 18:4Δ6,9,12,15 in neutral lipids rather than in PC, suggesting MsΔ6 desaturation was not lipid-dependent. In all Δ6 desaturase expression cultures, the 18:4Δ6,9,12,15 product was roughly equally distributed to PI/PS, PE, and diphosphatidyglycerol (CL).

When Δ5 desaturases were tested for the distribution of the desaturation product, 20:4Δ5,8,11,14, in various lipid classes, the product was detected in the neutral lipid fraction and in all phospholipids analyzed (Fig. 2B). The expression of MsΔ5, 20:4Δ5,8,11,14, resulted in the accumulation of about equal proportions in PC, PI/PS, PE, CL, and in the neutral lipid fraction. In contrast, 20:4Δ5,8,11,14 formed with expression of PtΔ5 accumulated predominately in PC and was detected only in low amounts in PI/PS, PE, or CL.

In contrast to the results for lipid-specific desaturases (here for comparison, PtΔ5, Fig. 2B), where desaturation products are mostly enriched in PC, as shown by Domergue et al. (13), the data indicate that fatty acids produced by MsΔ6 and MsΔ5 do not associate predominantly with PC and can be detected to roughly equal amounts in all lipid species analyzed. These results suggest that, similar to the acyl-CoA-specific Δ6 desaturation from O. tauri, MsΔ6 and MsΔ5 may use acyl-CoA esters as substrates rather than lipid-linked acyl groups.

Desaturase Products of MsΔ6 and MsΔ5 Are Detected More Rapidly in the CoA Pool Than Products of Lipid-dependent Desaturases—For direct verification of acyl-CoA dependence of MsΔ6 and MsΔ5, acyl-CoA profiles of the respective yeast expression cultures were determined. Control cultures expressing OtΔ6, PtΔ6, or PtΔ5 were tested in parallel. Data presented were obtained by the method described by Domergue et al. (19). Exogenous substrates (18:3Δ9,12,15 for Δ6 desaturases or 20:3Δ8,11,14 for Δ5 desaturases) were added to induced yeast cultures at an A600 of 1.5–2.0. In cultures not supplied with exogenous fatty acid substrates, the patterns of total fatty acids and of fatty acids bound to CoA reflected only yeast-enriched fatty acids (data not shown). Within 5 min of adding exogenous 18:3Δ9,12,15 substrate to cultures expressing MsΔ6, OtΔ6, or PtΔ6, 18:3Δ9,12,15 was detected as a new peak in the total fatty acid pools (Fig. 3, left) and in the acyl-CoA pools as a shoulder of 16:1Δ9-CoA in the acyl-CoA chromatogram (see supplemental Fig. 2). In addition, a new peak representing the 18:4Δ6,9,12,15 product of Δ6 desaturation appeared simultaneously in the acyl-CoA pool of yeast expressing MsΔ6 and OtΔ6 but not in that of yeast expressing PtΔ6 (Fig. 3, right). The 18:4Δ6,9,12,15 product was not detectable in the total fatty acid pools of any culture at this early time point, and 18:4Δ6,9,12,15 appeared in the total fatty acid profiles of cultures expressing MsΔ6, OtΔ6, and PtΔ6 only at time points exceeding 1 h after application of 18:3Δ9,12,15. After 1 h, trace amounts of 18:4Δ6,9,12,15 appeared also in the acyl-CoA pool of yeast-expressing PtΔ6. The appearance of the Δ6 desaturase product in the acyl-CoA pool before its appearance in the total fatty acid pool suggests that MsΔ6 desaturates 18:3Δ9,12,15 to 18:4Δ6,9,12,15 in an acyl-CoA-dependent manner, consistent with data on OtΔ6 presented before (19). Reciprocally, the delayed appearance of 18:4Δ6,9,12,15-CoA product in the acyl-CoA pool with expression of PtΔ6 indicates that desaturation by PtΔ6 occurred on fatty acids linked to phospholipids rather than on those associated with CoA.

