THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 34, Issue of August 24, pp. 31561–31566, 2001

Printed in U.S.A.

Identification of a Δ4 Fatty Acid Desaturase from *Thraustochytrium* sp. Involved in the Biosynthesis of Docosahexanoic Acid by Heterologous Expression in *Saccharomyces cerevisiae* and *Brassica juncea**

Received for publication, April 4, 2001, and in revised form, June 7, 2001
Published, JBC Papers in Press, June 7, 2001, DOI 10.1074/jbc.M102971200

Xiao Qiu‡§¶, Haiping Hong‡§, and Samuel L. MacKenzie‖

From the ‡Department of Research and Development, Bioriginal Food and Science Corporation, Saskatoon, Saskatchewan, S7J 0R1, Canada; and the ‖National Research Council of Canada, Plant Biotechnology Institute, Saskatoon, Saskatchewan, S7N 0W9, Canada

The existence of Δ4 fatty acid desaturation in the biosynthesis of docosahexanoic acid (DHA) has been questioned over the years. In this report we describe the identification from *Thraustochytrium* sp. of two cDNAs, *Fad4* and *Fad5,* coding for Δ4 and Δ5 fatty acid desaturases, respectively. The Δ4 desaturase, when expressed in *Saccharomyces cerevisiae*, introduced a double bond at position 4 of 22:5(*n*-3) and 22:4(*n*-6) resulting in the production of DHA and docosapentanoic acid. The enzyme, when expressed in *Brassica juncea* under the control of a constitutive promoter, desaturated the exogenously supplied substrate 22:5(*n*-3), resulting in the production of DHA in vegetative tissues. These results support the notion that DHA can be synthesized via Δ4 desaturation and suggest the possibility that DHA can be produced in oilseed crops on a large scale.

Long chain polyunsaturated fatty acids (LCPUFAs)[1] such as docosahexanoic acid (DHA) 22:6(4,7,10,13,16,19) are essential components of the cell membranes of various tissues and organelles such as retina and brain in mammals. For instance, over 30% of fatty acids in brain phospholipid are 22:6(*n*-3) and 20:4(*n*-6) (1). In retina, DHA accounts for more than 60% of the total fatty acids in the rod outer segment, the photosensitive part of the photoreceptor cell (2). Clinical studies show that DHA is essential for the growth and development of the brain in infants and for maintenance of normal brain function in adults (3). DHA also has significant effects on photoreceptor function involved in the signal transduction process, rhodopsin activation, and rod and cone development (2). In addition, some positive effects of DHA are also found on diseases such as hypertension, arthritis, atherosclerosis, depression, thrombosis, and cancer (4). Therefore, an appropriate dietary supply of this fatty acid is vital for humans to stay healthy. It is particularly important for infants, young children, and senior citizens to have an adequate intake of these fatty acids from the diet because the fatty acids cannot be synthesized efficiently in the body (5).

DHA is a fatty acid of the (*n*-3) series, according to the location of the last double bond at the methyl end. It is synthesized via alternating steps of desaturation and elongation. Starting with 18:3(9,12,15), the biosynthesis of DHA involves Δ6 desaturation to 18:4(6,9,12,15), followed by elongation to 20:4(8,11,14,17) and Δ5 desaturation to 20:5(5,8,11,14,17). Beyond this point there are some controversies about the biosynthesis. The conventional view is that 20:5(5,8,11,14,17) is elongated to 22:5(7,10,13,16,19) and then converted to 22:6(4,7,10,13,16,19) by the final Δ4 desaturation (6–7). However, Sprecher and co-workers (8–11) recently suggested an alternative pathway for DHA biosynthesis that is independent of Δ4 desaturase, involving two consecutive elongations, a Δ6 desaturation in microsome and a two-carbon shortening via limited β-oxidation in peroxisomes.

Thraustochytrids are a common type of marine microheterotroph and are taxonomically aligned with the heterokont algae (12). They are unusual in that they can produce substantial amounts of long chain polyunsaturated fatty acids such as DHA and docosapentanoic acid (DPA) 22:5(*n*-6) (13). Thus, thraustochytrids can serve as model organisms for studying the mechanism underlying the biosynthesis of LCPUFAs.

Based on the information that the so-called "front-end" microsomal desaturases share a similar primary structure (14), we undertook an RT-PCR approach to cloning genes that are involved in the biosynthesis of DHA. Using this strategy, we have identified two cDNAs from thraustochytrids that encode front-end desaturases. One is a Δ5 desaturase that introduces a double bond at the 5 position of 20:3(8,11,14). The other is a Δ4 fatty acid desaturase that could introduce a double bond at the 4 position of 22:5(7,10,13,16,19) and 22:4(7,10,13,16), resulting in the production of DHA and DPA, respectively. Introduction of the full-length desaturase into *Brassica juncea* under the control of the 35S promoter resulted in the production of DHA in leaves when exogenously supplied with 22:5(*n*-3) substrate. These results support not only the Δ4 desaturation pathway in DHA biosynthesis, but also the possibility of large-scale DHA production in oilseed crops.

