Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis[S]

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Abstract **In algae, the biosynthesis of docosahexaenoic acid (22:6ω3; DHA) proceeds via the elongation of eicosapentaenoic acid (20:5ω3; EPA) to 22:5ω3, which is required as a substrate for the final Δ4 desaturation. To isolate the elongase specific for this step, we searched expressed sequence tag and genomic databases from the algae *Ostreococcus tauri* and *Thalassiosira pseudonana*, from the fish *Oncorhynchus mykiss*, from the frog *Xenopus laevis*, and from the sea squirt *Ciona intestinalis* using as a query the elongase sequence PpPSE1 from the moss *Physcomitrella patens*. The open reading frames of the identified elongase candidates were expressed in yeast for functional characterization. By this, we identified two types of elongases from *O. tauri* and *T. pseudonana*: one specific for the elongation of (Δ6-)C18-PUFAs and one specific for (Δ5-)C20-PUFAs, showing highest activity with EPA. The clones isolated from *O. mykiss*, *X. laevis*, and *C. intestinalis* accepted both C18- and C20-PUFAs. By coexpression of the Δ6- and Δ5-elongases from *T. pseudonana* and *O. tauri*, respectively, with the Δ5- and Δ4-desaturases from two other algae we successfully implemented DHA synthesis in stearidonic acid-fed yeast.[jlr] This may be considered an encouraging first step in future efforts to implement this biosynthetic sequence into transgenic oilseed crops.**—Meyer, A., H. Kirsch, F. Domergue, A. Abbadi, P. Sperling, J. Bauer, P. Cirpus, T. K. Zank, H. Moreau, T. J. Roscoe, U. Zähringer, and E. Heinz. **Novel fatty acid elongases and their use for the reconstitution of docosahexaenoic acid biosynthesis. *J. Lipid Res.* 2004.** 45: **1899–1909.**

Supplementary key words *Ciona intestinalis* • *Oncorhynchus mykiss* • *Ostreococcus tauri* • polyunsaturated fatty acids • *Thalassiosira pseudonana* • *Xenopus laevis*

Docosahexaenoic acid (DHA) is a fatty acid with 22 carbon atoms and 6 methylene-interrupted *Z*-double bonds ($22:6^{\Delta4,7,10,13,16,19}$). Some human tissues such as brain, testis, and retina are characterized by membrane lipids carrying high proportions of this long-chain polyunsaturated fatty acid (LCPUFA), and many clinical studies have pointed out the crucial role of DHA in the development and functions of these tissues (1). In addition, DHA as well as the other LCPUFAs arachidonic acid (ARA; $20:4^{\Delta5,8,11,14}$) and eicosapentaenoic acid (EPA; $20:5^{\Delta5,8,11,14,17}$) are precursors of different classes of eicosanoid effectors involved in the regulation of many important functions in mammals. In view of the major roles attributed to LCPUFAs in human physiology, the reactions contributing to their biosynthesis have recently attracted growing interest (2). Because of its relevance for human nutrition, the biosynthetic sequence known in most detail is that realized in the mammalian liver and known as the Sprecher pathway (3). Moreover, LCPUFA biosynthesis was also studied in various organisms from phylogenetically divergent groups, such as algae, fungi, and lower plants. DHA is synthesized de novo in several microalgae as well as in some fungi, whereas in mammals its synthesis starts from the essential fatty acid α-linolenic acid (ALA; $18:3^{\Delta9,12,15}$). Another route for DHA biosynthesis that will not be further described here is relying on polyketide synthase systems. It is found in some marine bacteria and primitive eukaryotes like the thraustochytrid protist *Schizochytrium* (4).

The currently best known routes for DHA biosynthesis are depicted in simplified form in **Fig. 1**. In the liver of

Abbreviations: ALA, α-linolenic acid; ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EST, expressed sequence tag; GLA, γ-linolenic acid; LA, linoleic acid; LCPUFA, long-chain polyunsaturated fatty acid; ORF, open reading frame; STA, stearidonic acid.

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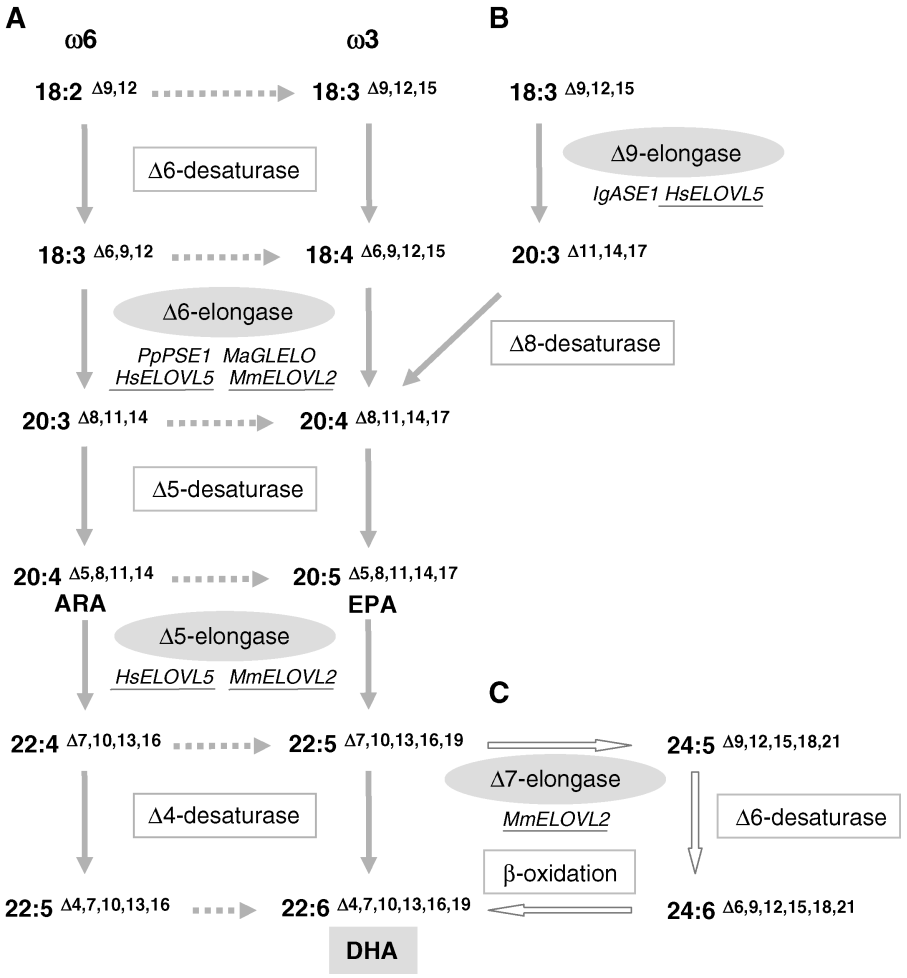