Changes in total fatty acids and the acyl-CoA pool with expression of the Δ5 desaturases MsΔ5 and PtΔ5 were analyzed before the addition of exogenous 20:3Δ8,11,14 substrate and afterward at defined times of 4, 8, and 24 h because these desaturases were not as efficient as the Δ6 desaturases, and Δ5-desaturation products were only detectable at those later time points. Within 1 h of adding exogenous 20:3Δ8,11,14 substrate to cultures expressing MsΔ5 or PtΔ5, 20:3Δ8,11,14 was detected as a new peak in the total fatty acids and in the acyl-CoA pools of all cultures. After 1 h, a new peak representing the 20:4Δ5,8,11,14-CoA product of Δ5 desaturation appeared in the acyl-CoA pool of yeast expressing MsΔ5 or PtΔ5. Simultaneously, the desaturation product 20:4Δ5,8,11,14 appeared in the total fatty acid pool of yeast expressing PtΔ5 but not in that of yeast expressing MsΔ5. In MsΔ5 cultures only at times exceeding 24 h after substrate addition, the 20:4Δ5,8,11,14 product was detectable in the fatty acid pool in trace amounts (data not shown).

Desaturase Products of MsΔ6 and MsΔ5 Are More Efficiently Elongated Than Those of Lipid-dependent Desaturases—To provide further evidence that MsΔ6 and MsΔ5 may be acyl-CoA-dependent enzymes, the desaturases were individually coexpressed with acyl-CoA elongases. The rationale of this
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experiment is that fatty acid elongation taking place in the acyl-CoA pool (13) should be more efficient with fatty acids desaturated within the acyl-CoA pool than with those desaturated by lipid-dependent enzymes, which require additional acyltransferase activities. The efficiencies of the combined desaturations/elongations were compared with those resulting from expression of the known desaturases OtΔ6, PtΔ6, or PtΔ5, in the same context.

The Δ6 desaturases were individually coexpressed in yeast with the Δ6 elongase PSE1 of the moss *P. patens* (24). As shown in Fig. 4A, the desaturation product of MsΔ6 and OtΔ6, 18:4Δ6,9,12,15 was elongated to the final elongation product 20:4Δ8,11,14,17, whereas the Δ6 desaturation product (18:2Δ6,9,12,15) did not accumulate and was barely detectable. An additional elongation product, 20:3Δ11,14,17, derived from the direct elongation of supplied 18:3Δ9,12,15, which appears prominently in the CoA pool due to the activation by CoA of the supplied fatty acid, is consistent with the previously reported elongation of Δ9-desaturated fatty acids by PSE1 (24, 33). In contrast to the situation with MsΔ6, the expression of PtΔ6 resulted in the accumulation of the Δ6 desaturation product, and only a smaller portion (ca. 60%, Fig. 4A) of the 18:4Δ6,9,12,15 was elongated to 20:4Δ8,11,14,17. It is important to note that supplied 18:3Δ9,12,15 was elongated to 20:3Δ11,14,17 with higher efficiency with expression of PtΔ6 than with expression of MsΔ6 or OtΔ6. These data indicate that supplied CoA-bound 18:3Δ9,12,15 was a better substrate for PSE1 than 18:4Δ6,9,12,15 formed by the lipid-dependent PtΔ6 desaturase despite of a preference of PSE1 for Δ6 fatty acid substrates. In contrast, 18:4Δ6,9,12,15 generated by MsΔ6 or OtΔ6 was efficiently elongated by PSE1 (97 and 96%, respectively: Fig. 4A) and competed with supplied 18:3Δ9,12,15 substrate for elongation activity, as evidenced by lower accumulation of 20:3Δ11,14,17 in these cultures.

The Δ5 desaturases MsΔ5 and PtΔ5 were coexpressed in yeast with the Δ5 elongase from *O. tauri*, OtELO2 (25). The expression cultures were supplied with 20:4Δ8,11,14,17, the ω3 substrate of the Δ5 desaturases, because the *O. tauri* Δ5 elongase prefers ω3 fatty acid substrates over ω6 substrates (25). As shown in Fig. 4B, the desaturation product of MsΔ5 EPA was almost completely elongated to 22:5Δ7,10,13,16,19 (elongation ~90%; Fig. 4B), whereas with the lipid-dependent desaturase, PtΔ5, accumulation of the lipid-bound desaturation intermediates, here EPA, was prominent, and the elongation efficiency was low (about 24%; Fig. 4B). Although additional lipid-bound desaturation cannot be excluded, the biochemical characterization of the *M. squamata* desaturases provides evidence that the two newly identified enzymes act on acyl-CoA substrates.