EXPERIMENTAL PROCEDURES

Materials—Thraustochytrium sp. (ATCC 21685) was purchased from the American Type Culture Collection (Manassas, VA) and grown in medium (12) at 24 °C for 7 days. Biomass was harvested by centrifugation and used for RNA isolation.

Construction and Screening of the cDNA Library—The total RNA

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§ Present address: National Research Council of Canada, Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, SK, Canada S7N 0W9.

¶ To whom correspondence should be addressed. Tel.: 306-975-9558; Fax: 306-975-4839; E-mail: xqiu@pbi.nrc.ca.

[1] The abbreviations used are: LCPUFAs, long chain polyunsaturated fatty acids, DHA, docosahexanoic acid; cyt, cytochrome; DPA, docosapentanoic acid; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; EPA, eicosapentanoic acid; FAMEs, fatty acid methyl esters; GC, gas chromatography; LC, liquid chromatography; MS, mass spectrometry, HPLC, high performance liquid chromatography.

was isolated from the above materials according to Qiu and Erickson (15). The cDNA library was constructed from the total RNA (16) and screened according to standard methods (17).

RT-PCR—For RT-PCR experiments, the single strand cDNA was synthesized by Superscript II reverse transcriptase (Life Technologies, Inc.) from the total RNA and was then used as the template for the PCR reaction with two degenerate primers. The PCR amplification consisted of 35 cycles of 1 min at 94 °C, 1.5 min at 55 °C, and 2 min at 72 °C followed by an extension step at 72 °C for 10 min. The amplified products from 800 to 1000 base pairs were isolated from agarose gel, purified using a kit (QIAEX II gel purification, Qiagen), and subsequently cloned into TA cloning vector pCR® 2.1 (Invitrogen). The cloned inserts were then sequenced by the PRISM DyeDeoxy Terminator cycle sequencing system (PerkinElmer Life Sciences).

Expression of Fad4 and Fad5 in Yeast (Saccharomyces cerevisiae)—The open reading frames of *Fad4* and *Fad5* were amplified by PCR using the Precision Plus enzyme (Stratagene) and cloned into a TA cloning vector (pCR® 2.1, Invitrogen). Having confirmed that by sequencing the PCR products were identical to the original cDNAs, the fragments were then released by a *Bam*HI-*Eco*RI double digestion and inserted into the yeast expression vector pYES2 (Invitrogen) under the control of the inducible promoter *GAL1*.

Yeast strain InvSc2 (Invitrogen) was transformed with the expression constructs using the lithium acetate method, and transformants were selected on minimal medium plates lacking uracil.

Transformants were first grown in minimal medium lacking uracil and containing glucose at 28 °C. After overnight culture, the cells were centrifuged, washed, and resuspended in distilled water. Minimal medium containing 2% galactose, with or without 0.3 mM substrate fatty acids in the presence of 0.1% Tergitol, was inoculated with the yeast transformant cell suspension and incubated at 20 °C for 3 days and then 15 °C for another 3 days.

Transformation of B. juncea and Exogenous Fatty Acid Treatment—The hypocotyls of 5–6-day seedlings of *B. juncea* were used as explants for inoculation with the *Agrobacterium tumefaciens* that hosts a binary vector with full-length Fad4 cDNA under the control of the 35S promoter. The subsequent co-cultivation and regeneration were essentially according to Radke *et al.* (18). The 20-day transgenic seedlings were used for exogenous fatty acid treatment. The seedling was divided into three parts: leaves, stems, and roots. Each was cut into small pieces and placed in a 24-well titer plate. To each well, 2 ml of 0.05% sodium salt of docosapentanoic acid (NuCheck Prep Inc., Elysian, MN) was added. The plate was then incubated at 24 °C for 4 h with gentle shaking. After incubation, the plant tissues were washed three times with water and then used for fatty acid analysis.

Fatty Acid Analysis—Two milliliters of methanolic KOH (7.5% w/v KOH in 95% methanol) was added to the materials, sealed in a 12-ml glass culture tube, and heated to 80 °C for 30 min. 0.5 ml of water was added, and the sample was extracted twice with 2 ml of hexane to remove the nonsaponifiable lipids. The remaining aqueous phase was then acidified by adding 1 ml of 6 N HCl and extracted twice with 2 ml of hexane. The hexane phases were combined and dried under a stream of nitrogen. Two milliliters of 3 N methanolic HCl (Supelco Inc., Supelco Park, Bellefonte, PA) was added, and the mixture was heated at 80 °C for 10 min. After cooling to room temperature, 1 ml of 0.9% NaCl was added, and the mixture was extracted twice with 2 ml of hexane. The combined hexane was evaporated under nitrogen. The resulting fatty acid methyl esters (FAMEs) were analyzed by GC according to Qiu *et al.* (16).