Fig. 1. ω6 and ω3 pathways for the synthesis of long-chain polyunsaturated fatty acids in eukaryotes. ω6-18:2 and ω3-18:3 can be converted to docosahexaenoic acid (DHA) by the consecutive action of desaturases and elongases, either starting with a Δ6 desaturation (A) or with a Δ9 elongation (B). In mammals, the Δ4 double bond in DHA is the result of a more complicated series of reactions that involve the elongation to a C24 fatty acid, a second Δ6 desaturation, and the final chain shortening in peroxisomes (the so-called Sprecher pathway; C). ω3-desaturases (dashed arrows), which link the ω6 and ω3 pathways in many eukaryotes, are missing in mammals. Examples of cloned elongases involved in DHA synthesis have been included in the figure. Although some elongases are specific for a single step (e.g., the moss PpPSE1 and the fungal MaGLELO), others are nonspecific or multifunctional and show up at several steps (e.g., the human HsELOVL5 and the murine MmELOVL2; underlined).

mammals, a Δ6-desaturase, a Δ6-elongase, and a Δ5-desaturase successively convert ALA via stearidonic acid (STA; $18:4^{\Delta 6,9,12,15}$) and $20:4^{\Delta 8,11,14,17}$ to EPA in the so-called ω3 pathway. In parallel, the same set of enzymes accepts the other essential fatty acid linoleic acid (LA; $18:2^{\Delta 9,12}$) to form ARA in the ω6 pathway. It is important to note that because of the absence of an ω3-desaturase in mammals, the intermediates of the ω6 and ω3 pathways are not interconvertible. In mammalian liver, EPA is then elongated twice without an intervening desaturation. Recent data suggest that the same elongase (ELOVL2) accepts EPA as well as Δ7-22:5 (i.e., the reaction product of its own first elongation cycle), leading to Δ9-C24:5 (5). At this point, the Δ6-desaturase that is responsible for the formation of Δ6-C18-PUFAs gets involved a second time and inserts a Δ6 double bond in Δ9-C24:5, leading to the synthesis of Δ6-C24:6 (5, 6). Finally, Δ6-C24:6 is transferred from the endoplasmic reticulum membranes to the peroxisomes for one round of β-oxidative chain shortening and release of DHA. These final steps, from EPA to DHA, are attributable to the absence of a Δ4-desaturase activity in mammals and are characteristic for the Sprecher pathway. Labeling studies (7) have shown that this sequence is also present in fish, suggesting that this route for DHA biosynthesis is not restricted to mammals.

Up to ARA and EPA, the biosynthetic pathways found in mosses, fungi, and algae are basically identical to the mammalian sequence. Also in these organisms, two front-end desaturases (Δ6 and Δ5) and one Δ6-elongase convert either LA or ALA into ARA or EPA, respectively, in the ω6 and ω3 pathways (Fig. 1A). In addition, some algae can use an alternative pathway, in which LA and ALA are elongated

to the corresponding Δ11-C20-PUFA (Fig. 1B), necessitating a subsequent Δ8-desaturation (8). Because many of these organisms possess an ω3-desaturase, they do not show the strict separation of ω6 and ω3 pathways typical for mammals. In addition, these organisms may use acyl chains of phospholipids as substrates for the desaturation reactions, whereas acyl-CoA thioesters are required for elongation reactions (9). In contrast, in mammals all of the intermediates are kept in the form of acyl-CoA thioesters. Most importantly for this study, the primary de novo producers of DHA appear to follow a simplified route for DHA synthesis in which a Δ5-elongase and a Δ4-desaturase are responsible for the conversion of EPA to DHA (10–12).

Elongase complexes comprise four activities: the β-ketoacyl-CoA synthase, the ketoacyl-CoA reductase, the hydroxyacyl-CoA dehydratase, and the enoyl-CoA reductase. From these, the first enzyme is considered to be rate limiting and specificity controlling with regard to chain length and pattern of double bonds. ELO-type sequences involved in LCPUFA biosynthesis were cloned from the moss *Physcomitrella patens* (13), the fungus *Mortierella alpina* (14), the nematode *Caenorhabditis elegans* (15), the alga *Isochrysis alpina* (8), and different mammals [for a recent review, see ref. (16)]. Because of their close relation to the enzymes ScELO1, ScELO2, and ScELO3 from *Saccharomyces cerevisiae* (17, 18), they are thought to code for β-ketoacyl-CoA synthase activities and are often referred to as "elongases," although biochemical data supporting the actual condensing activity are still missing.

Yeast and animal cells were used for the expression of most of the elongases catalyzing the elongation steps shown in Fig. 1. Most of the sequences characterized to date are of mammalian origin, but few of them have been studied in sufficient detail to answer all questions regarding regioselectivity and chain length selectivity. Therefore, it remains unclear which substrates other than those indicated in Fig. 1 could also be accepted by these enzymes. Among the few sequences of nonmammalian origin studied in more detail, none was shown to be specific for the elongation of EPA. The Δ5-elongases cloned to date were all isolated from mammals and rather unspecific, in that they carried out multiple elongation reactions not restricted to C20-PUFAs, as shown in Fig. 1.

As mentioned above, the synthesis of LCPUFAs in mammals depends on the dietary supply of LA and ALA, and ω6- and ω3-LCPUFAs are each precursors of antagonistic eicosanoid effectors. Typical Western diets are characterized by very high ratios of LA/ALA that are far above the recommended value of ~5 and thus favor the synthesis of ARA at the expense of EPA and DHA (19). In addition, because ALA appears to be rapidly degraded by β-oxidation, it seems best to include an appropriate mixture of LCPUFAs in the diet. As this growing demand cannot be met by farmed or landed fish (20) and none of the oilseeds produces LCPUFAs, the implementation of LCPUFA biosynthesis into oilseed crops by modern biotechnology would provide a truly sustainable source of these valuable fatty acids. Because the biosynthesis of DHA according to the Sprecher pathway is clearly too complicated to be reconstituted by gene technology, the alternative route relying on the use of a Δ5-elongase and a Δ4-desaturase is most promising. Whereas all of the desaturases, including the Δ4-desaturase, and the Δ6-elongase have already been isolated from various organisms, an elongase specific for the conversion of a C20-PUFA to a C22-PUFA has not been cloned yet. To reconstitute the simpler pathway of DHA biosynthesis, we started experiments to clone a specific C20-PUFA-elongase restricted in its action to a single elongation cycle to produce a C22-PUFA. Such an activity may become particularly relevant in transgenic plants, in which other potential substrates may be present in excess, leading to complicated mixtures of elongated products.

EXPERIMENTAL PROCEDURES

Identification and cloning of putative elongase sequences

To identify novel elongases, we used the Δ6-elongase sequence from *P. patens* (PpPSE1) as the query in a tBLASTn (21) search and identified putative elongase expressed sequence tag (EST) clones from *Xenopus laevis* (GenBank accession number BC044967), *Ciona intestinalis* (GenBank accession number AK112719), and *Oncorhynchus mykiss* (GenBank accession number CA350786). The *C. intestinalis* clone was kindly provided by S. Fujiwara (22), the clone from *O. mykiss* was a gift from C. E. Rexroad (United States Department of Agriculture, Agricultural Research Service, National Center for Cool and Cold Water Aquaculture), and the *X. laevis* clone was purchased from the American Type Culture Collection (ATCC 6844054). The open reading frames (ORFs) were amplified by PCR (primers XlELO, CiELO, and OmELO; **Table 1**) and cloned into the yeast expression vector pYES2.1/V5-His-TOPO (XlELO and CiELO) or pYES3CT (OmELO) from Invitrogen, yielding pXlELO, pCiELO, and pOmELO.