Side-by-side Assessment of Acyl-CoA-dependent Versus Lipid-dependent EPA Biosynthetic Pathways Reconstituted in Yeast—In the next step we tested whether the coexpression of the CoA-dependent *M. squamata* desaturases would lead to more effective biosynthesis of EPA than with lipid-dependent desaturases. To test this hypothesis, we compared the capacity to accumulate EPA from yeast expressing MsΔ6 together with PSE1 and MsΔ5 with that of yeast coexpressing PtΔ6 with the PSE1 and PtΔ5. All cultures were supplemented with ω-3-linolenic acid. Fatty acid products formed in all cultures were identified as ω3–18:4Δ6,9,12,15, 20:3Δ11,14,17, ω3–20:4Δ8,11,14,17, and ω3–20:5Δ5,8,11,14,17 (EPA, Fig. 4C). Most importantly, in both expression combinations, the biosynthesis of EPA was established, and EPA accumulated to 0.24 and 0.7% that of the total fatty acids, respectively. EPA accumulation through the CoA-dependent pathway was, therefore, to 2–3 times higher than that with expression of the lipid-dependent enzymes (Fig. 4C) even though the MsΔ5 desaturase was not as efficient as the PtΔ5 desaturase (for comparison see Fig. 1, B and C). A possible explanation is that flux from the substrate, 18:3Δ9,12,15 to EPA was more effective in the CoA-dependent pathway than in the lipid-dependent pathway, where the Δ6 desaturation product accumulated to high levels and was not available for further conversion.

Thus, whereas the use of lipid-dependent desaturation is limiting here in yeast, as described previously for *linseed* by Abbadi et al. (12), a more efficient interplay of the acyl-CoA-dependent enzymes may avoid the LPCAT bottleneck and may push the intermediates toward the end product, EPA. However, EPA accumulation in yeast was overall lower than expected. A possible explanation is that yeast cofactors may be different from those present in algae naturally harboring the enzymes used and that the functionality of the enzymes may, thus, be restricted in the heterologous system. The existence of 20:3Δ11,14,17 resulted from the direct elongation of the substrate 18:3Δ9,12,15 by PSE1 as mentioned above. This byproduct accumulated to higher amounts in the lipid-dependent expression cultures than observed with the CoA-dependent EPA biosyn-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** Interplay of desaturases with the respective elongases indicates acyl-CoA-dependent desaturation by MsΔ6 and MsΔ5. Yeast cultures coexpressing either MsΔ6, OtΔ6, or PtΔ6 with the elongase PSE1 or MsΔ5 or PtΔ5 with the elongase OtELO2 were analyzed for their fatty acid composition. A, elongation efficiencies (product × 100/(educt + product)) of the Δ6 elongase provided with its Δ6-desaturated substrate (18:3Δ9,12,15) by the respective Δ6 desaturase enzymes. B, elongation efficiencies of the Δ5 elongase provided with its Δ5-desaturated substrate (20:4Δ8,11,14,17) by the respective Δ5 desaturase enzymes. As controls the corresponding empty vectors and the elongases were expressed separately. In the absence of the respective desaturation products, only elongation of the feeding substrate was detected (data not shown). C, establishment of an exclusively acyl-CoA-dependent pathway for EPA biosynthesis in yeast. EPA accumulation in yeast expressing MsΔ6 together with PSE1 and MsΔ5 were compared with yeast expressing PtΔ6, PSE1, and PtΔ5. As control, the corresponding empty vector combination was also expressed (data not shown). The yeast cultures were grown in the presence of 250 μM 18:3Δ9,12,15. Data shown are representative for six independent experiments.
thesis pathway. In addition, the fatty acid 20:4\(\Delta 5,11,14,17\) was detected in both expression combinations, which is in agreement with the substrate specificities determined before (see Table 1).