GC/MS analysis of FAMEs was performed in standard electrospray ionization mode using a Fisons VG TRIO 2000 mass spectrometer (VG Analytical, UK) controlled by MassLynx version 2.0 software coupled to a GC 8000 Series gas chromatograph (16). LC/MS analysis of free fatty acids was performed using an HP1100 HPLC system (Hewlett-Packard Co.) coupled to a Quattro LC mass spectrometer (Micromass, UK). The HPLC system comprised a binary pump, autosampler, column heater (25 °C), and a Genesis C18 LC column (2.1 × 100 mm, 4 μm; Jones Chromatography, UK). Gradient elution was performed using 0.07% acetic acid in water (solvent A) and 0.07% acetic acid in 90:10 acetonitrile:water (solvent B), starting at 85% of B. After 2 min B was increased to 100% at the rate of 1.5% per min and held for 10 min, giving a total run time of 24 min (including column equilibration). The mass spectrometer was operated in negative electrospray ionization mode, and the combined LC/MS system was controlled by MassLynx version 3.3 software (Micromass Inc.).

RESULTS

Identification of cDNAs Coding for the Front-end Desaturase—Thraustochytrium is a unicellular marine eukaryotic microorganism that has recently received scientific attention because of its ability to produce LCPUFAs such as DHA and DPA (13). We were interested in the molecular information underlying the biosynthesis of those fatty acids. To identify genes coding for desaturases involved in the biosynthesis of LCPUFAs in *Thraustochytrium* sp., a PCR-based cloning strategy was adopted. Two degenerate primers were designed to target the heme-binding motif of the cytochrome b_5-like domain in front-end desaturases and the third conservative histidine motif in all microsomal desaturases, respectively. The rationale behind the design was that the desaturases involved in DHA biosynthesis in *Thraustochytrium* sp., if there were any, should have a primary structure similar to other front-end desaturases such as Δ5 and Δ6 desaturases, *i.e.* the N-terminal extension of the cytochrome b_5-like domain in the desaturase. Using this strategy we identified two cDNA fragments from *Thraustochytrium* sp. that encode fusion proteins containing a cytochrome b_5-like domain in the N terminus.

To isolate full-length cDNA clones, the two inserts were used as probes to screen a cDNA library of *Thraustochytrium* sp., which resulted in the identification of several cDNA clones in each group. Sequencing of all the clones identified two full-length cDNAs that were named *Fad4* and *Fad5*. The open reading frame of *Fad4* is 1560 base pairs and codes for 519 amino acids with a molecular mass of 59.1 kDa. *Fad5* is 1230 base pairs in length and codes for 439 amino acids with a molecular mass of 49.8 kDa. Sequence comparison showed that there is a 16% amino acid identity between the two deduced proteins, and FAD4 contains an additional 80 amino acids between the second and third conserved histidine motifs (Fig. 1).

A homology search indicates that both FAD5 and FAD4 share amino acid sequence similarity to the front-end desaturases in the publilc data bases. Highly homologous sequences include human Δ5 and Δ6 desaturases (19–20), the Δ8 desaturase from *Euglena gracilis* (21), the Δ5 and Δ6 desaturases from *Mortierella alpina* (22–24), the Δ5 desaturase from *Dictyostelium discoideum* (25), a bifunctional Δ6 acetylenase/desaturase from the moss *Ceratodon purpureus* (26), and the Δ6 desaturase from *Physcomitrella patens* (27). Alignment of those homologous sequences indicates that the homology occurs mainly in the cytochrome b_5-like domain and in the three conserved histidine-rich motif areas. It was noted that, compared with all other homologous enzymes, FAD4 has a longer domain occurring between the second and third conserved histidine motifs. In the second conserved histidine motif, there are three rather than two amino acids between the first and second histidine (data not shown). It remains to be determined whether these differences are related to the catalytic specificity of the enzyme.

Phylogenetic analysis of the homologous sequences indicates that FAD4 distinguishes itself as one of the most deeply branching, although it may cluster with the Δ5 desaturase from *M. alpina* and *D. discoideum*. On the other hand, FAD5 is clearly grouped within a main branch of the front-end desaturases, which includes various Δ5 and Δ6 desaturases from humans and microorganisms (Fig. 2). These results may suggest the possible different functions of FAD4 and FAD5 as those of desaturases involved in the biosynthesis of LCPUFAs.

Expression of Fad4 and Fad5 in Yeast—To confirm the function of the two genes, both full-length cDNAs were expressed in the yeast strain InvSc2 under the control of the inducible promoter. Fig. 3 shows that with supplementation of the me-

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FAD4 - MTVGYDEEIPFEQVRAHNKPDDAWCAIHGHVYDVTKFASVHPGGDIIL-L -50
       |  |           |    |          |          ||||  ||  |
FAD5 - MGKGSEGRSAAREMTAEANGDKRKTILIEGVLYDATNFK-HPGGSIINFL -50

FAD4 - AGKEATVLYETYHVRGVSDAVLRKYRIGKLPDGQGGANEKEKRTLSGLSS -100
         |          |          ||    ||        |     |
FAD5 - EGEAGVDATQAYREFHQRSGKADKY-LKSLPKLDAS---KVESRFSAKEQ -96

FAD4 - ASYYTWNSDFYRVMRERVVARLKERGKARRGGYELWIKAFLLLVGFWSSL -150
         |       |      | |                              |  |
FAD5 - ARRDAMTRDYAAFREELVAEGYFDPSIPHMI-----YRVVEIVALFALSF -141