A bacterial artificial chromosome library of *Ostreococcus tauri* OTHH0595 was constructed and sequenced as previously described (23) and used for tBLASTn with PpPSE1 as the query. Two ORFs showing significant similarity to the elongase and apparently lacking introns were amplified by PCR with specific primers (OtELO1 and OtELO2). Fresh cells that were grown in K medium (Sigma-Aldrich) at 23°C in the light and frozen once were used as template. The PCR products were cloned into the pYES2.1/V5-His-TOPO yeast expression vector, yielding pOtELO1 and pOtELO2, respectively.

PpPSE1 was also used to blast a genomic library of *Thalassiosira pseudonana* (Department of Energy Joint Genome Institute), yielding two putative elongase clones (PQI68798 and PQI119277). *T. pseudonana* SAG 1020-1b (Sammlung für Algenkulturen, Göttingen, Germany) was grown in the light at 23°C in f/2 medium, and the genomic DNA was isolated using the DNeasy Kit (Qiagen). The genomic DNA was used for PCR (primers Tp687GENOMIC and Tp119GENOMIC), and the PCR products were cloned into pGEM-T (Promega) and sequenced. GENESCAN (Arabidopsis algorithm) identified in each clone two exons separated by one intron. The ORF of PQI68798 was amplified by PCR, whereby the 5′ primer was constructed in such a way that it was complementary to exon I (91 bp) and to the 5′ end of exon II (23 bp); therefore, it was missing the part coding for the 142 bp intron (primer Tp687ELO). The 5′ primer of exon I was altered to approximate optimal yeast codon usage without changing the translated sequence. The putative ORF of PQI119277 was also constructed by PCR, taking into account the yeast codon usage. In a first step, both exons were amplified with primers (Tp119EI and Tp119EII)

TABLE 1. PCR primers used in this work

Gene/Clone	Primer	Oligonucleotide Sequence
Xl*ELO*	XlELO-5′	5′-AGGATCCATGGCCTTCAAGGAGCTCACATC-3′
	XlELO-3′	5′-CCTCGAGTCAATGGTTTTTGCTTTTCAATGCACCG-3′
Ci*ELO*	CiELO-5′	5′-TAAGCTTATGGACGTACTTCATCGT-3′
	CiELO-3′	5′-TCAGATCTTTAATCGGTTTTACCATT-3′
Om*ELO*	OmELO-5′	5′-AAGCTTACATAATGGAGACTTTTAA-3′
	OmELO-3′	5′-GGATCCTTCAGTCCCCCCTCACTTCC-3′
Ot*ELO1*	OtELO1-5′	5′-CCAAGCTTACATAATGAGTGGCTTACGTGCACCCAA-3′
	OtELO1-3′	5′-CCCTCGAGTCACTGCTGTTTTTTCTGGAGCTTA-3′
Ot*ELO2*	OtELO2-5′	5′-CCAAGCTTACATAATGAGCGCCTCCGGTGCGCT-3′
	OtELO2-3′	5′-CCCTCGAGTTAGTCAATTTTTCGAGATCGCGTG-3′
PQI119277	Tp119GENOMIC-5′	5′-ATTGGCGTAATTCTTCGGGG-3′
	Tp119GENOMIC-3′	5′-GTGCTTGTCAAAGTAGAATAAG-3′
PQI68798	Tp687GENOMIC-5′	5′-TCAACCCTCAATACAAAGC-3′
	Tp687GENOMIC-3′	5′-GTGGATGGAAGCTGTTAA-3′
Tp*ELO1*	Tp687ELO-5′	5′-ATGGACGCTTACAACGCTGCTATGGACAAGATTGGTGCTGCTATTATTGACTGGTCTGATC-CCGATGGAAAGTTCCGTGCCGATAGAGAGGACTGGTGGCTCTGCGACTTCCGT-3′
	Tp687ELO-3′	5′-CTAAGCACTCTTCTTCTTTTTGGGTGC-3′
Tp*ELO2*	Tp119EI-5′	5′-ATGTGTTCTCCACCACCATCTCAATCCAAGACTACCTCCTTGTTGGCTAGATACACCAC-CGCCGCCCTCCTCCTCCTCACCCTCACAACGTGGTGCCACTTCGCCTTCCCAGCCGCC-3′
	Tp119EI-3′	5′-CGTGTGGTGGTAGATGTGGAGGAAGGAGACCTGGTCCATTTTCCCCCTCAACAC-3′
	Tp119EII-5′	5′-TTTATGGTGTTGAGGGGGAAAATGGACCAGGTCTCCTTCCTCCACATCTACCACCACACG-3′
	Tp119EII-3′	5′-CTACATGGCACCAGTAACACG-3′

that created a 54 bp overlapping sequence. The overlapping products subsequently served as templates for the second PCR using the 5′ primer of exon I and the 3′ primer of exon II. The ORFs of PQI68798 and PQI119277 were cloned into pYES2.1/V5-His-TOPO, yielding pTpELO1 and pTpELO2, respectively.

pXlELO, pCiELO, pOmELO, pOtELO1, pOtELO2, pTpELO1, and pTpELO2 were then used for the transformation of *S. cerevisiae* 334 (24) or INVSc1 (Invitrogen).

Expression in yeast

For functional expression of the elongases, precultures were grown at 30°C in minimal medium with 2% raffinose lacking the respective amino acid or base for vector selection. Five milliliters of the medium were inoculated with precultures (2 days old) to an optical density at 600 nm of 0.05, and expression was induced with 2% galactose. Expressions were carried out for 4 days at 20°C in the presence of exogenously supplied fatty acids of commercial origin (250–500 μM). Pinolenic acid ($18:3^{\Delta5,9,12}$) was part of a fatty acid mixture isolated from *Larix decidua* seeds (25). For the coexpression of elongases and desaturases, the yeasts were additionally transformed with the Δ5-desaturase from *Phaeodactylum tricornutum* (26) and the Δ4-desaturase from *Euglena gracilis* (11). Yeasts harboring the empty vectors (pYES2, pYES3CT, and pESC-Leu) were used as controls.

Fatty acid analysis

Yeast cells were sedimented by centrifugation and directly used for transmethylation of fatty acids. Fatty acid methyl esters were routinely analyzed by gas liquid chromatography as described previously (11), whereas detailed structural identities of new fatty acids were determined by GC-MS (27).