The establishment of an acyl-CoA-dependent pathway for EPA production in yeast (Fig. 4C) provided proof of concept data for the feasibility of the chosen approach. Because results obtained with the yeast model cannot be readily transferred to the situation in plants, the next set of experiments aimed at establishing acyl-CoA-dependent EPA production in *Arabidopsis* seeds.

**Establishment of Acyl-CoA-dependent EPA Biosynthesis in A. thaliana**—To test whether acyl-CoA-dependent EPA biosynthesis could be established in plants, Ms\(\Delta 6\) and Ms\(\Delta 5\) were coexpressed with PSE1 in plants, subsequently referred to as triple-Ms plants. For direct comparison of the efficiency of EPA production by different enzymes, a construct containing Ot\(\Delta 6\) and Ot\(\Delta 5\) combined with PSE1 was also tested, which will be referred to as triple-Ot. To provide a side-by-side control against the efficiency of lipid-dependent EPA biosynthesis, Pt\(\Delta 6\) and Pt\(\Delta 5\) were coexpressed with PSE1 in plants, which reflect the approach previously reported (2, 36) and which will be referred to as triple-Pt plants. The plant transformation constructs encoded each gene under the control of the seed-specific USP promoter (26). The fatty acid analysis of individual T2 seeds of triple-Ms, triple-Ot, and triple-Pt plants showed several new fatty acids that were identified as \(3–18:4\)\(\Delta 6,9,12,15\), \(3–20:3\)\(\Delta 11,14,17\), \(3–20:4\)\(\Delta 8,11,14,17\), and EPA (Fig. 5, B–D) and for triple-Ot and triple-Pt additionally as \(6–18:3\)\(\Delta 6,9,12\), \(6–20:3\)\(\Delta 8,11,14\), and ARA (Fig. 6, B–D), indicating that in triple-Ms plants the \(3\) pathway and in triple-Ot and triple-Pt plants both the \(6\) and the \(3\) pathways had been successfully established. The fatty acid content of the endogenous fatty acids serving as substrates for the VLCPUFA pathway, \(18:3\)\(\Delta 9,12,15\) and \(18:2\)\(\Delta 9,12\), respectively, are more or less unaltered in the transgenic *Arabidopsis* seeds (Fig. 5A and 6A, respectively). Among the new fatty acids produced in the triple-Ms and the triple-Ot seeds, \(3–20:4\)\(\Delta 8,11,14,17\) is most abundant. In triple-Pt seeds, the most abundant fatty acids were \(6–18:3\)\(\Delta 6,9,12\), \(6–3\)\(\Delta 20:3\)\(\Delta 11,14,17\), and at least the first desaturation product of the \(6\) pathway, \(18:4\)\(\Delta 6,9,12,15\). The accumulation of both \(6\)-desaturated fatty acids in the triple-Pt plants is consistent with results obtained with this construct in tobacco, rapeseed, or linseed (2, 36) and confirms the presence of the bottleneck in lipid-dependent VLCPUFA biosynthesis. In contrast, in the triple-Ms and triple-Ot no or only minor accumulation of the \(6\) desaturation product \(18:4\)\(\Delta 6,9,12,15\) was observed, and nearly all \(18:4\)\(\Delta 6,9,12,15\) was elongated to the respective \(5\) product with very high efficiency (97% conversion) by the \(6\) elongase in case of the triple-Ms plants. These results indicate that the acyl-CoA and PC pool bottleneck has been successfully avoided by the use of strictly CoA-dependent desaturation (triple-Ms and triple-Ot). The

