A Sphingolipid Desaturase from Higher Plants

IDENTIFICATION OF A NEW CYTOCHROME \( b_5 \) FUSION PROTEIN* (Received for publication, May 27, 1998, and in revised form, July 20, 1998)

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A recently cloned cDNA from sunflower seeds for a fusion protein composed of an N-terminal cytochrome \( b_5 \) and a domain similar to membrane-bound acyl lipid desaturases. For a functional identification, homologous cDNAs from Brassica napus and Arabidopsis thaliana were expressed in Saccharomyces cerevisiae, and sphingolipid long chain bases were analyzed. The expression of the heterologous enzyme resulted in significant proportions of new \( \Delta^{9-9}-\text{cis}/\text{trans-phytosphingenes} \) that accompany the residual \( \text{C}_{18} \)-phytosphinganine predominating in wild-type yeast cells. These results represent the first identification of a gene coding for a sphingolipid desaturase and for a stereounselective desaturase showing \textit{trans}-activity from any organism. Furthermore, this fusion protein is a new member of the cytochrome \( b_5 \) superfamily. The formation of the two regioisomeric phytosphingenes in the transformed yeast sheds new light on the factors controlling regioselectivity.

Sphingolipids are ubiquitous membrane components in eukaryotic cells and in a few bacteria (1). The hydrophobic moiety is a long chain base (LCB); \( 2 \)-amino-1,3-dihydroxynalkane) carrying \( N \)-acylated fatty acids of 14–26 carbon atoms to form a ceramide. Depending on the source, this basic structure can be modified by differences in chain length, degree of unsaturation, methyl branching, and insertion of additional hydroxy groups. Complex sphingolipids such as cerebrosides and phytoglycolipids are generated by the addition of various sugar residues and other polar head groups to the ceramide.

In animal cells, these membrane lipids and in particular their catabolites serve as intra- and intercellular messengers regulating cell growth, differentiation, apoptosis, and pathogenic defense (2, 3). On the other hand, far less is known about the role of sphingolipids in plants and fungi. However, the lethality of \textit{Saccharomyces cerevisiae} mutants defective in LCB biosynthesis (4) and the toxicity of compounds interfering with their synthesis in plants suggest that they are essential also in these organisms (5).

Very recently, genes for the ceramide hydroxylation were identified (6, 7), but the genes responsible for the desaturation of the LCB were still unknown. Enzymatic studies with animal systems showed that the \( \Delta^{4-4} \)-trans double bond is inserted into free ceramide (N-acylated sphinganine) and that this conversion is catalyzed by a desaturase and not by a dehydrogenase or an oxidase (8, 9). In contrast to animal and yeast cells, plant sphingolipids contain additional C-8-unsaturated LCB of \textit{cis}- and \textit{trans}-configuration, which might be involved in chilling resistance (10), but nothing is known about the molecular mechanism responsible for their formation.

Here we describe the first cloning of a sphingolipid desaturase from plants that catalyzes the formation of unsaturated LCBs.

**EXPERIMENTAL PROCEDURES**

**Chemicals—Phytosphinganine, 4-trans-phytosphingine, and sphinganine were purchased from Sigma. \( \beta \)-erythro-sphinganine was purified from the \( \beta \)-threo isomer as dinitrophenyl derivative (11).**

**DNA Amplification from Brassica napus—A \( \lambda \) ZAP cDNA library of developing siliques of \( B. \) napus cv. Ascar (12) was used for PCR amplification of a 571-bp DNA fragment with the degenerated primers 5\textsuperscript{-}G/G/C/A/T/G/C/TGGTGGAA/AG/TG/G/TG-3\textsuperscript{1} (forward) and 5\textsuperscript{-}G/G/G/A/G/G/G/A/G/G/A/G/TG/G/TG/TG/G/TG-3\textsuperscript{1} (reverse). The \( 5' \)-end was amplified with the T3 primer (Stratagene) and the specific reverse primer 5\textsuperscript{-}TATAGGCGTTCAATTCCGAC-3\textsuperscript{1} derived from the 5\textsuperscript{-} and 3\textsuperscript{-} untranslated regions were used in PCR experiments, and a full-length clone of 1502 bp was isolated and sequenced. mRNA isolation, DNA synthesis and tailing, PCR amplification, and DNA sequencing were carried out as described (13).**