RESULTS

Isolation of elongase genes from *Xenopus*, *Ciona*, *Oncorhynchus*, *Ostreococcus*, and *Thalassiosira*

The majority of elongase sequences available in databases originate from mammals (i.e., human, mouse, and rat), and among those functionally characterized, none was shown to be specific for the elongation of Δ6-C18- or Δ5-C20-PUFAs. Because the Pp*PSE1* gene from the moss *P. patens* is known to code for a specific Δ6-elongase, we decided to use its translated sequence as the query in a tBLASTn search to identify PUFA-elongases in EST and genomic databases of nonmammalian organisms. The fish *O. mykiss*, the frog *X. laevis*, the sea squirt *C. intestinalis*, and the two DHA-producing algae *O. tauri* and *T. pseudonana* were selected as candidate organisms. Among several identified ORFs of interest, we could amplify seven putative elongase clones. The actual numbers of amino acids (272–358) representing the various ORFs as well as their identity compared with the other elongases cloned in the present study are summarized in **Table 2**. Most of the proteins were only 19–26% identical to PpPSE1, with the exception of OtELO1, which showed a significantly higher identity (42%). Interestingly, the identity between OtELO1 and OtELO2 is only 20%. It should be mentioned that Tp*ELO1* and Tp*ELO2* from *T. pseudonana* were constructed by PCR after a GENESCAN analysis to delete putative introns and, therefore, may not represent the ORFs translated in the alga. The deduced amino acid sequences of the newly cloned proteins all contained seven to nine putative transmembrane helices as well as the various motifs (16) that are typical for this group of elongases (KxxE/DxxDT, the extended histidine box QxxFLHxYHH, the tyrosine box NxxxHxxMYxYY, and TxxQxxQ) (**Fig. 2**). Lysine residues close to the C terminus that may function as endoplasmic reticulum retention signals were clearly seen in three sequences (XlELO, OtELO1, and TpELO1). The phylogenetic alignment of the currently cloned elongases together with previously characterized enzymes will be discussed below.

Functional expression in yeast

The functions of the proteins encoded by the isolated genes were verified by expression studies in *S. cerevisiae*

TABLE 2. Elongase genes cloned in this work

Organism	Source	Open Reading Frame	Accession Number	Peptide Length (Amino Acids)	Identity with PpPSE1	Other Identities
Xenopus laevis	Expressed sequence tag	Xl*ELO*	AY605098	302	25%	39% CiELO, 32% OmELO, 24% OtELO2
Ciona intestinalis	Expressed sequence tag	Ci*ELO*	AY605099	289	26%	36% OmELO, 27% OtELO1, 24% OtELO2
Oncorhynchus mykiss	Expressed sequence tag	Om*ELO*	AY605100	295	24%	22% OtELO2, 22% OtELO1, 21% TpELO2
Ostreococcus tauri	Genomic	Ot*ELO1*	AY591335	292	42%	23% TpELO1, 20% OtELO2, 20% TpELO2
Ostreococcus tauri	Genomic	Ot*ELO2*	AY591336	300	21%	24% XlELO, 23% TpELO2, 20% TpELO1
Thalassiosira pseudonana	Genomic	Tp*ELO1*	AY591337	272	23%	22% CiELO, 20% OmELO, 20% XlELO
Thalassiosira pseudonana	Genomic	Tp*ELO2*	AY591338	358	19%	20% CiELO, 20% XlELO, 17% TpELO1

comprising incubations with exogenous fatty acids followed by GC-MS analysis of total fatty acid methyl esters. To identify which ORF could encode a Δ5-C20-specific elongase, each ORF was expressed in the presence of either Δ6- or Δ5-polyunsaturated fatty acids (STA or EPA, respectively). Low basal elongation of STA, but no elongation of EPA, was obtained with yeast transformed with the empty vectors (data not shown). All cloned elongases were active in yeast and produced novel fatty acids whose structures were confirmed by GC-MS. Using either STA or EPA as exogenously supplied substrates, the seven elongases could be separated into three different groups: Δ6-C18-PUFA-elongases (TpELO1 and OtELO1), Δ5-C20-PUFA-elongases (TpELO2 and OtELO2), and bifunctional enzymes accepting both C18- and C20-PUFAs (OmELO, XlELO, and CiELO). Results obtained with one member of each group are given in **Fig. 3**. On expression of TpELO1 (and of OtELO1; data not shown, but see **Fig. 4**), STA was very efficiently converted to 20:4ω3, whereas EPA was not elongated. In contrast, when OtELO2 (and TpELO2; data not shown, but see Fig. 4) was expressed, EPA was very efficiently elongated, whereas STA elongation did not exceed that of the control strain. When OmELO was expressed, STA was successively elongated to 20:4ω3 and 22:4ω3, whereas EPA was elongated to 22:5ω3. Yeast expressing XlELO or CiELO were also able to convert both STA and EPA (data not shown, but see Fig. 4). As shown in Fig. 3, OmELO was in addition active on $16:1^{\Delta 9}$, $18:1^{\Delta 9}$, and $18:1^{\Delta 11}$, as indicated by the increase/presence of $18:1^{\Delta 11}$, $20:1^{\Delta 11}$, and $20:1^{\Delta 13}$. Starting from $16:1^{\Delta 9}$, OmELO was in fact capable of five successive elongation steps leading to the synthesis of $26:1^{\Delta 19}$.

Substrate specificity of the different elongases

We then investigated to what extent these regioselective specificities persisted when fatty acid substrates of different structures, regarding both chain length and position of double bonds, were supplied. The results (Fig. 4) show that TpELO1 and OtELO1 were exclusively active with C18-PUFAs. Although the activity of TpELO1 was restricted to the Δ6-unsaturated fatty acids γ-linolenic acid (GLA) and STA, OtELO1 elongated GLA and STA very efficiently but also to a minor extent Δ9-C18-PUFAs (LA and ALA) and a Δ5-C18-PUFA (pinolenic acid). The activity of the Δ5-C20-PUFA-elongases (TpELO2 and OtELO2) was restricted to C20-PUFAs, with EPA being most efficiently elongated in both cases. None of these enzymes elongated Δ8-20:3ω6, but both TpELO2 and OtELO2 were active on Δ5-20:4ω3 (2% and 13% conversion, respectively). The most active clone, OtELO2, also displayed activity with Δ8-20:3ω3 (7% conversion), indicating that the presence of an ω3 double bond of the C20-PUFAs may play an important role in substrate recognition. For example, OtELO2 elongated 11% of ARA but 56% of EPA.

In contrast, the three elongases from animals elongated almost all LCPUFA substrates offered, although with different efficiencies. The *X. laevis* XlELO mainly converted Δ6-C18- and Δ5-C20-PUFAs (e.g., 24% of STA and 10% of EPA), but it was also very active with the Δ11 fatty acid 20:3ω3 (13% conversion). CiELO from the sea squirt was most active with pinolenic acid (38% conversion; see supplemental data) and least active with STA. OmELO was the most efficient among the three enzymes and elongated most substrates by more than 20% (Fig. 3). However, OmELO did not elongate ARA or EPA to C24-PUFAs; therefore, it does not seem to be involved in the Sprecher pathway.