![FIGURE 5. \(\omega 3\) fatty acid contents of transgenic *Arabidopsis* plants. Single seeds were analyzed from three independent lines each of triple-Ms, triple-Ot, and triple-Pt Arabidopsis plants and compared with the fatty acid content of wild type *Arabidopsis* seeds (control). A, seed endogenous fatty acid 18:3\(\Delta 9,12,15\). B, the \(\Delta 6\) desaturation product 18:4\(\Delta 6,9,12,15\). C, the \(\Delta 6\) elongation product 20:4\(\Delta 8,11,14,17\). D, the final \(\Delta 5\) desaturation product 20:5\(\Delta 8,11,14,17\). The values are the mean ± S.D. of 13–16 individual seeds. n.d., not detectable.](http://example.com/figure5.png)
low proportions of $\omega 3-20:5^{\Delta 5,8,11,14,17}$ (EPA) in the triple-Ms or triple-Ot plants indicate that the $\Delta 5$ desaturase activity of Ms$\Delta 5$ and, even more that of Ot$\Delta 5$, still limit the accumulation of EPA in these seeds. This result is consistent with the proof of concept data from yeast expression studies, where Ms$\Delta 5$ also showed only low desaturation efficiency, and Ot$\Delta 5$ was nearly inactive.

The expression of the lipid-dependent enzymes in Arabidopsis seeds showed that LPCAT activity with non-native $\Delta 6$-polyunsaturated fatty acids was limiting in Arabidopsis, as previously observed in linseed (12). This observation led to the hypothesis that the selectivity of the LPCATs might be a ubiquitous problem in vascular plants. By avoiding the LPCAT activity in an exclusively acyl-CoA-dependent pathway for VLCPUFA production, no $\Delta 6$ intermediates were accumulating. The accumulation of EPA was nonetheless rather low both in yeast and in Arabidopsis seeds. The low accumulation even with acyl-CoA-dependent enzymes is likely a consequence of inefficient $\Delta 5$-desaturation after successful elimination of the LPCAT bottleneck (compare Fig. 1B with Figs. 5 and 6). Future studies will be directed toward the isolation of more efficient acyl-CoA-dependent $\Delta 5$ desaturases, which currently have not been identified.

Although the changes in the total fatty acids of seeds of triple-Ms plants show that the accumulation of $\Delta 6$ desaturation intermediates has been successfully avoided using the enzymes from M. squamata, another important concern for VLCPUFA production is the incorporation into TAG. To test whether the newly formed fatty acids accumulated in TAG or in membrane phospholipids, the distribution of the various fatty acid products of the newly established biosynthetic pathways was analyzed, and the percentages of each product associated with different lipids were calculated. An initial analysis of the lipid composition of different transgenic seeds indicated that all transgenic lines accumulated similar proportions of TAG (Table 2). The comparison of plants expressing the triple-MS

| Lipid class of glycerolipids | % Composition of glycerolipids | % Distribution of EPA | % Distribution of ARA |
|-----------------------------|-------------------------------|-----------------------|-----------------------|
| Triple-Ms plants            |                              |                       |                       |
| TAG                         | 94.2%                        | 92.4%                 | n.d.                  |
| PC                          | 3.1%                         | 7.6%                  | n.d.                  |
| PI                          | 1.2%                         | n.d.                  | n.d.                  |
| PE                          | 1.4%                         | 90.0%                 | 72.9%                 |
| Triple-Ot plants            |                              |                       |                       |
| TAG                         | 96.7%                        | 90.0%                 | 72.9%                 |
| PC                          | 1.8%                         | 10.0%                 | 11.5%                 |
| PI                          | 0.7%                         | n.d.                  | 1.2%                  |
| PE                          | 0.8%                         | n.d.                  | 14.4%                 |
| Triple-Pt plants            |                              |                       |                       |
| TAG                         | 92.3%                        | 72.8%                 | 60.7%                 |
| PC                          | 4.1%                         | 22.3%                 | 27.3%                 |
| PI                          | 1.6%                         | n.d.                  | 3.8%                  |
| PE                          | 1.8%                         | 4.9%                  | 8.2%                  |