**DNA Amplification from Arabidopsis thaliana—A BLAST search (14) in the non-redundant data base of the GenBank\textsuperscript{TM} EST Division revealed some \( A. \) thaliana EST clones\textsuperscript{a} aligning with the \( B. \) napus amino acid sequence amplified above. Specific primers derived from the \( A. \) thaliana EST clones EMBL accession no. T42569 (forward) and \( 5'-\text{GC}G\text{GATGGCGAAAGTCTTAC-3'} \) and EMBL accession no. F13717 (reverse) 5\textsuperscript{-}TATTGCCATGATTCCACACA-3\textsuperscript{3}- and 5\textsuperscript{-}CAAATTCTGATGATGCATC-3\textsuperscript{3}') derived from the \( 5' \)- and 3\textsuperscript{-}untranslated regions were used in PCR experiments, and a full-length clone of 1502 bp was isolated and sequenced. mRNA isolation, DNA synthesis and tailing, PCR amplification, and DNA sequencing were carried out as described (13).**

**Plasmid Construction and Yeast Transformation—A 1.2-kilobase pair FAD2 sequence was amplified from cDNA of developing \( B. \) napus cv. Drakkar embryos by PCR with oligonucleotide primers (5\textsuperscript{-}CCGG-**

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‡ The abbreviations used are: LCB, long chain base(s); PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; bp, base pairs; EST, expressed sequence tag.

\[5\textsuperscript{-}G/G/C/A/T/G/C/TGGTGGAA/AG/TG/G/TG-3\textsuperscript{1} \]

\[5\textsuperscript{-}G/G/G/A/G/G/G/A/G/G/A/G/TG/G/TG/TG/G/TG-3\textsuperscript{1} \]

\[5\textsuperscript{-}TATAGGCGTTCAATTCCGAC-3\textsuperscript{1} \]

\[5\textsuperscript{-}CAAATTCTGATGATGCATC-3\textsuperscript{3'} \]

\[5\textsuperscript{-}TATTGCCATGATTCCACACA-3\textsuperscript{3'} \]

\[5\textsuperscript{-}TATTGCCATGATTCCACACA-3\textsuperscript{3'} \]

\[5\textsuperscript{-}CAAATTCTGATGATGCATC-3\textsuperscript{3'} \]
Fig. 1. Sequence alignments of the sphingolipid desaturase from rapeseed (BnDES8), Arabidopsis (AtDES8), and sunflower (HaDES?) with the \( \Delta^6 \)-acyl lipid desaturase from borage (BoDES6). The N-terminal domain up to position 121 of the sunflower protein is homologous to the hydrophilic part of cytochrome \( b_5 \), with the conserved amino acids underlined. The three highly conserved histidine regions characteristic for acyl lipid desaturases are indicated by boxes. Identical amino acids are shaded.

**Table 1:**

| Strain | Sequence | Identity |
|--------|----------|----------|
| BnDES8 | --------- | 100%     |
| AtDES8 | --------- | 100%     |
| HaDES? | --------- | 100%     |

**Legend:**

- **BnDES8:** sequence from rapeseed
- **AtDES8:** sequence from Arabidopsis
- **HaDES?:** sequence from sunflower
- **BoDES6:** sequence from borage

**Notes:**

- The sequences were aligned using ClustalW software.
- Identical amino acids are shaded.
- The conserved histidine regions are boxed.
RESULTS

PCR Cloning and Sequence Analysis—In previous experiments, we had cloned a cDNA from sunflower that codes for a fusion protein between an N-terminal cytochrome b_{5} and a putative membrane-bound desaturase domain, but so far a biochemical function had not been assigned to this C-terminal domain (13). In continuation of these investigations, we carried out additional PCR experiments with cDNA from developing B. napus embryos and detected another cDNA closely related to the previously cloned sunflower sequence (13). We isolated a full-length clone of 1594 bp corresponding to an open reading frame of 449 amino acids. Estimates of gene copy number indicated 6–8 gene copies/haploid genome of B. napus. This number corresponds to the copy numbers found for other lipid desaturases in B. napus, which all are present as small gene families (25).