Phylogenetic relationships

Several elongase phylograms have been constructed before, but because of the limited number of functionally assigned sequences, only a few questions regarding the branching pattern could be addressed (13, 16, 28). The elongase enzymes studied here significantly increase the number of available sequences with known specificities. To see how the resulting branching correlates with substrate specificity and phylogenetic relationships of the organisms, we created an unrooted phylogenetic tree comprising all of the elongases cloned and functionally characterized to date by the neighbor-joining method using Tree View. **Figure 5** shows that two major groups (with exceptions that will be discussed below) could be identified. The first group comprises elongases involved in saturated fatty acid (SFA) and MUFA elongation and does not contain any sequence from the present study. The second group contains the three new animal elongase sequences (XlELO, OmELO, and CiELO; marked by asterisks) together with mammalian elongase sequences involved in

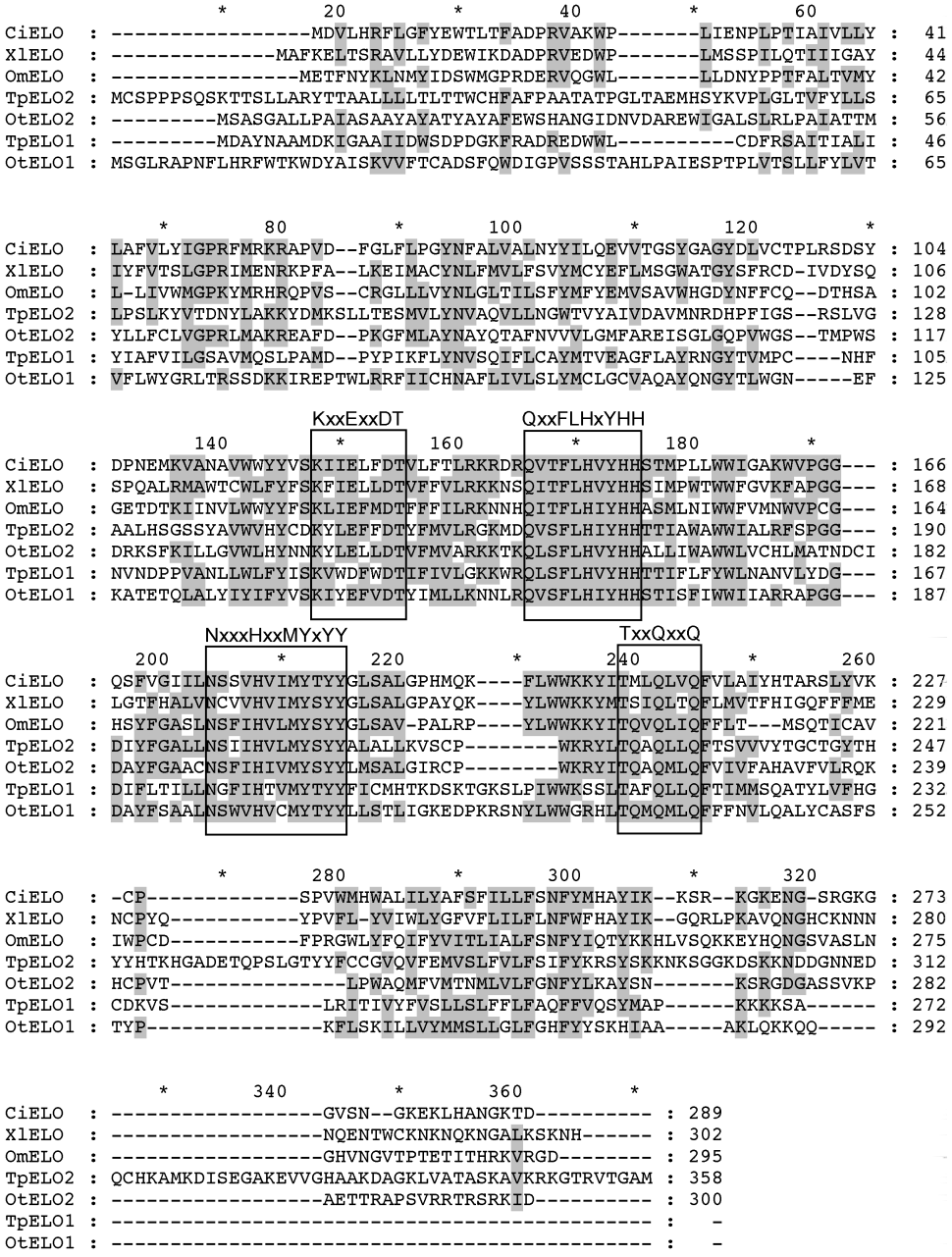

Fig. 2. Amino acid sequences of elongases characterized in the present study. The alignment was obtained using the CLUSTAL X program (gap opening 10, gap extension 0.05). Conserved amino acids are shown on a gray background, whereas conserved motifs found in all ELO-type sequences are framed. The nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank sequence data bank (see Table 2).

PUFA metabolism. A representative member of this group is the murine MmELOVL2, which elongates Δ6-C18-, Δ5-C20-, and Δ7-C22-PUFAs (29). The three new elongases expand the substrate specificity to include Δ8, Δ9, and Δ11 coverage, suggesting that the members of this group generally display multiple regioselectivities. The remaining one-step PUFA-elongases do not fall into one of the two groups mentioned, but the algal elongases TpELO1 and OtELO1 together with the moss PpPSE1 and the fungal MaGLELO may form a putative third group of elongases restricted to the elongation of Δ6-C18-PUFAs (encircled by a dotted line in Fig. 5). Among these, TpELO1 shows the deepest separation from the other members. The two algal enzymes specific for the elongation of C20-PUFAs (TpELO2 and OtELO2) are not closely related to each other, and despite their identical specificity, it is questionable whether they can be considered members of a common branch. Similarly, at present it is not possible to conclude that the unique Δ9-elongase from *Isochrysis galbana* (IgASE1) (8) forms a separate branch. Although there are some exceptions in some of these groups (see Discussion), Fig. 5 suggests that a first approximation of