The values are the mean ± S.D. of 13–16 individual seeds. n.d., not detectable.
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and triple Ot-constructs indicates a preferential incorporation of CoA-bound ω3 fatty acids over ω6 fatty acids in TAG (Table 2). The distribution of ARA and EPA in different lipid classes of Arabidopsis seeds showed that the VLCPUFA produced by acyl-CoA-dependent pathways accumulated overall to a higher degree in TAG than those produced by the lipid-linked pathway (Table 2). These observations invite some speculation on the routes of TAG biosynthesis in Arabidopsis seeds. Based on the fact that, in contrast to lipid-bound VLCPUFAs, CoA-bound VLCPUFAs were preferentially incorporated into TAG rather than into phospholipids, we suggest that TAG is rather produced by an acyl-CoA:diacylglycerol acyltransferase than by a phospholipid:diacylglycerol acyltransferase enzyme in Arabidopsis seeds. It is interesting to note that incorporation of ω3 products into TAG was significantly higher than that of ω6 products (compare in Table 2). A possible explanation for the higher incorporation of ω3 products into TAG would be a preference of the acyl-CoA:diacylglycerol acyltransferase and/or phospholipid:diacylglycerol acyltransferase for the conversion of ω3-substrates.

From plant transformation studies it is widely known that the insertion sites of transgenes in the plant genome play an important role for biotechnological efficiency. In particular, multi-gene constructs with more than one promoter require certain distances between different promoter-gene-terminator packages. The low efficiency of EPA accumulation in the transgenic Arabidopsis plants reported here may, thus, in part be a consequence of less than optimal transgene insertion and positional effects. Another possible explanation for low product accumulation are so-far unknown cofactors or other accessory proteins required by the algal enzymes for optimal functionality that are missing in the plants, as was already mentioned for yeast.

In summary, VLCPUFA can be produced in plants using an exclusively acyl-CoA-dependent pathway that avoids rate-limiting shuttling of fatty acid intermediates between lipids and coenzyme A. Although technical constraints, such as low catalytic activities of the Δ5 desaturases or low expression levels of transgenes in plants still limit VLCPUFA accumulation in the experiments reported here, the data show that in principle there is no requirement for acyl-shuttling into lipids. VLCPUFA produced by an acyl-CoA-dependent fashion are in fact more efficiently incorporated into TAGs than those produced from the lipid-bound fatty acid substrates, suggesting that acyl-CoA-dependent VLCPUFA biosynthesis may offer advantages over other approaches to improve the composition of plant oils.