Based on substantial similarity of some EST clones of A. thaliana (EMBL accession numbers T42569 (36–102), N37558 (42–204), F13728 (122–210), T42806 (375–405), F13717 (430–458) (numbers in parentheses refer to the amino acid positions given in Fig. 1) to the B. napus sequence, we also carried out PCR experiments with an A. thaliana ZAP cDNA library. A full-length clone of 1678 bp was obtained, which corresponds to an open reading frame of the same length as the B. napus sequence. At the N terminus, both open reading frames are nine amino acids shorter than the sunflower protein, but they also show seven highly conserved amino acid residues characteristic for cytochrome b_{5} (13). The two predicted polypeptides of 51–52 kDa showed 76% identity to each other and 65% identity to the sunflower protein (Fig. 1).

The Cytochrome b_{5}-Desaturase Fusion—Acy1 lipid desaturases catalyze the oxygen- and electron donor-dependent insertion of double bonds into fatty acid residues. The immediate electron donor for many microsomal desaturases is cytochrome b_{5}. Therefore, a fusion between cytochrome b_{5} and a desaturase as found in these new sequences may have a functional advantage. A fused cytochrome b_{5} was detected in the Δ^{3}-acyl-CoA desaturase from yeast (13, 26) and a red alga (27), in the Δ^{2}-acyl lipid desaturase from plants (28, 29) and the nematode Caenorhabditis elegans (30), as well as in the sphingolipid α-hydroxylase of yeast (7) and in the TU-36B gene of Drosophila melanogaster (31). Therefore, the desaturase-like proteins encoded by the two above mentioned sequences represent new members of the cytochrome b_{5} superfamily (Fig. 2). The heme-protein nature of the N-terminal domain from the homologous sunflower fusion protein has been confirmed by redox absorbance difference spectra of the recombinant cytochrome b_{5} expressed in Escherichia coli (13), and the characteristic histidine boxes conserved in all lipid desaturases (32) are also present in this new class of desaturases. The highest similarity (58–60% identity) was found for the Δ^{3}-desaturase from borago (28) (Fig. 1), but only limited resemblance (≤22% identity) exists to the Δ^{3}-desaturase from the moss Physcomitrella patens (29) and the nematode C. elegans (30).

Functional Expression in S. cerevisiae and Fatty Acid Analysis—In order to elucidate the catalytic function of these new fusion proteins, they were expressed in yeast cells, which provide a membrane-bound redox system suitable for functional cooperation with heterologous desaturases (33). As a control, we inserted a full-length cDNA clone for the microsomal Δ^{2}-desaturase from B. napus into a yeast-E. coli shuttle vector under the transcriptional control of the inducible yeast GAL1 promoter. Galactose-induced cultures of transformed yeast cells were used to prepare fatty acids from membrane lipids for subsequent analysis (18). S. cerevisiae clones harboring the B. napus cDNA encoded a Δ^{3}-acyl lipid desaturase from borago (28) (Fig. 1), but only limited resemblance (≤22% identity) exists to the Δ^{3}-desaturase from the moss Physcomitrella patens (29) and the nematode C. elegans (30).
The B. napus FAD2 gene were capable of forming 11% of \( \Delta_9,12-C_{16} \)- and \( \Delta_9,12-C_{18} \)-dienoic fatty acids, proving and confirming the capability to express a microsomal \( \Delta_12 \)-desaturase of plant origin (33). Therefore, the two newly isolated cDNAs coding for the fusion proteins of \( A. \) thaliana and \( B. \) napus were ligated into the same site of the vector described above, and the derived plasmids pBnDES8 and pAtDES8 were transformed into \( S. \) cerevisiae. Fatty acid analysis of transformed cells harboring pBnDES8, pAtDES8, or uncut pYES2 (negative control) showed identical wild-type patterns for all three cultures. To confirm that the monoenoic fatty acids (16:1, 18:1) observed in the two transgenic strains are in fact the same regioisomers as formed in wild type yeast, i.e. palmitoleate and oleate with \( \Delta_9 \) double bonds, we carried out a detailed localization of the double bond in each of these two fatty acids from these strains. For this purpose, the fatty acid methyl ester fraction was subjected to a modified von Rudloff oxidation converting the original double bond into a pair of vicinal trimethylsilyloxy groups. Upon GC-MS, the derivatives of the two monounsaturated fatty acids in all three strains were identified as methyl 9,10-di-(trimethylsilyloxy)-hexadecanoate (retention time 20.15 min) and methyl 9,10-di-(trimethylsilyloxy)-octadecanoate (retention time 23.73 min). Fragmentation between the vicinal trimethylsilyloxy groups results in characteristic fragments for the carboxyl ends (C-1–C-9 at \( m/z = 5259 \) from both \( \Delta_9-16:1 \) and \( \Delta_9-18:1 \)) and for the methyl ends (C-10–C-16 at \( m/z = 5259 \) for 16:1; C-10–C-18 at \( m/z = 2572 \) for 18:1). The negative result of a single ion monitoring experiment to detect corresponding fragment ions for carboxyl and methyl ends resulting from \( \Delta_9-16:1 \) (\( m/z = 217 \) and 229) and \( \Delta_9-18:1 \) (\( m/z = 217 \) and 257) excluded the formation of these particular regioisomer (for discussion, see below). The analysis of pyrrolidides also showed added linoleic acid (\( \Delta_9,12,18:3 \)) into their membrane lipids (34), did not show any \( \Delta_9 \)- or \( \Delta_6 \)-desaturation activity with a polyunsaturated substrate. These results indicated that the new fusion proteins are not involved in the desaturation of fatty acids.