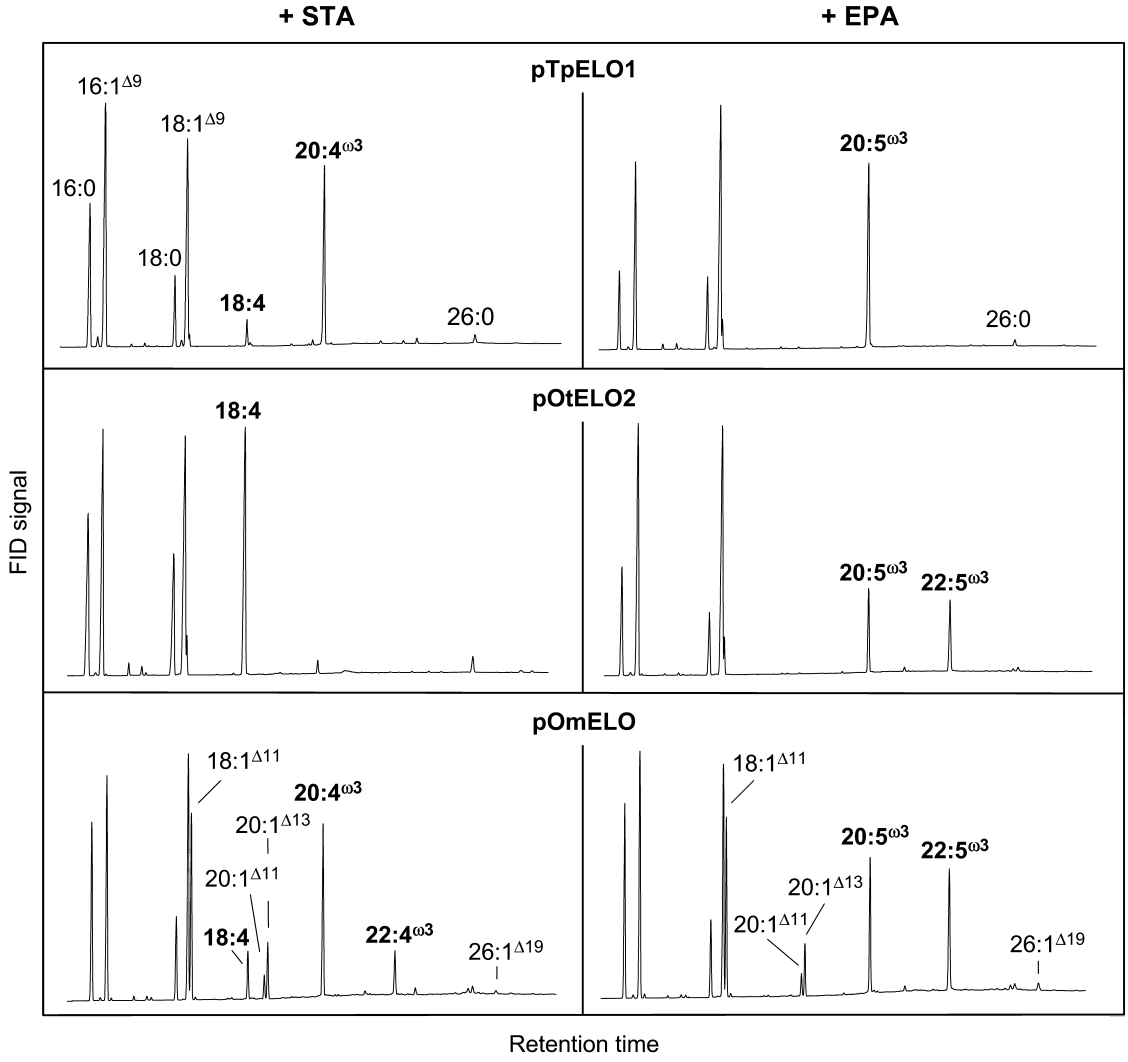

Fig. 3. Elongation of stearidonic acid (STA) and eicosapentaenoic acid (EPA) in transgenic yeast expressing the elongases TpELO1, OtELO2, or OmELO. Transgenic yeasts were incubated for 4 days in the presence of exogenously supplied STA or EPA. Fatty acid profiles were analyzed by gas-liquid chromatography. These fatty acid profiles reflect results obtained in three to five independent expression experiments. FID, flame ionization detector.

the substrate specificity of elongases cloned in the future may be deduced from their mapping in this phylogram.

Synthesis of DHA in yeast

Because the production of DHA was the major reason for the isolation of new elongase genes, we tried to implement its biosynthesis into yeast. For this purpose, we coexpressed one of the new Δ6-elongases, the Δ5-desaturase from the diatom *P. tricornutum* (26), a new Δ5-elongase, and the Δ4-desaturase from *E. gracilis* (11) in a single strain that was grown in the presence of STA. To compare the relevance of using multifunctional or highly specific elongases for the production of DHA, we used either the OmELO elongase (which can catalyze both elongation steps) or the two most active and specific elongases, TpELO1 and OtELO2. DHA synthesis was observed in both yeast strains (**Fig. 6**). STA was first elongated to 20:4ω3 either by OmELO or TpELO1; the elongated product was subsequently Δ5-desaturated to EPA (20:5ω3), which was then further elongated by the Δ5 elongation activity of OmELO or OtELO2. The resulting 22:5ω3 was finally converted to DHA by the Δ4-desaturase. The DHA structure was confirmed by GC-MS analysis (data not shown). The only by-product observed in the strain expressing the two algal elongases was 22:4ω3, which can be ascribed to the "leaky" substrate specificity of OtELO2 (Fig. 4). On the other hand, the strain expressing the bifunctional OmELO showed a dramatic increase in $18{:}1^{\Delta 11}$ as well as significant proportions of $20{:}1^{\Delta 11}$, $20{:}1^{\Delta 13}$, and $26{:}1^{\Delta 19}$. They all were most likely formed by OmELO-catalyzed elongation of the yeast monounsaturated fatty acids (Fig. 3).

The enzyme combinations with the bifunctional fish elongase or the specific algal elongases were similarly efficient concerning DHA yield. DHA represented ~0.5% of the total fatty acids in both yeast strains. However, the formation of unwanted by-products (i.e., fatty acids that are

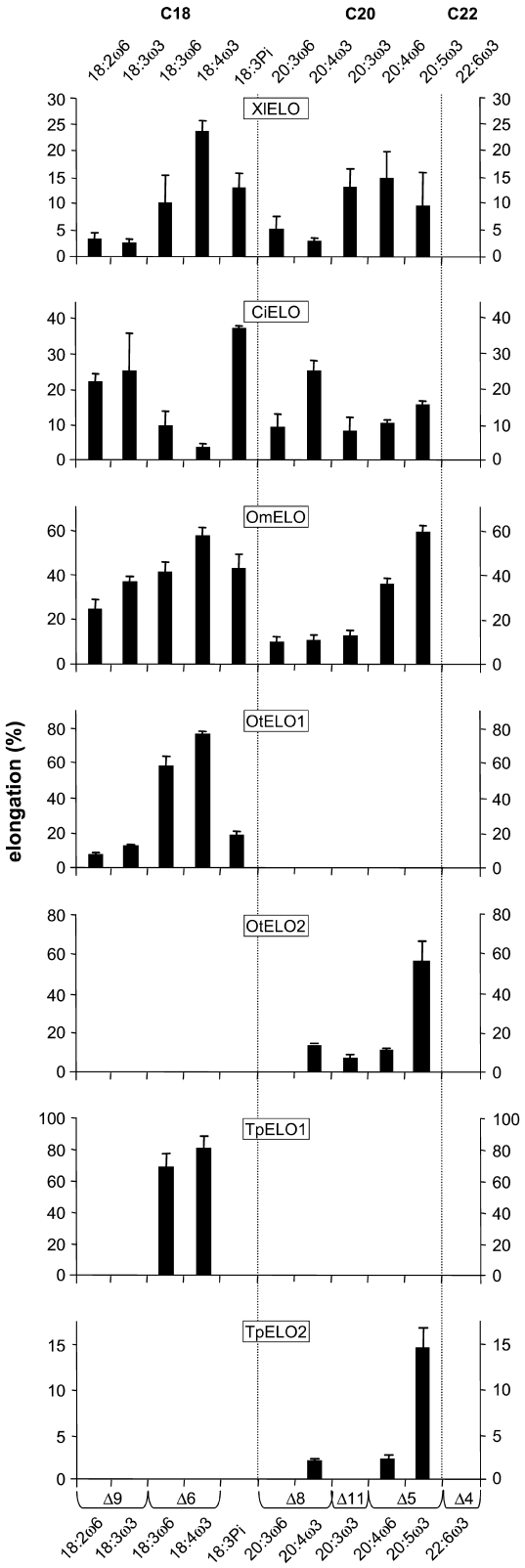

Fig. 4. Specificities of functionally expressed elongase enzymes. All transformed yeasts were separately incubated with 250–500 μM of the C18, C20, and C22 fatty acids listed on the abscissa for 4 days, except for pinolenic acid [18:3$^{\Delta 5,9,12}$ (18:3Pi)], which was part of a fatty acid mixture isolated from *Larix* seeds. Fatty acid profiles were analyzed by gas-liquid chromatography. The elongation is given as [product/(substrate + product) × 100]. Each value is the mean ± SD from three to five independent experiments.