FAD4 - YWMCTLDPSFGAILAAMSLGVFAAFVGTCIQHDGNHGAFAQSRWVNKVAG -200
         |    |         | |  |         |   || |    |
FAD5 - WLMSKASPTSLVLGVVMN-G-IAQGRCGWVMHEMGHGSFTGVIWLDDRMC -185

FAD4 - WTLDMIGASGMIWEFQHVLGHHPYTNLIEEENGLQKVSGKKMDTKLADQE -250
              | ||  |  ||   ||   |  |
FAD5 - FYGVGCGMSGHYWKNQHSK-HHAAPNRLEHDVDLNT-------------- -226

FAD4 - SDPDVFSTYPMMRLHPWHQKRWYHRFQHIYGPFIFGFMTINKVVIQDVGV -300
            |          |       | |          |
FAD5 - LPLVAFNERVVRKVKPGSLLALWLRVQ------AYLFAPVSCLLIGIGWT -270

FAD4 - VLRKRLFQIDAECRYASPMYVARFWIMKALTVLYMVALPCYMQGPWHGLK -350
                              ||       |
FAD5 - LYLHPRYMLRTKRHMEFVWIFARYIGWFSIMGALGYSPGT---------- -310

FAD4 - LFAIAHFTCGEVLATMFIVNHIIEGVSYASKDAVKGIMAPPKIMHGVTPM -400
              |   |    |                          |   |   ||
FAD5 - --SVGMYLCSFGLGCIYIFLQF----------------AVSHIHLPVINP -342

FAD4 - NNTRKEVEAEASKSGAVVKSVPLDDWAVVQCQTSVNWSVGSWFWNHFSGG -450
              |  |                            ||  |  ||
FAD5 - EDQLHWLEYAADHT--------------------VNISTKSWLVIWWMSN -372

FAD4 - LNHQIEHHLFPGLSHETYYHIQDVFQSTCAEYGVPYQHEPSLWTAYWKML -500
       || ||||||||         |          ||         |
FAD5 - LNFQIEHHLFPTAPQFRFKEISPRVEALFKRHNLPY-YDLPYTSAVSTTF -421
                 <---
FAD4 - EHLRQLGNEETHESWQRAA -519
         |   |
FAD5 - ANLYSVGHSVGADT-KKQD -439
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FIG. 1. **Comparison of FAD4 and FAD5 protein sequences of *Thraustochytrium* sp.** The *vertical bar* indicates amino acid identity. The conservative motifs such as the cytochrome b_5 heme-binding domain and the three histidine boxes are *shaded*. The *two arrows* indicate the binding locations of the two degenerate primers.

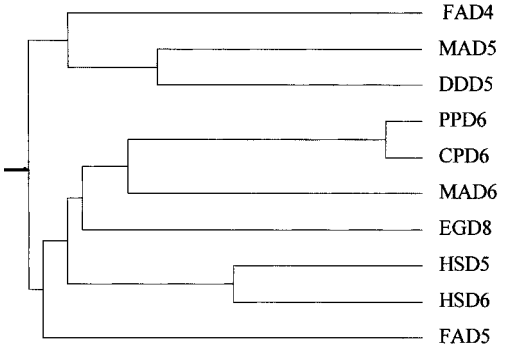

FIG. 2. **Phylogenetic analysis of FAD4 and FAD5 and homologous enzymes.** The dendrogram represents the results of cluster analysis of amino acid sequences. Sequences used for the analysis were obtained from: *EGD8*, Δ8 desaturase from *E. gracilis* (21); *MAD6* and *MAD5*, Δ6 and Δ5 desaturases from *M. alpina* (22–24); *DDD5*, Δ5 desaturase from *D. discoideum* (25); *HSD5* and *HAD6*, *Homo sapiens* Δ5 and Δ6 desaturases (19–20); *PPD6*, Δ6 desaturases from *P. patens* (27); *CPD6*, a bifunctional Δ6 acetylenase/desaturase from *C. purpureus* (26); *FAD4* and *FAD5*, this report.

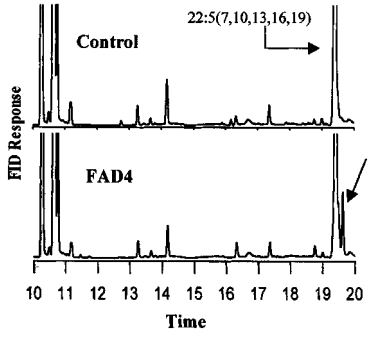

FIG. 3. **GC analysis of FAMEs from yeast strain Invsc2 expressing FAD4 with exogenous substrate 22:5(n-3).**

dium with 22:5(7,10,13,16,19), yeast cells containing *Fad4* cDNA had an extra fatty acid compared with the vector control. The peak had a retention time identical to the DHA standard. LC/MS analysis of the free fatty acid showed that it yields deprotonated molecular ions (m/z = 279) identical to the DHA standard in negative ion electrospray (data not shown). Moreover, GC/MS analysis of the FAME confirmed that the spectrum of the peak is identical to that of the DHA standard (Fig. 4). These results indicate that FAD4 is a Δ4 fatty acid desaturase that is able to introduce a double bond at position 4 of the 22:5(7,10,13,16,19) substrate, resulting in the Δ4 desaturated fatty acid DHA 22:6(4,7,10,13,16,19).