**LCB Analysis**—To investigate a possible modification of sphingolipid LCB, yeast cells were directly subjected to strong alkaline hydrolysis to liberate the free LCB from their complex sphingolipids (19). The extracted LCB were converted into dinitrophenyl derivatives and analyzed by reversed-phase HPLC.
whereas sphinganine and C_{20}-phytosphinganine are only minor components under nonstressed growth conditions (35, 36). Yeast cells transformed with the empty vector (control) showed the wild-type LCB pattern (Fig. 3B). Transformants expressing pAtDES8 and pBnDES8 accumulated in addition to phytosphinganine new LCB in yields of 59 and 31%, respectively (Fig. 3C and D). Their shorter retention times in reversed-phase HPLC suggested that they may be unsaturated derivatives of C_{18}-phytosphinganine.

GC-MS Analysis of Phytosphingenine Regioisomers—Unequivocal proof for the presence of phytosphingamines was achieved by GC-MS (23). Peracetylated LCBs were resolved into three components and identified as 2-N-acetamido-1,3,4-tri-O-acetyl-1,3,4-trihydroxyoctadecane (retention time 15.72 min) and two isomeric 2-N-acetamido-1,3,4-tri-O-acetyl-1,3,4-trihydroxyoctadecenes (retention times 15.30 and 15.45 min; both with m/z = 483 [M^+] and m/z = 423 [M-60]^+). For the localization of the double bond, N-acetylated trihydroxybases were subjected to two different degradation protocols, i.e. to lead tetracetate and to von Rudloff oxidation (22). Lead tetracetate fragmentation/reduction resulted in alcohols, which were separated by GC-MS as their nicotinates into three peaks. The first (retention time 16.30 min) was a saturated alcohol, the second was identified as a derivative of 6-pentadecenol (retention time 15.72 min, m/z = 331 [M^+] and fragments at m/z = 192, 206, 218, and 232), and the third was nicotinate of 5-pentadecenol (retention time 15.95 min, m/z = 331 [M^+] and fragments at m/z = 192, 204, 218, and 232). The von Rudloff oxidation, which yields aliphatic acids from the alkyl portion between the double bond and the terminal methyl group, resulted in methyl esters of nonanoic (7.5 min) and decanoic (9.0 min) acid in relative proportions of 1:3. These two independent sequences prove the formation of two regioisomeric phytosphingamines with a Δ^8 or a Δ^9 double bond in relative proportions of 3:1.

NMR Analysis of Phytosphingenine Stereoisomers—For assignment of the double bond stereochemistry (cis/trans), the peracetylated LCB fraction was separated by preparative silver nitrate TLC into three components with R_F values of 0.46 (125 mg), 0.44 (300 mg), and 0.38 (100 mg), which were used for recording ^1H NMR spectra. The component with the highest R_F value of 0.46 turned out to be the acetylated phytosphinganine (data not shown). The spectrum (Fig. 4) of the fraction with R_F = 0.44 displayed nonresolved multiplet signals for olefinic protons at 5.308 ppm, which showed cross-peaks in the COSY experiment to the α-methylene protons at 1.879 ppm characteristic for trans-configuration. In the fraction with R_F = 0.38 the olefinic signals were shifted to higher field (5.275 ppm), whereas the α-methylene protons appeared at lower field (1.927 ppm) as typical for a cis-double bond (37).