not part of the ω3 pathway shown in Fig. 1A and that, therefore, lead to "dead end" products) was largely reduced in the strain expressing the specific elongases TpELO1 and OtELO2. The total fatty acid profiles presented in Fig. 6 suggest that the low yield of DHA was largely attributable to the low Δ5-desaturation activity. Because the activity of the *P. tricornutum* desaturase was shown to be restricted to fatty acids acylated at the *sn*-2 position of phosphatidylcholine (9), the substrate availability for the Δ5-desaturase may be the limiting factor. In the present experiments, the Δ5-desaturase substrate 20:4$^{\Delta 8,11,14,17}$ results from the elongation of STA and is therefore produced as acyl-CoA ester before being transferred to the various lipids found in yeast (9). Although some of it will be acylated at the *sn*-2 position of phosphatidylcholine, all of the 20:4$^{\Delta 8,11,14,17}$ that is channeled elsewhere, such as in the neutral lipids, remains unavailable for the Δ5-desaturase.

DISCUSSION

Using the *P. patens* PpPSE1 sequence, which is known to code for a specific Δ6-elongase, as query in a tBLASTn search in EST and genomic databases, we were able to isolate seven PUFA-elongase clones from the fish *O. mykiss* (Om*ELO*), the frog *X. laevis* (Xl*ELO*), the sea squirt *C. intestinalis* (Ci*ELO*), the prasinophyte alga *O. tauri* (Ot*ELO1* and Ot*ELO2*), and the diatom *T. pseudonana* (Tp*ELO1* and Tp*ELO2*). The deduced amino acid sequences were 19–42% identical to that of the moss elongase, with OtELO1 being the most similar. All seven elongases were active when expressed in yeast and enabled us to carry out a detailed analysis of the substrate specificities and to conclusively annotate OtELO1 and TpELO1 as Δ6-C18-PUFA-elongases, OtELO2 and TpELO2 as Δ5-C20-PUFA-elongases, and OmELO, XlELO, and CiELO as bifunctional C18/C20-PUFA-elongases. To our knowledge, OtELO2 and TpELO2 represent the first examples of Δ5-C20-PUFA-elongases.

The seven new elongases studied in this work originate from widely divergent organisms and display very diverse substrate specificities. In an unrooted phylogenetic tree of 29 functionally characterized elongases, the new enzymes grouped into two of three major clades. One clade comprises elongases specific for SFAs and MUFAs and includes the well-characterized elongases ScELO1–ScELO3 from *S. cerevisiae* (17, 18), MaELO from the oleaginous fungus *M. alpina* (GenBank accession number AAF71789), and two elongases from the nematode *C. elegans*, CeELO1 and CeELO2. CeELO2 is specific for C16:0 (30), whereas CeELO1 from the same organism has a preference for Δ6-C18-PUFAs (15). In the context of this clade, the CeELO1 enzyme represents a functional exception that may be ascribed to a local and independent invention of PUFA elongation after the duplication of an ancestral SFA/MUFA-elongase. Similarly, the Δ5- and Δ6-desaturases from *C. elegans* also group together, although the Δ5- and Δ6-desaturases from a single organism (such as *M. alpina*) are usually found in different clades (31).

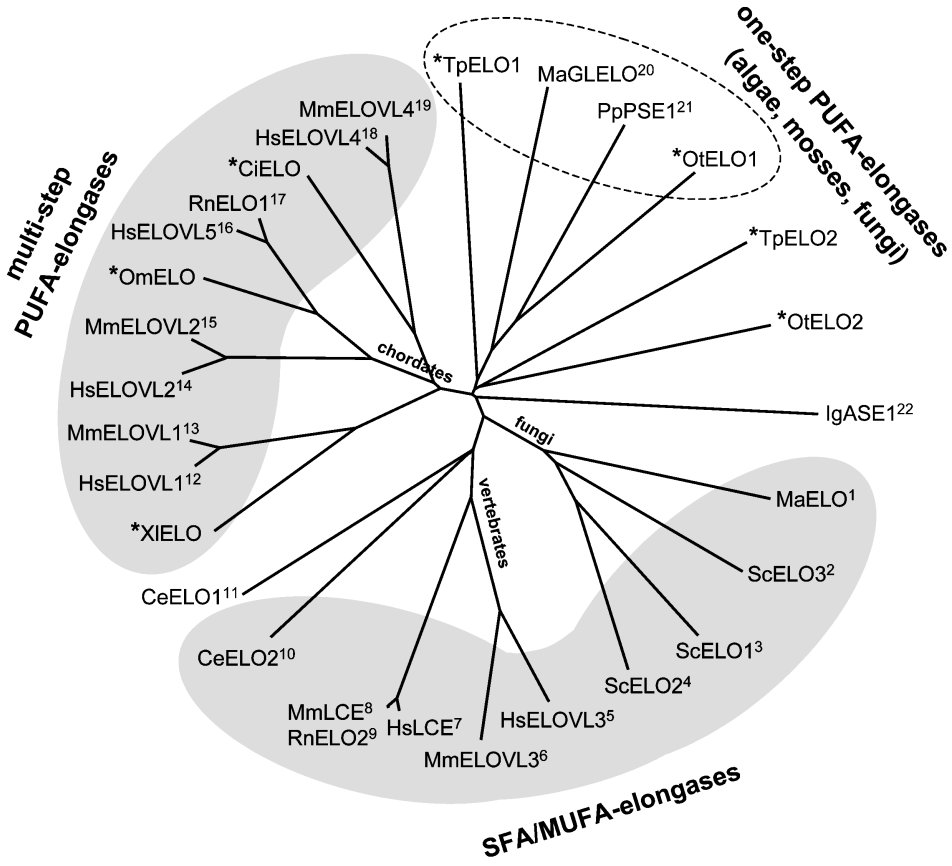

Fig. 5. Unrooted phylogram of functionally characterized ELO-type sequences. The alignment was generated by the CLUSTAL X program, and the unrooted phylogram was constructed by the neighbor-joining method using Tree View. The DDBJ/EMBL/GenBank accession numbers of the different protein sequences are as follows: (1) AAF71789, (2) NP_013476, (3) NP_012339, (4) NP_009963, (5) AAG17875, (6) NP_031729, (7) NP_076995, (8) AAL14239, (9) BAB69888, (10) NP_503114, (11) AAF70462, (12) XP_002040, (13) NP_062295, (14) NP_060240, (15) NP_062296, (16) NP_068586, (17) NP_599209, (18) NP_073563, (19) AAG47667, (20) AAF70417, (21) AAL84174, and (22) AAL37626. The enzymes characterized in the present study are marked by asterisks. The branch comprising the four Δ6-specific, single-step elongases is marked by a dotted line to show its preliminary delineation.