To further study the substrate specificity of FAD4, a number of substrates including 18:2(9,12), 18:3(9,12,15), 20:3(8,11,14), and 22:4(7,10,13,16) were supplied separately to the yeast transformants. The results indicated that FAD4 could also use 22:4(7,10,13,16) as a substrate (Fig. 5) to produce another Δ4 desaturated fatty acid, DPA 22:5(4,7,10,13,16)(Fig. 6). The rest of the fatty acids examined were not effective substrates.

As compared with the control, the yeast transformant containing *Fad5* cDNA, when supplemented with 20:3(8,11,14),

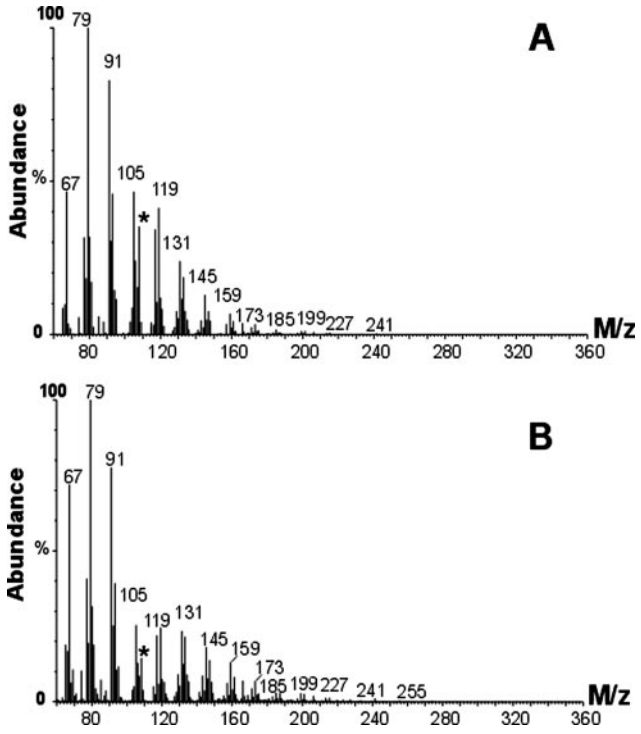

FIG. 4. **GC/MS analysis of FAMEs of the new peak in Fig. 3.** *A*, the FAD4 product. *B*, the 22:6(*n*-3) standard. *Asterisk* indicates the diagnostic ion for (*n*-3) fatty acid at *m/z* = 108.

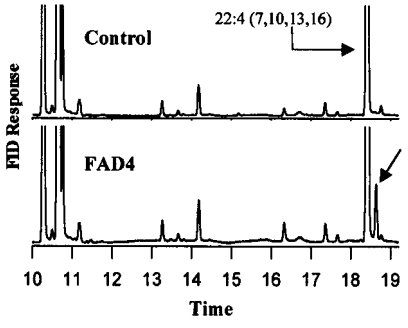

FIG. 5. **GC analysis of FAMEs from yeast strain Invsc2 expressing FAD4 with exogenous substrate 22:4(*n*-6).**

was found to contain one extra fatty acid having a retention time consistent with 20:4(5,8,11,14). Comparison of the GC/MS spectra indicated that the spectrum was identical to that of an arachidonic acid standard 20:4(5,8,11,14) (data not shown), indicating that FAD5 is a Δ5 desaturase.

Expression of FAD4 in B. juncea—To determine whether *Thraustochytrium* FAD4 is functional in oilseed crops, we transformed *B. juncea* with the construct containing FAD4 under the control of the constitutive Cauliflower Mosaic Virus 35S promoter. Eight independent transgenic plants were obtained. In *B. juncea* there is no Δ4 fatty acid desaturase substrate available. Thus to examine the activity of the transgenic enzyme in the plants, the 22:5(*n*-3) substrate must be exogenously supplied (see "Experimental Procedures"). In this experiment, both wild type and transgenics were applied with an aqueous solution of sodium docosapentaneate. It was found that exogenously applied substrates were readily taken up by the roots, stems, and leaves of both types of plants, but converted into DHA only in transgenics. Leaves have a higher level of production of DHA than roots and stems. In leaves, the exogenous substrate was incorporated to a level of 10–20% of the total fatty acids and Δ4 desaturated fatty acid 22:6(*n*-3) was produced in a range of 3–6% of the total fatty acids (Fig. 7).