In view of these data, it is obvious that HPLC separates the cis/trans-stereoisomers, but not the Δ^8/Δ^9-regioisomers of phytosphingamines (Fig. 3, C–E). The smaller peak with shorter retention time contains the two cis-Δ^8/Δ^9-phytosphingamines, whereas the larger peak following closely behind contains the two trans-Δ^8/Δ^9-phytosphingamines. The cis/trans ratios of phytosphinganine, calculated from HPLC analysis, are 1:3 in transformants harboring pAtDES8 and 1:6.7 in pBnDES8 transformants (Fig. 3, C and D). Considering all data, the relative proportions of the different phytosphingamines

![Fig. 4](image-url)

**Fig. 4.** ^1H NMR spectra of peracetylated phytosphinganine stereoisomers recorded at 600 MHz in CDCl_3. Shown are the diagnostic signals of the olefinic (5.2–5.4 ppm; left) and the α-methylene protons (2.0–1.8 ppm; right) used for the assignment to the trans-Δ^8- (A) and cis-Δ^8,9-phytosphinganine (B) isomers. The LCB were recovered from transgenic yeast cells expressing a sphingolipid desaturase from A. thaliana and resolved by preparative argentation TLC in the form of peracetylated derivatives. The N- and O-acetate signals are characterized by appropriate labeling.
Desaturase

$\Delta_8$-Desaturase

$\Delta_8$-Desaturase

C 4-Hydroxylase

$t18:1^8$

$d18:1^8$

$t18:0$

$C4$-Hydroxylase

Plant

Desaturase

$\Delta_6$

$\Delta_8$

$\Delta_8$-Desaturase

$\Delta_8$-Desaturase

Sphingolipid Desaturase from Plants

Fig. 5. Working hypothesis for the biosynthesis of phytosphinganine in plant as well as in yeast cells expressing a heterologous sphingolipid desaturase from plants. The desaturase measures the $\Delta^8$ distance from the oxygen-functionalized carbon atom next to the double bond to be introduced. When expressed in yeast and depending on the hydroxylation status of C-4, the sphingolipid desaturase from higher plants introduces a C-8 (left branch) or a C-9 double bond (right branch) of cis- and trans-configuration into LCB leading to the formation of $\Delta^8$- and $\Delta^9$-phytosphinganine (t18:1$^8$ and t18:1$^9$). In higher plants, the desaturation of phytosphinganine (t18:0) seems to be blocked (right branch), because only C-8-unsaturated LCB have been shown to occur (10). $R$, different fatty acyl residues of the basic ceramide structure.

$\Delta^8$-Desaturase (28), but in contrast to the fatty acid desaturases, the LCB desaturase lacks stereospecificity. Therefore, this desaturase is the first trans-desaturase to be cloned. Furthermore, its activity does not depend on the presence of an additional cis-double bond in allylic position as required by most other acyl lipid desaturases including the $\Delta^6$-desaturase. Because of the sequence similarity of the LCB desaturase to $\Delta^6$-acyl group desaturases and the possibility that a $\Delta^6$-hexadecenoyl-CoA could be used by the yeast serine palmitoyl transferase for a direct formation of an unsaturated $\Delta^6$-LCB, we carried out a detailed analysis of the location of double bonds in monounsaturated C16 and C18 fatty acids of the two transgenic yeast strains. Both strains contained only the $\Delta^6$-regioisomers as found in wild type and thus rule out the possibility that the $\Delta^6$-double bond in LCB is carried over from the $\Delta^8$-unsaturated fatty acid precursor. Despite the fact that the sphingolipid desaturase accepts a satu-