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REFERENCES

1. Simopoulos, A. P. (1991) Am. J. Clin. Nutr. 54, 438–463
2. Simopoulos, A. P. (2002) J. Am. Coll. Nutr. 21, 495–505
3. Goyens, P. L., Spilker, M. E., Zock, P. L., Katan, M. B., and Mensink, R. P. (2006) Am. J. Clin. Nutr. 84, 44–53
4. Hites, R. A., Foran, J. A., Schwager, S. J., Knuth, B. A., Hamilton, M. C., and Carpenter, D. O. (2004) Environ. Sci. Technol. 38, 4945–4949
5. Graham, I. A., Larson, T., and Napier, I. A. (2007) Curr. Opin. Biotechnol. 18, 142–147
6. Napier, J. A. (2007) Annu. Rev. Plant Biol. 58, 295–319
7. Qi, B., Fraser, T., Mugford, S., Dobson, G., Sayanova, O., Butler, J., Napier, J. A., Stobart, A. K., and Lazarus, C. M. (2004) Nat. Biotechnol. 22, 739–745
8. Qi, B., Beaudoin, F., Fraser, T., Stobart, A. K., Napier, J. A., and Lazarus, C. M. (2002) FEBS Lett. 510, 159–165
9. Wallis, J. G., and Browse, J. (1999) Arch. Biochem. Biophys. 365, 307–316
10. Michaelson, L. V., Lazarus, C. M., Griffiths, G., Napier, J. A., and Stobart, A. K. (1998) J. Biol. Chem. 273, 19055–19059
11. Sayanova, O., Haslam, R., Qi, B., Lazarus, C. M., and Napier, J. A. (2006) FEBS Lett. 580, 1946–1952
12. Abbadi, A., Domergue, F., Bauer, J., Napier, J. A., Welti, R., Zähringer, U., Cirpus, P., and Heinz, E. (2004) Plant Cell 16, 2734–2748
13. Domergue, F., Abbadi, A., Ott, C., Zank, T. K., Zähringer, U., and Heinz, E. (2003) J. Biol. Chem. 278, 35115–35126
14. Wu, G., Truksa, M., Datla, N., Vrinten, P., Bauer, J., Zank, T., Cirpus, P., and Eui, X. (2005) Nat. Biotechnol. 23, 1013–1017
15. Kinney, A. J., Cahoon, E. B., Danmude, H. G., Hitz, W. D., Liu, Z. B., and Kolar, C. W. (2004) WO 2004/074167 A2
16. Tocher, D. R., Leaver, M. J., and Hodgson, P. A. (1998) Prog. Lipid Res. 37, 73–117
17. Okayasu, T., Nagao, M., Ishibashi, T., and Imai, Y. (1981) Arch. Biochem. Biophys. 206, 21–28
18. Sprecher, H., Chen, Q., and Yin, F. F. (1999) Lipids 34, 153–156
19. Domergue, F., Abbadi, A., Zähringer, U., Moreau, H., and Heinz, E. (2005) Biochem. J. 389, 483–490
20. Ausubel, F. M., Brent, R. E., Kingston, D. D., Seidmann, J. R., Smith, J. A., and Struhl, K. (1993) Current Protocols in Molecular Biology, Green Publishing Associates and John Wiley and Sons Inc., New York
21. Manton, I., and Parke, M. (1960) Butcher’s Marine Biol. Ass. UK 39, 275–278
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor NY
23. Donahue, T. F., and Cigan, A. M. (1999) Methods Enzymol. 185, 366–372
24. Zank, T. K., Zähringer, U., Beckmann, C., Pohnert, G., Boland, W., Holz, H., Reski, R., Lerchl, J., and Heinz, E. (2002) Plant J. 31, 255–268
25. Meyer, A., Kirsch, H., Domergue, F., Abbadi, A., Sperling, P., Bauer, J., Cirpus, P., Zank, T. K., Moreau, H., Roscoe, T. J., Zähringer, U., and Heinz, E. (2004) J. Lipid Res. 45, 1899–1909
26. Baumlein, H., Boerjan, W., Nagy, I., Bassuner, R., Van Montagu, M., Inze, D., and Wobus, U. (1991) Mol. Gen. Genet. 225, 459–467
27. Hornung, E., Korfei, M., Pernstich, C., Struss, A., Kindl, H., Fulda, M., and Wobus, U. (1991) Biochim. Biophys. Acta 1013–1017
28. Butte, W., Reimann, H. H., and Walle, A. J. (1982) Clin. Chem. 28, 1778–1781
29. Larson, T. R., and Graham, I. A. (2001) Plant J. 25, 115–125
30. Napier, J. A., Sayanova, O., Sperling, P., and Heinz, E. (1999) Trends Plant Sci. 4, 2–4
31. Sperling, P., Schmidt, H., and Heinz, E. (1995) Eur. J. Biochem. 232, 798–805
32. Shanklin, J., and Cahoon, E. B. (1998) Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 611–641
33. Domergue, F., Lerchl, J., Zähringer, U., and Heinz, E. (2002) Eur. J. Biochem. 269, 4105–4113
34. Sayanova, O., Haslam, R., Venegas-Caleron, M., and Napier, J. A. (2006) Planta 224, 1269–1277
35. Garcia-Maroto, F., Manas-Fernandez, A., Garrido-Cardenas, J. A., and Alonso, D. L. (2006) Phytochemistry 67, 540–544
36. Abbadi, A., Fulda, M., Feussner, I., Orsini, J., Weyen, J., Frauen, M., and Leckband, G. (2007) in Proceedings of the 12th International Rapeseed Congress Sustainable Development in Cruciferous Oilseed Crops Production–Vol. II Biotechnology (Meng, J., Lu, C., Zhao, J., Liu, C., and Hua, W., eds) pp. 152–155, Science Press U. S. A. Inc., Monroe Junction, NJ
37. Clough, S. J., and Bent, A. F. (1998) Plant J. 16, 735–743