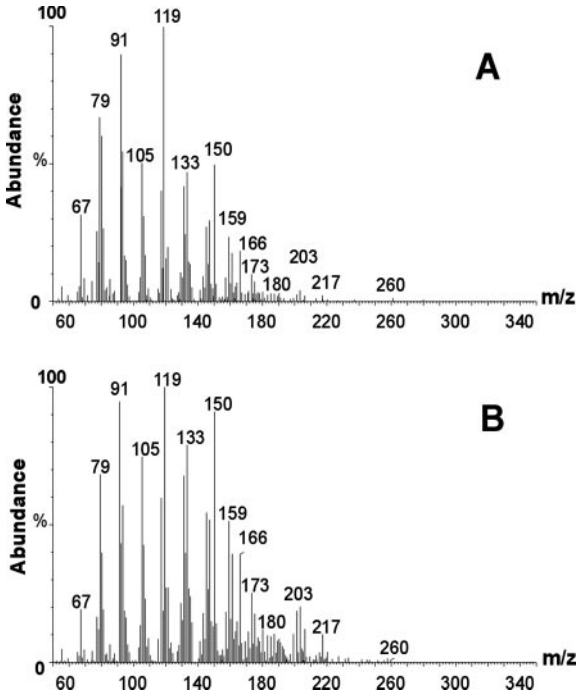

FIG. 6. **GC/MS analysis of FAMEs of the new peak in Fig. 5.** *A*, the FAD4 product. *B*, 22:5(*n*-6) standard.

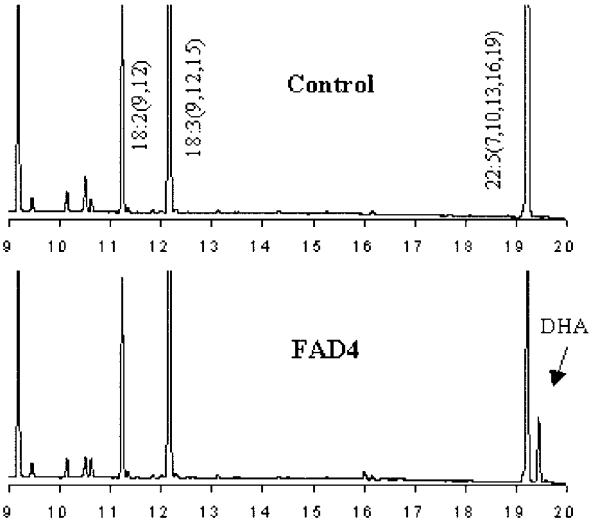

FIG. 7. **GC analysis of leaf FAMEs from *B. juncea* expressing FAD4 with exogenous substrate 22:5(*n*-3).**

These results indicate that the Δ4 fatty acid desaturase from *Thraustochytrium* sp. is functional in oilseed crops.

DISCUSSION

It is known that biosynthesis of LCPUFAs such as eicosapentanoic acid (EPA) and arachidonic acid involves a series of desaturation and elongation steps (6). Various front-end desaturases in the pathway, such as Δ5 and Δ6 desaturases, have recently been identified from plants (28–29), humans (19, 20, 30), animals (31–33), and eukaryotic microorganisms (22–24, 34). They all share a similar primary structure with the N-terminal fusion of the cytochrome b_5-like domain. By taking advantage of this information, we isolated a cDNA encoding a new desaturase by the PCR approach using two degenerate primers targeting the heme-binding motif in the cytochrome b_5-like domain and the histidine-rich motif in microsomal desaturases. Thus, a new member is added to the superfamily of cytochrome b_5 fusion proteins.

Very recently, several elongases in the biosynthetic pathway of long chain polyunsaturated fatty acids have also been identified from an oily fungus (35), a nematode worm (36), and mammals (37). These elongases are part of the complex involving the elongation of 18:4(*n*-3) and 18:3(*n*-6). Thus, in the biosynthetic pathway of LCPUFAs such as DHA, only one step (from 20:5(*n*-3) to 22:6(*n*-3)) remains to be determined. In fact, this is the most controversial step. The conventional view is that this step is catalyzed by an elongation and then a microsomal Δ4 desaturation resulting in the introduction of a double bond at position 4 of 22:5(*n*-3). However, the presence of a microsomal Δ4 desaturase has not been demonstrated over the years (38, 8). Thus, an alternative view held by Sprecher's recent studies (8–11, 40–44) proposed a Δ4 desaturase-independent pathway. According to the proposal, synthesis of DHA from EPA involves two consecutive elongations to a 24-carbon fatty acid, Δ6 desaturation of the 24:5 acid in the microsomes and a 2-carbon unit shortening in peroxisomes. Infante and Huszagh (39, 45, 46) recently also proposed that 22:6(*n*-3) and 22:5(*n*-6) are synthesized by respective separate (*n*-3)- and (*n*-6)-specific Δ4 desaturases. The 22:6(*n*-3) is synthesized in the mitochondria via a channeled carnitine-dependent pathway involving an (*n*-3)-specific Δ4 desaturase, whereas 22:5(*n*-6) is synthesized by both mitochondrial and microsomal systems.

In this study, we report the identification of cDNA (*Fad4*) from *Thraustochytrium* sp. coding for a desaturase that can introduce a Δ4 double bond into 22:5(*n*-3), resulting in the production of DHA. This result provides unambiguous evidence of the existence of polyunsaturated fatty acid Δ4 desaturase in *Thraustochytrium* sp. Our data also demonstrate that the Δ4 desaturase from *Thraustochytrium* sp., like other front-end microsomal desaturases, can desaturate both (*n*-3) and (*n*-6) substrates, *i.e.* 22:5(*n*-3) and 22:4(*n*-6), implying that both families of fatty acids share a common desaturation system. However, whether the *Thraustochytrium* sp. desaturase is located in microsomes or in mitochondria remains to be determined. Given that the Δ4 desaturase shares similar structural features with other front-end microsomal desaturases, including an N-terminal fusion of the cytochrome b_5-like domain and three conservative histidine motifs, we predict that the Δ4 desaturase might be localized in the same site.