The animal elongases OmELO, CiELO, and XlELO cloned in the present work fall into another clade comprising chordate elongases specific for PUFAs but displaying multiple regioselectivities. One apparent exception in this group is MmELOVL1 (Scc1) from mouse, which can complement yeast mutants deleted in the SFA/MUFA-elongase ELO3 (32). Because MmELOVL1 is most closely related to the PUFA-specific human HsELOVL1 (33), the activity displayed by MmELOVL1 in yeast mutants may represent a normally minor activity just sufficient for complementation, whereas its preferred substrates may be PUFAs, which has never been assayed to our knowledge. The inclusion of the elongase CiELO from the sea squirt *C. intestinalis* in this clade emphasizes that this branch is not restricted to PUFA-elongases from vertebrates but rather comprises animals from different chordate groups including, in addition to vertebrates, the primitive tunicate *Ciona*.

A third clade is made up of elongases specific for a single step in PUFA biosynthesis, the elongation of Δ6-C18-PUFAs. At present, this clade includes only four enzymes, the algal OtELO1 and TpELO1, the moss PpPSE1 (13), and the fungal MaGLELO (14). Additional sequences to be found in the future will show whether the delineation of this clade can persist. According to the phylogram, the Δ5-elongases of the two algae, OtELO2 and TpELO2, are not closely related to each other or to the Δ6-elongase of the same algae, pointing to ancient gene duplications.

The elongase sequences collected to date suggest that the evolution of C18-PUFA elongation up to DHA was realized by following two alternative strategies. In one case, two different and specific enzymes for the consecutive Δ6 and Δ5 elongations evolved that have only been detected in algae. Because elongation stopped at a chain length of C22, the development of a Δ4-desaturase was necessary. In the second group, exemplified by mammals, a single multifunctional elongase enzyme, accompanied by secondary copies, was developed. This development resulted in an extended "unspecificity" accepting in addition to C18- and C20- also C22-PUFA substrates, which in turn

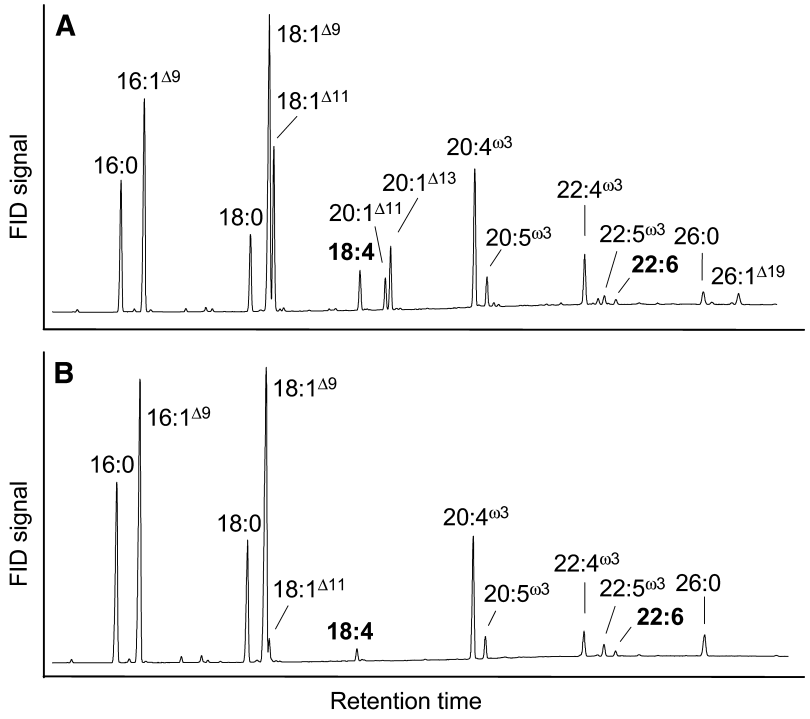

Fig. 6. Implementation of DHA synthesis in *S. cerevisiae* starting from STA. A: A yeast strain coexpressing the bifunctional elongase OmELO, the Δ5-desaturase from *P. tricornutum* (26), and the Δ4-desaturase from *E. gracilis* (11). B: A yeast strain coexpressing the same two desaturases used in A together with the elongases TpELO1 and OtELO2. The cultures were grown for 4 days in the presence of exogenously supplied STA. The fatty acid profile was analyzed by gas-liquid chromatography. These fatty acid profiles reflect results obtained in three independent expression experiments.

made DHA biosynthesis independent of a Δ4-desaturase (Sprecher pathway; Fig. 1C). This pathway is also realized by nonmammalian vertebrates such as fish, but it is not clear whether all chordates follow this pathway to produce DHA.

With regard to the biotechnological implementation of DHA production in oilseed crops, the pathway involving Δ4 desaturation is the preferred choice for several reasons. First, in contrast to the Sprecher pathway, this route does not lead to the synthesis of C24-PUFA intermediates and, therefore, does not depend on the peroxisomal β-oxidation machinery. Second, various reasons regarding the future acceptance of transgenic foods require the use of nonmammalian organisms as gene sources to clone all of the sequences required for an assembly of LCPUFA biosynthesis in transgenic oilseeds. Furthermore, the broad substrate specificities of the mammalian elongases would favor the formation of unwanted by-products with unknown physiological effects. This is particularly unacceptable in the background of transgenic plants, in which several enzymes with different ranges of substrate specificities have to cooperate in a processive manner. The use of enzymes restricted to a single step in the DHA biosynthetic pathway might provide a means to significantly reduce these by-products.

The synthesis of ARA and EPA from GLA and ALA, respectively, has already been successfully demonstrated in transgenic yeast (15, 26), but the experimental proof of a reconstitution of DHA production was still missing. Using the Δ5-desaturase from *P. tricornutum* and the Δ4-desaturase from *E. gracilis*, together with the Δ6- and Δ5-elongases from *O. tauri* and *T. pseudonana*, we were able to implement DHA synthesis starting from STA in yeast using enzymes of exclusively algal origin. We could also compare the efficiency of this pathway with that involving the bifunctional elongating enzyme from the fish *O. mykiss*. Both combinations were able to convert STA via EPA into DHA. There was no difference in DHA yield, but several different fatty acids that are not intermediates of the pathway were found in the yeast expressing the fish elongase. The only detectable by-product in the strain expressing the algal elongases was $22{:}4^{\Delta 10,13,16,19}$, which resulted from the elongation of $20{:}4^{\Delta 8,11,14,17}$, underlining the advantage of using specific single-step elongases.

In summary, we present here the cloning and functional characterization of the first C20-specific PUFA-elongases. In addition, the successful reconstitution of DHA synthesis in yeast represents an encouraging step in the process of establishing transgenic, DHA-producing oilseed plants.

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