Discussion

In view of the fact that only one additional heterologous gene is expressed in the transformed yeast cells, obvious questions arising concern the origin of both the cis/trans-stereoisomers and the $\Delta^9/\Delta^8$-regioisomers. The presence of cis- and trans-double bonds in the newly formed products could be ascribed either to the activity of a genuine yeast cis/trans-isomerase similar to fatty acid isomerases found in other organisms (38) or to the lack of absolute stereospecificity of the desaturase itself as known from some monooxygenases (39). Reinvestigation of the trihydroxybases from A. thaliana sphingolipids by HPLC and GC-MS confirmed that cis- and trans-isomers are present in $\Delta^8$-phytosphingamines and that the double bond is restricted to C-8. In addition, the cis/trans ratio of A. thaliana phytosphingamines (Fig. 3E) is similar to that found in each of the $\Delta^8$- and $\Delta^9$-phytosphingamines extracted from pAtDES8 transformants (Fig. 3C). This suggests that formation of both stereoisomers may be catalyzed by a single desaturase of plant origin, which does not show absolute stereospecificity as typical for all the fatty acid desaturases cloned so far.

The presence of two regioisomeric $\Delta^6$- and $\Delta^9$-double bonds in phytosphinganine can be explained as follows. The plant LCB desaturase has a regioselectivity comparable with that of a fatty acid desaturase, which introduces a double bond in the $\Delta^6$-position. The acyl desaturase measures this distance from the oxygen-functionalized carbon atom ($\Delta$-desaturase) of the acyl group, which is usually the carboxyl carbon (C-1) of the fatty acid. On the other hand, this carbon atom does not necessarily have to carry an additional carbonyl oxygen. Desaturation of alkenyl glycerol ethers has shown that the oxygen of an ether bridge is sufficient to identify C-1 of the 9-cis-alkenyl chain for correct alignment and regioselective insertion of the second double bond by the $\Delta^{12}$-desaturase (40). Therefore, we assume that the LCB desaturase measures the $\Delta^8$-position from the oxygen-carrying carbon atom next to the alkyl chain in the LCB (Fig. 5). In plants “C-1” can only be C-3 of sphinganine and sphingosine (4-trans-sphinganine), resulting in the new double bond at C-8, whereas formation of $\Delta^8$-phytosphinganine by hydroxylation at C-4 can only occur subsequent to C-8 desaturation of sphinganine. The reason for this order of events is not at present understood. On the other hand, in the transformed yeast, the strict sequence of C-8 desaturation followed by C-4 hydroxylation does not exist, and the heterologous plant LCB desaturase also has access to phytosphinganine. In this case, C-4 and not C-3 of the LCB is used as “C-1” for alignment of the alkyl chain. Insertion of the double bond in the fixed distance gives $\Delta^6$- instead of $\Delta^8$-phytosphinganine. This picture (Fig. 5) explains the sequence similarity between the LCB $\Delta^8$-desaturase and the fatty acid $\Delta^6$-desaturase (28), but in contrast to the fatty acid desaturases, the LCB desaturase lacks stereospecificity. Therefore, this desaturase is the first trans-desaturase to be cloned. Furthermore, its activity does not depend on the presence of an additional cis-double bond in allylic position as required by most other acyl lipid desaturases including the $\Delta^6$-desaturase. Because of the sequence similarity of the LCB desaturase to $\Delta^6$-acyl group desaturases and the possibility that a $\Delta^6$-hexadecenoyl-CoA could be used by the yeast serine palmitoyl transferase for a direct formation of an unsaturated $\Delta^6$-LCB, we carried out a detailed analysis of the location of double bonds in monounsaturated C16 and C18 fatty acids of the two transgenic yeast strains. Both strains contained only the $\Delta^6$-regioisomers as found in wild type and thus rule out the possibility that the $\Delta^6$-double bond in LCB is carried over from the $\Delta^8$-unsaturated fatty acid precursor. Despite the fact that the sphingolipid desaturase accepts a satu-
rated substrate, it is not related to the other desaturase groups that use saturated substrates (acyl-ACP-, acyl-CoA-, des-C-, and the senescence-induced desaturase (41)). Its similarity to the Δ^5-acyl group desaturase suggests that structural features controlling regioselectivity rather than those allowing proton abstraction from saturated substrates were more suitable for evolutionary modification. On the other hand, in plants, LCBs controlling regioselectivity rather than those allowing proton introduction of double bonds are far more widespread than the rare introduction of Δ^5-double bonds into acyl groups. Therefore, the evolutionary correlation between these two groups of desaturases (i.e., whether one evolved from the other or whether both represent independent lines from a common ancestor) is an interesting but open question.

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