The production of DHA has recently drawn tremendous attention because of its beneficial effect on human health. Currently the major sources for DHA are oils from fish and alga. Although fish oil is a traditional source for the fatty acid, it is a by-product from the fishing industry and is usually handled in such a manner that it is badly oxidized when sold. Furthermore, the supply of the oil is highly variable with the years and is jeopardized by shrinking fish population in the oceans. The algal source for DHA has recently been developed, but it is expensive because of the low yield and the high costs of extracting the oil from materials.

Genetic engineering of oilseed crops will be a cost-effective means for the large scale production of this specialty fatty acid. All of the genes (except the one for Δ4 desaturation involved in the DHA biosynthesis) have already been isolated. Here we are adding the missing one in the biosynthetic pathway, which is functional in plants. This result indicates the possibility of producing fish oil in plants. Because of better control over the oil production process with the plant-based oil compared with fish oil, the quality will be higher. Such an improved quality of oil and cost-effective production will have great potential in the nutraceutical industry.

In summary, we have identified two cDNA sequences that encode Δ4 and Δ5 polyunsaturated fatty acid desaturases using the RT-PCR approach. The Δ4 desaturase, when expressed in yeast, can use both (*n*-3) and (*n*-6) substrates to synthesize the Δ4 desaturated fatty acids, DHA and DPA. Thus, the biosynthesis of DHA from α-linolenic acid in *Thraustochytrium* sp. can be represented as follows: Δ6 desaturation of 18:3(*n*-3) to 18:4(*n*-3), elongation of 18:4(*n*-3) to 20:4(*n*-3), and Δ5 desaturation of 20:4(*n*-3) to EPA 20:5(*n*-3), followed by another elongation of EPA, and the final Δ4 desaturation of 22:5(*n*-3). The synthesis of DPA from linoleic acid 18:2(*n*-6) takes place via an analogous pathway. Functionality of the Δ4 desaturase of *Thraustochytrium* in *B. juncea* suggests the possibility of large-scale DHA production in oilseed crops.

Acknowledgments—We thank Nagamani Datla for technical assistance and Stephen Ambrose, Plant Biotechnology Institute, National Research Council of Canada, for GC/MS and LC/MS analysis.

REFERENCES

1. Crawford, M. A., Costeloe, K., Ghebremeskel, K., Phylactos, A., Skirvin, L. & Stacey, F. (1997) *Am. J. Clin. Nutr.* **66,** 1032S-1041S
2. Giusto, N. M., Pasquare, S. J., Salvador, P. I., Roque, M. E. & Ilincheta de Boschero, M. G. (2000) *Prog. Lipid Res.* **39,** 315–391
3. Martinetz, M. (1992) *J. Pediatr.* **120,** S129-S138
4. Horrocks, L. A. & Yeo, Y. K. (1999) *Pharmacol. Res.* **40,** 211–215
5. Spector, A. A. (1999) *Lipids* **34,** S1–S3
6. Horrobin, D. F. (1992) *Prog. Lipid Res.* **31,** 163–194
7. Gurr, M., I,. (1992) *Lipid Biotech.* **4,** 141–143
8. Sprecher, H., Luthria, D. L., Mohammed B. S. & Baykousheva, S. P. (1995) *J. Lipid Res.* **36,** 2471–2477
9. Sprecher, H., Chen, Q. & Yin F. Q. (1999) *Lipids*, S153–S156
10. Sprecher, H. (2000) *Biochim. Biophys. Acta* **1486,** 219–231
11. Voss, A., Reinhart, M., Sankarappa, S. & Sprecher, H. (1991) *J. Biol. Chem.* **266,** 19995–20000
12. Weete, J. D., Kim, H., Gandhi, S. R., Wang, Y. & Dute R. (1997) *Lipids* **32,** 839–845
13. Lewis, T. E., Nichols, P. D. & McMeekin, T. A. (1999) *Mar. Biotechnol.* **1,** 580–587
14. Napier, J. A., Sayanova, O., Sperling, P. & Heinz E. (1999) *Trends Plant Sci.* **4,** 2–4
15. Qiu, X. & Erickson, L. (1994) *Plant Mol. Biol. Repr.* **12,** 209–214
16. Qiu, X., Reed, D. R., Hong, H. P., Mackenzie, S. L & Covello, P. S. *(2001) Plant Physiol.* **125,** 847–855
17. Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, pp. 8.46–8.52, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
18. Radke, S. E., Turner, J. C. & Facciotti, D. (1992) *Plant Cell Rep.* **11,** 499–505
19. Cho, H. P., Nakamura, M. T. & Clarke, S. D. (1999) *J. Biol. Chem.* **274,** 471–477
20. Cho, H. P., Nakamura, M. & Clarke, S. D. (1999) *J. Biol. Chem.* **274,** 37335–37339
21. Watts, J. L. & Browse, J. (1999) *Arch. Biochem. Biophys.* **362,** 175–182
22. Huang, Y., Chaudhary, S., Thurmond, J. M., Bobik, E. G., Yuan, L., Chan, G., Kirchner, S. J., Mukerji, P. & Knutzon, D. S. (1999) *Lipids* **34,** 649–659
23. Knutzon, S. D., Thurmond, J. M., Huang, Y. S., Chaudhary, S., Bobik, E. G., Jr., Chan, G., Kirchner, S. J. & Mukerji, P. (1998) *J. Biol. Chem.* **273,** 29360–29366
24. Michaelson, L. V., Lazarus, C. M., Griffiths, G. & Napier, J. A. (1998) *J. Biol. Chem.* **273,** 19055–19059
25. Saito, T., Morio, T. & Ochiai, H. (2000) *Eur. J. Biochem.* **267,** 1813–1818
26. Sperling, P., Lee, M., Girke, T., Zaehringer, U., Stymne, S., and Heinz, E. (2000) *Eur. J. Biochem.* **267,** 3801–3811
27. Girke, T., Schmidt, H., Zahringer, U., Reski, R. & Heinz, E. (1998) *Plant J.* **15,** 39–48
28. Sayanova, O., Smith, M. A., Lapinskas, P., Stobart, K. A., Dobson, G., Christie, W. W., Shewry, P. R. & Napier, J. A. (1997) *Proc. Natl. Acad. Sci.* U. S. A. **94,** 4211–4216
29. Sperling, P., Zahringers, U. & Heinz, E. (1998) *J. Biol. Chem.* **273,** 28590–28596
30. Leonard, A. E., Kelder, B., Bobik, E. G., Chuang, L., Parker-Barnes, J. M., Thurmond, J. M., Kroeger, P. E., Kopchick, J. J., Huang, Y. & Mukerji, P. (2000) *Biochem. J.* **347,** 719–724
31. Napier, J. A., Hey, S. J., Lacey, D. J. & Shewry, P. R. (1998) *Biochem. J.* **330,** 611–614
32. Michaelson, L. V., Napier, J. A., Lewis, M., Griffiths, G., Lazarus, C. M. & Stobart, K. A. (1998) *FEBS Lett.* **439,** 215–218
33. Wallis, J. G. & Browse, J. (1999) *Arch. Biochem. Biophys.* **365,** 307–316
34. Sakuradani, E., Kobayashi, M. & Shimizu, S. (1999) *Gene* (*Amst.*) **238,** 445–453
35. Parker-Barnes, J. M., Das, T., Bobik, E., Leonard, A. E., Thurmond, J. M., Chaung, L., Huang, Y. & Mukerji, P. (2000) *Proc. Natl. Acad. Sci.* U. S. A. **97,** 8284–8289
36. Beaudoin, F., Michaelson, L. V., Hey, S. J., Lewis, M. J., Shewry, P. R., Sayanova, O. & Napier, J. A. (2000) *Proc. Natl. Acad. Sci.* U. S. A. **97,** 6421–6426
37. Leonard, A. E., Bobik, E., Dorado, J., Kroeger, P. E., Chuang, L., Thurmond, J. M., Parker-Barnes, J. M., Das, T., Huang, Y. & Mukerji, P. (2000) *Biochem. J.* **350,** 765–770
38. Mohammed, B. S., Sankarappa, S., Geiger, M. & Sprecher, H,. (1995) *Arch.*

Biochem Biophys. **317,** 179–184
39. Infante, J. P. & Huszagh, V. A. (2000) *FEBS Lett.* **468,** 1–5
40. Luthria, D. L., Mohammed, B. S. & Sprecher, H. (1996) *J. Biol. Chem.* **271,** 16020–16025
41. Sprecher, H. (1996) *Nutrition* **12,** S5–7
42. Mohammed, B. S., Luthria, D. L., Bakousheva, S. P. & Sprecher, H. (1997) *Biochem. J* **326,** 425–430
43. Sprecher, H., Chen, Q. & Yin, F. Q. (1999) *Lipids* **34,** S153–6
44. Innis, S. M., Sprecher, H., Hachey, D., Edmond, J. & Anderson, R. E. (1999) *Lipids* **34,** 139–149
45. Infante, J. P. & Huszagh, V. A. (1998) *FEBS Lett.* **431,** 1–6
46. Infante, J. P. & Huszagh, V. A. (1997) *Mol. Cell. Biochem.* **168,** 101–115

Identification of a Δ4 Fatty Acid Desaturase from *Thraustochytrium* sp. Involved in the Biosynthesis of Docosahexanoic Acid by Heterologous Expression in *Saccharomyces cerevisiae* and *Brassica juncea*

Xiao Qiu, Haiping Hong and Samuel L. MacKenzie

J. Biol. Chem. 2001, 276:31561-31566.
doi: 10.1074/jbc.M102971200 originally published online June 7, 2001
